EPICUTICULAR WAX CHEMISTRY, MORPHOLOGY, AND PHYSIOLOGY IN SAND BLUESTEM, *ANDROPOGON GERARDII* SSP. *HALLII,* AND BIG BLUESTEM, *ANDROPOGON GERARDII* SSP. *GERARDII*

by

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Abstract

Plant epicuticular wax (ECW) isolates internal tissues from harsh external conditions increasing drought tolerance. Beta-diketone-rich ECW reflect light and result in a glaucous phenotype that may ameliorate the thermal environment of the leaf. The overall goal is to characterize the form and function of ECW in two closely related, but phenotypically divergent grasses. *Andropogon gerardii* ssp. *gerardii*, big bluestem, is a non-glaucous, agronomically and ecologically dominant grass in the United States while *Andropogon gerardii* ssp. *hallii*, sand bluestem, is a glaucous subspecies restricted to dry, sandy soils. The objectives are to contrast sand and big bluestem ECW chemistry, morphology, and physiology to determine the distinctions in ECW resulting in the glaucous phenotype and determine the effect this has on leaf optical qualities and permeability. Gas chromatography mass spectrometry (GC-MS) and scanning electron microscopy (SEM) were used to examine ECW chemistry and micromorphology. It was hypothesized that beta-diketones and beta-diketone tubules where present only in leaves of sand bluestem and that the ECW was more reflective and abundant and the cuticle was less permeable. Beta-diketones and tubular ECW were absent in big bluestem and common on sand bluestem's surface, although less than 20% of ECW was beta-diketones. Functional implications of ECW phenotypes were investigated by comparing minimum conductance (G_{min}), wax load, reflectance, and transmittance. Reflectance, with and without ECW, and G_{min} were measured with an infrared gas analyzer and a spectroradiometer, respectively. Sand bluestem had twice the ECW in mg cm^2 (P=.01) and three times lower G_{min} in ms^{-1} 10^{-5} (P=.02). Partial least squares

(PLS) models were trained to predict subspecies from reflectance spectra and were able to distinguish the subspecies. These experiments indicate that in comparison to big bluestem, increased reflectance is a property uniquely imparted to sand bluestem by ECW and the presence of beta-diketones determines the distinction. Glaucous crop species have shown higher yield under drought and extreme weather, including drought, is expected to become more common. Therefore, this study of glaucous waxes, may be applied in engineering drought tolerance.

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Chapter 1: Literature review

1.0 Introduction to the cuticle, the epicuticle, and β-diketone-rich wax

Water resistant, waxy cuticles coat plant epidermal cells isolating their internal environment from harsh external conditions in both the earliest known land plants and their modern day descendants (Edwards et al. 1996). Cuticles predate the presence of stomata by tens of millions of years and are considered a key adaptation, allowing colonization of the land by plants (Edwards et al. 1996, Raven 2002). In the evolution of the stomate, the cuticle is considered a prerequisite structure (Raven 2002). The cuticle and the epicuticle, an additional waxy layer exterior to the cuticle, function as a unit on the surface of plants. The hydrophobic, extracellular matrix prevents loss of water and discriminates against diffusion of CO_2 (Riederer 2006). In modern land plants, hydrophobic plant waxes give the stomatal aperture greater control over water and gas exchange (Raven 2002). As a result, land plants can adjust rapidly to seasonal and diurnal shifts in temperature and water availability (Raven 2002). Additionally, cuticular and epicuticular wax can reduce exposure to radiation, protect against pathogens, reduce wettability, self-heal after damage, and function as a self-cleaning surface (Riederer 2006). Although cursory studies indicate great variety in structure and chemical composition, most knowledge of these ancient and critical structures is based on examination of a small number of species (Jeffree 2006).

As previously described, epicuticular wax (ECW) is exterior to the cuticle. The epicuticle is a region of mostly aliphatic molecules that self-organize into crystals on the basis of ECW chemical composition (Jeffree 2006). This adds a dimensionality to the surface of the plant that can be altered by regulation of wax synthesis (Koch et al,

2010). Changes in crystal shape can affect optical properties including reflection. The presence of β-diketones in ECW is known to result in the formation of β-diketone tubules (Barthlott et al. 1998; Meusel et al. 2000). In turn, β-diketone rich ECW is milky white and reflective (Holmes and Kellier 2002; Koch et al, 2010). This form of reflection is known as glaucousness and is defined as a property of ECW three-dimensional structure rather than wax amount or thickness (McIntosh et al. 2003).

1.1 Sand bluestem is locally adapted

This study compares the epicuticular waxes of two closely related prairie grasses that differ in ECW as well as habitat. Sand bluestem is a close relative of *Andropogon gerardii* ssp. *gerardii* Vitman (big bluestem) that is locally restricted to sandy soils (Barnes 1984, 1986; Wipff 1996). Evidence for its recent divergence from big bluestem includes broadly overlapping range (Fig. 1.1) and ability to hybridize (Barnes 1984, 1986; Peters and Newell 1961). The range of sand bluestem, *A. gerardii* ssp. *hallii* (Hack.) Wipff, extends from Arizona, Utah, and Montana east to Indiana with a high occurrence in the Sandhills of Nebraska (Fig. 1.1; Barnes 1984, 1986). Big bluestem distribution overlaps with sand bluestem on the western border but extends farther east. In areas where both subspecies occur, sand bluestem populations are isolated from big bluestem on the basis of soil composition and soil moisture. For example, in a study of its occurrence in the Sandhills, *A. gerardii* ssp. *hallii* plants were observed on high sandy ridges and slopes (Fig. 1.2; Barnes 1984, 1986). Big bluestem was found nearby, but populated low loamy meadows where soil moisture was observed to be higher due to proximity to the underlying water table (Fig. 1.2; Barnes 1984, 1986). The overlapping

range indicates that sand bluestem is ecologically rather than geographically isolated from big bluestem.

Both sand bluestem and big bluestem are interfertile, obligately out-crossing, wind-pollinated subspecies (Barnes 1984, 1986; Wipff 1996). Hybrids have been observed in the wild (Barnes 1984, 1986) and can be generated through controlled crosses (Peters and Newell 1961). The ability to hybridize and the abundance of naturally occurring morphologically intermediate plants has led taxonomists to debate the status of sand bluestem, alternately classifying sand bluestem as a species, variety, subvariety, subspecies, or forma (Boe et al. 2004). In this thesis, the classification of subspecies was used in accordance with Wipff (1996) and ITIS (2012). Due to the frequency of natural introgression, Wipff (1996) argued that this rank is warranted and four characters (awn length, inflorescence cilia length, ligule length, and rhizome internode length) can be used to distinguish the two subspecies. Taken together, the current range and ability to hybridize support the premise that sand and big bluestem are closely related and locally restricted by water availability and soil type.

Sand bluestem and big bluestem are restricted to the specific soil types and they appear morphologically distinct. Despite the lack of reproductive isolation, sand bluestem from arid, sandy soils and big bluestem from loamy soils display a high degree of constitutive morphologic divergence, especially the glaucous, leaves of sand bluestem (Barnes 1984, 1986; Wipff 1996). In an area populated by both subspecies, twenty-four traits have been scored on specimens collected over a transect passing from high sand dune to low lying meadow at the Derner Ranch in the Eastern Sandhills, Nebraska (Barnes 1984, 1986). Extreme values for four traits (rhizome internode length,

awn length, cilia length on the midrib and pedicel of the spikelet, and ligule length) were used to calculate a hybrid index and were significantly correlated (Fig. 1.2). After comparison with hybrid index scores of relatively isolated populations (Fig. 1.3), Barnes recommended that these traits be used to differentiate the subspecies. In 1996, Wipff argued, with some success that values established in the Derner Ranch experiment, for these four traits in isolated populations could serve as a key to identify subspecies. Additionally, glaucous leaf waxes are known to be a characteristic of sand bluestem (Tulloch and Hoffman 1979) while big bluestem leaves are non-glaucous, or bright green-yellow and relatively waxless (Peters and Newell 1961).

Sand bluestem morphology may confer an adaptive advantage in the coarse, dry substrate, and sparsely vegetated terrain of the Sandhills. Absorptance measurements in the Derner transect experimentally confirmed the restriction of the glaucous phenotype to sandy dunes. Along the Derner Ranch transect, leaves of dune bluestems were more glaucous with an absorptance of 10-15% less at 625 nm (Barnes 1986). Both subspecies had lower rates of survival in nonnative soils in a reciprocal transplant experiment where sand bluestem plants were grown on loamy meadow soils and big bluestem plants were grown on elevated sandy dunes (Barnes 1984, 1986). Glaucous plant waxes have contributed to significant increases in plant performance in field trials designed to evaluate the effects of glaucousness (Johnson et al. 1983; Richards et al. 1986; Merah et al. 2000; Monneveux et al. 2004). Additionally, both subspecies had lower rates of survival in non-native soils in a reciprocal transplant experiment where sand bluestem plants were grown on loamy meadow soils and big bluestem plants were grown on elevated sandy dunes (Barnes 1984, 1986). The results of the reciprocal

transplantation and reflectance quantification experiments demonstrate that big and sand bluestem are locally adapted to loamy soils and sandy dunes, respectively, and that glaucous foliage may be an adaptation of sand bluestem to arid environments.

1.2 The chemistry of the sand bluestem epicuticle is known only in cultivars and not investigated in big bluestem

One trait associated with sand bluestem has been characterized on a biochemical scale. The thick bluish ECW layer of the Goldstrike sand bluestem cultivar has been analyzed by Tulloch and Hoffman (1979) and found to be rich in β-diketones. Grass waxes tend to fall into one of two categories, primary alcohol-type or β-diketone-type, according to the dominant chemical class present in the ECW (Jeffree 2006). In fact, *A. gerardii* ssp. *hallii* is one organism in which β-diketone-type wax chemistry has been studied in great length (Tulloch and Hoffman 1979). Tulloch and Hoffman (1979) found that 67% of the wax extract was β-diketones, and 16% of the extract was hydroxy β-diketones. Of the β-diketones, hentriacontane-12,14-dione was the most prevalent but 10,12 and 14,16 diones were also identified. Preliminary analysis of the Garden County, NE accession (similar to a cultivar) of sand bluestem also indicates that the primary components in cuticular wax are β-diketones, mostly hentriacontane-12,14-dione. In total, β-diketones and hydroxy β-diketones were 58.6% of the epicuticular extract (n=3 with a standard error of 8.1%) (Ravenek et al. unpublished data).

The epicuticle of the ecologically dominant and wide-ranging big bluestem has not, to our knowledge, been chemically described. Partial descriptions of discrete chemical classes, as in Broadi et al. (2002) or Conte et al. (2003), have described aspects of wax chemistry important to digestibility or for use as biomarkers respectively.

These data are based on isolated chemical characteristics relevant to agronomic quality or fidelity as a biomarker and not a full chemical profile. Preliminary analysis of the Kaw cultivar of big bluestem (Ravenek et al. unpublished data), indicates that it is significantly different from the composition of the Garden County accession with regard to both diketones and primary alcohols. It conforms to the other dominant category of grass waxes, in particular those rich in primary alcohols (Jeffree 2006). This chemical phenotype is defined in the following sections as primary alcohol-type. Primary alcohols were 75.0% of the epicuticular extract (n=3 with a standard error of 11%). The most abundant compound was an aliphatic primary alcohol, 31 carbons in length (C31 primary alcohol). This thesis fills gaps in knowledge of ECW through more detailed characterization of ECW chemistry, form, and function in these divergent grasses especially the ECW of big bluestem.

1.3 Chemical composition of wax determines cuticular morphology and morphology determines reflectance properties

Plant waxes are mixtures of mostly straight chain, aliphatic, compounds. On the plant surface, these mixtures self-organize into amorphous layers or epicuticular crystals (Kunst et al. 2006). Total chemical composition plays a role in the final crystalloid architecture but select chemical classes tend to play a dominant role (Barthlott et al. 1998; Jeffree 2006). The presence of β-diketones is frequently dominant to primary alcohols (Barthlott et al. 1998; Jeffree 2006). Therefore, waxes with the two chemical phenotypes, primary alcohol-type and β-diketone-type, generally have platelet and β-diketone tubule morphological phenotypes, respectively (Jeffree 2006).

β-diketone tubules have a consistent morphology. β-diketone tubules are up to ~5 µm long with a diameter of ~2 µm with hollow centers and forked ends (Barthlott et al. 1998). They generally appear alone or occur with coiled rodlets (Meusel et al. 2000). Muesel et al. (2000) found that the presence of coiled rodlets correlates with asymmetrical positions of the ketones along the polyketone's carbon chain. Straight β-diketone tubules without coiled rodlets were found on waxes composed of β-diketones with 12-14, 14-16, or 16-18-diones. Muesel et al. (2000) also confirmed that waxes rich in β-diketones form these β-tubules both *in vitro* and *in vivo*, indicating that of β-diketone tubules undergo self-assembly.

The most common wax micromorphology is the platelet-dominated cuticle (Barthlott et al. 1998). Platelets vary greatly in shape and size. Barthlott et al. 1998, described entire platelets ranging from 1-3 µm while non-entire platelets range from 1-10 µm in width. Platelets often co-occur with straight rodlets, which are solid structures with a length: width ratio not exceeding 50:1, and are not as indicative of wax chemical composition as β-diketone tubules (Barthlott et al. 1998). However, platelet phenotypes are noted as characteristic of primary alcohol-rich waxes (Jeffree 2006).

In contrast to non-glaucous epicuticles, tubule-rich ECWs have leaf optical properties that can increase reflection of harmful UV radiation (Holmes and Kellier 2002; Reicosky and Hanover 1978; Clark and Lister 1975). Two species of *Eucalyptus* with β-diketone tubule-type waxes were shown to reflect approximately 25 to 35% of UV wavelengths, while ECW of two relatively waxless *Eucalyptus* species reflected at most

5% of UV light (Holmes and Kellier 2002). In *Picea pungens,* reflectance of 350nm wavelengths was 25.6% on glaucous needles and 10.1% on non-glaucous or glabrous needles (Reicosky and Hanover 1978). A decline in UV reflectance was also noted after mechanical removal of tubule-rich, glaucous cuticular wax from blue spruce needles (Clark and Lister 1975).

1.4 Optical properties influence performance under drought and light stress

In many grasses, the distribution of β-diketone-type and primary alcohol-type ECW varies over time, organ, and position. Unlike the generally glaucous leaves and sheaths observed on sand bluestem, many glaucous lines of agronomically important grasses accumulate β-diketones primarily on the vertical tissues highest in the canopy (uppermost leaves and sheaths) (Fisher and Wood 1978; Johnson et al. 1983; Richards et al. 1986; Merah et al. 2000; Monneveux et al. 2004). These tissues are described as glaucous while the vegetative leaves have high levels of primary alcohols and are described as non-glaucous. In glaucous spring wheat and durum wheat lines, Tulloch (1973) found that β-diketone synthesis was observed to peak at 66 days from germination, shortly before anthesis when the flag leaf had developed fully; while, alcohol content peaked between 37 and 44 days from germination. Tulloch (1973) reported an inverse relationship between levels of β-diketones and alcohols. Tulloch (1973) also noted that β-diketones accumulated in greater quantities on the upper most leaf sheaths and flag leaf (Tulloch 1973). Because peak β-diketone accumulation

occurs at anthesis on tissue near developing floral organs, Tulloch (1973) argued these ECWs function to protect reproductive organs and developing seeds.

In fact, the tubule-rich ECWs of many monocot, crop species have been correlated with increased yield and decreased reflectance Fisher and Wood 1978; Johnson et al. 1983; Richards et al. 1986; Merah et al. 2000; Monneveux et al. 2004). However, a correlation of amount of ECW with conductance has been weak and water use efficiency (WUE) estimates vary with measurement technique. Fisher and Wood (1978), Johnson et al. (1983), Richards et al. (1986), Merah et al. (2000), and Monneveux et al. (2004) reported increased grain yield in glaucous lines compared to their near isogenic, non-glaucous counterparts in a variety of monocot crops, especially in rainfed and irrigated field trials. Johnson et al. (1983) failed to find significant differences in extremely droughted plots. Fisher and Wood (1978) found waxiness of spring wheat lines was one of three traits best able to predict yield under drought. Positive correlations between glaucous phenotypes and superior yield under water-limited conditions are often unrelated to total wax load (Johnson et al. 1983; Premachandra et al. 1993) or conductance (Premachandra et al. 1993; Merah et al. 2000). High long term WUE has been inferred in C_3 grasses by low stable isotope discrimination, which reflects increased stomal closure. Stable isotope discrimination experiments often report lower long-term WUE in glaucous plants in spite of increased yield under drought (Febrero et al. 1998; Merah et al. 2000; Monneveux et al. 2004); however, infrared gas exchange (IRGA) often indicates higher instantaneous WUE (Premachandra et al. 1993; Richards et al. 1986) under drought. The source of the discrepancy may stem from the fact that stable isotope discrimination and IRGAs

measure stomatal closure and assimilation to transpiration rate respectively. These studies indicate glaucousness may impart a fitness advantage, although the precise mechanism remains elusive, especially in arid climates like the Sandhills.

A functional explanation for the observed yield advantage under drought was suggested by Richards et al. (1986), who observed that interior leaf temperatures decreased in β-diketone tubule phenotypes of up to 0.7 °C in a field experiment comparing glaucous and non-glaucous durum wheat under drought conditions. Though increased reflection of longer (visible and near-infrared (NIR)) wavelengths decreased photosynthetic rate, the decline in temperature decreased internal vapor pressure enough to increase overall transpiration efficiency in glaucous tissues. The ratio of photosynthetic to transpiration rate was higher in the ears of glaucous lines. Richards et al. (1986) concluded that the inverse relationship between abaxial flag leaf and leaf sheath reflectance and the tissue temperature was responsible for an added three days of seed filling during drought. Taken together, this work in near isogenic lines indicates that glaucous waxes contribute to greater yield and higher reflectance potentially lowering leaf temperature.

1.5 β-diketone synthesis is well characterized in barley and putative homologs have been mapped

The chemical composition of most grass cuticles is dominated by either primary alcohols or β-diketones. Synthesis of wax β-diketones occurs in phylogenetically divergent species (Jeffree 2006, Bianchi 1995). However, the enzymatic steps of wax β-diketone biosynthesis have only been dissected in barley (von Wettstein-Knowles

1995). Samuels et al. (2008) reviewing typical wax synthesis pathways indicated *de novo* synthesis and elongation occurs in plastids by the Fatty Acid Synthase (FAS) complex. The growing hydrocarbon chain undergoes successive rounds of condensation, reduction, and dehydration resulting in C16 to C18 hydrocarbons. These wax precursors undergo further elongation in the cytosol in the Fatty Acid Elongase (FAE) complex. Again elongation occurs through rounds of condensation, reduction, and dehydration. These C18 to C34 chains can then be modified via decarbonylation or reduction pathways to form a subspecies of chemical classes including primary alcohols (Samuels et al. 2008). Alternatively, in barley, von Wettstein-Knowles (1972, 1995) describes what appears to be a modification of the FAE pathway (Figure 1.4). C16 and C18 hydrocarbons can undergo successive rounds of condensation without subsequent chemical reductions and dehydrations resulting in the incorporation of ketones into the chain followed by further elongation. The end product is a very long chain β-diketone. The complex reported by von Wettstein-Knowles to synthesize β-diketones in barley is the CER-CQU enzyme complex. The genes *cer-cqu*, currently named *gsh6, gsh1,* and *gsh8* respectively, have been mapped to barley chromosome 2HS. Wettstein-Knowles (1972, 1995) identified, three putative enzymatically distinct regions. The CER-Q domain is believed to have a role upstream in the synthesis pathway indicating it may be responsible for initial binding of the substrate and has β-ketoacyl synthase activity (elongation activity) (Von Wettstein-Knowles 1972, 1995; Figure 1.4). CER-C is putatively involved in the successive rounds of elongation without dehydration and reduction (incorporation of ketones into the chain) (Von Wettstein-Knowles 1972, 1995;

Figure 1.4). CER-U is a putative hydroxylase associated with synthesis of hydroxy-β-diketones (Von Wettstein-Knowles 1972, 1995; Figure 1.4).

Putative orthologs to *gsh6, gsh1,* and *gsh8* have been discovered in many cereals (Lui et al. 2007; Simmonds et al. 2008; Yoshiya et al. 2011). In spring wheat the presence of an orthologous dominant, epistatic inhibitor to all wax deposition has been found at the *Iw2* loci was found on chromosome 2DS (Lui et al. 2007) and a dominant glaucous gene, *W1,* on chromosome 2BS (Simmonds et al. 2008). These two genes have functional duplicates at *W2* (2DS) and *Iw1* (2BS) (Simmonds et al. 2008). Additional orthologous, glaucouness loci in grasses include *wal* on rye chromosome 7RL and *gl2* on maize chromosome 2S (Lui et al. 2007; Simmonds et al. 2008; Yoshiya et al. 2011). Many of the species in which orthologs have been identified have highly repetitive or polyploid genomes. Though a great deal of evidence supports the β-diketone synthesis pathway in barley, it is important to remember that this trait is polyphyletic and may have evolved through the modification of an unknown enzyme complex or at the level of transcriptional, post-transcriptional, translational, and/or post-translational regulation in *Andropogon*.

1.6 Conclusions

Sand and big bluestem represent an excellent study system for evaluating the contrasting ECW phenotypes (primary alcohol wax phenotype in big bluestem and the β-diketone wax phenotype in sand bluestem). These *Andropogon* subspecies are important and well studied agronomically as well as ecologically in the Central United States (Epstein et al. 1998). However, the chemical nature of the wax phenotypes has

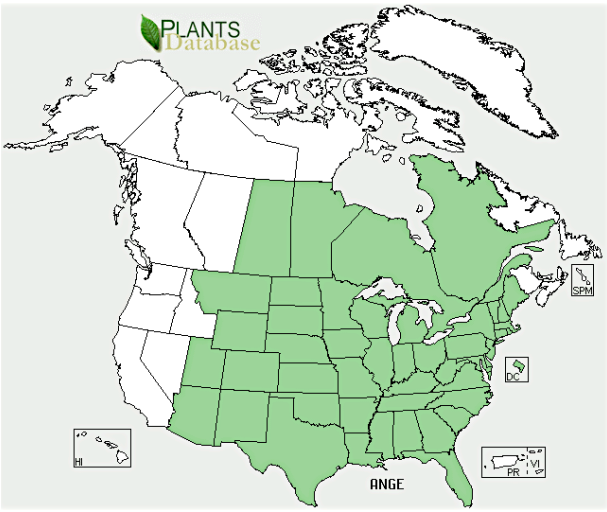
not been evaluated in wild populations, a gap this thesis will address. The potential for introgression in their natural range and restriction of glaucousness to sand bluestem vegetative leaves, coupled with the evidence that glaucousness can increase performance under drought indicates that glaucousness may be an adaptation in *A. gerardii* ssp. *hallii* to dry, sandy soils. If this provides a performance benefit to *A. gerardii* ssp. *hallii*, knowledge of the effect on plant tissues may inform efforts to improve glaucous lines of monocot crops (Tulloch 1983; Jenks 1992). Reflectance, conductance, and quantity are all cuticular properties that influence plant function. However, of these properties only absorptance, through decreased reflectance, has been studied in the bluestem system by Peters and Newell (1961) and Barnes (1986). Their studies suggest hybridizations cause a continuum from glaucous to non-glaucous phenotypes. However the studies provide no specifics biochemical or quantitative details. Therefore, in the following chapter ECW chemistry, micromorphology, and physiology (including non-stomatal conductance, quantification of ECW, and optical properties) will be evaluated, in order to examine the molecular basis of these different wax phenotypes and their ramifications for whole plant physiology in bluestems.

Figures

Figure 1.1 Map of the distribution of big bluestem across the United States (from USDA plants database)

The shaded counties are populated with big bluestem (left) or sand bluestem (right).

big bluestem



sand bluestem

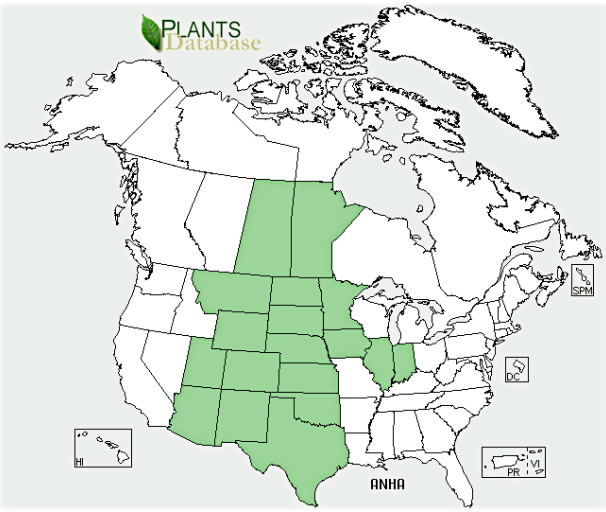


Figure 1.2 Histogram of hybrid index scores, with count of individual plants on the y-axis, in the mixed, sand and big bluestem, Derner Ranch population (modified from Barnes 1984)
In the histogram of hybrid index scores from a single site in which both subspecies of sand and big bluestem occur, a score of 40 indicates extreme sand bluestem morphology and a score of 0 indicates extreme big bluestem morphology. Decreasing soil moisture and increasing distance from the water table distinguish the meadow (green), transition (transparent blue), and dune (white) sectors of the Derner transect.

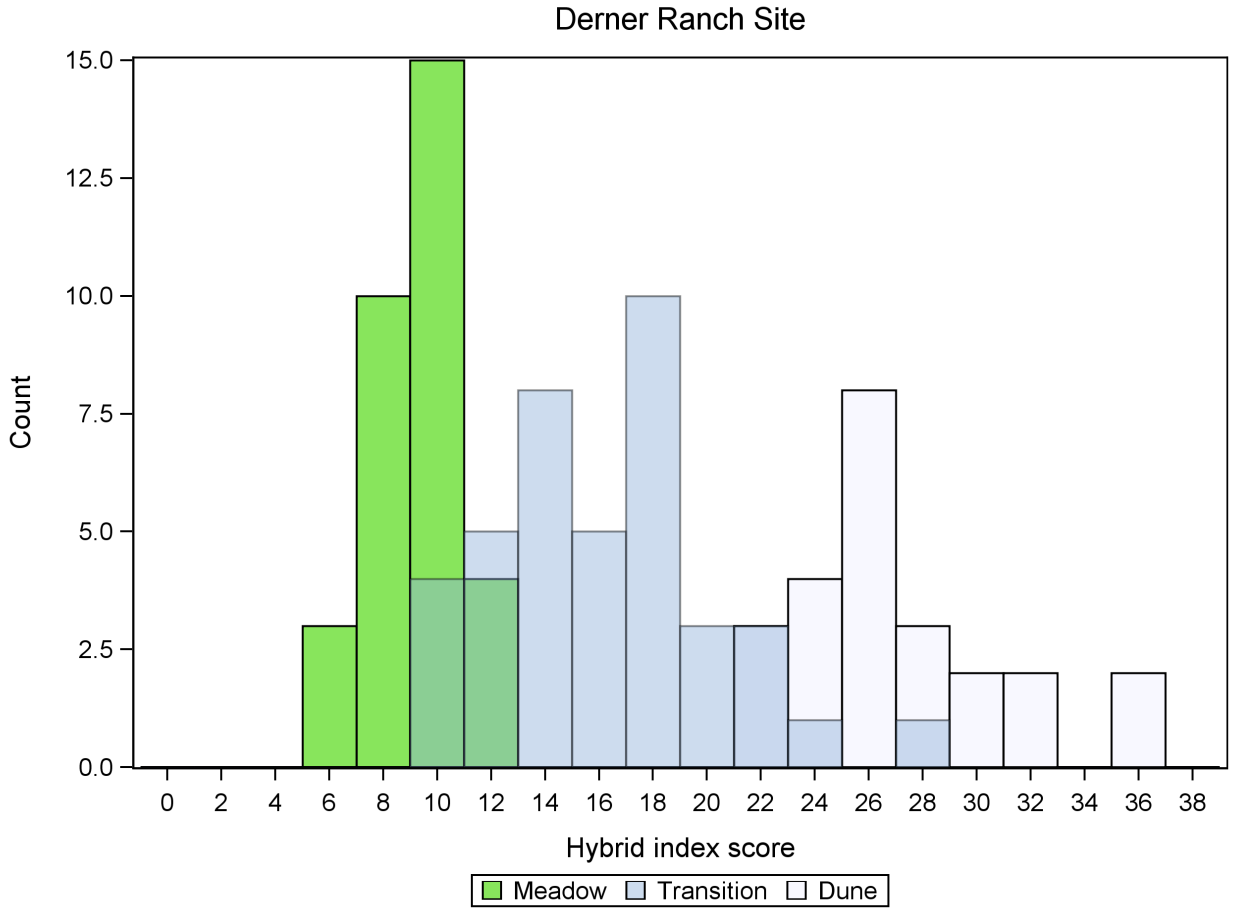


Figure 1.3 Histogram of hybrid index scores, with count of individual plants on the y-axis, in isolated Arapaho, sand bluestem, and Nine-mile, big bluestem, populations (modified from Barnes 1984)
Possible hybrid index scores range from 0-40. A score of 40 indicates extreme sand bluestem morphology and a score of 0 indicates extreme big bluestem morphology. The histogram of two isolated populations shows that index scores for plants collected from the Arapaho population of sand bluestem (white) and scores for plants at the isolated Nine-mile population of big bluestem (green).

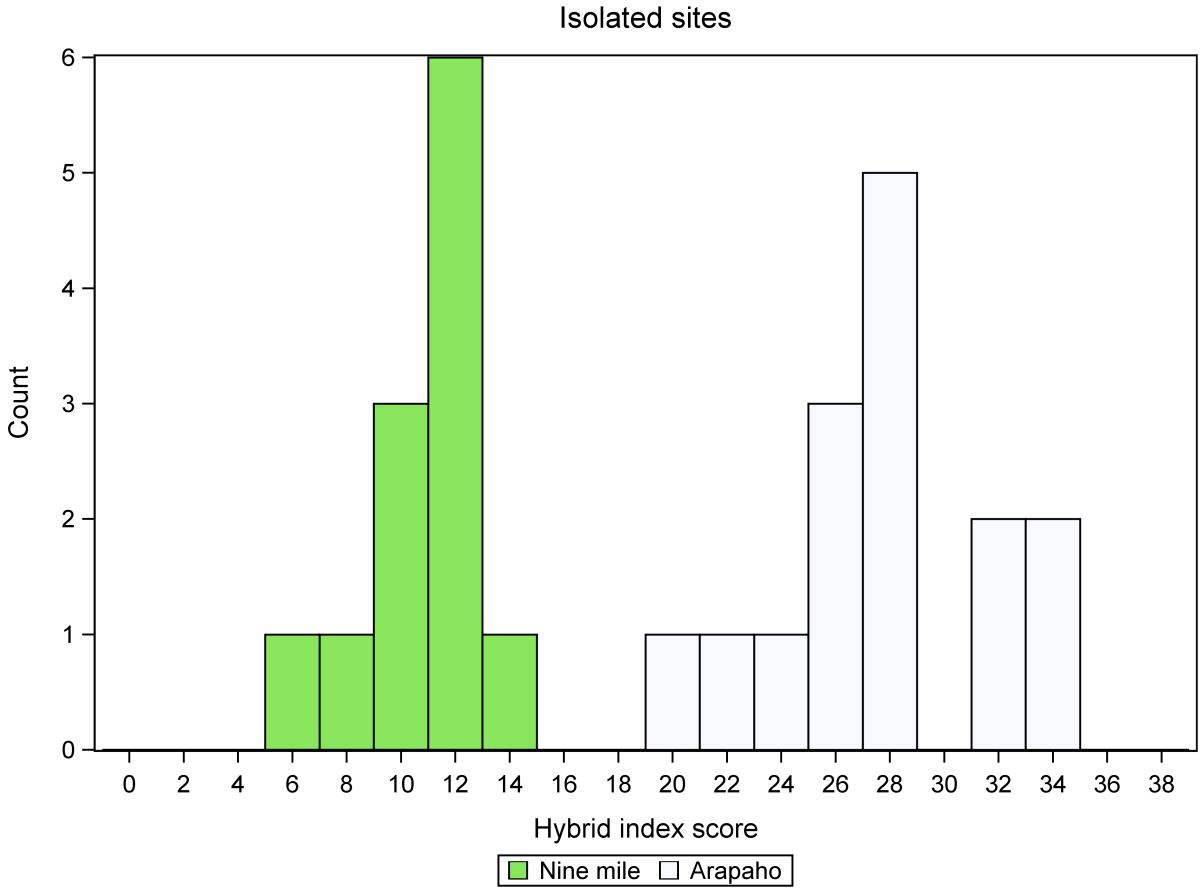
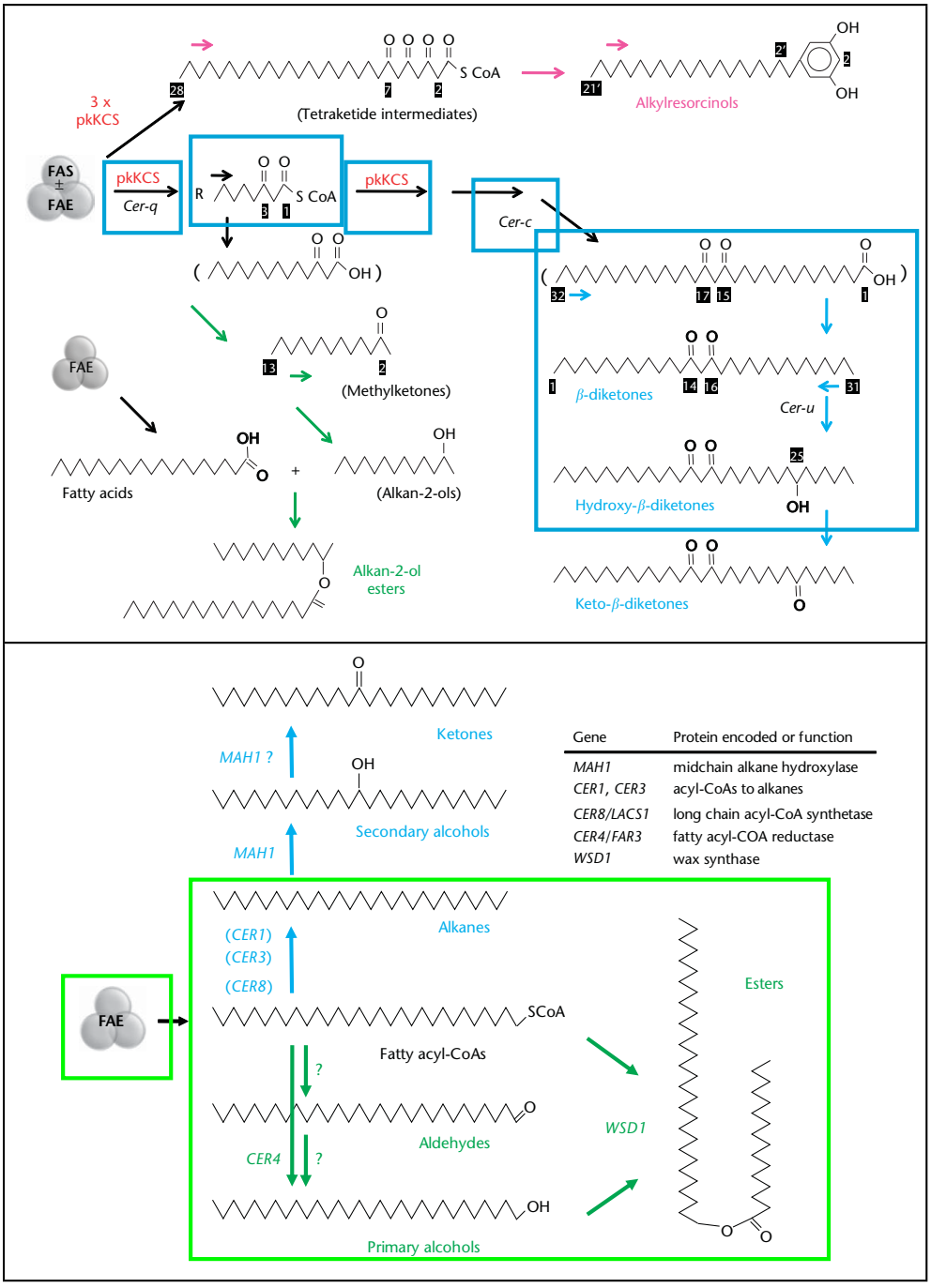


Figure 1.4 Wax synthesis and enzymes and products
Products of the β-diketone pathway that have been previously identified in sand bluestem cultivars (in blue boxes) and products from pathways described in *Arabadopsis* that have also been identified in sand bluestem cultivars (in green boxes) are shown. *Cer-q, Cer-c,* and *Cer-u* are shown (top panel) influencing substrate specificity, performing two successive condensation reactions, and a hydroxylation reaction respectively (Modified from von Wettstein-Knowles 2012).



Gene	Protein encoded or function
MAH1	midchain alkane hydroxylase
CER1, CER3	acyl-CoAs to alkanes
CER8/LACS1	long chain acyl-CoA synthetase
CER4/FAR3	fatty acyl-COA reductase
WSD1	wax synthase

Chapter 2 - Chemistry, micromorphology, and physiology of epicuticular wax in non-glaucous *Andropogon gerardii* ssp. *gerardii* and glaucous *Andropogon gerardii* ssp. *hallii*

Abstract

Waxy cuticles are critical to protection of the plant's internal, cellular environments from external conditions. Plant waxes mediate gas exchange, water-loss, and optical properties. This research focuses on two grass varieties contrasting in epicuticular wax: big bluestem (*Andropogon gerardii*), a non-glaucous wide-ranging prairie grass and sand bluestem (*A. gerardii ssp. hallii*) an interfertile, glaucous (bluish white) subspecies restricted to water-limited sand dunes. The objective of this study was to characterize and contrast epicuticular wax (ECW) of big bluestem and sand bluestem and relate the amounts and classes of ECW to morphology and physiology. The research may give insights into the role of waxes in adaptation to arid environments and drought tolerance. It was hypothesized that β-diketones and β-diketone tubules would be detected only in sand bluestem and that the cuticle of sand bluestem would be less permeable and have more ECW. In these experiments ECW was characterized with gas chromatography mass spectrometry (GC-MS) and related to micromorphology using scanning electron microscopy, non-stomatal conductance, and leaf optical properties. The ECW of sand bluestem was ~20% β-diketones with more ECW (P=.01), the chemical responsible for β-diketones tubule formation, while β-diketones were absent in big bluestem leaves. Sand bluestem leaves exhibited large, ~4 µm long, β-diketone tubules while these were absent in big bluestem. Only platelets, ~1 µm long, and rodlets were observed in big bluestem. Minimum conductance (G_{min}), an estimate of cuticular permeability, was averaged from repeated measurements of total conductance for dark-adapted plants and was significantly lower in sand bluestem. Reflectance and

transmittance of leaves with intact ECW and after removal of ECW with chloroform was measured with a spectroradiometer and integrating sphere from 400-850 nm. ECW affected reflectance and transmittance only in sand bluestem, thus lowering spectral absorptance of light and potentially ameliorating leaf thermal conditions. In summary, sand bluestem exhibits contrasting ECW, with β-diketone waxes, large distinctive tubules, lower G_{min}, and decreased absorptance. This research indicates multiple potential mechanisms whereby the cuticle of sand bluestem is critical for drought tolerance.

2.0 Introduction

Big bluestem, *Andropogon gerardii* ssp. *gerardii* Vitman [= *Andropogon gerardii* Vitman], is a dominant grassland species native to the central United States (Boe et al. 2004). Sand bluestem, *Andropogon gerardii* ssp. *hallii* (Hack.) Wipff [= *Andropogon hallii* Hack.], is a fully interfertile subspecies native to Nebraska's Sand Hills (Fig. 2.1) and sandy soils in the central United States (Boe et al. 2004). These subspecies exhibit strikingly different epicuticles. The purpose of this research is to evaluate the function, morphology and chemistry of the different epicuticles of these two subspecies from contrasting habitats, and shed light on the possible adaptive significance. On vegetative leaves, sand bluestem has a glaucous cuticle (Tulloch and Hoffman 1979) while the cuticle of big bluestem is non-glaucous (Ravenek et al. unpublished data). Early work by Tulloch and Hoffman 1979 on sand bluestem and preliminary data by Ravenek et al. indicate contrasting epicuticular wax (ECW) chemistry between cultivars of these subspecies. In the experiments described here, the gap in understanding of the role of ECW in contributing to wax morphology and physiology differences between these subspecies is addressed. Chemical analysis using gas chromatography mass spectrometry (GC-MS) was performed on epicuticular extracts detailing products of wax synthesis to address the lack of knowledge of big bluestem's ECW composition and to directly compare with sand bluestem. Physiological experiments were conducted to fill in gaps in the published record of the optical qualities, micromorphology, and cuticular conductance of leaf tissue to assess the degree to which these differences in leaf surfaces affect basic plant function. Optical properties of ECWs of *A. gerardii* have not been described quantitatively with the exception of a report of absorptance values at

625 nm (Barnes 1986). The goal of this investigation is to elucidate the nature of the glaucous/non-glaucous phenotypes in *A. gerardii* ssp. *gerardii* and *A. gerardii* ssp. *hallii* to examine potential adaptive advantages waxes may confer to these agronomically and ecologically important grassland subspecies (Epstein 1998). Big bluestem is a major forage grass in the Great Plains, and thus contributs to a billion dollar cattle industry in Kansas (USDA NASS 2007). To that end, in this Chapter wax micromorphology and plant physiology (conductance and reflectance) is linked with wax chemistry and quantity to better characterize these divergent phenotypes.

This research specifically contrasts the epicuticles of big bluestem and sand bluestem because glaucousness is a defining distinction between these subspecies (Boe et al. 2004; Wipff 1996) and is a functionally important trait in plant adaptation to water and temperature stress (Johnson et al. 1983; Richards et al. 1986; Merah et al. 2000; Monneveux et al. 2004). Glaucousness is a trait defined by highly reflective bluish-white appearance (McIntosh 2003). Glaucous ECWs have properties that can alter internal leaf temperature and increase reflection of harmful UV radiation (Holmes and Kellier, 2002; Reicosky and Hanover, 1978; Clark and Lister, 1975; Richards et al. 1986). Glaucousness has been shown to significantly increase grain yield under drought for many monocot crop species including wheat, barley, and sorghum (Johnson et al. 1983; Richards et al. 1986; Merah et al. 2000; Monneveux et al. 2004). However, these were artificially induced mutations or introgressed traits rather than naturally occurring adaptations. With regard to *A. gerardii*, taxonomical descriptions of sand bluestem have often qualitatively described sand bluestem as glaucous (Boe et al. 2004; Wipff 1996) and it is known to be a trait that varies continuously between sand bluestem and hybrid

offspring and big bluestem (Peters and Newell 1961). In this research ECWs of the subspecies complex of sand and big bluestem are evaluated to better understand the role of waxes in plant form and function, and to help elucidate the potential adaptive significance of waxes.

2.1 Background and hypotheses

Glaucous and non-glaucous phenotypes in grasses are generally associated with primary alcohol-rich and β-diketone-rich ECW, respectively (Jeffree 2006). Chemical composition of epicuticular waxes can influence ECW crystalline micromorphology (e.g. certain chemical classes tend to produce tubules rather than platelets (Barthlott et al. 1998; Meusel et al. 2000). Waxes, which are rich in β-diketones, are densely covered in β-diketone tubules, and these tubules, in turn, increase surface reflectance or glaucousness (Barthlott et al. 1998; Meusel et al. 2000; Koch et al. 2004; Koch et al. 2010). Primary alcohol-rich wax is the more common of the two chemical phenotypes (Jeffree 2006) and is not associated with increased surface reflectance (Koch et al. 2010). The ability to synthesize β-diketone-rich wax is a polyphyletic trait in monocots (Jeffree 2006). Although both primary alcohol-rich and β-diketone-rich waxes are common phenotypes with distinct effects on leaf optical properties, the occurrence of both phenotypes in phylogenetically close lineages is understudied within wild grasses. The ECW of sand bluestem was hypothesized to have more ECW overall and to be rich in β-diketones with corresponding β-diketone tubules on the surface. In contrast, big bluestem was hypothesized to be platelet dominated due to primary alcohol-rich ECW.

With regard to cuticular permeability, it was hypothesized that sand bluestem would have a less permeable cuticle based on prior correlations between cuticular

permeability and adaptation to water limited environments (Richards et. al. 1986; Schreiber et. al 1996)**.** Waxes can influence non-stomatal, cuticular conductance. Cuticular permeability is quite low; however, it has been hypothesized that this can contribute to survival in extremely water-limited environments (Richards et. al. 1986; Schreiber et. al 1996). Ideally, permeability measurements should be made on stomate-free surfaces. Sand bluestem has stomates on both ad- and abaxial surfaces (Barnes 1986). Therefore, minimum conductance was measured after stomates were estimated to have closed. Cuticular permeability is difficult to measure on tissue with stomates because stomates contribute to total conductance at a greater rate, even during the night (Caird, 2007). Minimum conductance, G_{min}, was used as an estimate of cuticular conductance as recommended by Kerstiens (1996). If the cuticle of sand bluestem is adapted to a more arid microclimate in this respect, cuticular conductance would be expected to be lower in sand bluestem.

It was hypothesized that sand bluestem's ECWs would be more reflective and this would be indicated by an increase in reflectance when ECW was intact as compared to reflectance after ECW removal. The solubility of ECW in non-polar solvents allows measurement of surface optical properties with and without ECW. Additional steps were taken in these experiments to identify regions of the spectra where the ECW of sand bluestem had the greatest influence on reflectance values using partial least squares analysis (PLS). It was hypothesized the change in reflectance after removal on ECW would be great enough that the PLS model could differentiate big bluestem from sand bluestem specimens.

Degree of glaucousness is known to be intermediate in hybrid offspring of big and sand bluestem (Peters and Newell 1961). Therefore, our populations were evaluated on a hybrid index and this was related to reflectance using a partial least squares (PLS) model. Sand bluestem and big bluestem are obligate outcrossing, wind pollinated, and interfertile (Barnes 1984, 1986; Wipff 1996). Because of the potential for introgression, individuals from all populations were scored on a hybrid index similar to an index used in prior field and hybridization experiments (Peters and Newell 1961; Barnes 1984). The results of previous experiments indicate that hybrids can be identified based on intermediate morphology in a few key traits and that this technique can be applied in the field or greenhouse. Because of careful selection of isolated source populations, no population would be expected to span both the big bluestem and sand bluestem spectrum of hybrid index scores but score variability allows the evaluation of the relationship between ECW reflectance and introgression.

2.2 Methods

Seed collection and growth: Sand bluestem was grown from seeds collected in Valentine National Wildlife Refuge and at the Barta Brothers' Ranch, University of Nebraska in Nebraska. All big bluestem was grown from seeds collected in Saline Experimental Range and Webster Reservoir State Park in Kansas (Table 2.1; Fig. 2.1). Populations are referred to as Saline, Webster, Valentine and Barta Brothers', respectively. Plants grown for this experiment are referred to as cohort I. These plants were used for ECW extraction and scanning electron microscopy (SEM). Seeds were germinated and grown in a green house in Metromix360 with daily watering. Diurnally, temperatures cycled from 20 °C (night) to 38 °C (day). Light levels ranged between

~400 and ~900 photosynthetic photon flux density (PPFD) midday on cloudless days. Artificial lights supplemented sunlight on cloudy days resulting in light levels of ~100 PPFD. Safer® Insect Killing Soap was sprayed in order to treat a mild thrip infestation on 25 June 2010 and 5 July 2010.

Cohort II was grown in August 2011. Seeds the sand bluestem populations of the Gudmundsen Sandhills Laboratory-University of Nebraska, Arapaho Prairie, and Cimmaron National Grassland along with the big bluestem populations from Relic Prairie, and Cedar Bluffs State Park (Table 2.1) were grown in a greenhouse. Seeds from the four populations in cohort II were also grown for a total of nine populations. In total, seventy-nine individuals were grown from seed. Populations are referred to as Gudmundsen, Arapaho, Cimmaron, Relic, and Cedar Bluffs respectively. These plants were used for wax quantification, cuticular conductance estimation, and reflectance measurement. Seeds were germinated and grown in a green house in Metromix510 and watered daily. The green house was heated to ~ 30 °C (day) and ~ 25 °C (night). Average light levels in full sun were ~780 photosynthetic photon flux density (PPFD). Because natural light levels were low during these months, 14 hrs of flourescent light was provided.

Micromorphology: Leaf tissue of twelve ~8 months old plants from cohort I (Saline, Webster, Valentine and Barta Brothers' populations) was removed. Images of fresh leaves (two leaves per individual from three individuals of each population) were taken under high vacuum at 3.0 KV using a secondary electron detector fitted to a using a secondary electron detector fitted to a S-3500N Scanning Electron Microscope manufactured by Hitachi Science Systems, Ltd, Hitachinaka, Ibaraki Pref., Japan.

Specimens were prepared by rapidly freezing the tissue and maintaining an ambient temperature of -190 degrees Celsius during imaging. Two randomly selected sites from the adaxial surface of each leaf were captured at 200x, 2000x, and 4000x magnifications. Structures in profile from each quality image were measured for crystal length using Quartz PCI software (Quartz Imaging Corp. Vancouver, BC Canada). Scale and surface micromorphology were analyzed qualitatively according to Barthlott et al. (1998) following the Barthlott classification system for crystal dimensions.

Wax composition: Mature leaf tissue (approximately 200 mg) was collected from thirty-two ~8 month old plants from cohort I and submerged in chloroform for 30 s. One leaf per individual from eight individuals from the Saline, Webster, Valentine and Barta Brothers' populations was used for extractions. Extracts were processed at the KSU Lipidomics Facility by Thilani Samarakoon and Richard Jeannotte (P.I. Ruth Welti). Two microliters of pentadecane-d_{32} with a concentration of 0.01039 g mL^{-1} was added as an internal standard. Samples were derivatized at 65°C over 30 min using 45 µL of BSTFA/TCMS (N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane) and 5 µL pyridine. Derivatization prevents compounds from being identified intact (e.g. breaks ester bonds).

After derivatization, a 6890N GC (Agilent Technologies) with A DB-5ms capillary column coupled to a 5975N quadrupole mass selective detector (Agilent Technologies) was used to analyze the samples. The column flow rate was 1 mL min -1 and helium was used for the carrier gas. The operating pressure of the front inlet was 18.97 psi and the temperature was 280 °C. An Agilent 7683 autosampler was used to inject 2 μL of the sample. This was done in the splittless mode. The gas chromatograph the temperature

ramp was, initially 80 °C holding for 2 min, then ramped at 5 °C min -1 to 320 °C, and held finally for 15 min. Mass spectrometer quad temperature was set to 150 °C and the MS source temperature was set to 250 °C. The data were processed using Agilent Chemstation software and Automated Mass Spectral Deconvolution and Identification System. The Nikolau lab, specifically post-doc Zhihong Song (P.I. Basil Nikolau), searched an in-house mass-spectral library of ~300 ECW related compounds and a NIST08 spectral library to identify peaks. Composition is reported as the relative percent of each major chemical class and chain length.

Wax composition statistical analysis: In these experiments, steps were taken to insure that chemical composition was reported in a manner that reflects plant-plant, inter-population, and inter-subspecies variability. Because variation could exist between plants, populations, or varieties, Nora Bello of the K-State department of Statistics helped to develop a statistical model to estimate intra vs inter subspecies sources of variance. If, for example, one population of sand bluestem deviated from the overall trend for sand bluestems, the difference between sand and big bluestem cuticular wax composition may have been insignificant. Therefore, a model was developed that also indicates the covariance parameter for population. If the covariance parameter for population was large, further analysis of differences based on source population, rather than subspecies, may have been warranted.

Accordingly, a nested mixed model was used for least squares means (LSM) estimation with the experimental unit (EU) defined as population nested within subspecies where possible. Equation in appendix A.1 is the general model for predicting percentage of total extract. Statistics based of this model were generated with PROC

GLIMMIX (SAS v9.2, SAS Institute, Cary, NC). Optimization was performed with the Newton Raphson method with ridging. Variance components were estimated with restricted maximum likelihood method. In many cases, the covariance parameter for the random effects was estimated as zero causing inflation of the denominator degrees of freedom (ddf) for the fixed effects. These random effects were left in the model as they reflect the experiment design. Therefore, the containment method was used when necessary to estimate sufficiently conservative ddf. Some subsets of data do not contain all of the variable classes of the general model. These subsets were evaluated using similar but reduced models where needed.

In order to model chemical composition by chemical class, the percentages of alcohols, alkanes, aldehydes, fatty acids, and unknown molecules in the ECW were modeled with a single model. Chain length was not known for the last category and was dropped from the model. A log transformation was needed to avoid violating model assumptions. In this model, two heterogeneous variance components were fitted to avoid violating model assumptions; one for alcohols and one for the remaining four groups.

Diketones, triterpenes, and hydroxy β-diketones were only detected in the sand bluestem extracts. Therefore, percent composition of these three groups was estimated using a second model that excludes subspecies and nests chemical class and individual plant within the fixed effect of population. This model required a probit transformation.

In terms of analysis of individual compounds, four full models were used for alcohols, alkanes, aldehydes, and fatty acids of specific chain lengths. The model for C26-C32 alcohols required a probit transformation. Log transformations were used for

the three remaining of these models. For diketones C31-C33, subspecies was dropped from the model and a probit transformation was used. The remaining compounds had no known chain length or no variation in chain length and were only detected in sand bluestem. They were fit to a model with only compound name as the fixed effect with plant-to-plant variability as a random effect and a probit transformation was used.

Quantification of total mass of ECW: Wax weight in mg was taken after a 30 s extraction in 100% chloroform. Leaf area of the adaxial side of each leaf was calculated from photographs. This area was considered half of the total leaf surface area; therefore, total surface area = 2 x adaxial area in cm^2. An ANOVA was performed with PROC GLIMMIX (SAS v9.2, SAS Institute, Cary, NC). Population was defined as the experimental unit (n=2) and nested as a random effect within subspecies for Valentine and Barta Brothers' Ranch populations of sand bluestem and Webster and Saline populations of big bluestem, respectively. Three plants of each population were subsampled.

Minimum conductance: G_{min}, was calculated from minimum conductance readings for eighteen ~6 month old plants (cohort II) according to Kerstiens (1996) and Anfodillo (2002) after environmental conditions were changed to induce stomatal closure (low relative humidity and an extended period of dark adaptation). A Licor 6400xt Portable Photosynthesis and Fluorescence System (LI-COR, Lincoln NE, USA) was used to record conductance; pressure was kept at 98+/- 1 kPa with temperature at 25 C +/- 2 C. During the course of the hour, water vapor was removed from the infrared gas analyzer (IRGA) chamber. G_{min} was calculated from conductance values measured every 90 s after 24 hrs of dark adaption and 30 min in the chamber. The LI-6400 records total

conductance in terms of the mole fraction of water vapor using equation 2. This was converted to a vapor-based concentration gradient using to the equation 3 (equation 3 is derived from Kerstiens (1996); Anfodillo (2002)). Population was defined as the experimental unit and nested as a random effect within subspecies for all nine populations from cohort II of sand bluestem and big bluestem. Two plants of each population (Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers') were subsampled. After 30 min in the chamber the rate of decline in conductance decreased and readings stabilized. This was interpreted as evidence of stomatal closure. In order to determine if conductance values had stabilized, time (from 30 min to 45 min) and its interaction terms were used as an additional fixed effect in the model. Least squares means were generated and tested for differences using PROC GLIMMIX (SAS v9.2, SAS Institute, Cary, NC). The containment method was used prevent inflation of ddf in order to insure that population is the EU and plants from the same population are considered subsamples.

equation 2:

$$g_{tw} = E\,(1000 - (W_l + W_s)/2)$$

$$\frac{}{W_l + W_s}$$

where:

g_{tw} = total conductance (mol m^{-2} s^{-1})

E = transpiration (mmol m^{-2} s^{-1})

W_s = the water mole fraction within the chamber (mmol H_20 mol air^{-1})

W_l = the water mole fraction within the leaf (mmol H_20 mol air^{-1})

equation 3:

$0.41 \text{ mmol } m^{-2} s^{-1} = 1 \text{ m } s^{-1} 10^{-5}$

Leaf optical properties: Reflectance (*r*) and transmittance (*t*) values were obtained with a LI-1800-12 integrating sphere (LI-COR, Lincoln NE, USA) attached to a spectral radiometer (Jazz, Ocean optics, Dunedin FL, USA). Absorptance (*a*) of light is the result of both reflectance and transmittance (equation 4). Therefore both reflectance and transmittance were calculated for this experiment. Measurements were made on mature, attached leaves of ~5 month old plants from cohort II. Epicuticular waxes were removed with a 30 s immersion in 100% chloroform and the same tissue was immediately re-sampled. Nine populations, all populations from cohort II (Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers'), were examined. Four individuals were too small to fill a leaf clip with tissue and were excluded from the trial and a total of fourteen plants were scanned. Each of the scans was performed twice and averaged.

equation 4:

$a = 1 - (t + r)$

where:

a = absorptance

t = transmittance

r = reflectance

PLS leaf reflectance statistics: Partial least squares is a statistical tool introduced

in Wold (1975). In cases like spectral analyses, where the number of X-variables is large and highly correlated partial least squares (PLS) is a powerful tool for prediction. In experiments where the sample size is small PLS has been found to be especially useful. In Lindberg et al.1983, Wold used 16 observations of absorbance to predict concentration of compounds from spectra of ocean samples. Wold et al. (1989) found 10 samples useful for prediction from spectra (Chin et al. 2003). PLS has also been found to be useful in discriminating between two classes when the variance is equivalent (Reeves and Delwiche 2008). Therefore, this tool was applied to train a model to predict identity of plant from leaf reflectance spectra in order to estimate degree to which ECW influences changes in reflectance. Sand bluestem was expected to have a more reflective epicuticle and therefore, these efforts were undertaken to evaluate the overall degree and the specific regions of the spectra that are most altered.

Reflectance was evaluated using PROC PLS (SAS v9.2, SAS Institute, Cary, NC). A model was created that predicted subspecies (binary classes of either big or sand bluestem) from reflectance spectra between 400 and 800 nm. Three types of reflectance values were tested separately for a total of three models. The first type of reflectance measurement was the reflectance of the unaltered leaf surface, the second was the reflectance of the leaf surface after wax removal, and the third was the difference between a leaf with wax and the same leaf with ECW removed. Leave-one-out cross validation was used to reduce the dependence of the model on any overly influential observation. The number of latent factors left in the final model was determined to be the number of factors with the highest R^2 and a Root Mean PRESS statistically not significantly higher than the lowest PRESS. R^2 of model variables and Y-

variables, root mean square deviation (RSMD), and predicted and actual values of Y were reported. Two general models were tested. Fourteen observations with big bluestem and sand bluestem as categorical Y-variables (labeled subspecies) were used in the model. Each model was then trained to predict Y from reflectance values of either leaves with or without ECW, or the difference between the two spectra, in order to gain a full understanding of the contribution of ECW to leaf reflectance.

Characterization of intergradation of morphological traits: Three floral characteristics were scored on fifty-three plants, in this experiment considered fifty-three replicates, from the larger cohort, cohort II (Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers'), in an effort to assess level of introgression of source populations for comparison with variance in reflectance spectra. The index is based on the four-character hybrid index tested in both wild and common garden individuals by Barnes (1984) and used as a key to identify the subspecies (Wipff 1996). Rhizomes were developing but none had adventitious roots so the fourth characteristic of his hybrid index was omitted from the index. Hybrid score (measurements made according to Hybrid index 2; Barnes 1984) was calculated with equation 5. Pairwise comparisons between scores were corrected for heterogeneous variance by fitting separate covariance parameters for each population. The results of the pairwise comparisons were adjusted for multiple comparisons with the Tukey-Kramer method. Statistics were generated with PROC GLIMMIX (SAS v9.2, SAS Institute, Cary, NC).

equation 5:

Hybrid Score = 10 ((ligule *ind.* - liguleBB *extreme*) / (liguleSB *extreme* - liguleBB

extreme) + (awn *BB extreme* - awn *ind.*) / (awn *BB extreme* - awn *SB extreme)* + (inflorescence cilia *ind.* - inflorescence cilia *BB extremes*) /(inflorescence cilia *SB extremes* - inflorescence cilia *BB* $_{extremes}$))

where:

ind = the length in mm of the plant feature

extreme = the extreme values for that plant feature

2.3 Results

Sand bluestem and big bluestem differ with regard to chemical composition of the ECW. Of the chemical classes detected diketones, hydroxy β-diketones, and triterpenes were only detected in sand bluestem (Fig. 2.2). Diketones were estimated to be more prevalent than hydroxy β-diketones and triterpenes (Adj P = 0.0005; Adj P = 0.0012 respectively). In this study, β-diketones accounted for only 17.6% (95% CI: 12.2, 24.4), hydroxy β-diketones for 1.6% (95%CI: 0.8, 2.8). Alcohols, alkanes, aldehydes, fatty acids, and unknown compounds were detected in both subspecies (Fig. 2.2). For both sand and big bluestem, alcohols were found to be the most abundant class. There is evidence that sand bluestem ECW has less alcohols (Adj P = 0.0007) and aldehydes (Adj P = 0.0002). More (4.5%) of the unidentified (unknown) isolates (Adj P = 0.0045) were detected in sand bluestem (Fig. 2.2). Overall, unidentified compounds represented a small fraction, less than 7% of the ECW extract. No evidence was found for a difference between subspecies in relative amount of alkanes and fatty acids. The corresponding t-values and degrees of freedom for the test of differences for simple effect least squares means for subspecies are listed in Table B.1. For alcohols, alkanes,

aldehydes, fatty acids, and unknown compounds there is evidence for an interaction between the effect of chemical class and subspecies ($p < 0.0001$), indicating sand bluestem and big bluestem produce different ratios of these chemical classes in their ECWs. If accumulation of alcohols, alkanes, aldehydes, and fatty acids was unaffected by the accumulation of diketones, hydroxy β-diketones, and triterpenes then no interaction should be detected (i.e. each chemical that is class common to both subspecies should be reduced to the same degree in sand bluestem by the presence of ketones and triterpenes). In terms of quantity of ECW, more ECW in mg cm^2 bluestem was found on the surface of sand bluestem leaves (P=0.0145) (Fig. 2.3). The covariance parameter for population converged to 0. The corresponding t-values and degrees of freedom for test of differences for least squares means for subspecies is listed in Table B.2.

The most common molecule in the ECW of both is C32 alcohol followed by C33 diketone in sand bluestem and C30 alcohol in big bluestem (Fig. 2.4). Within the alcohols more C26 and C28 (Adj P = 0.0007; Adj P = 0.0089 respectively) alcohol was detected in sand bluestem while less C32 alcohol was detected (Adj P = 0.0002). More C28, C30, and C32 aldehydes were found in sand bluestem ECW (Adj $P < 0.0002$ for all three). No significant differences between proportional content of C27-C31 alkane were found between sand or big bluestem. Among the fatty acids, only C32 was a significantly smaller portion of the ECW in sand bluestem (AdjP = .0096). The corresponding t-values and degrees of freedom for test of differences for simple effect least squares means for subspecies is listed in Table B.3.

Based on micromorphology, chemical composition may vary slightly by population, but wax structures generally conform to either β-diketone tubules or primary alcohol platelets (Fig. 2.5). Tubules on sand bluestem specimens had an average length:width ratio of 4.3 (± SD 1.0) µm: 0.3 (± SD 0.1) µm (n=57) and a forked tip characteristic of β-diketone tubules (Fig. 2.5). Individuals from both sand bluestem populations had dense, uniform tubule covered surfaces, but straight β-diketone tubules were observed on Valentine specimens, while, β-diketone tubules and coiled rodlets were observed on Barta Brothers'. Primarily, platelets and short rodlets were observed on Saline and Webster big bluestem specimens (Fig. 2.5). Crystalline shape was variable on big bluestem specimens (rare structures were up to 7.1 *u*m) but average height of platelets and granules was 1.0 (± SD 0.6) µm (n=32).

Cuticular conductance estimates (G_{min}) estimates were lower for sand bluestem indicating that sand bluestem cuticles are less permeable than those of big bluestem. The LSM estimate of G_{min} for each individual plant is derived from the 10 readings taken after adaptation to dark and low RH. These values were considered to represent cuticular conductance at maximal stomatal closure when they appeared stable over time (e.g. conductance values were no longer falling). In fact, the effect of time and the interaction of time and subspecies was nonsignificant, P = 0.8127; P = 0.6382 respectively. There is evidence that sand bluestem has lower G_{min} (P = 0.0236) (Fig. 2.6). The covariance parameter for population converged to 0, indicating that no difference between the populations was detected in this experiment. The corresponding t-values and degrees of freedom the tests of differences of subspecies least square means are listed in Table B.4.

Sand bluestem and big bluestem also differed in reflectance and transmittance across the span of 400-850 nm. With regard to optical properties, decreases in reflectance and an increases in transmittance were noted in the averaged spectra of sand bluestem plants after removal of ECW (Fig. 2.7). Big bluestem plants had no change in reflectance spectra across the span of 400-850 nm (Fig. 2.7).

The contrast between sand bluestem and big bluestem is also apparent when the spectra, with and without wax, are analyzed using PLS. In all models using reflectance of leaves with intact ECW or the difference in spectra after ECW removal as X-variables the predicted value of the sand bluestem plants did not overlap the predicted value of big bluestem plants (Fig. 2.8). This indicates the PLS model discriminates the two varieties well when using reflectance of ECW for predictors of group. For the model designed to predict subspecies, the highest R^2 was 87.1% when reflectance of leaves with intact ECW and the difference in leaf reflection after ECW removal was used for prediction (Table 2.2). The lowest R^2 for both models was the analysis using reflectance of only leaves with the ECW removed. When the glaucous and non-glaucous ECW was removed, the model failed to discriminate between leaf spectra. For the model predicting subspecies $R^2 = 24.8\%$ (Table 2.2). Variable importance plots score X-variables by their usefulness to the explanatory power of the model. For both prediction of subspecies and hybrid index score from reflectance spectra of change in leaf reflectance after removal of ECW, the variable importance plot indicates that all spectra below 700 nm were above 1 and important to the prediction model. In general, all models using reflectance spectra that involved the effect of ECW on reflectance for prediction were best at explaining the variance in Y-values.

The hybrid index was used to characterize intergradation of morphological traits. Two of the characteristics used to calculate the hybrid index score are floral characteristics. Only 12 of the 14 plants that were scanned flowered; therefore, the PLS predicting hybrid index score from spectra had two fewer observations. A total of 53 individuals flowered. Overall, index estimates for sand bluestem individuals ranged from 15.74-20.8 on a scale from 0-30, where 0 indicates big bluestem morphology and 30 indicates sand bluestem morphology. Big bluestem estimates only ranged from 4.94-6.72. Pairwise comparisons of all populations indicate that no big bluestem population differs significantly from any other big bluestem population. There is evidence of three tiers of populations within the sand bluestem (Fig. 2.9). Barta Brothers' had a very similar index score to Arapaho such that Barta Brothers' was estimated at only 0.178 (Adj P = 1.0000) more points than Arapaho. However, the Barta Brothers' population had much higher standard error due to high within population variability (SE = 1.2559 for Barta Brothers' compared to SE = 0.3854 for Arapaho). The corresponding t-values and degrees of freedom for test of differences for least squares means for subspecies is listed in Table B.6. Though variance in hybrid index score was recorded, no population had individuals that overlapped with values in the reciprocal subspecies.

2.4 Discussion

2.4.1 Contrasting epicuticular chemistry among subspecies

The results demonstrate β-diketones were only detected in the leaf ECW of sand bluestem. Furthermore, the two subspecies differed in amount of wax. Thus, these

lines of evidence support the hypotheses that β-diketones should be unique to sand bluestem and sand bluestem should have more ECW. This is also supported by other findings. Approximately five times more cuticular wax was observed on the surface of the Garden county sand bluestem accession than on the Kaw cultivar (Ravenek et al. unpublished data). Similarly, the cuticle of sand bluestem was thicker (Fig. 2.3), however, the increase in thickness was not as pronounced. Surprisingly, the proportion of β-diketones detected in the sand bluestem ECW was lower than cultivar experiments (Tulloch and Hoffman 1979; Ravenek et al. unpublished data). The detection of β-diketones and hydroxy β-diketones in sand bluestem agrees with previous studies of sand bluestem cultivars. Goldstrike sand bluestem cultivar was analyzed by Tulloch and Hoffman (1979) and found to be rich in β-diketones. However, Tulloch and Hoffman (1979) found ~60% more of the ECW extract was β-diketones. Preliminary analysis of a Garden County accession of sand bluestem also indicates that the primary component in the vegetative leaf ECW is β-diketones (Ravenek et al. unpublished data). In total, β-diketones and hydroxy β-diketones represented 40% more of the epicuticular extract (Ravenek et al. unpublished data) than in this study. Both previous studies were conducted on cultivars rather than wild collected seeds. Pedigree, age, and environmental cues may contribute to the observed differences in proportions of β-diketones (Nawrath 2006; Giese 1975; Richards et al. 1986). The ability to observe β-diketone tubules on the surface of plants when β-diketones account for a small proportion of the total ECW supports the idea that total chemical composition plays a role in the final crystalloid architecture but certain compounds tend to play a dominant role even when they are not the most abundant chemical (Jeffree 2006). Though the

proportion of β-diketones and hydroxy β-diketones in sand bluestem ECW was notably low, with regard to previous experiments (Tulloch and Hoffman 1979; Ravenek et al. unpublished data), the dominant chemical hypothesis may provide an explanation for the abundance of diketone tubules observed (Jeffree 2006). Conversely, the absence of diketones in ECW of big bluestem supports the big bluestem micromorphology results.

Comparison of the differences between proportions of β-diketones with alcohols and aldehydes suggests the reductive pathway may be less active in sand bluestem than big bluestem (von Wettstein-Knowles 2012). In contrast to β-diketones, alcohols and aldehydes, both products of the reductive pathway, were significantly reduced in sand bluestem when compared to big bluestem, although C26 alcohol was more abundant in sand bluestem. The difference in proportion of most alcohols and aldehydes may be due to a lower activity of the reductive pathway relative to to the β-diketone pathway in big bluestem compared to sand bluestem. The reductive pathway of plants is thought to produce aldehydes as precursors in primary alcohol synthesis (von Wettstein-Knowles 2012). If this were the case, possibly precursors to the reductive pathway are being used by the β-diketone pathway, thus explaining the negative correlation between primary alcohol synthesis and β-diketone and hydroxy β-diketone synthesis (Tulloch 1979).

In addition to the differences in diketones, triterpenes were detected only in sand bluestem suggesting an additional advantage of the ECW of sand bluestem. The triterpenes α-amyrin and β-amyrin, pentacyclic triterpenoids, are made from lupenyl cation precursors by the 2,3-Oxidosqualene cyclases α-Amyrin synthase and β-Amyrin synthase respectively (Haralampidis et al. 2002). This finding suggests that at least one

additional pathway differs between sand and big bluestem. The presence of triterpenes suggests a possible defensive advantage to the sand bluestem epicuticle. Triterpenes, including the α-amyrin and β-amyrin detected in sand bluestem, can inhibit insect feeding behavior and protect plant tissues from predation (Eigenhrode and Espelie 1995). Compositional analysis of sand and big bluestem suggests that the sand bluestem epicuticle may differ from the big bluestem epicuticle in ways other than those related to glaucousness. Specifically, increased triterpene accumulation and reduced wax synthesis via the reductive pathway as β-diketone and hydroxy β-diketone synthesis is undertaken in sand bluestem.

2.4.2 Wax morphology corroborates chemistry

Wax morphology, indicated by SEM, corroborates the hypothesis that β-diketones are absent in big bluestem leaf epicuticles and present in sand bluestem ECWs. β-diketone tubules were only characteristic of sand bluestem micromorphology, in this study. Furthermore, the sand bluestem micromorphology in this study is similar to the β-diketone tubule-rich surface of the Garden County accession observed by Ravenek et al. (unpublished data). Additionally, the measurements of β-diketone tubules presented here were within the dimensions described in Barthlott et al. (1998) for other species. Barthlott et al. defines β-diketone tubules as approximately 0.5 to 5.0 µm with a diameter of 0.2 to 0.3 µm with hollow centers and forked ends. They have been correlated with the presence of β-diketones in ECW composition in 113 *Eucalypt* species in experiments dating back forty years (Hallam 1964,1967; Hallam and Chambers 1970) and, more recently, in a large number of monocot and dicot species (Jeffree 2006). Meusel et al. 2000 confirmed that waxes rich in β-diketones form these

characteristic β-diketone tubules both *in vitro* and *in vivo*, indicating that this pattern of crystallization is an inherent quality of β-diketone-rich waxes as observed in sand bluestem.

Additionally, the absence of tubules on big bluestem specimens may also be informative. Small quantities of β-diketones can cause β-diketone tubules to monopolize the surface of the ECW (Jeffree 2006; Meusel et al. 2000; Koch et al. 2004; Koch et al. 2010); therefore, absence of recognizable β-diketone tubules on big bluestem plants in this study suggests the absence of the chemical in these big bluestem populations. In contrast to sand bluestem, leaf micromorphology of big bluestem suggests a primary alcohol-rich epicuticle, though this phenotype is not as predictable from ECW composition (Barthlott et al. 1998). Platelet-type epicuticular waxes were correlated with primary alcohol-rich waxes in over 200 *Eucalypt* species (Hallam 1964,1967; Hallam and Chambers 1970); however, morphology of platelets is highly variable (Barthlott et al. 1998). The structures identified as platelets on Saline and Webster populations had dimensions within the described range of primary alcohol-type platelets (Barthlott et al. 1998). Barthlott et al. (1998), described entire platelets ranging from 1-3 µm while non-entire platelets range from 1-10 µm in width. Taken together, the ECW chemistry and micromorphology of leaf waxes in these experiments supports the hypotheses that presence and absence of β-diketones results in the respective glaucous sand bluestem and non-glaucous big bluestem leaf phenotypes.

2.4.3 Wax chemistry and morphology putatively confer contrasting physiologies

In addition to differences in composition, sand bluestem's ECW is potentially less permeable, which has been previously correlated to adaptation to arid environments

(Kerstiens 1996; Schreiber et al. 1996). Specifically, the most useful correlation found in Schreiber et al. 1996 was that plants originating from drier microclimates (e.g. tropical epiphytes and mediterranean climates) had less permeable cuticles. This indicates that the lower permeability noted in sand bluestem cuticles may be an adaptation to arid soils. Sand bluestem's wax was thicker in this study; however, no correlation between increased thickness and decreased permeability has been found (Kerstiens 1996; Richards et al. 1986; Anfodillo et al. 2002) and the inverse has been observed (Riederer and Schreiber 2001). In effect, the results of prior experiments suggest that the estimated lower permeability of sand bluestem's cuticle may be an adaptation to xeric environments.

The reflective property of sand bluestem ECW may be indicative of adaptation to an arid climate by ameliorating the leaf thermal environment. Both big and sand bluestem have ECW on vegetative leaves but, in agreement with the hypothesis, only the ECW of sand bluestem detectably decreased total absorptance (Fig. 2.7). The combination of increased reflection and decreased transmission results in a decrease in leaf absorptance and is consistent with the estimate of a 10-15% decrease in absorptance for sand bluestem compared to big bluestem (Barnes 1986). The decrease in reflectance from glaucous ECW also agrees with previous experiments that test optical qualities of glaucous leaves (Holmes and Kellier, 2002; Reicosky and Hanover, 1978; Clark and Lister, 1975; Richards et al. 1986). The general increase of up to 20% reflectance across the measured spectra in sand bluestem agrees with an observed increase of 8-15% (Richards et al. 1983) that correlated with lower leaf temperatures and higher yield under drought. Non-glaucous big bluestem is known to be susceptible

to high temperatures (Nippert et al. 2009). As a whole, the averaged reflectance spectra suggest that sand bluestem has a more reflective cuticle that may provide benefits in terms of leaf thermal properties and represent an adaptation to an arid climate.

Reflectance is known to vary between subspecies, and these data support the supposition that glaucous ECW is potentially, in terms of reflectance, the most influential leaf feature in the measured wavelengths, and that sand bluestem ECW is increasingly reflective in the lower, harmful UV wavelengths (Holmes and Kellier, 2002; Reicosky and Hanover, 1978; Clark and Lister, 1975; Richards et al. 1986). For the PLS model predicting two categorical variables, big and sand bluestem, the lack of overlap of predicted subspecies values in Fig. 2.8, top row, for models using intact leaves with ECW and the effect of ECW (or the difference in spectra after ECW removal) differ greatly such that the effect of the ECWs on reflectance of leaves may be a useful tool for distinguishing subspecies. The lack of ability of the model to distinguish spectra from either subspecies after ECW removal was surprising because cell size, pigment concentration, trichomes, and thickness can also alter leaf reflectance (Knapp and Carter 1998). This suggests that other leaf features do not distinguish optical properties of the subspecies to as great a degree as ECW. The poor performance of the model in predicting subspecies from leaves with wax removed also indicates that the ECW may be responsible for the ability of the trained PLS models to predict subspecies. Additionally, the results of variable importance plot for wavelengths below 700 nm, coupled with the tendency for averaged reflectance to increase as wavelength decreases in sand bluestem, suggest that, like the glaucous waxes of many species (Holmes and Kellier, 2002; Reicosky and Hanover, 1978; Clark and Lister, 1975;

Richards et al. 1986), the glaucous ECW of sand bluestem increases reflectance especially in the lower visible and UV wavelengths. Taken together, the ability of increased reflectance of ECW in sand bluestem relative to big bluestem appears to be the primary distinction between these subspecies in terms of leaf optical qualities. Ultimately, the increase in reflectance in lower wavelengths may indicate a mechanism for adaptation to xeric climates by reflecting light harmful UV wavelengths (Holmes and Kellier, 2002; Reicosky and Hanover, 1978; Clark and Lister, 1975; Richards et al. 1986).

The results of the hybrid index indicate that, in agreement with the hypothesis, populations were distinctively composed of either sand and big bluestem individuals. Furthermore, the index values were reliable in relation to previous applications of the index (Barnes 1984) and broad subspecies distribution (Fig. 2.1). The finding of a greater range of hybrid index scores within sand bluestem populations agrees with a similar study using awn, cilia, ligule, and a fourth characteristic to calculate a hybrid index (Barnes 1984). In this study, no plants grown in cohort II had individuals with hybrid index scores that overlapped those of the reciprocal subspecies. In the Barnes study, within population variability was also found to increase as degree of hybridization increased. Additionally in this study, the observed variability in hybrid index score and population confidence interval (CI) is supported by published subspecies distributions (Potvin and Harrison 1984; Killion and Rothenberger 2011). Taken together, the agreement with prior applications of similar hybrid indexes and the agreement with distribution maps indicate that the index may be a useful tool for estimating introgression, and individuals in this study are well defined with regard to subspecies.

2.5 Summary

The objective of these experiments was to contrast the non-glaucous and glaucous phenotypes in wild big and sand bluestem populations and to assess the physiological ramifications of the associated ECWs. These results suggest the glaucous phenotype results from accumulation of β-diketones in the waxes of sand bluestem leaves that were absent in the vegetative leaves of big bluestem. Prior research suggests that the glaucous phenotype inherently alters reflectance properties, but has low correlation with cuticular conductance or thickness (Kerstiens 1996; Richards et al. 1986; Anfodillo et al. 2002). However, in this study, sand bluestem specimens had lower cuticular conductance and thicker wax, indicating that the sand bluestem cuticle may be adapted to drought in multiple ways. ECWs are present on both subspecies. However, the diketones in sand bluestem's ECWs are correlated with an abundance of β-diketone tubules on the leaf tissues that increase reflectance and decrease absorptance. PLS models were able to separate the subspecies from reflectance spectra suggesting that ECW may be the most influential leaf feature that distinguishes sand from big bluestem in terms of optical properties. This work highlights the relationship between morphology, chemistry and physiology of the epicuticle in native sand and big bluestem populations and the potential adaptive significance of ECW for the subspecies in their native environments.

Figures and Tables

Table 2.1 Collection sites for big and sand bluestem
The location of Barta Brothers' Ranch was taken from (http://snr.unl.edu/aboutus/where/fieldsites/fieldsites.asp).

Subspecies	Collection Site	County	Lat. N	Long. W
Big bluestem	Webster Reservoir State Park	Rooks, KS	39° 24'	99° 32'
Big bluestem	Saline Experimental Range	Ellis, KS	39° 02'	99° 14'
Big bluestem	Relic Prairie	Ellis, KS	38° 51'	99° 22'
Big bluestem	Cedar Bluffs State Park	Trego, KS	38° 45'	99° 46'
Sand bluestem	Valentine National Wildlife Refuge	Cherry, NE	42°59'	100°55'
Sand bluestem	Barta Brothers' Ranch	Rock & Brown, NE	42°14'	99°39'
Sand bluestem	Gudmundsen Sandhills Laboratory	Grant, NE	42° 05'	101° 26'
Sand bluestem	Cimmaron National Grassland	Morton, KS	37° 06'	102° 00'
Sand bluestem	Arapahoe Prairie (Nature Conservancy)	Arthur, NE	41°27'	101°53'

Table 2.2 R^2 and RMSD for PLS predictions of subspecies or hybrid index score from wax reflectance spectra between 400-800nm

A PLS model was trained to predict either subspecies, designated as -1 for big bluestem and 1 for sand bluestem, (top row) or hybrid index score (bottom row) from relative reflectance values for 949 wavelengths from 400-800 nm.

	Reflectance with wax intact			Reflectance after wax removal			Difference in reflectance		
	RMSD	R^2 of Y	R^2 of model	RMSD	R^2 of Y	R^2 of model	RMSD	R^2 of Y	R^2 of model
Subspecies	0.36	87.10	97.70	0.87	24.81	79.53	0.34	87.10	97.70
Hybrid index score	2.14	90.96	98.29	6.15	25.59	79.27	1.54	95.32	95.63

Figure 2.1 Collection sites (black circles) on a map showing the distribution of big and sand bluestem across Nebraska and Kansas (adapted from USDA plants database)
The shaded counties are populated with sand bluestem (left) or big bluestem (right). The nine source populations for seeds are indicated with circles.

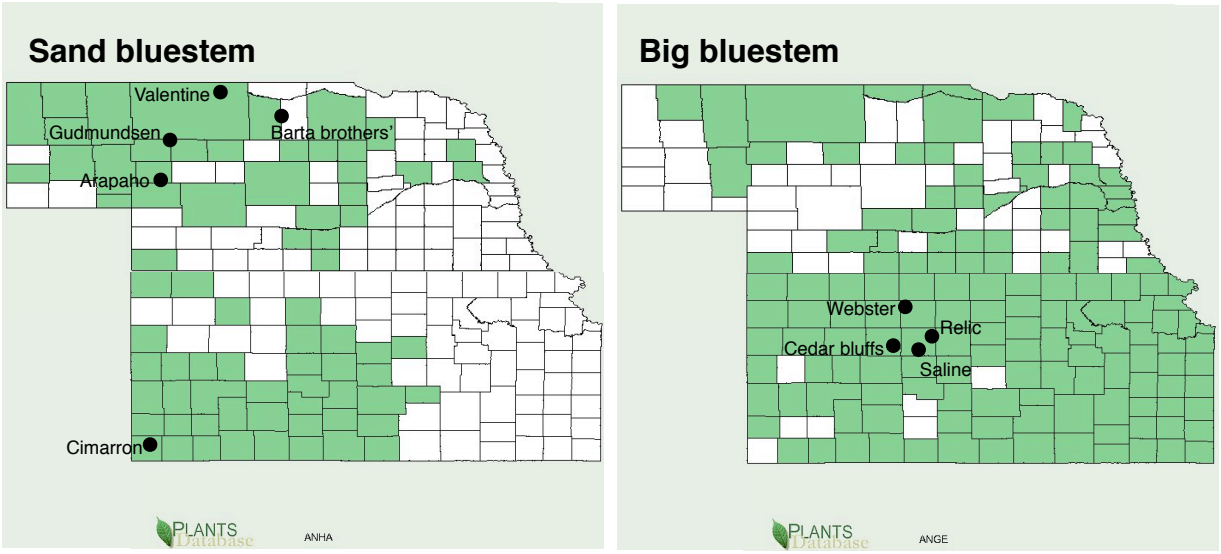


Figure 2.2 Back-transformed least squares means with 95% CI for chemical classes as percent of total composition in sand and big bluestem
The percentage of total ECW compounds of chemical classes is graphed for big bluestem (green) and sand bluestem (white). Bars indicate back-transformed LSM estimates for both subspecies of bluestem (classes found only in sand bluestem are indicated with 'SB'). Differences were tested for chemical classes found in both subspecies. Bonferonni adjusted P-values for one-tailed tests are indicated (** $P < .01$; *** $P < .001$). Eight plants from Saline, Webster, Valentine, and Barta Brothers' populations were subsampled.

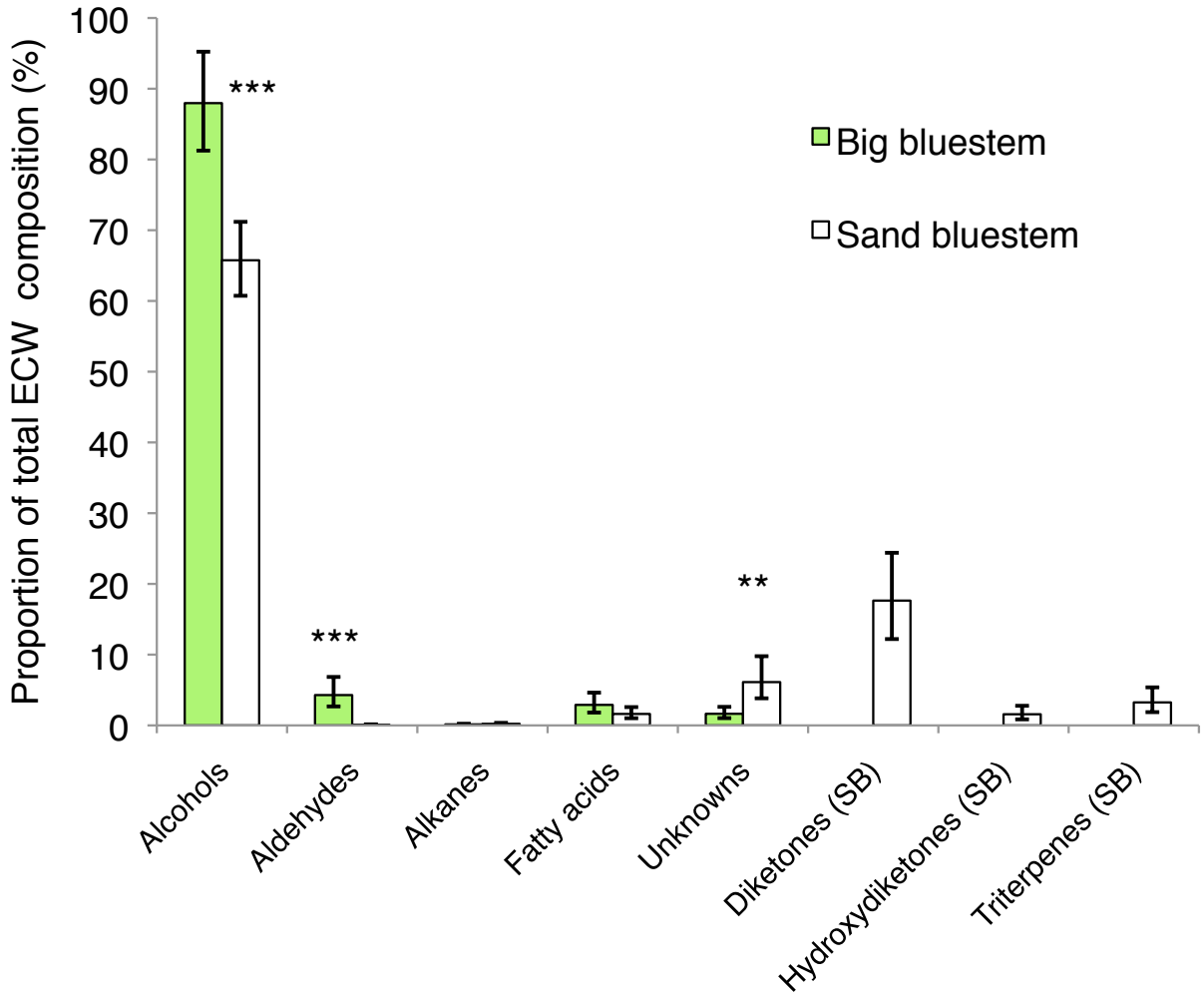


Figure 2.3 Least squares means estimates for sand and big bluestem with 95%CI for amount of epicuticular wax (ECW) in mg cm^{-2}
Wax weight in mg and divided by 2 x adaxial surface area. Three plants of each population (Valentine, Barta brothers', Webster, and Saline) were subsampled. The covariance parameter for population converged to 0. P-value is indicated (** P < .01).

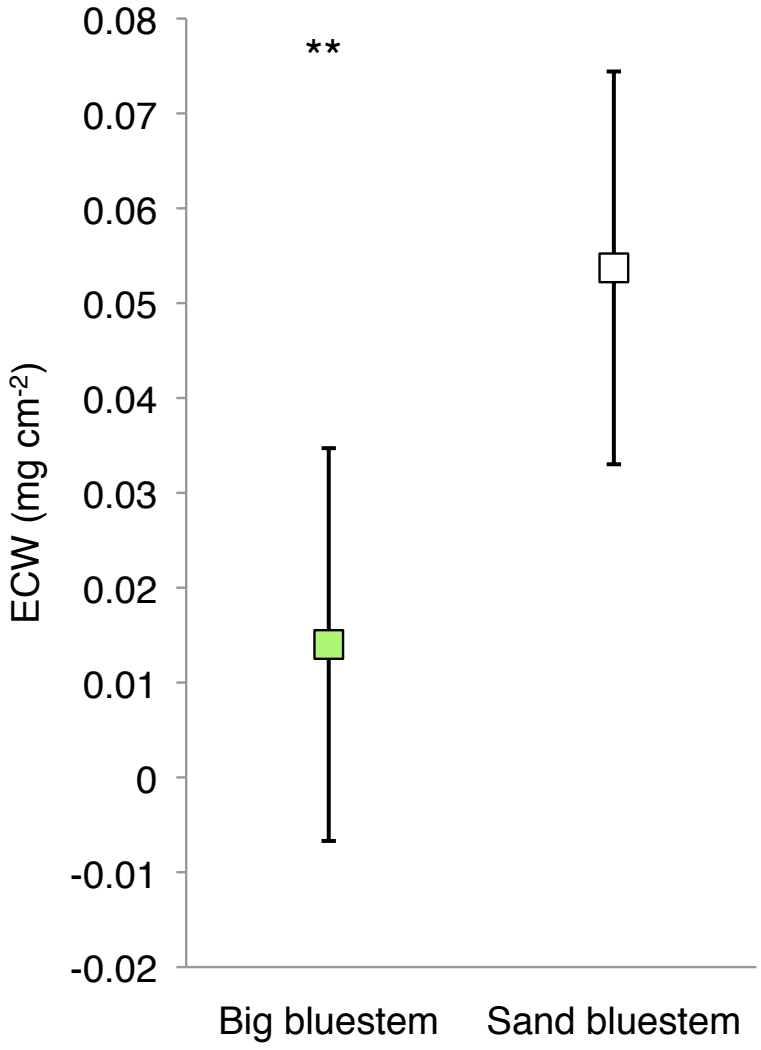


Figure 2.4 Back-transformed least squares means with 95% CI for epicuticular wax metabolites as a percent of total composition for big and sand bluestem
The percentage of total ECW compounds of different chain lengths is graphed for big bluestem (green) and sand bluestem (white). Bars indicate back-transformed LSM estimates for both subspecies of bluestem (chemicals found only in sand bluestem are indicated with the letters 'SB'). Bonferonni adjusted P-values for one-tailed tests are indicated above chemicals detected in both big and sand bluestem (* $P < .05$; ** $P < .01$; *** $P < .001$). Eight plants from Saline, Webster, Valentine, and Barta Brothers' populations were subsampled.

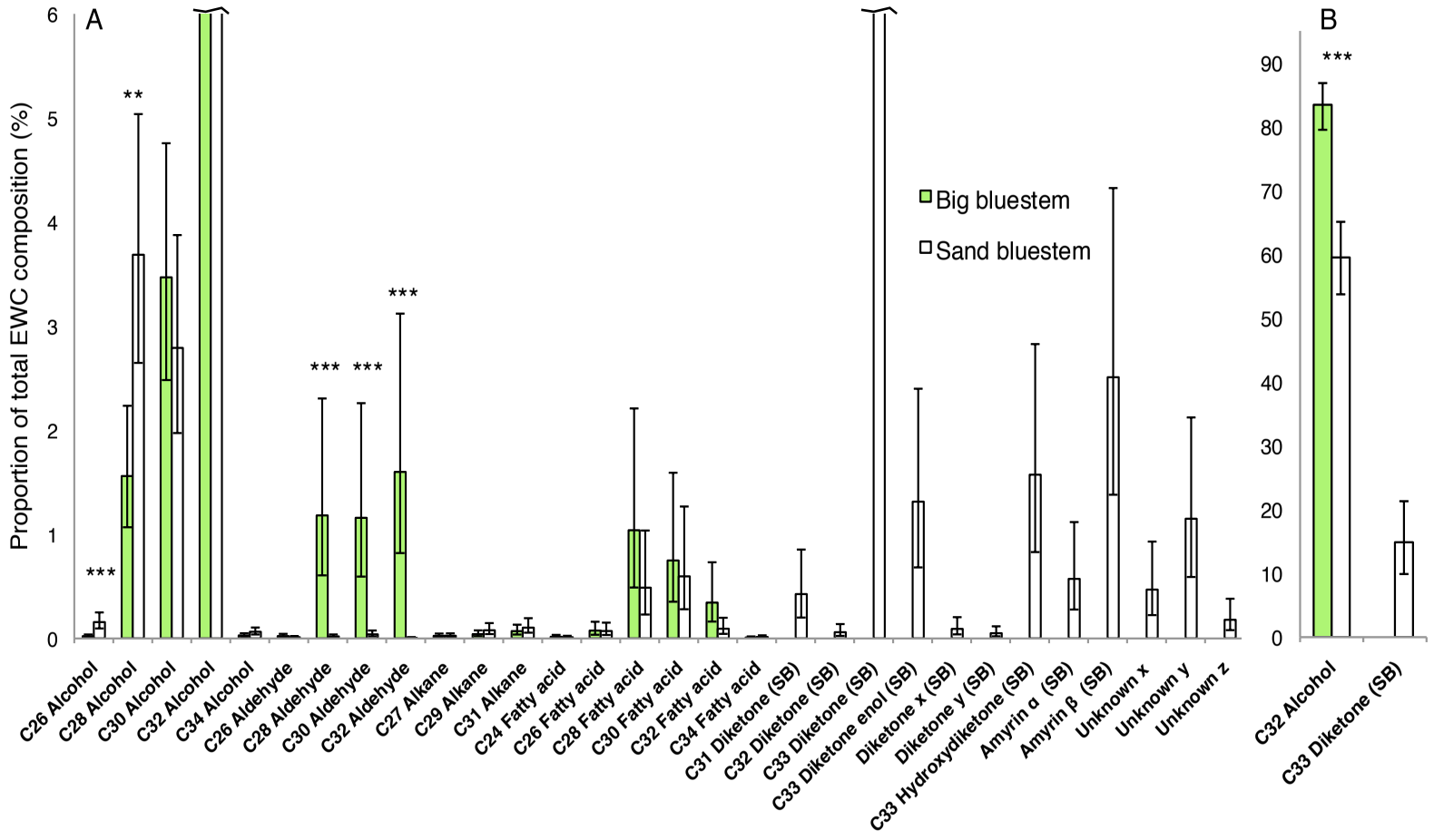

A
B
Proportion of total EWC composition (%)
Big bluestem
Sand bluestem
***
**
***
***
***
***
C26 Alcohol
C28 Alcohol
C30 Alcohol
C32 Alcohol
C34 Alcohol
C26 Aldehyde
C28 Aldehyde
C30 Aldehyde
C32 Aldehyde
C27 Alkane
C29 Alkane
C31 Alkane
C24 Fatty acid
C26 Fatty acid
C28 Fatty acid
C30 Fatty acid
C32 Fatty acid
C34 Fatty acid
C31 Diketone (SB)
C32 Diketone (SB)
C33 Diketone (SB)
C33 Diketone enol (SB)
Diketone x (SB)
Diketone y (SB)
C33 Hydroxydiketone (SB)
Amyrin α (SB)
Amyrin β (SB)
Unknown x
Unknown y
Unknown z
C32 Alcohol
C33 Diketone (SB)

Figure 2.5 Scanning electron microscope images of leaf epicuticles for sand and big bluestem

SEM images of glaucous, sand bluestem leaves, indicated with (SB), from an individual of the Valentine and Barta Brothers' populations, and big bluestem leaves, indicated with (BB), from an individual of the Webster and Saline populations (non-glaucous leaves). Images are of the middle portions of the adaxial leaf surfaces. Platelets and short rodlets were observed on Saline and Webster big bluestem specimens. Straight β-diketone tubules were observed on Valentine specimens; while, β-diketone tubules and coiled rodlets were observed on Barta Brothers' sand bluestem specimens.

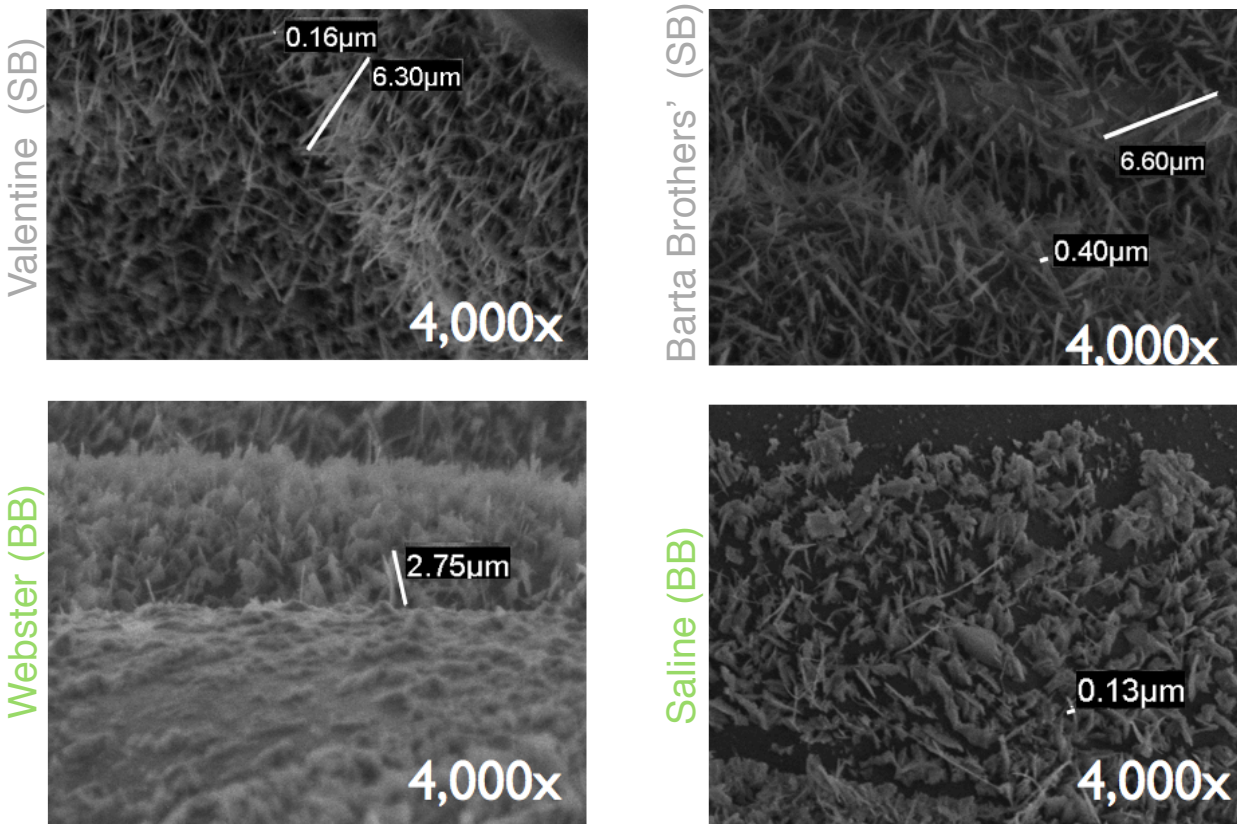


Figure 2.6 Least squares means estimates for sand and big bluestem with 95%CI for minimum conductance (G_{min}) in ms $^{-1}$ 10^{-5}

G_{min} was calculated from measurements taken after stomatal closure was induced. The estimate of G_{min} for each subspecies of bluestem was significant (P=0.0472). During the interval, from 30-43.5 min stomatal closure was environmentally induced, there was no significant effect or interaction involving time. The inset is an example of several complete logs of G_{min}. In the inset, 0-43.5 min is on the X-axis and G_{min} his on the Y-axis and the final 15 min interval is inscribed in red. Two plants of Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers' populations were subsampled. The covariance parameter for population converged to 0.

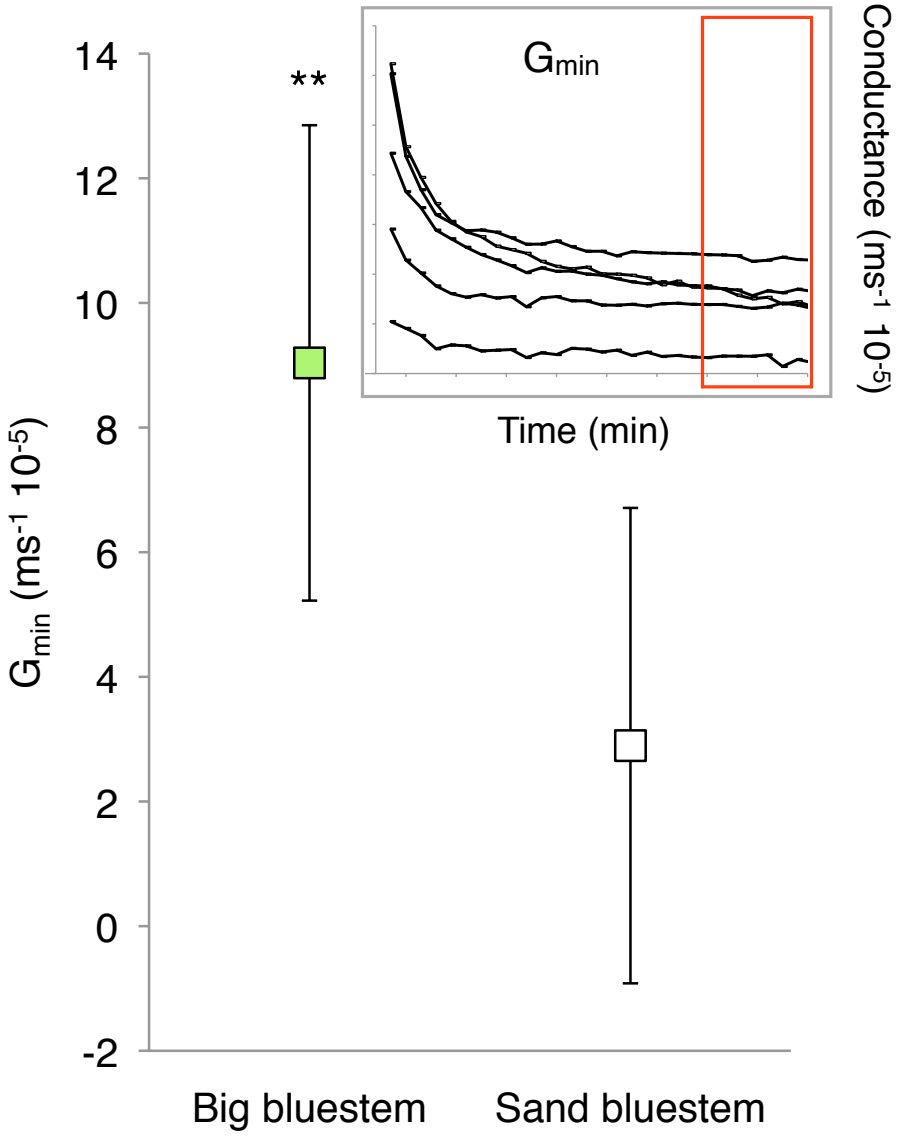


Figure 2.7 Averaged reflectance and transmittance from 350nm to 850nm
Adaxial reflectance (top) and transmittance (bottom) at mid-blade was measured using an integrating sphere with an attached spectroradiometer. Measurements were taken on attached leaves (solid lines), the same leaves where then immersed in chloroform for 30s and reflectance was again measured (dashed lines). Fourteen plants in total were measured. Values were averaged for plants from the Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers' populations.

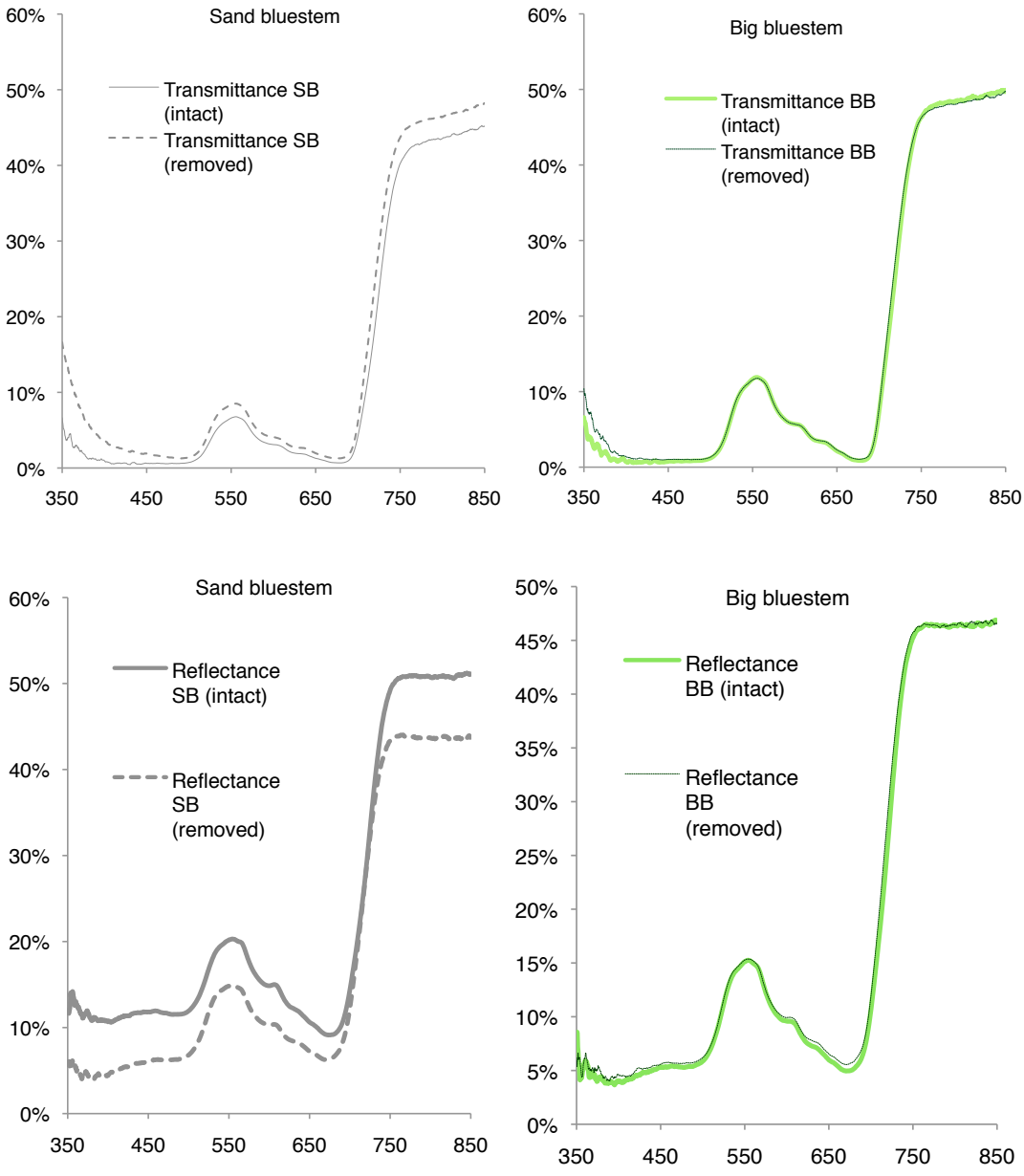


Figure 2.8 Predicted vs. actual subspecies and hybrid index scores from PLS models using either reflectance of leaves with intact epicuticular wax (ECW), removed ECW or the change in reflectance after ECW removal as independent variables

A PLS model was trained to predict either subspecies, designated as -1 for big bluestem and 1 for sand bluestem, (top row; from 14 plants) or hybrid index score (bottom row; from 12 plants) from relative reflectance values for 949 wavelengths from 400-800 nm. Predicted values are plotted on the X-axis versus actual values on the Y-axis. Plants classified as big bluestem are indicated with green markers and plants classified as sand bluestem are indicated with white markers. X-values were either the reflectance of leaves with intact ECW (first column), reflectance of leaves after removal of ECW (second column), or the difference in reflectance after ECW removal (third column).

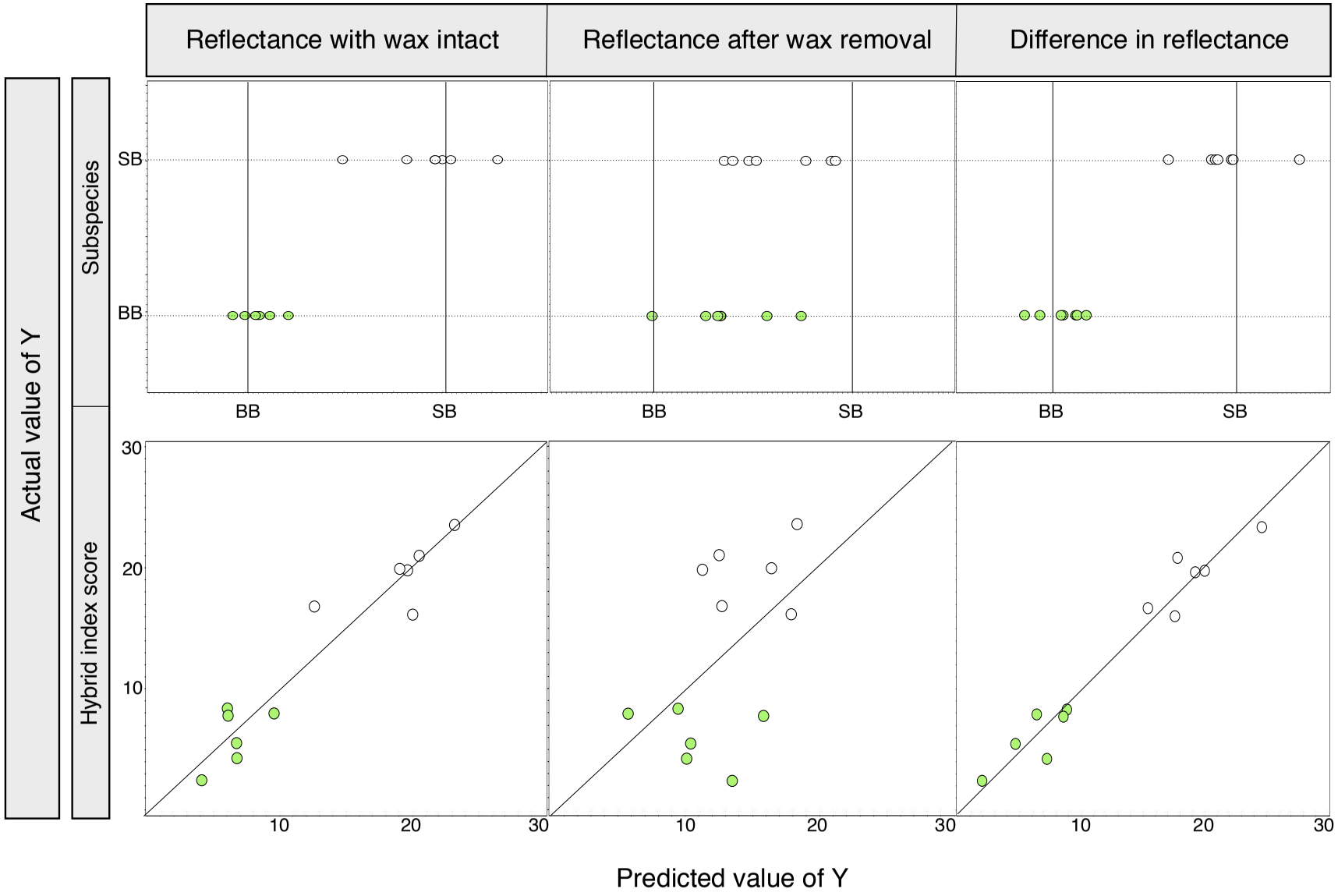
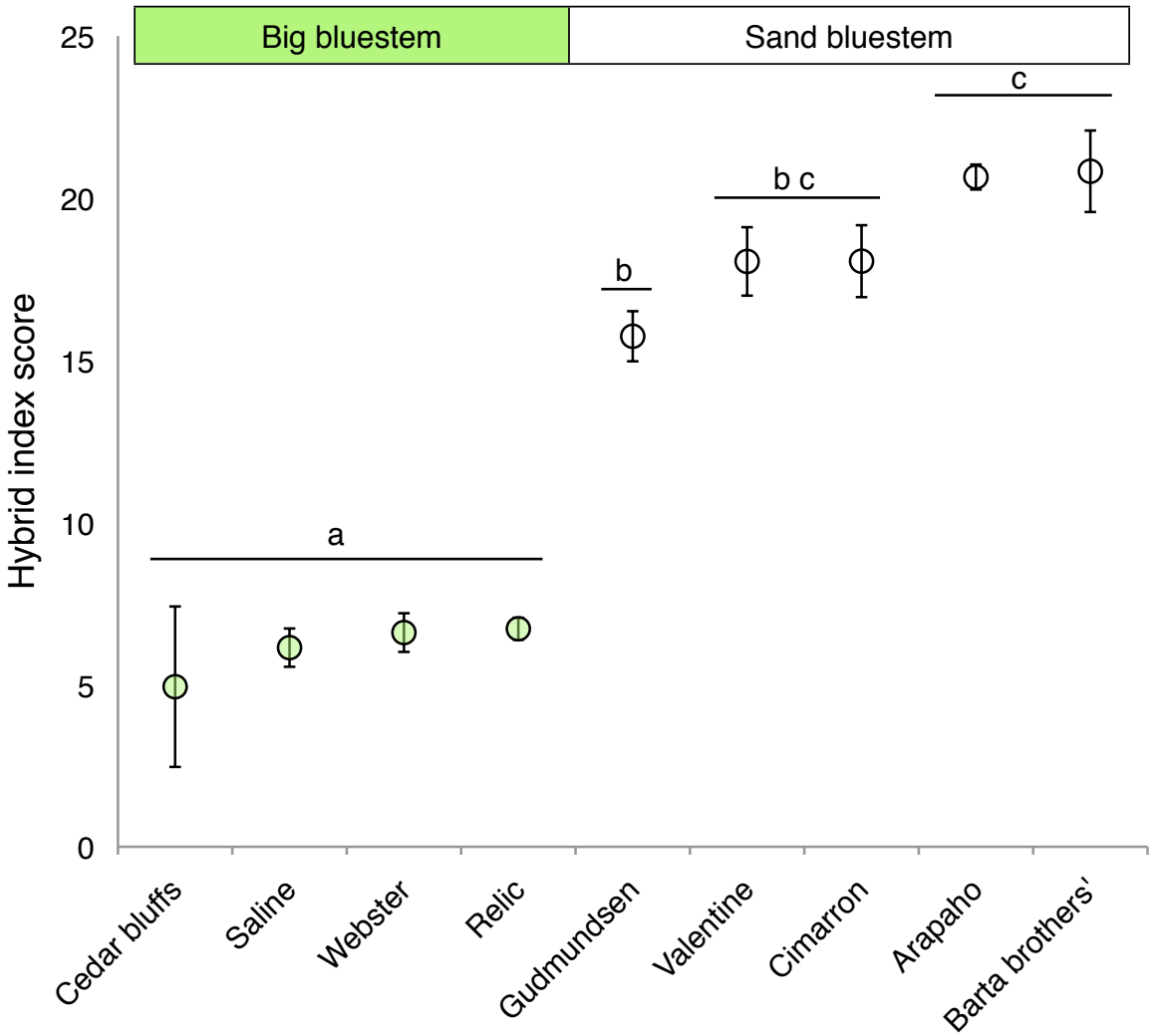

Reflectance with wax intact
Reflectance after wax removal
Difference in reflectance
Actual value of Y
Subspecies
SB
BB
BB
SB
BB
SB
BB
SB
Hybrid index score
30
20
10
10
20
30
10
20
30
10
20
30
Predicted value of Y

Figure 2.9 Least squares means with 95% CI of hybrid index score for nine populations of big and sand bluestem with letters to indicate the results of pairwise comparisons

Hybrid index scores are graphed by population (n=53). Horizontal bars indicate the results of pairwise comparisons of Saline, Webster, Valentine, Gudmundsen, Arapaho, Cimmaron, Relic, Cedar Bluffs, and Barta Brothers' populations. Three floral characteristics (awn length, cilia on the inflorescence length, and ligule length at mid-culm in mm) were scored relative to big and sand bluestem extreme values. A score near 0 indicates big bluestem morphology and score nearer to 30 indicates sand bluestem morphology. All big bluestem individuals scored below 10. Sand bluestem scores ranged from 13 to 24.



Chapter 3 - Conclusions and future directions

In Chapter 1, the epicuticle was introduced as a highly conserved structure that is the first point of contact between a plant's internal cellular environment and the dry, inhospitable exterior environment. Glaucousness was defined as a property of reflectance rather than abundance of wax; and, the molecular composition of the sand bluestem's glaucous cuticle was introduced. It has already been established that sand bluestem cultivars possess β-diketone and β-diketone tubules (Tulloch and Hoffman 1979; Ravenek et al. unpublished data). However the nature of big bluestem's cuticle was largely unknown. The value of the cuticle as a tool to increase yield was established in Chapter 1; and it was pointed out that the mechanism by which glaucousness can improve yield remains in question after conflicting studies report improved performance with or without improved values of WUE. The CER-CQU enzyme complex was described and the enzymatic steps of synthesis of β-diketones in barley were explained. Putative homologs for *cer-cqu* in monocots were described. Taken together, Chapter 1 addresses the importance of glaucousness to plant function and discusses the lack of a complete understanding of its evolution in grass lineages including big and sand bluestem.

In Chapter 2, a set of experiments were described that address questions including the nature of the glaucous and non-glaucous phenotype in native sand and big bluestem and the physiological ramifications of the two phenotypes. It was concluded that the presence and absence of β-diketones in sand and big bluestem, respectively, causes the divergent phenotypes, consistent with Tulloch and Hoffman (1979) and

Ravenek et al. unpublished data. However, the finding that sand bluestem has proportionally less β-diketones in leaf ECW could imply inherent differences between cultivars and wild populations, the result of different plant age (Tulloch 1973), or environmental conditions. However, the Ravenek et al. unpublished study was conducted in the same greenhouse environment. Sand bluestem ECW content was found to be greater, in agreement with the hypothesis that sand bluestem would have more ECW, but again not by the margin noted in the Garden country accession and Kaw cultivar by Ravenek et al. unpublished. Lower G_{min} was observed in sand bluestem consistent with adaptation to water limitation and in agreement with Richards et. al. (1986); Kerstiens (1996); and Schreiber et. al (1996). It was concluded from the results of the averaged spectra that only sand bluestem ECW changes optical properties. In agreement with the hypothesis that sand bluestem ECW would have higher leaf reflectance. The inability of the PLS model to distinguish spectra of either subspecies when ECW was removed was presented in support of prediction.

Additionally, the PLS model relating variance in hybrid index score to variance in glaucousness did support prior hypotheses that glaucousness can be used to establish hybrid index score (Peters and Newell 1961). The ability to distinguish subspecies and to distinguish subspecies based on the change in reflectance after wax removal, suggests that the ECW is largely the cause of the glaucous phenotype. Evidence suggests that no one population had individuals with hybrid index values overlapping the reciprocal subspecies. Taken together, these findings generally support the hypotheses and indicate multiple potential adaptations for the ECW of sand bluestem in comparison to big bluestem.

Additionally, a high proportion of the variability in hybrid index score is explained by a PLS model trained to predict index score with the effect of ECW on reflectance as predictor variables (Fig. 2.8 bottom right graph), indicating that effect of ECW on reflectance my be a useful tool for estimation of introgression. Hybrid index scored is currently the basis of the taxonomical distinction between big and sand bluestem. The hybrid index score is a time consuming measurement that can only be made at the final stage of growth in a season. Two of the measurements needed to calculate this score are floral characteristics. The reflectance of leaf tissue has been reported to be intermediate in natural and experimentally induced hybrids of sand and big bluestem (Barnes 1986; Peters and Newell 1961). In the PLS model designed to predict hybrid index value from the effect of ECW on vegetative leaf reflectance, 95% of the variability in hybrid index score was explained by a model using change in leaf reflectance as predictor variables. The ability of this model to explain variability in hybrid index score suggests that this may be a tool that could be developed to estimate introgression in field trials of these agronomically and ecologically important subspecies (Epstein et al. 1998).

In addition to the importance of studying the epicuticular phenotypes as phenotypes intimately tied to wax chemistry, this study creates the foundation for a comparison of two big and sand bluestem individuals from cohort II. Two individuals from this cohort were selected, one with the highest and one with the lowest index score in the Arapahoe and Saline population. RNA was extracted from developing leaf tissue and sequenced on a 454 Life Science/Roche, Sequencer FLX System, by Alina Akhunova at the KSU Integrated Genomics Facility. The catalog of epicuticular wax

synthesis products described in Chapter 2 will be used to search the sand bluestem transcriptome for wax synthesis genes that are unique or expressed at higher levels relative to big bluestem in sand bluestem. The hybrid index was used for this project in order to select a representative from each subspecies that is likely to be from an isolated population. Ultimately, these projects may help fill in gaps in research into ECW occurrence and function for wild plants that may be useful for research into both native and cultivated grasses.

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Appendix A - Statistic model

Equation A.1:

$$y_{ijklm} = \mu + c_l + s_i + p_{j(i)} + q_{k(ij)} + cp_{l(ij)} + sc_{il} + e_{ijklm}$$

where:

$p_{j(i)} \sim iid\ N(0,\sigma_p^2)$

$q_{k(ij)} \sim iid\ N(0,\sigma_q^2)$

$e_{ijkl} \sim iid\ N(0,\sigma e^2)$

μ = intercept

c_l = the differential effect of the *lth* chain length

s_i = differential effect of the *ith* subspecies

sc_{il} = the differential effect of the combination of the lth chain length and ith subspecies

$p_{j(i)}$ = the random effect of the *jth* population nested within the *ith* subspecies

$cp_{l(ij)}$ = the random effect of the *lth* chain length crossed with the *jth* population nested in the *ith* subspecies

$q_{k(ij)}$ = the random effect of the *kth plant* corresponding to the *jth* population nested within the *ith* subspecies

Appendix B - Supporting statistics for hypotheses tests

Table B.1 The results of the test of simple effects of subspecies least squares means estimates for proportion of ECW represented by each chemical class present in both subspecies

Simple Effect Comparisons of subspecies*chemical class Least Squares Means By chemical class with Bonferroni adjusted P-value						
CHEMICAL CLASS	Estimate	Standard Error	DF	t value	Pr > ltl	Adj P
ALCOHOL	0.291	0.049	8	5.980	0.000	0.001
ALDEHYDE	3.821	0.288	8	13.280	<.0001	0.000
ALKANE	-0.429	0.288	8	-1.490	0.174	0.616
FATTY ACID	0.583	0.288	8	2.030	0.077	0.331
UNKNOWN	-1.317	0.288	8	-4.580	0.002	0.009

Table B.2 The results of the test of simple effects of subspecies least squares means estimates for proportion of ECW represented by each chemical at all detected chain lengths

Simple Effect Comparisons of subspecies*CHAIN_LENGTH Least Squares Means By CHAIN_LENGTH with Bonferroni adjusted P-value						
SUBSPECIES	Estimate	Standard Error	DF	t value	Pr > ltl	Adj P
C26 Aldehydes	0.184	0.113	6	1.630	0.153	0.486
C28 Aldehydes	1.306	0.113	6	11.590	<.0001	<.0001
C30 Aldehydes	1.083	0.113	6	9.610	<.0001	<.0001
C32 Aldehydes	1.683	0.113	6	14.930	<.0001	<.0001
C24 FATTY ACIDS	0.290	0.467	9	0.620	0.551	0.992
C26 FATTY ACIDS	0.056	0.467	9	0.120	0.908	1.000
C28 FATTY ACIDS	0.757	0.467	9	1.620	0.142	0.600
C30 FATTY ACIDS	0.228	0.467	9	0.490	0.638	0.998
C32 FATTY ACIDS	1.303	0.467	9	2.790	0.022	0.126
C34 FATTY ACIDS	-0.435	0.467	9	-0.930	0.377	0.941
C26 ALCOHOL	-0.557	0.090	8	-6.180	0.000	0.001
C28 ALCOHOL	-0.366	0.090	8	-4.060	0.004	0.018
C30 ALCOHOL	0.096	0.090	8	1.070	0.316	0.850
C32 ALCOHOL	0.731	0.090	8	8.100	<.0001	<.0001
C34 ALCOHOL	-0.215	0.090	8	-2.380	0.044	0.087

Table B.3 The results of the test of differences of least squares means estimates for G_{min} for subspecies

Differences of subspecies Least Squares Means Adjustment for Multiple Comparisons: Tukey-Kramer					
Estimate	Standard Error	DF	t value	Pr > ltl	Adj P
6.141	2.555	7	2.400	0.047	0.047

Table B.4 The results of the pairwise comparisons of least squares means estimates for hybrid index score for populations

Differences of Population Least Squares Means for hybrid index score Adjustment for Multiple Comparisons: Tukey-Kramer							
POPULATION	POPULATION	Estimate	Standard Error	DF	t value	Pr > ltl	Adj P
ARAPAHO	BARTA BROTHERS'	-0.178	1.314	44	-0.140	0.893	1.000
ARAPAHO	CEDAR BLUFFS	15.714	2.503	44	6.280	<.0001	<.0001
ARAPAHO	CIMARRON	2.594	1.174	44	2.210	0.032	0.418
ARAPAHO	GUDMUNDSEN	4.909	0.864	44	5.690	<.0001	<.0001
ARAPAHO	RELIC	13.928	0.513	44	27.140	<.0001	<.0001
ARAPAHO	SALINE	14.5103	0.7052	44	20.58	<.0001	<.0001
ARAPAHO	VALENTINE	2.6020	1.1234	44	2.32	0.0253	0.3550
ARAPAHO	WEBSTER	14.0452	0.7099	44	19.79	<.0001	<.0001
BARTA BROTHERS'	CEDAR BLUFFS	15.8923	2.7740	44	5.73	<.0001	<.0001
BARTA BROTHERS'	CIMARRON	2.7720	1.6754	44	1.65	0.1051	0.7696
BARTA BROTHERS'	GUDMUNDSEN	5.087	1.475	44	3.450	0.001	0.031
BARTA BROTHERS'	RELIC	14.106	1.301	44	10.840	<.0001	<.0001
BARTA BROTHERS'	SALINE	14.688	1.388	44	10.580	<.0001	<.0001
BARTA BROTHERS'	VALENTINE	2.780	1.640	44	1.690	0.097	0.746

Differences of Population Least Squares Means for hybrid index score Adjustment for Multiple Comparisons: Tukey-Kramer							
POPULATION	POPULATION	Estimate	Standard Error	DF	t value	Pr > ltl	Adj P
BARTA BROTHERS'	WEBSTER	14.223	1.390	44	10.230	<.0001	<.0001
CEDAR BLUFFS	CIMARRON	-13.120	2.711	44	-4.840	<.0001	0.001
CEDAR BLUFFS	GUDMUNDSEN	-10.805	2.591	44	-4.170	0.000	0.004
CEDAR BLUFFS	RELIC	-1.786	2.497	44	-0.720	0.478	0.998
CEDAR BLUFFS	SALINE	-1.204	2.543	44	-0.470	0.638	1.000
CEDAR BLUFFS	VALENTINE	-13.112	2.689	44	-4.880	<.0001	0.001
CEDAR BLUFFS	WEBSTER	-1.669	2.544	44	-0.660	0.515	0.999
CIMARRON	GUDMUNDSEN	2.315	1.352	44	1.710	0.094	0.735
CIMARRON	RELIC	11.334	1.160	44	9.770	<.0001	<.0001
CIMARRON	SALINE	11.916	1.256	44	9.480	<.0001	<.0001
CIMARRON	VALENTINE	0.008	1.531	44	0.010	0.996	1.000
CIMARRON	WEBSTER	11.451	1.259	44	9.100	<.0001	<.0001
GUDMUNDSEN	RELIC	9.019	0.844	44	10.690	<.0001	<.0001
GUDMUNDSEN	SALINE	9.601	0.973	44	9.870	<.0001	<.0001
GUDMUNDSEN	VALENTINE	-2.307	1.308	44	-1.760	0.085	0.704
GUDMUNDSEN	WEBSTER	9.136	0.976	44	9.360	<.0001	<.0001
RELIC	SALINE	0.582	0.681	44	0.860	0.397	0.994
RELIC	VALENTINE	-11.326	1.108	44	-10.22	<.0001	<.0001
RELIC	WEBSTER	0.117	0.686	44	0.170	0.865	1.000
SALINE	VALENTINE	-11.908	1.209	44	-9.850	<.0001	<.0001
SALINE	WEBSTER	-0.465	0.839	44	-0.550	0.582	1.000
VALENTINE	WEBSTER	11.443	1.212	44	9.440	<.0001	<.0001