THE EFFECTS OF INHIBITING DHURRIN BIOSYNTHESIS IN SORGHUM

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Agronomy

West Lafayette, Indiana

August 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL

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I dedicate this dissertation to my family, who has supported me throughout my academic journey.

ACKNOWLEDGMENTS

I would like to acknowledge everyone that has supported me throughout this process. I am going to start with my personal support. I would like to thank Erin Marty, for keeping me sane on my worst days. For both my parents, Greg and Patty Gruss who has supported me throughout my entire life. My three brothers Donny, Greggory, and Elliott Gruss, without them I would not have my competitive personality and I knew if I could handle growing up with them, I could do this. Lastly, for all my friends that I have met here at Purdue that I was able to bounce ideas off of and be able to help each other through some of the tough times.

Now I would like to put out a special thank you to everyone who has helped me throughout my studies. Dr. Mitchell R. Tuinstra for taking me on as a student, making adaptations to help with anything I needed, guidance and support throughout this journey in completing my Ph.D. Dr. Keith D. Johnson introducing me to efficacy of forages and their uses. Andrew Linvill and Eugene Glover being ready to help whenever I needed even when it was very last minute. All the Tuinstra Tigers that helped me in the field, greenhouse, CEPF, setting up experiments, collecting data, and even killing Japanese beetles. My committee members in giving the advice and knowledge to complete my projects, and to look at questions in a different way. Dave Isenhour for the invaluable advice and encouragement to obtain my Ph.D.

Lastly, I would like to thank the Agronomy Department at Purdue. I have received so much support throughout my time here. Whether it was a getting candy from Connie Foster to make it through the end of the day, or to be able to talk through ideas with a fellow graduate students. It was a very supportive academic environment for me. Thank You.

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LIST OF ABBREVIATIONS

µl =	microliters
ACRE =	Agronomy Center for Research and Education at Purdue University
ADF =	acid detergent fiber
ASCRE =	Animal Science Center for Research and Education at Purdue University
bmr =	brown midrib
CC =	Chlorophyll Content
CCI =	Chlorophyll Content Index
CG =	Cyanogenic Glucosides
cm =	centimeters
CP =	crude protein
cyp79a1 =	C493Y mutation in the CYP79A1 enzyme
Dhr2 =	Dhurrinase2
dhr2-1 =	mutation in the Dhr2 enzyme
dm =	dry matter
dwg =	dry weight grams
dwg =	dry weight grams
EMS =	ethyl methosulfonate
FA =	Feigl Anger
FAW =	Fall Armyworm
fwg =	fresh weight grams
g =	grams
h =	hours
ha=	hectares
HCN =	Hydrogen Cyanide
HCNp =	Hydrogen Cyanide potential
IVDMD =	In vitro Matte Digestibility Procedure
KARE =	Kearny Agricultural Research and Extension Center at University of California
kg =	kilograms

kg ha^{-1} =	kilogram per hectare
LN =	liquid nitrogen
m =	meters
mg =	milligrams
N=	nitrogen
NIBC=	near-isogenic backcross
NIH=	near-isogenic hybrid
NIL=	near-isogenic line
NDF =	neutral detergent fiber
NDVI =	Normalized Difference Vegetation Index
nm =	nanometers
NUE=	nitrogen use efficiency
P=	Phosphorous
QTL =	quantitative trait loci
RCBD=	Randomized complete block design
RDP =	ruminant digestible protein
SbEMS =	*Sorghum bicolor* ethyl methosulfonate
SP =	soluble protein
TDN =	total digestible nutrients
UAV =	Unmanned Aerial Vehicle
UHPLC =	Ultra- High Performance Liquid Chromatography
WT =	wild-type

NOMENCLATURE

$CaCl_2$ =	Calcium Chloride
CO_2 =	Carbon Dioxide
H_2O =	Water
KH_2PO_4 =	Potassium Dihydrogen Phosphate
MeOH =	Methanol
$MgSO_4$- =	Magnesium Sulfate
Na_2CO_3 =	Sodium Carbonate
NaCl =	Sodium Chloride
NO_3 =	Nitrate

ABSTRACT

Dhurrin is a cyanogenic glucoside (CG), an important compound that can interplay with primary and secondary metabolism in sorghum. Dhurrin metabolism contributes to insect resistance, growth, nitrogen (N) metabolism, drought tolerance, and safety for animal consumption when used as a forage. Through chemical mutagenesis with ethyl methanesulfonate (EMS), a mutation in the gene encoding CYP79A1 (*cyp79a1*), the first enzyme in the biosynthetic pathway of dhurrin, was discovered that inhibits the production of dhurrin. The acyanogenic phenotype of this mutant could be a major benefit in reducing the risk of hydrogen cyanide (HCN) toxicity within animals; however, understanding the effects of inhibiting dhurrin biosynthesis is important in understanding metabolic tradeoffs that could occur. This dissertation describes research to assess impacts and tradeoffs of the dhurrin-free trait on susceptibility to Fall Armyworm [*Spodoptera frugiperda* (J.E. Smith)] (FAW) feeding, seedling growth, effects on post-flowering drought tolerance, cold stress and utilization as a forage. Insect susceptibility and seedling growth were examined using near-isogenic lines (NILs) within the greenhouse utilizing non-destructive phenotyping technologies for green plant area and in the field comparing total leaf area and dry weight. Post-flowering drought stress was induced within a greenhouse, growth chamber, and field environments. The *cyp79a1* mutation was tested in NILs, a near-isogenic backcross (NIBC) population, and near-isogenic hybrids (NIH), to understand the impacts of the *cyp79a1* mutation on the stay-green trait. Palatability as forage was examined by comparing the feeding preference of ruminant animals with multiple conventional hybrids and an experimental hybrid carrying the *cyp79a1* mutation. This preference was also examined using a set of NILs varying in the *cyp79a1* mutation. Safety was assessed in preference trials by testing for HCN release before grazing. To further our understanding of the benefits of sorghum as a forage, the dhurrin-free experimental hybrid was compared to seven conventional hybrids as a dry product. The dry sorghum product was tested for the release of HCN and dhurrin content. Lastly, the effects of low temperatures and frost were assessed for their effects on the production of dhurrin in cyanogenic and dhurrin-free sorghum genotypes.

Overall, the biosynthesis of dhurrin had a significant effect on the deterrence of FAW and on the growth of sorghum seedlings. Dhurrin-free lines were more susceptible to FAW feeding but also

exhibited a significantly higher growth rate. Dhurrin-free lines and hybrids only exhibited a slight increase in susceptibility to post-flowering drought stresses with only one dhurrin-free hybrid discovered to senesce faster than its wild-type NIH. Comparisons of the effects of dhurrin biosynthesis on stay-green in a NIBC population in Tx642 (B35), one of the most important sources of the stay-green trait, did not show any variation in chlorophyll concentration (CC) and normalized difference vegetation index (NDVI). Analyses of the impact of dhurrin on palatability as a forage showed that ewes preferred grazing on the dhurrin-free hybrids and NILs, showing that the ewes were able to detect the presence or absence of dhurrin while feeding. Experiments to assess the safety and stability of dhurrin in dried plant material demonstrated that dhurrin content did not change during drying and HCN was released after rehydration. Furthermore, high levels of HCN were immediately released when rumen fluid was added to dried plant materials containing dhurrin; however, no detectable HCN was released from dhurrin-free genotypes. Finally, sorghum plants exposed to freezing temperatures exhibited an increase in dhurrin content in conventional sorghum hybrids while no detectable dhurrin was noted within *cyp79a1* mutants.

Taken together, these studies demonstrate pleiotropic effects for the *cyp79a1* mutation. Dhurrin-free genotypes were more susceptible to insect herbivory and may be slightly more susceptible to post-flowering drought within the hybrids; however, these genotypes exhibited higher seedling growth rates, feeding preference by ewes, no release of HCN in fresh or dry plant material, and frost did not cause an increase in dhurrin content.

CHAPTER 1. REVIEW ON CYANOGENIC GLUCOSIDE: FOCUSING ON CHARACTERICTICS OF DHURRIN IN SORGHUM

1.1 Cyanogenic Glucosides

Plants produce a large number of secondary compounds that do not have a direct effect on the growth and metabolism but are important for protection, attraction of pollinators and many more beneficial purposes (Conn 1981). Cyanogenic glucosides (CGs) are secondary compounds found in over 2,500 plant species representing many plant families including Rosaceae, Gramineae, Fabaceae, and Leguminosae (E. E. Conn 1981a; Blomstedt et al. 2016). Plants containing CGs are cyanogenic, because hydrolysis of the CG releases hydrogen cyanide (HCN). Release of HCN from CG has been shown to be beneficial as a defense mechanism against herbivory. CGs are sometimes referred to as phytoanticipins (Osbourn 1996). CGs may play important roles within a plant but can cause toxicity in humans and animals, and potentially death when present in high concentrations.

HCN disrupts the respiratory pathway by inhibiting the final enzyme, chytochrome c oxidase, a metalloenzyme in the electron transport chain, not allowing the cell to utilize oxygen for respiration (Price 1985), ultimately leading to loss of consciousness, coma, and death ("ATSDR - Medical Management Guidelines (MMGs): Hydrogen Cyanide (HCN)" n.d.). Farmers and consumers must be very careful while harvesting and preparing these crops to avoid any HCN toxicity to the consumer, human or animal. There have been multiple incidences of toxicity in humans from the CGs in cassava linamarin and lotaustralin, because of improper preparation of the vegetable (Nzwalo and Cliff 2011). Sorghum produces the CG dhurrin. Dhurrin accumulation can reach up to one third of the dry weight in the upper portion of the plant during early growth stages (Halkier and Moller 1989; Adewusi 1990), easily reaching above 750 mg kg^{-1} of HCN which is considered very dangerous to cattle (Strickland et al. 2017).

Even though cyanogenic crops can be dangerous, they are important throughout the world. Many of today's crop plants are cyanogenic including clovers, cassava, barley, and sorghum (Jones 1998). It has been hypothesized that these plants may have been selected during crop development due to their ability to out compete their neighbors and were favored due to their resistance to insect feeding (Jones 1998). Cassava originated from South America and is now grown widely in Africa,

where it represents a large proportion of the caloric intake for the local population. Preparation of cassava is very important for the safety of the consumer (Nzwalo and Cliff 2011). Similarly, sorghum is widely grown in Africa and other arid to semi-arid environments because of its tolerance for high heat and drought and good adaptation to low-input environments in comparison to other cereals (Smith and Frederiksen 2000); however, care must be taken when harvesting the crop as a forage to avoid toxic levels of dhurrin (Smith and Frederiksen 2000; Adewusi 1990; Gray et al. 1968).

Biosynthesis, catabolism, and compartmentalization characteristics of CG metabolism are often similar across plant species. CGs also play similar roles in insect resistance and environmental adaptation across plant species (Poulton 1979; Gleadow and Møller 2014). These similarities can help identify the relevance and importance of CGs to plant defense, growth, and overall health.

1.2 Dhurrin in Sorghum

Dhurrin accumulation occurs in young plant tissues and can increase in older plants due to environmental stresses such as extreme drought, overfertilization, and frost (Halkier and Moller 1989; Wheeler et al. 1990; Smith and Frederiksen 2000; Gleadow and Møller 2014; Strickland et al. 2017). Dhurrin biosynthesis has been shown to play a role in both primary and secondary metabolism. Dhurrin has been reported to be a deterrent to insect feeding (Tattersall et al. 2001; Krothapalli et al. 2013), to play a role in N storage and partitioning in the plant increasing nitrogen use efficiency (NUE) (Blomstedt et al. 2018; Rosati et al. 2019), and to contribute to post-flowering drought tolerance (Burke et al. 2013; Hayes et al. 2016; Adeyanju et al. 2016; Varoquaux et al. 2019). Understanding the biosynthetic and catabolic pathways of dhurrin metabolism will contribute to our understanding of the mechanisms involved in these phenotypes.

1.2.1 Dhurrin Biosynthesis and Catabolism

CGs are derived from amino acids; aliphatic protein amino acids, aromatic amino acids, and aliphatic non-protein amino acid. The amino acidsl-valine, l-isoleucine, l-leucine, l-phenylalanin, l-tyrosine, and cyclopentenyl-glycine are used for the production of many different CGs (Gleadow and Møller 2014). Linamarin and lotaustralin from cassava are derived from l-valine and l-

isoleucine, respectively (Bromer et al. 1970; Jorgensen 2005). Dhurrin is derived from the amino acid l-tyrosine (Bromer et al. 1970).

The catabolism of CGs leads to the release of HCN. Beta-glucosidases and alpha-hydroxynitrilase facilitate the catabolism of CGs into HCN (Zagrobelny et al. 2004). There are a variety of β-glucosidases found in plants, animals, bacteria, and fungi (Hosël and Conn 1982). They catalyze the hydrolysis the aryl and akyl groups of beta-glucosides releasing a glycone and an aglycone moiety. β-glucosidases generally have broad specificity for the aglycone moiety, but are more specific for the glycone moiety of their substrate (Hosël and Conn 1982). Plant β-glucosidases can vary greatly in their substrate specificity based on the glycone moiety. Animal β-glucosidases have less specificity for a substrate, and **can** breakdown glucosides from plants (Singh et al. 2016), potentially including cyanogenic glucosides such as dhurrin. Cyanogenic glucosides upon hydrolysis by β-glucosidase will release an aglycone that can freely dissociate into HCN causing a very quick release of HCN (Zagrobelny et al. 2004; Gleadow and Møller 2014).

1.2.1.1 Biosynthesis of Dhurrin

The biosynthesis of dhurrin is controlled through a pathway that avoids any self-toxicity from a buildup of unwanted intermediates, or the accidental breakdown of dhurrin leading to the release of HCN. Dhurrin is derived from the amino acid L-tyrosine through enzymes forming a metabolon (Nielsen et al. 2008) beginning with two cytochrome P450 enzymes, CYP79A1 and CYP71E1. CYP79A1 catalyzes the conversion of L-tyrosine to (E)-p-Hydroxyphenylacetaldehydeoxime. This is the rate-limiting step, and CYP79A1 shows high substrate specificity for tyrosine. This product is converted to p-Hydroxymandelonitrile by CYP71E1 (Kahn et al. 1999) after which it is stabilized through glycosylation by UGT85B1 to form dhurrin (Jones et al. 1999). The three enzymes are co-regulated and co-located on chromosome 1 (Hayes et al. 2015a). The two chytochrome P450 enzymes are membrane bound proteins that attach to the ER membrane. UGT85B1 is localized towards the ER surface when the chytochrome P450s are present, forming an anchored metabolon with both CYP79A1 and CYP71E on the ER membrane (Nielsen et al. 2008). The metabolon is anchored through the CYP79A1 and CYP71E1 enzymes leading to the localized production of dhurrin. The formation of the metabolon inhibits the build-up of toxic intermediates or the binding of the wrong substrate to the intermediate enzymes which exhibit less

specificity compared to CYP79A1. The intermediate (E)-p-Hydroxyphenylacetaldehydeoxime, is a reactive oxygen species that can cause damage to the plant, and the p-Hydroxymandelonitrile can dissociate to form HCN if p-Hydroxybenzaldehyde is not stabilized by the glycosylation by UGT85B1 (Bak et al. 2000; Blomstedt et al. 2016).

1.2.1.2 Catabolism of Dhurrin

The catabolism of dhurrin is caused by two beta-glucosides isoenzymes, dhurrinase-1 (dhr1) and dhurrinase-2 (dhr2) and then followed by α-hydroxnitrile lyase. Dhr1 or Dhr2 cleaves the stabilizing glucose from dhurrin releasing the unstable intermediate alpha- hydroxymandelonitrile, which is unstable and can dissociate into HCN spontaneously or more quickly dissociate from the presence of ∝-hydroxynitile lyase (Kojima et al. 1979). The concentration of dhr1 and dhr2 are variable in different plant tissues (Cicek and Esen 1998), although both enzymes are located on chromosome 8 (Hayes et al. 2015a). Root tips generally contain a higher concentration of dhr1, while the shoots have a higher concentration of dhr2. The two enzymes vary in molecular weight, size, and structure, even though the enzymes both perform the same function (Cicek and Esen 1998).

1.2.1.3 Alternative and Detoxification Pathway for Dhurrin

To avoid self-toxicity, there is a detoxification pathway for HCN, and a proposed alternative pathway for the catabolism of dhurrin without the production of HCN. The proposed alternative pathways recycle dhurrin and HCN to produce ammonia and *p*-hydroxyphenylacetonitrile in the alternative pathway, and ammonia and asparagine or aspartate in the detoxification pathway (Bjarnholt et al. 2018). The production of ammonia could be very beneficial by providing N in a usable form to the plant supporting the hypothesis that dhurrin could be used for N storage in the plant (Blomstedt et al. 2018). Nielson et al. (2016) noted a peak of dhurrin production during seed development 25 days after pollination, but there was no release of HCN, because of the absence of the catabolic enzymes, dhr2 or dhr1, the developing seed. By the end of seed development there was a negligible amount of dhurrin within the seed, showing dhurrin was being utilized through an alternative pathway (Nielsen et al. 2016).

1.2.1.4 Dhurrin Compartmentalization

The compartmentalization of dhurrin and β-glucosidases is important to avoid unwanted catabolism of dhurrin into HCN. A study conducted by Kojima et al. (1979) showed the compartmentalization of dhurrin and the β-glucosidases to be at the tissue level of the plant. Dhurrin is stored in the epidermal cells in the leaf and shoot portions of the plant. The β-glucosidases are located within the mesophyll cells of the leaves and shoots (Kojima et al. 1979). This cellular compartmentalization reduces the risk of self-toxicity from the cyanogenesis of dhurrin within the plant tissue but allows for cyanogenesis to occur rapidly when cells are broken down through cell maceration, because of their close proximity when insects or animals chew and breakdown the cell walls. Previous studies had suggested that within the epidermal tissues dhurrin is stored within the vacuoles (Saunders and Conn 1978), but more recently Raman-spectroscopy has identified dhurrin to be localized within the cytoplasm and cell walls of the epidermal, cortical, and vascular tissue of a seedling (Heraud et al. 2018). Localization of the CG towards the surface could be for protection from insect feeding. Heraud et al. (2018) hypothesized that dhurrin localized in the vascular tissue, is for transport dhurrin to other parts of the plant. Translocation is possible with the modification of dhurrin into a diglucoside (Selmar et al. 1996), and CGs from other plants have been identified within the phloem (Neilson et al. 2013; Heraud et al. 2018).

1.2.2 **Acyanogenic Sorghum**

The production of HCN can be problematic in plants such as cassava, sorghum, or barley that are used for crop production. HCN can cause toxicity in the consumer whether it is in animals or humans (Way 1984; Price 1985). Some cyanogenic plant species such as white clover, eucalyptus, and birdsfoot trefoil have natural occurring acyanogenic lines, but no naturally occurring acyanogenic (dhurrin-free) plants have been identified for sorghum (Blomstedt et al. 2012). Blomstedt et al. (2012), Krothapoli et al. (2013), and Tuinstra et al. (2016) used EMS mutagenesis to disrupt and modify *cyp79a1* and *dhurrinase2*, sorghum genes involved in dhurrin metabolism. Disruption in the other enzymes could lead to stunted growth and development. Disruption of the CYP71E1 would result in the production of reactive oxygen species, 4-hydroxyphenylacetaldoxime, that could lead to self-toxicity (Bak et al. 2000). A nonfunctional UGT85B1 would lead to the unstable intermediate, 4-Hydroxymandelonitrile, that can

spontaneously dissociate to produce HCN (Bak et al. 2000; Blomstedt et al. 2016). CYP71E1 and UGT85B1 mutations would inhibit the production of dhurrin, but could also affect the growth and development of the mutant variety leading to stunted and slow growth as seen in transgenic tobacco (Bak et al. 2000; Blomstedt et al. 2016). Targeting mutations in genes for CYP79A1 or dhurrinase allows for disruption of dhurrin metabolism without a buildup of detrimental intermediates that could lead to stunted growth or an undesired phenotype.

Two of the EMS mutations in *cyp79a1* caused disruptions in dhurrin biosynthesis (Blomstedt et al. 2012; Tuinstra et al. 2013) and one EMS mutation in *dhr2* caused a disruption of the rapid catabolism of dhurrin (Krothapalli et al. 2013). The dhurrin-free sorghums were generated by P414L and C493Y mutations in the CYP79A1 enzyme (Blomstedt et al. 2012; Tuinstra et al., 2013). The *dhr2* allele reported by Krothapalli et al. (2013) was caused by a T194* (Tryptophan 194 to STOP) from a nonsense SNP mutation in the gene encoding the dhurrinase 2 enzyme, *dhr2-1* (Krothapalli et al. 2013).

The EMS mutant lines were examined for differences or in growth habit. Understanding the tradeoffs of inhibiting the biosynthetic or catabolic pathways is important in understanding how dhurrin could affect primary and secondary metabolism. The P414L mutant was observed to have a slower growth rate during the seedling stage and delayed flowering time (Blomstedt et al. 2012; Sohail 2020). There were no noticeable differences in biomass for the *dhr2-1* mutant (Krothapalli et al. 2013). This difference in early growth stages between the P414L mutant and *dhr2-1* mutant could be associated with the presence of dhurrin. Dhurrin accumulates in large amounts in the early stages of growth, and could be used as a N source for the plant (Busk and Moller 2002; Blomstedt et al. 2018; Rosati et al. 2019; Sohail et al. 2020).

Dhurrin biosynthesis has also been proposed as a key player in insect resistance (Krothapalli et al. 2013), N use efficiency (NUE) (Neilson et al. 2015; Blomstedt et al. 2018; Rosati et al. 2019), drought tolerance (Burke et al. 2013; Adeyanju et al. 2016; Hayes et al. 2016; Varoquaux et al. 2019), grain development (Nielsen et al. 2016), and HCN potential (HCNp). Disruptions in dhurrin metabolism could lead to tradeoffs in adaptation and productivity due to complex interactions in primary and secondary metabolism.

Dhurrin has been linked to resistance to insect herbivory (Tattersall et al. 2001; Krothapalli et al. 2013). As the insect chews on the plant, disruption of the plant tissue leads to catabolism of dhurrin

mediated by dhurrinase causing the release of HCN (Zagrobelny et al. 2004). HCN interacts with the respiratory system causing inhibition of the utilization of oxygen (Way 1984; Price 1985). The HCN released can have a bitter taste as shown in almonds that may be unfavorable and seen as toxic to herbivores such as insects (Arrázola et al. 2012; Gleadow and Møller 2014).

CG mediated insect resistance can occur in a variety of ways (Gleadow and Woodrow 2002). A major contributing factor is concentration of the CG within the plant with higher concentrations leading to greater resistance as shown in a study using Japanese Beetles with 24 *Prunus* species (Patton et al. 1997; Gleadow and Woodrow 2002). Plants containing CGs may be avoided when there is another food source (Gleadow and Møller 2014; Gleadow and Woodrow 2002). Early instars of *Zonocerua variegatus* fed on cassava when it was the only option, but did not feed on cassava when an alternative food source was available (Bernays et al. 1977). Another factor is the ability of the plant to release HCN even without a β-glucosidase present (Gleadow and Woodrow 2002). Krothapoli et al. (2013) showed increased susceptibility to fall armyworm damage in a *dhr2-1* mutant. The mutant had slower release of HCN compared to the wild-type making it more susceptible to FAW. Next, the feeding style of insects can also contribute to the levels of resistance. Aphids have the ability to feed without cell disruption by moving its stylet through the apoplast of the plant to the phloem, minimizing plant tissue damage, causing minimal to no release of HCN (Zhu-Salzman 2004; Gleadow and Woodrow 2002). Finally, the relationship of the insect with the plant, for example an opportunistic feeder versus a specialized feeder, can influence the efficacy of CGs in insect deterrence. Opportunistic herbivores may be deterred by cyanogenic plants (Cooper and Swain 1976), while there is no deterrence in specialists (Schappert and Shore 1991). Specialist insects may have evolved with the specific cyanogenic plants to be more tolerant of the CG or even sequester the CG for its own defense (Zagrobelny et al. 2004).

Sorghum contains high amounts of dhurrin during the early stages of plant growth with the highest concentrations detected in the youngest plant tissue (Halkier and Moller 1989; Busk and Moller 2002). Dhurrin peaks in concentration within the first few days after germination but retains relatively high for several weeks (Busk and Moller 2002). Recent studies of transgenic plants (Tattersall et al. 2001) and forward genetic studies using EMS mutagenesis (Krothapalli et al. 2013) showed the benefit for dhurrin and the quick release of HCN in increasing insect resistance in plants (Tattersall et al. 2001; Krothapalli et al. 2013). Tattersall and colleagues transformed the

biosynthetic pathway of dhurrin into Arabidopsis. The transgenic plants successfully produced dhurrin and exhibited increased resistance to *Phyllotreta nemorum*, flea beetle (Tattersall et al. 2001). In another experiment, Krothapalli and colleagues showed increased susceptibility to fall armyworm using sorghum plants without an active dhurrinase. These plants produced dhurrin but lacked the mechanism for the quick release of HCN (Krothapalli et al. 2013).

Resistance to insect feeding of cyanogenic plants may be affected by the concentration of the CG (Patton et al. 1997; Gleadow and Woodrow 2002). Dhurrin-free sorghum was developed through EMS mutagenesis using reverse (Blomstedt et al. 2012) and forward genetics (Tuinstra et al. 2013). These acyanogenic lines may be susceptible to increased level of insect herbivory. Krothapalli (2013) and Tattersall (2001) demonstrated the importance of dhurrin for deterrence to insect herbivory with flea beetle and fall armyworm, respectively. Resistance/susceptibility needs to be determined on potential plant pests on completely dhurrin-free sorghum lines.

Lepidoptera species that did not evolve with CG plants but have more recently been noted as a pest of sorghum (Day et al. 2017) would be good subjects to study dhurrin-free sorghum x insect interaction. They did not evolve with sorghum, so there may not be an unknown evolutionary preference or deterrence (Pentzold et al. 2015). Lepidoptera species have chewing mouthparts that would cause tissue disruption leading to the breakdown of dhurrin and release HCN. The Lepidoptera species, *Spodoptera frugiperda*, (Fall armyworm (FAW)) has a wide feeding distribution throughout the world, including Africa (Goergen et al. 2016), India (Chormule et al. 2019), South America, and the United States (Sparks 1979), infesting over 80 plant species including cotton, millet, corn, and sorghum (Day et al. 2017). The distribution and wide range of hosts make FAW a significant pest.

Overall, dhurrin can play large effect on insect resistance within sorghum and could be a tradeoff in commercializing dhurrin-free lines and hybrids. Dhurrin accumulating in large concentrations, and its quick breakdown through cell disruption are both important characteristics in resistance to insects (Zagrobelny et al. 2004; Krothapalli et al. 2013; Gleadow and Møller 2014).

1.2.3 **Stay Green**

The stay-green trait is one of the most important adaptation traits of sorghum (Rosenow et al., 1983; Borrell et al., 2000; Varoquaux et al., 2019). The stay-green phenotype allows the plant to

maintain green leaves and stalks under post-flowering drought stresses (Borrell et al., 2001). Stay-green genotypes remain photosynthetically active longer under drought conditions after flowering in comparison to senescent varieties leading to higher grain and forage yields (Borrell et al., 2000; van Oosterom et al., 2010; Thomas and Ougham, 2014).

Discoveries have been made about a link between N content and dhurrin metabolism. It has been noted that when N-fertilizer is applied to older sorghum plants, a spike in dhurrin concentration is observed (Busk and Moller 2002). This is not observed in seedlings because the biosynthesis of dhurrin is at full capacity at a young age when dhurrin is at its highest concentrations. It has been proposed that the increase in dhurrin following N applications is evidence that dhurrin is being used a storage sink for N (Busk and Moller 2002; Gleadow et al. 2016; Blomstedt et al. 2018). The breakdown of dhurrin to ammonia and p-hydroxyphenylacetic acid could provide an alternate pathway for utilizing the dhurrin pool (Bjarnholt et al. 2018). Ammonia is a usable source of N for the plant. This alternative pathway allows the plant to access the N stored in dhurrin, without the threat of self-toxicity from the breakdown of dhurrin to HCN.

Dhurrin as a N storage pool could be important for expression of the stay-green drought tolerance trait. Burke et al. (2013) was the first to describe a relationship between dhurrin and stay-green with the higher levels of dhurrin at the seedling stage being associated with stay-green under post-flowering drought stress. Follow-up studies suggested that dhurrin functions as an osmoprotectant involved in stay-green expression (Hayes et al. 2015b; Varoquaux et al. 2019). Initially, four QTLs for the stay-green trait were discovered in sorghum (Tuinstra et al. 1997; Xu et al. 2000; Kebede et al. 2001; Sanchez et al. 2002; Harris et al. 2006). These four QTL explained approximately 40% of the phenotypic variation and influenced plant N status (Harris et al. 2006), changes in root architecture (Mace et al. 2012), less tillering, and smaller leaves (Borrell et al. 2014), leading to better absorption of N, with less demand of N on vegetative growth (Borrell et al. 2014). However, the candidate genes for these QTL were not well understood. Recently, a fifth QTL for stay-green, Stg 5, was reported by Hayes et al. (2016) and was reported to explain 8-14% of the variation in stay-green derived from BTx642. Stg 5 is located on the SB101 region of the sorghum genome and is co-aligned with the QTL for the biosynthetic pathway of dhurrin (Hayes et al. 2016).

Dhurrin metabolism may play a role in drought tolerance and nitrogen metabolism (Burke et al., 2013; Hayes et al., 2016; Adeyanju et al., 2016; Blomstedt et al., 2018; Rosati et al., 2019);

however, it is still unclear how dhurrin contributes to environmental sustainability. Dhurrin could potentially be used as a source of N allowing the plant tissues to remain green longer. During grain fill, the grain acts as an N sink pulling N from the roots and other tissues. When the N demand is larger than the N being absorbed, N from the leaves, stems, and rachis can be mobilized for use in the grain (van Oosterom et al., 2010). Leaf dhurrin can represent 1-5% of the leaf N (Hayes et al. 2016). The remobilization of dhurrin could supply some of this N content, instead of relying on the breakdown of RUBISCO leading to leaf senescence (Borrell and Hammer, 2000). The remobilization of CG in other cyanogenic species has been identified and dhurrin has been located near the vascular bundle supporting this theory (Heraud et al. 2018).

More work is needed to clarify the role of dhurrin in the expression of the stay-green trait. If dhurrin plays a direct role in expression of stay-green, the development and use of dhurrin-free sorghum varieties could result in greater susceptibility to post-flowering drought stresses.

1.2.4 **Forage Sorghums**

Forage sorghum has some very important characteristics including production of high amounts of biomass (Marsalis et al. 2010), nutritious and highly digestible forage biomass (Porter et al. 1978; Grant et al. 1995), and excellent adaptation to drought-prone and low-fertility environments (Sanchez et al. 2002; Borrell and Hammer 2000). Sorghum also exhibits extensive genetic and phenotypic variation for many traits making it an ideal crop for utilization as a silage, hay, green chop, or pasture (Smith and Frederiksen 2000).

The discovery and development of brown midrib (bmr) sorghum types has had a dramatic impact on nutritional quality (Porter et al., 1978; Oliver et al. 2005a). The bmr trait reduces the amount of lignin, making the plant more digestible (Oliver et al. 2005a). The nutritional quality of bmr sorghum varieties has been shown to decrease the neutral detergent fiber of the dry matter in comparison to non-bmr forage sorghums (Oliver et al. 2005b). The greater digestibility of bmr sorghum silage supported milk production in mid-lactating cows similar to corn and alfalfa (Grant et al. 1995). The bmr sorghum varieties are a valuable forage crop especially in areas of water limitation where corn may not perform as well (Smith and Frederiksen 2000).

Although sorghum has many important forage production characteristics, some attributes are less desirable and hinder its overall value. C4 crops such as maize, pearl millet, and sorghum can

accumulate nitrates. Nitrate toxicity is caused by a high accumulation of nitrates within the rumen. The excess nitrates can enter the bloodstream and change the hemoglobin to metheglobin. Metheglobin is unable to carry oxygen throughout the body, potentially leading to death. In animals not adapted to nitrates, a high concentration of the nitrate ion is 0.93% on a dry basis. Increases in nitrate levels can be due to environmental conditions such as drought, herbicide damage, and N fertilization (Drewnoski et al. 2019). Nitrate accumulation needs to be monitored while using sorghum as a forage but can occur in other C4 crops (Smith and Frederiksen 2000; Drewnoski et al. 2019).

Unlike nitrates, dhurrin production is unique to sorghum. High dhurrin concentrations can lead to HCN toxicity in livestock through the breakdown of dhurrin leading to the release of HCN. As mentioned previously, HCN can inhibit cellular respiration (Price 1985). HCN potential (HCNp) has been described to be highest in the youngest parts of the plant and HCNp decreases as the plant parts mature (Loyd and Gray 1970). This leads to the highest concentration of dhurrin being concentrated at the upper portions of the plant. At the seedling stage, the upper ten percent of a plant can contain dhurrin content can reach up to 30 percent (Halkier and Moller 1989).

Dhurrin concentrations can vary greatly between varieties (Burke et al. 2013) and is affected by many factors, including drought stress (O'Donnell et al. 2013; Rosati et al. 2019), the age of the plant (Halkier and Moller 1989; Loyd and Gray 1970), temperature (Strickland et al. 2017), and fertilizer applications (Smith and Frederiksen 2000; Wheeler et al. 1990; Harms and Tucker 1973). Hayes et al. (2015) created a conversion panel of 700 converted lines that vary in dhurrin content. Loyd and Gray (1970) described differences among three forage sorghum types in dhurrin concentrations across time. Low dhurrin forage sorghums are available but no dhurrin-free genotypes have been discovered in the standing variation of sorghum (Duncan 1996; Gleadow et al. 2012). Dhurrin-free sorghums developed through EMS mutagenesis (Blomstedt et al. 2012; Tuinstra et al. 2013) could be a good tool to avoid HCN toxicity; however, more work is needed to understand the trade-offs between dhurrin production and adaptation to biotic and abiotic stresses.

1.2.5 **Forage Management of HCNp**

Good forage sorghum management practices should be followed to avoid HCN toxicity including delaying grazing or harvesting until the crop is 45 to 60 cm tall and avoiding fields that have been damaged by drought or frost (Strickland et al. 2017). Ensiling or chopping may help reduce HCNp by up to 50 percent (Smith and Frederiksen 2000; Strickland et al. 2017). Hay production is another option for sorghum forages but care still needs to be taken because removing the water can lead to concentrating dhurrin (Strickland et al. 2017; Smith and Frederiksen 2000). The stability of dhurrin during the drying process and storage is not well understood. Gleadow et al. (2012) reported that dhurrin was stable in tissues dried with high heat or drying at room temperature in a dark room, and was stable in long-term storage when stored in a desiccator. This suggests that HCNp could be maintained during the hay making process but drying at lower temperatures, or in direct sunlight, or processing plants with mechanical conditioning are not well understood. Usage of sorghum as a silage is the most commonly suggested method for processing sorghum if there are any concerns of high HCNp (Strickland et al. 2017). The chopping of silage would cause significant cell damage, and storage with a higher moisture content and low pH could cause the breakdown of dhurrin decreasing HCNp.

The use of dhurrin-free sorghum varieties could avoid any chance of HCN toxicity and be very beneficial; however, nitrate accumulations should still be carefully managed (Gleadow et al. 2016), Also, understanding the palatability, nutritional quality, and nitrate accumulation of the dhurrin-free sorghum is important to understand its value as a forage crop.

1.2.5.1 Environmental Effects of HCNp

Developing a better understanding of the genetic and environmental factors that influence dhurrin production in sorghum including nutrient fertility (Harms and Tucker 1973; Kempel et al. 2009; Ballhorn et al. 2013; Blomstedt et al. 2018), drought (O'Donnell et al. 2013; Vandegeer et al. 2013; Rosati et al. 2019), extreme temperatures (Wheeler et al. 1990; Stochmal and Oleszek 1997), and age of the plant (Loyd and Gray 1970; Halkier and Moller 1989) is important in using sorghum as a forage. Plants supplemented with high-N fertilizer applications generally have an increased amount of CG's in the plant. Stressful environments including extreme drought can also cause an increase in CG. Drought stress increased the CG content of young cassava leaves by 2.9 fold

(Vandegeer et al. 2013) and increased in dhurrin content in sorghum (Rosati et al. 2019). Cold temperatures can also increase CG content within cyanogenic white clover (Stochmal and Oleszek 1997; Vickery et al. 1987) and is thought to cause dhurrin accumulation (Strickland et al. 2017), although when tested by Wheeler et al. (1990) HCNp did not increase in cold stressed sorghum plants.

1.3 Conclusion and Discussion

Sorghum has many important characteristics that make it a valuable crop throughout the world. Sorghum also produces the CG, dhurrin, that has been associated with many aspects of crop production including insect resistance (Krothapalli et al. 2013; Tattersall et al. 2001), stay-green (Hayes et al. 2016; Burke et al. 2013), and potentially a N source (Gleadow et al. 2016; Bjarnholt et al. 2018). All are important characteristics in a successful crop; however, dhurrin can be problematic when using sorghum as a forage crop. Special care needs to be taken to avoid poisoning of livestock by minimizing the amount of dhurrin within the plant by avoiding young plants (Adewusi 1990), not harvesting sorghum from drought stressed fields (O'Donnell et al. 2013), or fields exposed to freezing temperatures (Gleadow and Møller 2014). Forage processing technologies also play an important role in managing HCNp with dhurrin content being affected by ensiling, hay-making, or green chopping (Strickland et al. 2017).

Many of the concerns for management of dhurrin could be avoided with utilization of dhurrin-free sorghum varieties. Production of dhurrin-free sorghum forages would decrease risk of toxicity to livestock due to HCN but more research is needed to understand the tradeoffs that may result from the removal of dhurrin from sorghum. More studies need to be done to understand the tradeoffs of dhurrin-free sorghum on insect susceptibility (Krothapalli et al. 2013; Tattersall 2001), drought tolerance (Burke et al. 2013; Hayes et al. 2016; Adeyanju et al. 2016; Varoquaux et al. 2019), N use and partitioning (Blomstedt et al. 2018; Rosati et al. 2019), and the preferential grazing of animals.

CHAPTER 2. SEEDLING GROWTH AND FALL ARMYWORM FEEDING PREFERENCE INFLUENCED BY DHURRIN PRODUCTION IN SORGHUM

Submitted to Journal of Theoretical and Applied Genetics.

2.1 Abstract

Cyanogenic glucosides (CGs) play a key role in host-plant defense to insect feeding; however, the metabolic tradeoffs between synthesis of CGs and plant growth are not well understood. In this study, genetic mutants coupled with nondestructive phenotyping techniques were used to study the impact of the CG dhurrin on fall armyworm [*Spodoptera frugiperda* (J.E. Smith)] (FAW) feeding and plant growth in sorghum [*Sorghum bicolor* (L.) Moench]. A genetic mutation in CYP79A1 that disrupts dhurrin biosynthesis was used to develop sets of near-isogenic lines (NILs) with contrasting dhurrin content in the Tx623 *bmr6* genetic background. The NILs were evaluated for differences in plant growth and FAW feeding damage in replicated greenhouse and field trials. Greenhouse studies showed that dhurrin-free Tx623 *bmr6 cyp79a1* plants grew more quickly than wild-type plants but were more susceptible to insect feeding based on changes in green plant area (GPA), total leaf area, and total dry weight over time. The NILs exhibited similar patterns of growth in field trials with significant differences in leaf area and dry weight of dhurrin-free plants between the infested and non-infested treatments. Taken together, these studies reveal a significant metabolic tradeoff between CG biosynthesis and plant growth in sorghum seedlings. Disruption of dhurrin biosynthesis produces plants with higher growth rates than wild-type plants but these plants have greater susceptible to FAW feeding.

2.2 Introduction

Plants produce many different types of metabolites that can deter insect feeding (Tattersall et al. 2001; Wittstock and Gershenzon 2002) including cyanogenic glucosides (CG). CGs are a family of compounds found in over 2,500 plant species (Jones 1998; Conn 1981; Gleadow and Woodrow 2002; Gleadow and Møller 2014), including sorghum, bitter almond (Hosël and Conn 1982), and cassava, and thought to play key roles in deterring insect feeding (Gleadow and Woodrow 2002).

CGs contribute to host-plant resistance to insects by releasing hydrogen cyanide (HCN) when the plant tissues are disrupted by feeding insects (Zagrobelny et al. 2004). HCN inhibits respiration and the utilization of oxygen (Way 1984; Price 1985). HCN also has a bitter taste (Arrázola et al. 2012) that is thought to deter herbivory leading to the deterrence of feeding in some insects (Gleadow and Møller 2014).

Feeding deterrence can vary in many ways (Gleadow and Woodrow 2002). One of the key factors controlling deterrence is the amount of CGs within the plant tissues with higher concentrations leading to greater deterrence, as shown in a study of Japanese Beetle feeding in 24 *Prunus* species (Patton et al. 1997). Alternate food sources can also influence feeding patterns as insects will generally avoid feeding on plant tissues with high concentrations of CGs if an alternative food source is available (Gleadow and Møller 2014). Bernays et al. (1977) showed that early instars of *Zonocerua variegatus* would feed on cyanogenic cassava if there were no other choices, but feeding was deterred if another food source was available. The ability of the plant to release HCN is an additional factor that influences insect deterrence. Krothapalli et al. (2013) demonstrated that the β-glucosidase that catalyzes rapid HCN release in sorghum, dhurrinase2, played a role in insect feeding deterrence, even in the presence of wild-type levels of CG accumulation. Feeding style also influences the role of CGs in host-plant resistance. Tissue disruption is generally required for HCN release. CGs generally have little to no effect on aphids that feed by injecting the stylet through the apoplast of the plant to the phloem, minimizing plant tissue damage (Zhu-Salzman 2004; Gleadow and Møller 2014). The relationship of the insect with the plant can also influence the role of CGs on insect feeding. Opportunistic herbivores may be deterred by cyanogenic plants (Cooper and Swain 1976), while specialists are not deterred because these insects may have evolved tolerance to the CG or have the ability to sequester the CG for defense (Zagrobelny et al. 2004).

Dhurrin is an important CG of sorghum.

The highest concentrations of dhurrin in sorghum plants are found during early growth stages and within the youngest plant tissues. Dhurrin concentrations peak within the first few days after germination, and then concentrations decline over time (Busk and Moller 2002; Halkier and Moller 1989). Halkier and Moller (1989) found that dhurrin composed up to 30 percent of plant dry weight in the top ten percent of a seedling during germination.

Genetic studies have demonstrated that dhurrin contributes to the deterrence of insect feeding in plants. Tattersall et al. (2001) transformed the biosynthetic pathway for dhurrin into Arabidopsis and showed increased resistance to *Phyllotreta nemorum,* flea beetle. Krothapalli et al. (2013) used chemical mutagenesis to create a knockout of dhurrinase2, the β-glucosidase that catalyzes the release of HCN from dhurrin and showed increased feeding by FAW. Other mutations that disrupt dhurrin biosynthesis have also been identified (Blomstedt et al. 2012; Tuinstra et al., 2016). More work is needed to quantify the impact these mutations have on insect feeding.

Lepidoptera insects and other species that did not evolve as a pest of sorghum may be good subjects to study the role of dhurrin in deterrence of feeding (Pentzold et al. 2015). Lepidoptera species have chewing mouthparts that cause the lead to the hydrolysis of dhurrin= and release of HCN. FAW have a wide feeding distribution throughout North and South America (Sparks 1979) and currently are emerging as a significant new pest in Africa (Goergen et al. 2016) and Asia (Chormule et al. 2019), infesting over 80 plant species, including cotton, millet, corn, and sorghum (Day et al. 2017). The distribution and wide range of hosts make FAW a significant pest.

Analyses of host-plant resistance and insect feeding generally rely on destructive methods (Krothapalli et al. 2013; Tattersall et al. 2001) or a ranking system (Diawara et al. 1990) to quantify differences among plant samples. Destructive sampling can be a challenge because each sample provides insight into a just single time point, and many samples are required to develop an understanding of changes in feeding preference over time. Alternatively, the ranking scale approach can be used to assess feeding damage over time, but these measures are subjective and can vary based on the individual ranking the samples. New methods are needed to quickly and accurately phenotype insect feeding characteristics. Nondestructive imaging techniques have shown promise in efforts to classify plant phenotypes in an efficient manner with lower overall costs, time, and labor (Araus and Cairns 2014; Hairmansis et al. 2014). Image based phenotyping systems have been used to calculate relative growth rate of sorghum based on changes over time (Neilson et al. 2015). Image based systems could be beneficial in quantifying feeding damage based on changes in plant biomass from insect herbivory.

Dhurrin accumulation and turnover play key roles in primary and secondary metabolism by influencing sorghum responses to insect feeding and adaptation to abiotic stresses. In this study, genetic mutants coupled with high-throughput image-based phenotyping techniques were used to

explore tradeoffs for manipulating dhurrin metabolism in sorghum under conditions of FAW infestations. Specific objectives were to (1) evaluate the sensitivity of nondestructive plant imaging techniques for quantifying feeding damage in sorghum and (2) evaluate NILs contrasting for dhurrin accumulation for differences in plant growth and feeding damage following infestation by FAW in greenhouse and field trials.

2.3 Materials and Methods

2.3.1 Insects

FAW were obtained from Benzon Research (Carlisle, PA) at the $2^{nd}/3^{rd}$ instar for the first round of the greenhouse experiments and at the 1^{st} instar stage for the ssecond round within the greenhouse and in all field experiments.

2.3.2 Imaging Platform

An ARIS TopView Phenotyping System (ARIS, Eindhoven, Netherlands) was used in plant imaging studies. ARIS uses seven channels to calculate the GPA, hue, saturation, and other factors. The ARIS system calculates the GPA (mm^2) based on pixel count. Calibration studies were conducted to evaluate the use of GPA and changes in GPA over time to quantify insect feeding damage.

Three sorghum genotypes were evaluated in the first set of calibration studies: (1) Tx623 (reference genome), (2) SbEMS932 *dhr2-1* (mutation in the dhurrinase2 enzyme described by Krothapalli et al. (2013)), and (3) SbEMS2447 *cyp79a1* (C493Y mutation in the CYP79A1 enzyme described by Tuinstra et al. (2016)). Both mutations were in the Tx623, reference genome, background. Sorghum plants were grown in plastic Cone-tainers (Hummert International, St. Louis, MO) with a single plant per container for approximately three weeks. The Cone-tainers were filled with the Sun-Grow germination mix. After two weeks, the plants were fertilized with Miracle-Gro garden fertilizer at a rate of 350 ppm of nitrogen, 100 ppm of phosphorous, and 200 ppm of potassium. Each plant was tested for repeatability of GPA measurements by first measuring 58 sorghum plants then measuring them again after 1 h keeping the plant orientation consistent from image to image. After imaging the second time, total leaf area (mm^2) of each plant was determined by removing

all the leaves and leaf tips at the collar, imaging using an RGB camera, and processing imagery with Image J software (https://imagej.net/) for comparison with GPA.

GPA measured by ARIS was compared with total leaf area (mm^2) and total dry weight (g) of plants before and after FAW feeding using the same three genotypes described above. Sixteen plants of each genotype were grown to the 4-leaf stage and randomly placed in racks for evaluation. One rack was placed in each of four different enclosures; three infested with FAW and one with no insects. The insect treated enclosures were infested with approximately 150 FAW at the 2nd instar stage. FAW were starved for two hours before infestation. Infestation occurred for 36 hours with ARIS GPA (mm^2) taken at 0, 12, 24, and 36 h. After 36 h, each plant was harvested and total leaf area was measured by removing all the leaves and leaf tips at the collar, imaged using an RGB camera, and images processed using Image J software. After imaging, dry weights were determined by drying the plant material in a paper envelope at 60°C for one week.

2.3.3 **Greenhouse Trials**

Greenhouse studies were conducted in a facility at Purdue University in West Lafayette, IN. Sorghum plants were grown in cone-tainers for approximately three weeks as described for the calibration studies with a single plant per container. The first study evaluated FAW feeding preference for (1) Tx623 and (2) SbEMS 2447 *cyp79a1*. The experiment was conducted using a randomized complete block design with repeated measures each genotype represented by 16 plants in each of four enclosures. Plants of similar size at the 3-leaf stage were used in the study. One enclosure was not infested with FAW and the other three were infested with approximately 150 FAW at the 2nd instar with the insects starved for two hours before infestation. FAW were allowed to feed for 36 hours with ARIS GPA (mm^2) measurements taken at 0, 12, 24, and 36 h. At 36 hours, total leaf area (mm^2) and total dry weight (g) were measured for each plant as described in the calibration studies, Chapter 2.3.2.

A second greenhouse study was designed to compare insect feeding in sorghum NILs contrasting for the *cyp79a1* mutation. *cyp79a1* was backcrossed twice to Tx623 *bmr6* to produce Tx623 *bmr6 cyp79a1*. FAW feeding preference was compared in Tx623, reference genome, Tx623 *bmr6*, and Tx623 *bmr6 cyp79a1*. Two NILs differ for the bmr6 trait, and two NILs differ for the C493Y trait allowing tests for the effects of both traits. The experiment was conducted using a randomized

complete block with each genotype represented by 16 plants in each of four enclosures using plants of similar size at 3-leaf stage. One enclosure was not infested with FAW and the other three insect treated enclosures were infested with approximately 150 FAW at the 1st instar stage to avoid any increased tolerance in the insects due to age (Figure 2.1). The duration of feeding was increased to 96 hours to provide an opportunity for significant insect damage to occur in each enclosure with ARIS GPA taken every 24 hours. After 96 hours total, dry weight (g) of each plant was determined as described in the calibration studies.

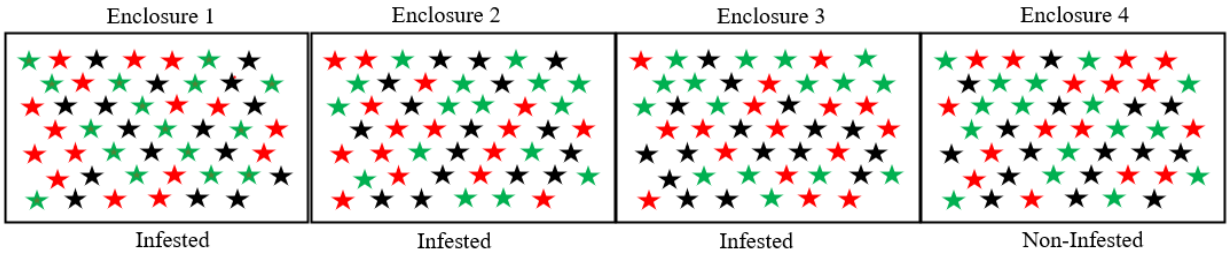


Figure 2.1: Represents the design of the feeding trial. The stars represent individual plants within each enclosure, the different colors of the stars represent the different genotypes in each enclosure.

2.3.4 **Field Trials**

Field trials were conducted in 2019 and 2020 using the NILs Tx623, Tx623 *bmr6*, and Tx623 *bmr6 cyp79a1*. The genotypes were overplanted and thinned to 35,000 plants ha^{-1} in 0.76 x 3.1 m plots to plants of similar size and leaf stage with five replications in 2019 and six replications in 2020 in a split plot design with FAW infestation as whole plots and genotype as subplots. Two rounds of this experiment were completed in the summer of 2019 in June and July, and the third round was completed June 2020. All three rounds were planted at the Purdue Agronomy Center for Research and Education. In 2019, plants were infested with FAW at the 3- to 4-leaf stage using a bazooka applicator with 4-5 insects per plant at the 1st instar stage for round 1 and 6-7 insects per plant for round 2. Two replications were harvested at 5 days post-inoculation and 3 replications harvested at 10 days. Twenty plants per plot were measured for dry weight (g). A subsample of four plants were measured for total leaf area (cm^2) using a LI-3100C Area Meter (LI-COR Biosciences, Lincoln, NE). A similar experiment was conducted in 2020 with six replications. Plants at the 3-leaf to 4-leaf stage were treated with FAW at the 1st instar stage on June 23, 2020 using a bazooka applicator at a rate of 4-5 insects per plants. Plots were harvested on day 12 with

a longer infestation time due to cool weather and slow feeding rates. Ten plants were harvested per plot and measured for total dry weight (g) and four plants were measured for total leaf area (cm^2).

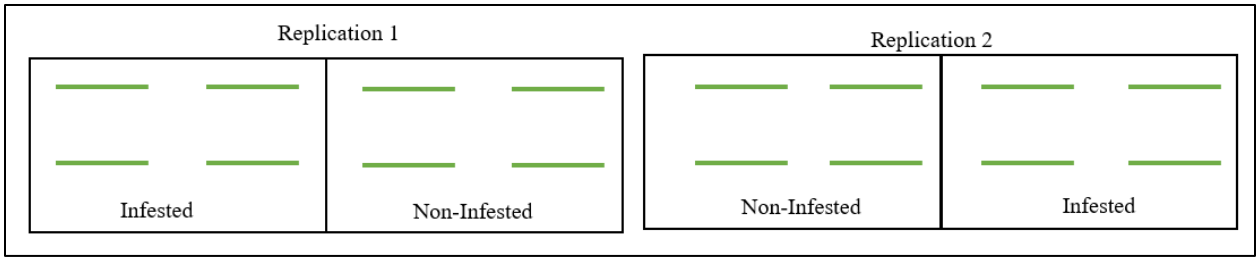


Figure 2.2: Is an example of two replications from the field trials. The green line represents a plot of plants, each plot is a specific genotype thinned to 35,000 plants ha^{-1}.

2.3.5 **Statistical Analysis**

Consistency of the GPA measurements by ARIS was tested using the two sets of measurements for GPA conducted an hour apart and evaluated using a paired t-test with a null hypothesis that the sample means were equal. Regression analyses were used to compare GPA to total leaf area (mm^2) and dry weight (g).

Outliers for each dataset was detected using boxplots. The boxplots were designed to describe the dependent variable; green plant area, dry weight, or total leaf area, based on the entry, treatments, and hours if captured. The influential points within the boxplots were screened to see if it could be a sampling error to be identified as an outlier. The second round of the 2019 field trials had four identified outliers. Each was almost two grams larger than the next highest value within the same genotype and treatment, and up to 7 g higher than the average for the genotype and treatment. Identified outliers were removed from the dataset. This was a less than one percent of the data within each dataset.

A mixed effects model was used to determine the effects of the insect treatments on the different genotypes in the experiments within the greenhouse. Fixed effects were entry (the NILs within the boxes and the field plots), treatment, hours, and their interactions. The random effects were replications nested within hours. Least square means were compared using Tukey's test with Kenward-Rogers degrees of freedom.

A mixed effects model was used to determine the effects of the insect treatments on the different genotypes in the experiments within the field experiment. Fixed effects were entry, treatment, days, and interactions for 2019, while days was not a factor in 2020. Replication was evaluated as a random effect. Each experiment was analyzed separately and in a combined analysis. Least square means were compared using Tukey's test with Kenward-Rogers degrees of freedom. The field experiment in 2020 explaining the dry weight and the two rounds conducted in the field in 2019 explaining the dry weights were transformed with a square root transformation to fit a normal distribution. The transformed data was used to be screened for outliers, to build the mixed effects model and Tukey's Test.

2.4 Results

2.4.1 Nondestructive plant imaging to quantify feeding damage

Calibration studies were conducted to determine the repeatability of GPA measurements from the ARIS TopView Phenotyping System. The average GPA measurements for the first set of measurements was 3155.6 mm^2, and the second set of measurements average was 3165.2 mm^2. Repeated measures were compared using a paired t-test and showed the values were not statistically different. Figure 2.3 displays the mask created to calculate GPA across time and insect feeding. Comparisons of GPA with total leaf area measured by destructive sampling and FAW feeding showed an R^2 of 0.86. Comparisons of the GPA with total dry weight showed similar results with an R^2 of 0.77. Pearson's Correlation showed a correlation of 0.93 compared to total leaf area and a correlation of 0.88 with dry weight (Fig. 2.4). The correlation between the GPA and the plant fresh weight was not as high with an R^2 of 0.61 (not shown).

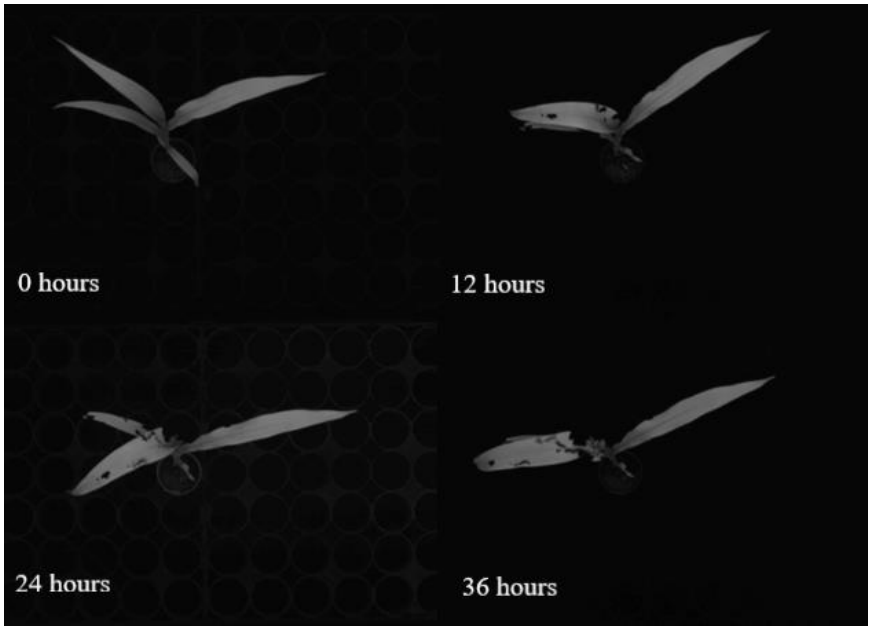


Figure 2.3. Infested sorghum plant imaged at 0, 12, 24, and 36 h after FAW infestation.

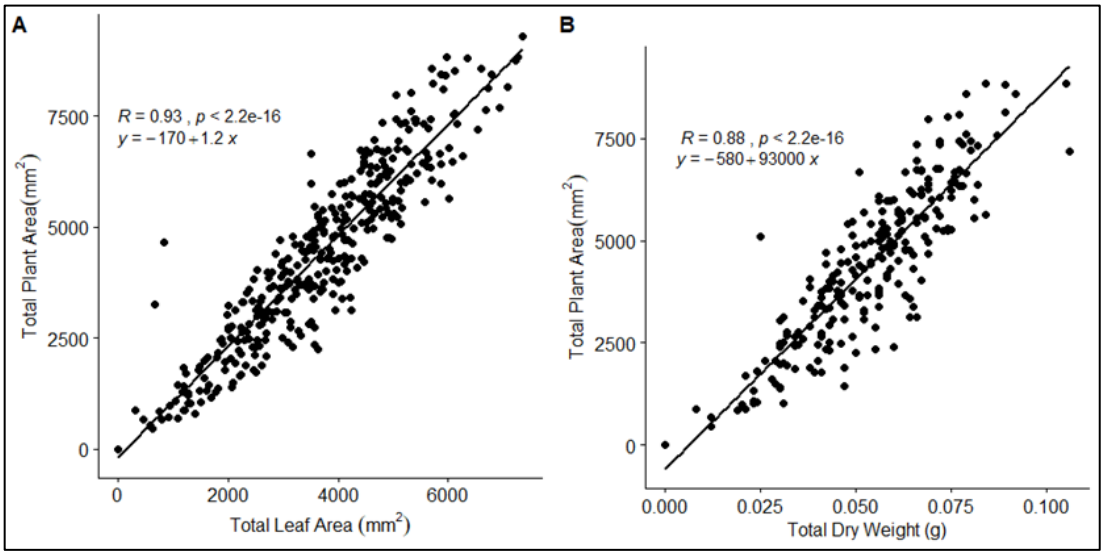


Figure 2.4: Linear regression model comparing (A) ARIS total plant area (mm^2) to total leaf area (mm^2) and (B) ARIS total plant area (mm^2) to total dry weight (g).

2.4.2 **FAW feeding preference of sorghum mutants**

The first round of greenhouse trials focused on an analysis of the original mutant SbEMS 2447 *cyp79a1* and Tx623 for differences in GPA and dry weight during and after FAW infestation. Figure 2.5A shows differences in GPA of mutant and wild-type plants under infested and non-

infested conditions over time. Btx623 GPA decreased over time with FAW feeding in the infested plants and GPA increased slightly over time with growth of plants in the non-infested treatment. The mutants exhibited lower GPA (Fig. 2.5A) and lower dry weights than BTx623 (Fig. 2.5B) under infested and non-infested conditions.

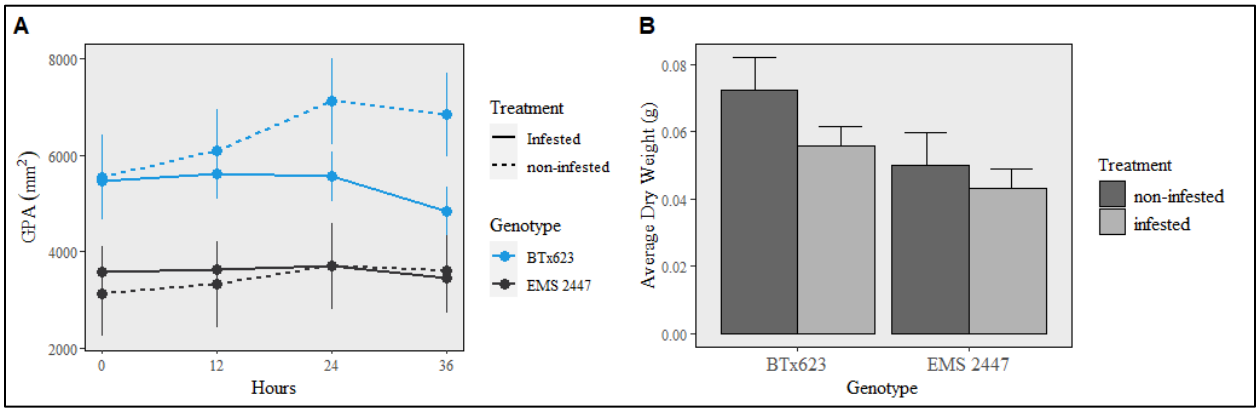


Figure 2.5: Effects of the *cyp79a1* mutation on FAW feeding in SbEMS 2447 and Tx623 wild-type comparing GPA over time (A) and the average dry weight at 36 h (B).

2.4.3 **Dhurrin biosynthesis influences sorghum growth and FAW feeding preference**

NILs of Tx623 contrasting for *cyp79a1* and *bmr6* mutations were compared for plant growth and susceptibility to FAW feeding under infested and non-infested conditions in greenhouse trials. Under non-infested conditions, the GPA for each of the three NILs increased over time as the plants maintained rapid growth (Fig. 2.6A). The dhurrin-free Tx623 *bmr6 cyp79a1* plants exhibited significantly higher GPA (Fig. 2.6A) and dry weight (Fig. 2.6B) than Tx623 and Tx623 *bmr6* plants at 96 h under non-infested conditions. Conversely, GPA remained steady or decreased over time in the sorghum NILs infested with FAW (Fig. 2.6A). The GPA of the infested Tx623 *bmr6 cyp79a1* plants decreased over time as the feeding trial progressed (Fig. 2.6A). Further analyses of GPA indicated that the insects began to devour enough plant material to produce a difference in plant area for Tx623 *bmr6 cyp79a1* between 48 and 72 h while Tx623 and Tx623 *bmr6* did not exhibit a difference until 96 h (Fig. 2.6A). The Tx623 *bmr6 cyp79a1* plants exhibited the largest difference in GPA (Fig. 2.6A) and dry weight (Fig. 4B) between the infested and non-infested treatments at 96 h demonstrating differential feeding preference for dhurrin-free plants when FAW had free-choice between the three genotypes. Although more susceptible to FAW

feeding, the dhurrin-free plants were still larger than wild-type plants in the infested treatments due to higher growth rates (Fig. 2.6).

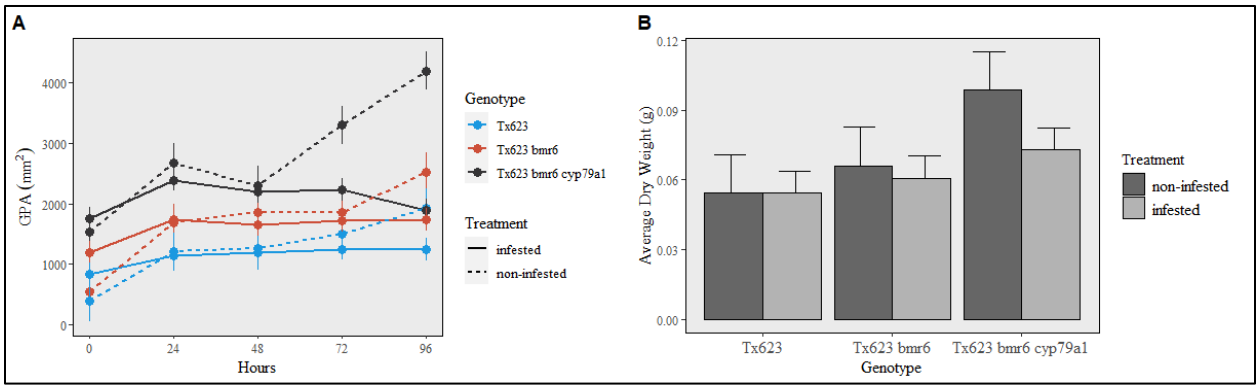


Figure 2.6: Effects of the cyp79a1 and bmr6 mutations on FAW feeding in sorghum NILs contrasting in dhurrin production based on changes in GPA over time (A) and the average dry weight at 96 h (B).

The sorghum NILs were also evaluated for variation in plant growth and feeding damage under FAW infested and non-infested conditions in three field trials conducted in 2019 and 2020. The FAW treatment effects on total leaf area and dry weight were significant in each study with infested plants exhibiting similar or lower total leaf areas and dry weights than non-infested plants for each genotype (Table 2.1). Genotype by treatment interaction effects were observed for total leaf area in one study in 2019 and for dry weight in both trials conducted in 2019 (Table 2.1). Tx623 *bmr6 cyp79a1* was the only NIL in the study that exhibited significant differences in total leaf area and dry weight between infested and non-infested treatments in all three studies (Table 2.1). These differences demonstrate that more FAW feeding occurred in the dhurrin-free Tx623 *bmr6 cyp79a1* as compared to Tx623 and Tx623 *bmr6*. Although more susceptible to FAW feeding, the dhurrin-free plants did not exhibit differences in leaf area or dry weight from the wild-type plants in the FAW infested treatments due to increased size of the dhurrin-free plants prior to infestation.

Table 2.1 Average leaf area and dry weight values of near isogenic sorghum lines contrasting for brown midrib (bmr6) and dhurrin biosynthesis (*cyp79a1*) mutations in field trials conducted in 2019 and 2020. Differences between values are shown using Tukey's Test. Analyses of variance were used to test significance of Entry, Treatment, and Entry x Treatment interactions.

		Total Leaf Area (cm^2)					
Entry	**FAW Treatment**	**2019-1[a]**		**2019-2**		**2020**	
Tx623 *bmr6 cyp79a1*		251	a	568	a	1069	a
Tx623 *bmr6*	No	108	b	501	ab	995	ab
Tx623		241	ab	467	ab	973	abc
Tx623 *bmr6 cyp79a1*		149	b	366	bc	887	bc
Tx623 *bmr6*	Yes	90	b	368	bc	834	bc
Tx623		188	ab	543	ab	785	c
Significance[b]	Entry	***					
	Treatment	**		*		***	
	Entry*Treatment			**			
		Total Dry Weight (g $plant^{-1}$)					
Entry	**FAW Treatment**	**2019-1**		**2019-2**		**2020**	
Tx623 *bmr6 cyp79a1*		1.57[c]	a	3.26	a	9.93	a
Tx623 *bmr6*	No	0.84	b	2.59	b	9.93	ab
Tx623		1.58	a	2.60	b	9.12	abc
Tx623 *bmr6 cyp79a1*		0.99	bc	2.35	bc	8.31	c
Tx623 *bmr6*	Yes	0.60	b	2.18	bc	8.70	abc
Tx623		1.12	bc	3.02	ab	9.34	abc
Significance	Entry	***		**			
	Treatment	***		**		***	
	Entry*Treatment			***			

[a] For the 2019 data, the day 5 values are not presented because there were no significant effects.
[b] **=0.05, **=0.01, ***=0.001*
[c] All average values are based on the non-transformed data.

2.5 Discussion

Many studies have demonstrated the value of image-based sorghum phenotyping technologies for assessment of plant growth and development (Neilson et al. 2015; Batz et al. 2016; Thapa et al. 2018; Masjedi et al. 2020). In this study, the ARIS phenotyping platform was shown to be useful for collecting nondestructive measurements of GPA in sorghum. Estimates of GPA were consistent over time and compared favorably with estimates of total leaf area calculated using ImageJ software and total dry weight for insect feeding damage. Unlike destructive measurements of insect feeding based on changes in dry weight and leaf area, the ARIS platform was useful for taking nondestructive measurements of the same plant over a time period when the insects were feeding. The platform was able to detect a decrease in leaf area compared to the non-infested plants due to feeding within and along the leaf margins. This image-based technique also avoids challenges with subjective ratings, provides for measurements at multiple time points, and is quick and easy to use. The opportunity for taking multiple readings per plant was particularly useful for measuring plant-insect interactions among genotypes. This provided unique insights into the feeding patterns of the insects throughout the infestation period.

FAW is generally a pest of sorghum at later stages of development when the whorl has developed or the panicle has emerged (Diawara et al. 1990). In this study, sorghum plants were infested at earlier stages of development to study FAW feeding preference when dhurrin content is much higher than observed at later stages of development. Wiseman and Gourley (1982) showed that seedling plants have consistent patterns of resistance at the whorl stage and can be used to study patterns of susceptibility and resistance to FAW feeding. Comparisons of infested and non-infested plants demonstrated significant differences in GPA and dry weight with infested plants consistently smaller than the non-infested plants in each round of the experiment. The infested plants were consistently smaller due to FAW feeding. Interactions between genotypes and infestation treatments provided evidence for differences in susceptibility to FAW feeding. FAW feeding studies in greenhouse trials produced plant damage more quickly than in field studies. This could be due to a more consistent and favorable environment for plant and insect development within the greenhouse. Rain, wind, cool nights, and predators such as birds may have slowed FAW feeding damage in the field studies.

NILs contrasting for *bmr6* responded to the FAW feeding in field and greenhouse trials with a pattern of response to FAW infestation similar to studies described by Dowd and Sattler (2015). The wild-type and *bmr6* plants had similar dry weights in the infested and non-infested treatments at 96 h. These observations suggest that the plants were growing at a similar rate and with similar FAW feeding over time. NILs contrasting for *cyp79a1* exhibited a different pattern of plant growth and feeding preference. The *cyp79a1* plants generally produced higher GPA and dry weights under non-infested conditions compared to wild type plants in greenhouse and field trials. This observation suggests that disruption of dhurrin biosynthesis releases finite carbon and N pools to support a higher growth rate of vegetative tissues. This differs from what was reported for a P414L mutation in the CYP79A1, totally cyanide deficient mutant1, that had reduced growth (Blomstedt et al. 2012) and altered flowering times (Sohail et al. 2020). The pattern of plant growth shifted after infestation by FAW. The infested *cyp79a1* plants were consistently smaller than the non-infested plants and showed the largest reduction in GPA and dry weight over time following infestation by FAW. This demonstrates that the wild-type plants were more resistant to insect feeding than dhurrin-free plants. Tattersall et al. (2001) noted an increase in insect resistance when introducing dhurrin biosynthesis into Arabidopsis. Krothapalli et al. (2013) showed that sorghum plants having reduced capacity to release HCN due to a genetic mutation in *dhurrinase2* exhibited greater susceptibility to FAW. In both cases, the insect species had chewing mouthparts that are necessary to release HCN during feeding.

Manipulation of dhurrin metabolism may provide a strategy to increase sorghum growth rate when infestations of insects such as FAW are controlled; however, dhurrin metabolism is complex and reported to play a role in NUE (Neilson et al. 2015; Blomstedt et al. 2018; Rosati et al. 2019) and drought tolerance (Burke et al. 2013; Adeyanju et al. 2016; Hayes et al. 2016; Varoquaux et al. 2019). Follow-up studies are needed to examine the metabolic tradeoffs that influence sorghum productivity and sustainability with variable N and water inputs.

2.6 Conclusions

Dhurrin metabolism plays an important role in sorghum resistance to FAW feeding. Given the tremendous flux of carbon and N through this metabolic hub, dhurrin production also influences plant growth and development. This study demonstrated that a genetic mutation that disrupts

dhurrin biosynthesis also positively impacts plant growth and development in non-infested growing conditions. Taken together, these studies reveal a significant metabolic tradeoff between CG biosynthesis and plant growth in sorghum seedlings. These results suggest that it may be possible to manipulate dhurrin metabolism to optimize sorghum productivity in diverse production environments.

CHAPTER 3. EXPRESSION OF STAY-GREEN IN DHURRIN-FREE SORGHUM

3.1 Abstract

Sorghum (Sorghum bicolor L. (Moench)) is naturally adapted to water-limited and low-nitrogen environments. In this study, a genetic mutation in CYP79A1, the first step in the biosynthetic pathway of dhurrin, was used to study the role of dhurrin accumulation in stay-green drought tolerance of sorghum. Near-isogenic lines (NIL), near-isogenic backcross (NIBC) populations and near-isogenic hybrids (NIH) were evaluated for differences in stay-green drought tolerance in controlled and field-testing environments. Chlorophyll concentration (CC) and normalized vegetative differential index (NDVI) were measured by proximal sensing and used to quantify variation in stay-green among genotypes. Comparisons of NILs in Tx623 *bmr6*, and NIBC progeny from the stay-green donor Tx642 indicated differences in CC and NDVI between plants in the drought and well-watered treatments but no significant differences between NILs and NIBC within treatments. Analyses of NIHs in controlled environment and field trials demonstrated similar patterns with some evidence for a genetic background interaction effect. One set of NIHs exhibited variation in CC and NDVI in the drought plants within the field. Taken together, these studies indicate that dhurrin production may contribute to the expression of stay-green, but the effects are small and genotype specific.

3.2 Introduction

Sorghum (Sorghum bicolor L. (Moench) is an important grain and forage crop that is naturally adapted to water-limited and low-nitrogen environments (Sanchez et al., 2002; Borrell and Hammer, 2000). Geneticists and plant breeders have worked for decades to improve the adaptation and performance of this crop under environmentally stressful and variable conditions (Rosenow et al., 1983). The stay-green trait is one of the most important adaptation traits of sorghum (Rosenow et al., 1983; Borrell et al., 2000; Varoquaux et al., 2019). The stay-green phenotype is the ability of a plant to maintain green leaves and stalks under post-flowering drought stresses (Borrell et al., 2001). Stay-green genotypes remain photosynthetically active longer under drought conditions after flowering in comparison to senescent varieties leading to higher grain and forage yields

(Borrell et al., 2000; van Oosterom et al., 2010; Thomas and Ougham, 2014). Outside of increased yields, stay-green also has been shown to help increase resistance to stalk rots and lodging (Rosenow et al., 1983; Thomas and Ougham, 2014; Adeyanju et al., 2016), both of which are a concern during the grain fill period.

Genetic mapping studies have shown that stay-green exhibits oligogenic inheritance including four major QTL that have been identified in multiple populations (Tuinstra et al., 1998; Crasta et al., 1999; Subudhi et al., 2000; Tao et al., 2000; Xu et al., 2000). These loci influence plant nitrogen status (Harris et al., 2006), leaf size and tiller number (Borrell et al., 2014), as well as root architecture (Mace et al. 2012); however, candidate genes for stay-green are still poorly understood. Burke et al. (2013) observed that the expression of stay-green under post-flowering stress was strongly correlated with dhurrin accumulation at the seedling stage. Follow-up studies suggested that dhurrin functions as an osmoprotectant involved in stay-green expression (Burke et al., 2015; Varoquaux et al., 2019). Hayes et al. (2016) showed that genes for dhurrin biosynthesis co-segregate with the stay-green drought tolerance trait.

Although numerous studies have reported that dhurrin metabolism contributes to stay-green drought tolerance and nitrogen metabolism (Burke et al., 2013; Hayes et al., 2016; Adeyanju et al., 2016; Blomstedt et al., 2018; Rosati et al., 2019), it is not clear how this metabolic hub contributes to environmental sustainability in variable environments. During the grain-filling period, the grain acts as a nitrogen (N) sink, pulling N from vegetative plant parts, including stems, rachis, and the leaves. This occurs when the N-demand is larger than the N being absorbed by the roots from the soil (van Oosterom et al., 2010). Leaf N content is correlated with RUBISCO content (Ookawa et al., 2004), and RUBISCO is necessary for photosynthesis. Leaf senescence begins when proteins, such as RUBISCO, are broken down as a source of N for grain development (Borrell and Hammer, 2000). Dhurrin may provide a source of N that delays the onset of leaf senescence.

Chemical mutagenesis of sorghum with ethyl methanesulfonate (EMS) has been used to create mutations in genes involved in dhurrin metabolism (Blomstedt et al., 2012; Krothapalli et al., 2013; Skelton, 2014; Tuinstra et al., 2016;). These mutations provide valuable tools for studying the role of dhurrin in biotic and abiotic stress tolerance (Blomstedt et al., 2012; Krothapalli et al., 2013; Neilson et al., 2013; Blomstedt et al., 2018; Rosati et al., 2019; Gruss and Tuinstra, 2021). In this

study, near-isogenic sorghum lines and hybrids with contrasting dhurrin production characteristics were used to study the impact of dhurrin accumulation on expression of the stay-green trait. Specific objectives included comparisons of (1) near-isogenic lines (NILs), (2) near-isogenic progeny (NIPs) from backcross populations, and (3) near-isogenic hybrids (NIHs) with contrasting dhurrin accumulation patterns for differences in stay-green and post-flowering drought stress tolerance in controlled environment and field trials.

3.3 Materials and Methods

3.3.1 Genetic Material

Sets of NILs, NIBC progeny, and NIHs with contrasting dhurrin production characteristics were developed to test hypotheses about the relationship between dhurrin and stay-green drought tolerance using a C493Y mutation in the CYP79A1 enzyme that completely disrupts dhurrin biosynthesis (Tuinstra et al., 2016). The C493Y mutation in CYP79A1 allele (*cyp79a1*) was created by chemical mutagenesis using EMS treatment of BTx623 seeds followed by selection using the Feigl Anger (FA) assay to select for dhurrin free plants (Feigl and Anger, 1966).

Sets of NILs were developed in the B Tx623, B Tx399, Excel S238 and MR732 genetic backgrounds (Table 2.1) A/B Tx623 *bmr6 cyp79a1* were developed by crossing and backcrossing B Tx623 *bmr6* (Oliver et al., 2006; Pedersen et al., 2006) to B Tx623 *cyp79a1* with selection for dhurrin production using the FA assay to produce B 17WL1690. B 17WL1690 is a Tx623 *bmr6 cyp79a1* type that does not accumulate dhurrin (Table 2.1). A 17WL1690 was developed by backcrossing B 17WL1690 to A Tx623 followed by at least four generations of backcrossing.

A/B Tx399 *bmr6 cyp79a1* was developed by crossing and backcrossing B Tx623 *cyp79a1* to B Tx399 *bmr6* (Oliver et al., 2006; Pedersen et al., 2006) with selection for dhurrin production using the FA assay to produce B 16/17GH1. B 16/17GH1 is a Tx399 *bmr6 cyp79a1* type that does not accumulate dhurrin (Table 2.1). A 16/17WL1 was developed by backcrossing B 16/17GH1 to A Tx399 followed by at least four generations of backcrossing.

Excel S235 is an expired PVP sudan-type pollinator that is used to make sorghum x sudan hybrids for forage production. Excel S235 was crossed to a plant from a heterogeneous R-line breeding population segregating for the *cyp79a1* and *bmr6* alleles followed by pedigree breeding with

selection for dhurrin production using the FA assay to produce Excel S235 *bmr6 cyp79a1 bmr6 cyp79a1*. Excel S235 *bmr6 cyp79a1* is a sudan-type pollinator parent with narrow leaves and thin stems (Table 2.1). MR732 is a caudatum-type sorghum pollinator that produces tall, silage-type sorghum hybrids. MR732 was crossed and backcrossed to a plant from a heterogeneous R-line breeding population segregating for the *cyp79a1* alleles followed by pedigree breeding with selection for dhurrin production using the FA assay to produce MR732 *cyp79a1*.

Tx642 (B35) represents a key source of stay-green for global sorghum breeding programs (Tao et al. 2000; Harris et al. 2007). The cyp79a1 mutation is also being incorporated into BTx642, the most important stay-green trait donor in sorghum. Tx642 was derived from IS12555, a durra sorghum from Ethiopia (Xu et al. 2000; Sanchez et al. 2002). Tx642 exhibits high dhurrin production characteristics at the seeding stage (Burke et al., 2013). Tx642 was crossed and backcrossed to Tx623 *cyp79a1* to produce a population of backcross progeny (BC1F2) segregating for dhurrin production in the Tx642 genetic background (Table 2.1). BC1F2 plants with contrasting dhurrin production characteristics were identified by FA testing.

Sets of near-isogenic lines, backcross progeny, and hybrids with contrasting dhurrin production characteristics were developed to test hypotheses about the relationship between dhurrin and stay-green drought tolerance using a C493Y mutation in CYP79A1 that completely disrupts dhurrin biosynthesis (Tuinstra et al., 2016). Near-isogenic hybrids were developed by intercrossing sets of A-lines and R-lines with contrasting dhurrin production characteristics with R-lines (Table 3.1). Excel S235 *bmr6 cyp79a1* was crossed to A Tx623 and A Tx623 *bmr6 cyp79a1* to produce NIHs with contrasting brown-mid rib and dhurrin production characteristics. Excel S235 *bmr6 cyp79a1* was also crossed to A Tx399 and A Tx399 *bmr6 cyp79a1* to produce second set of NIHs with contrasting brown-mid rib and dhurrin production characteristics. MR732 *cyp79a1* was crossed to A Tx623 and A Tx623 *bmr6 cyp79a1* to produce NIHs with contrasting dhurrin production characteristics. MR732 *cyp79a1* was also crossed to A Tx399 and A Tx399 *bmr6 cyp79a1* to produce second set of NIHs with contrasting brown-mid rib and dhurrin production characteristics (Table 3.1).

Table 3.1: Near-isogenic sorghum parent lines, backcross progeny, and hybrids

Type	Genotype	Source	***Brown midrib***	***Dhurrin***	Gen
NILs	BTx623	BTx623	*No*	*Yes*	Inbred
	ATx623	ATx623	*No*	*Yes*	Inbred
	BTx623 *bmr6*	BTx623 *bmr6*	*Yes*	*Yes*	Inbred
	BTx623 *bmr6 cyp79a1*	B17WL1690 *bmr6 cyp79a1*	*Yes*	*No*	BC1F6
	ATx623 *bmr6 cyp79a1*	A17WL1690 *bmr6 cyp79a1*	*Yes*	*No*	BC4
	BTx399	BTx399	*No*	*Yes*	Inbred
	ATx399	ATx399	*No*	*Yes*	Inbred
	BTx399 *bmr6 cyp79a1*	B16/17GH1 *bmr6 cyp79a1*	*Yes*	*No*	BC1F6
	ATx399 *bmr6 cyp79a1*	A16/17GH1 *bmr6 cyp79a1*	*Yes*	*No*	BC4
	Excel S235 *bmr6 cyp79a1*	17WL1525 *bmr6 cyp79a1*	*Yes*	*No*	F8
	MR732 *cyp79a1*	17WL1821 *cyp79a1*	*No*	*No*	BC1F6
NIBC	Tx642 *cyp79a1*	18WL9082-1	*No*	*Segregating*	BC1F2
NIH	(ATx623 x Excel S235)-F1	(ATx623 x 17WL1525)-F1	*No*	*No*	F1
	(ATx623 x Excel S235)-F1 *bmr6 cyp79a1*	(A17WL1690 x 17WL1525)-F1 *bmr6 cyp79a1*	*Yes*	*Yes*	F1
	(ATx399 x Excel S235)-F1	(ATx399 x 17WL1525)-F1	*No*	*No*	F1
	(ATx399 x Excel S235)-F1 *bmr6 cyp79a1*	(A16/17GH1 x 17WL1525)-F1 *bmr6 cyp79a1*	*Yes*	*Yes*	F1
	(ATx623 x MR732)-F1	(ATx623 x 17WL1821)-F1	*No*	*No*	F1
	(ATx623 x MR732)-F1 *cyp79a1*	(A17WL1690 x 17WL1821)-F1 *cyp79a1*	*No*	*Yes*	F1
	(ATx399 x MR732)-F1	(ATx399 x 17WL1821)-F1	*No*	*No*	F1
	(ATx399 x MR732)-F1 *cyp79a1*	(A16/17GH1 x 17WL1821)-F1 *cyp79a1*	*No*	*Yes*	F1

3.3.2 **Controlled Environment Studies**

Controlled environment trials were conducted within greenhouses located at Purdue University and in growth chambers in Purdue's Ag Alumni Seed Phenotyping Facility (AAPF) at Purdue University, West Lafayette, IN. These trials utilized similar methods and experiment designs. Pots were filled with a 1:1 mixture of Profile Porous Ceramic Greens Grade (Buffalo Grove, IL) and Metro-Mix® 360 from Sun Gro (Agawam, MA) for a total of six liters of the soil mixture per pot. Seeds were planted into plastic cone-tainers (Hummert International, St. Louis, MO) in Sun Gro propagation mix (Agawam, MA) for seedling germination. Uniform plants were selected after ten to twelve days of growth and transplanted into the larger pots and placed into the controlled environments. After two weeks in the controlled environment, plants were staked to maintain upright growth.

The first greenhouse study examined differences in plant growth and post-flowering drought tolerance of NILs Tx623 *bmr6 cyp79a1* and Tx623 *bmr6.* The experiment utilized a completely randomized design with six replications. In first round, the plants were maintained at saturation on a pot weight basis until flowering and fertilized with 90 mg kg^{-1} N. The flowering date for each plant was recorded as days after sowing (DAS). After flowering, drought treatments were applied to half of the plants while the remaining plants were maintained with full irrigation. The drought treatment was 10% volumetric water content (VWC) of the soil and fully irrigated plants were maintained at 35% VWC or higher. Each day, soil moistures for a sample of drought and irrigated treatment plants were measured using HydroSense II handheld soil moisture probe (Campbell Scientific, Logan, UT) with irrigation applied as needed to maintain the target VWC. On day three of the drought treatment, chlorophyll concentration index (CCI) was measured on each plants using an Apogee MC-100 Chlorophyl Concentration Meter (Apogee Instruments, Logan, UT). CCI for each plant was measured every other day for two weeks with measurements of leaf 1, 3, and 5 counting from the top. A SVC HR1042i handheld leaf clamp (Spectra Vista Corporation, Poughkeepsie, NY) was used to measure spectral reflectance between 338-2,521 nm with 1,024 channels and calculate normalized differential vegetative index (NDVI) greenness on leaf 1 and 4 counting from the top of the plant. After 14 days, the plants in the drought treatment exhibited severe symptoms of drought stress and were hand harvested to determine fresh weight (FW)

biomass (g). The fresh plant material was then dried at 60℃ to determine dry weight (DW) biomass (g).

The second greenhouse study examined differences in plant growth and post-flowering drought tolerance of NIBC progeny with contrasting dhurrin production characteristics from Tx642. Comparisons of NIBC representing wild-type and *cyp79a1* progeny were conducted in the same manner as the first experiment of the NILs. All the plants were watered to saturation until flowering. Flowering dates were determined for each plant. Drought treatments were imposed after flowering with drought treatments maintained at 10% VWC and fully irrigated plants maintained at >35% VWC. Measurements of CCI, NDVI, percent moisture and dry biomass (g) were recorded as described for the first study.

The third controlled environment study examined differences in plant growth and post-flowering drought tolerance of NIHS in growth chamber studies conducted in the Ag Alumni Seed Phenotype Facility using NIHs. The experiment included four hybrids, (ATx623 x Excel)-F1 *bmr6 cyp79a1*, (ATx623 x Excel)-F1, (ATx623 x MR732)-F1 *cyp79a1*, and (ATx623 x MR732)-F1, evaluated under post-flowering drought and fully irrigated treatments using a completely randomized design with eleven replications. The soil in the pots and planting protocol were similar to the previously described experiments. All the plants were well watered until flowering. When one-half the plants were flowering, drought treatments were applied using a weight-based system to maintain a 12.5% VWC for the drought treatment and >35% VWC for the fully irrigated pots. CCI was collected each week on leaf 1 and leaf 4 counting from the top collared leaf. At 1, 5, 12, 26, and 35 d after initiating the drought treatments, plants were imaged using a side-scan hyperspectral camera imaging the widest side of each plant. NDVI was calculated from the images on a whole plant basis. The hyperspectral camera was a Middleton MSV 500 spectral camera (Middleton, WI) with a range from 400 to 1000nm with a spectral resolution of 1.2 nm. Every two weeks some plants were destructively harvested to measure percent moisture and dry biomass (g) on a plant basis for a total of six weeks. Four replications after two and four weeks were harvested, with the remaining three replications being harvested at six weeks.

3.3.3 **Field Trials**

The field location was planted on June 10, 2019 at a population of 24,711 plants hectare^{-1}. The Kearney Agricultural Research and Extension Center (KARE) in Parlier, CA. The site has Hanford fine sandy loam soils with silty substratum (Soil Survey Staff, Natural Resources Conservation Service, and Department of Agriculture n.d.). The plants were planted in two row plots that were 6.1 m long with 1.5 m alleys, and 0.76 m row spacing. There were eight hybrids with five replications, and two treatments laid out in a split plot design, split between drought and fully irrigated (Figure 3.1).

Fully Irrigated

Row \ Range	1	2	3	4	5	6	7	8	9	10	11	12
1	B	B	B	B	B	B	B	B	B	B	B	B
2	B	B	B	B	B	B	B	B	B	B	B	B
3	B	B	1001	1006	1011	1016	1021	1026	1031	1036	B	B
4	B	B	1001	1006	1011	1016	1021	1026	1031	1036	B	B
5	B	B	1002	1007	1012	1017	1022	1027	1032	1037	B	B
6	B	B	1002	1007	1012	1017	1022	1027	1032	1037	B	B
7	B	B	1003	1008	1013	1018	1023	1028	1033	1038	B	B
8	B	B	1003	1008	1013	1018	1023	1028	1033	1038	B	B
9	B	B	1004	1009	1014	1019	1024	1029	1034	1039	B	B
10	B	B	1004	1009	1014	1019	1024	1029	1034	1039	B	B
11	B	B	1005	1010	1015	1020	1025	1030	1035	1040	B	B
12	B	B	1005	1010	1015	1020	1025	1030	1035	1040	B	B
13	B	B	B	B	B	B	B	B	B	B	B	B
14	B	B	B	B	B	B	B	B	B	B	B	B

Drought Treatment

Row \ Range	1	2	3	4	5	6	7	8	9	10	11	12
1	B	B	B	B	B	B	B	B	B	B	B	B
2	B	B	B	B	B	B	B	B	B	B	B	B
3	B	B	1041	1046	1051	1056	1061	1066	1071	1076	B	B
4	B	B	1041	1046	1051	1056	1061	1066	1071	1076	B	B
5	B	B	1042	1047	1052	1057	1062	1067	1072	1077	B	B
6	B	B	1042	1047	1052	1057	1062	1067	1072	1077	B	B
7	B	B	1043	1048	1053	1058	1063	1068	1073	1078	B	B
8	B	B	1043	1048	1053	1058	1063	1068	1073	1078	B	B
9	B	B	1044	1049	1054	1059	1064	1069	1074	1079	B	B
10	B	B	1044	1049	1054	1059	1064	1069	1074	1079	B	B
11	B	B	1045	1050	1055	1060	1065	1070	1075	1080	B	B
12	B	B	1045	1050	1055	1060	1065	1070	1075	1080	B	B
13	B	B	B	B	B	B	B	B	B	B	B	B
14	B	B	B	B	B	B	B	B	B	B	B	B

Figure 3.1: Experiment design for the field location (B=border). They are two row plots with each row labeled with the plot number, and each range is 6.1 m. The colors represent the five different replications.

All the plants had sufficient water through furrow irrigation at the KARE location until half the plants were flowering. Flowering notes were collected on a plot basis based on DAS. The drought treatment was applied on August 13, 2019. Irrigation was turned off for the drought treatments, while furrow irrigation remained consistent for the well-watered plants till harvest. At the KARE site, there was no recorded rain during the drought treatment, with the average temperatures recorded as a high of 35 °C and low of 17 °C ("Weather, Physical & Biological Data" 2020).

On August 13 and 33 days later on September 16, four plants from each of the plots were measured for CCI on the top collared leaf and fourth leaf counting from the top of the plant down, for a total of eight measurements per plot. Two plants from each plot were measured using the HR1024i, using the first leaf and the fourth leaf counting from the top down, a total of four measurements per plot. NDVI was calculated from the wavelengths, as described in the greenhouse using the NILs. Due to some sampling constraints on August 13, there was only two replications in the fully irrigated treatment, causing an unbalanced design for the spectrometer data. Biomass information was determined using a biomass harvester on a h^{-1} basis. The moisture content of the plots was also determined.

Lastly, CCI does not have a linear relationship with CC, because of leaf structure, such as the thickness or thinness of the leaves, and the stacking of the chlorophyll within the leaf. Using the equation CC=$-8 + 29(CCI)^{0.80}$, the CC in absolute units (μmol m^{-2}) for sorghum was calculated (Parry, Blonquist, and Bugbee 2014) for all the trials and used for comparisons.

3.3.4 **Statistical Analysis**

Data was first examined using boxplots to understand the trends and potential outliers in the data. To identify the outliers and influential data points four times Cook's distance from the average was used in combination with boxplots to identify the outliers within the datasets. The identified outliers were removed from the datasets.

Next, the data was examined for normality and equal variances. Not all the data fit the assumption of normality and equal variance. The NDVI data and moisture data from CA was not normally distributed and both were transformed using a square root transformation. To account for unequal variances there were two types of mixed effects models built using R studio. The first was a mixed

effects model with assumption of equal variances, and the second was a linear mixed effects model that accounted for variation in variances for a specific factor.

Both models were built to examine the effects the drought treatment had on the genotypes and hybrids. The fixed effects were the treatments, genotypes/hybrids, the days of drought and their interactions, while the explanatory variables included CC, NDVI, biomass, and moisture content. Within the greenhouse the random effects were on the plant basis, while in the field the plots were the random effects, because in the greenhouse multiple measurements were taken consistently on the same plant. While in the field it was analyzed on plot basis.

The second model was set up as described above with an extra descriptor, the factor to account for the variance differences, generally variation in variances was due to days after the drought or the genotype.

Comparisons were made using Tukey's post hoc test with a p-value < 0.05 for significance.

3.4 Results

3.4.1 Near-Isogenic Lines

Comparisons of NILs with contrasting dhurrin production characteristics in controlled environment trials indicated no significant differences in flowering date with BTx623 *bmr6* flowering 96 ± 3 DAS and BTx623 *bmr6 cyp79a1* flowered 98 ± 4 DAS, (p-value < 0.05). Analyses of CC in these trials demonstrated significant differences between drought treatments, days of drought, and the interaction drought treatment x days of drought. Further analyses of the interaction effects showed that all plants exhibited similar CC values at the start of the drought treatments but CC decreased over time in plants in the post-flowering drought treatment while exhibiting little change in the fully irrigated treatment (Table 3.2). Comparisons of NILs within each of the drought treatments indicated no significant differences in response between Tx623 *bmr6 cyp79a1* and Tx623 *bmr6* in either of the drought treatment groups (Table 3.2).

Table 3.2: Variation in CC of near isogenic sorghum lines with contrasting dhurrin biosynthesis characteristics in the Tx623 bmr6 genetic background under post-flowering drought and fully irrigated conditions in controlled environment trials.

		CC μmol m^{-2} Days after Drought													
Treatment	Genotype	0		3		5		7		9		11		13	
Drought	Tx623 *bmr6* *cyp79a1*	527	a	306	a	219	a	170	a	194	a	124	a	153	a
	Tx623 *bmr6*	564	a	340	a	250	a	166	a	172	a	104	a	128	a
Full Irrigation	Tx623 *bmr6* *cyp79a1*	544	a	529	a	530	b	495	b	481	b	438	b	516	b
	Tx623 *bmr6*	578	a	539	a	536	b	550	b	525	b	521	b	566	b

A similar pattern of expression for treatments and genotypes was observed for the NDVI phenotype. NDVI values were similar for plants in each treatment at the onset of the post-flowering drought period but NDVI was significantly reduced in drought stressed plants after 13 days of treatment (Table 3.3). Tx623 *bmr6 cyp79a1* and Tx623 *bmr6* responded similarly within each of the drought treatments and no significant differences were detected between NILs within treatments (Table 3.3). The NILs also exhibited similar moisture and dry weights in each drought treatment (Table 3.3) except Tx623 *bmr6 cyp79a1* was slightly larger than Tx623 *bmr6* in the full irrigation treatment (Table 3.3).

Table 3.3: Variation in NDVI, moisture contents, and dry weights of near isogenic sorghum lines with contrasting dhurrin biosynthesis characteristics in the Tx623 bmr6 genetic background under post-flowering drought and fully irrigated conditions in controlled environment trials.

		NDVI		Moisture (%)	DW (g)
Treatment	Genotype	0 days	13 days	13 days	13 days
Drought	Tx623 *bmr6 cyp79a1*	0.65 a	0.18 a	59 a	195 a
	Tx623 *bmr6*	0.68 b	0.13 a	55 a	199 a
Full Irrigation	Tx623 *bmr6 cyp79a1*	0.65 ab	0.70 b	68 b	271 b
	Tx623 *bmr6*	0.68 b	0.65 b	69 b	217 a

3.4.2 **Near-Isogenic Backcross**

Analysis of NIBC progeny in the Tx642 background showed that the wild-type plants flowered slightly later at 102 ± 5 DAS than *cyp79a1* plants at 96 ± 4 DAS (p-value < 0.05). Analyses of CC in these NIBCs indicated significant effects due to drought treatment, days of drought, drought treatment x days of drought interaction, the interaction genotypes x days of drought and the interaction between genotype, drought treatment and days of drought. CC was relatively stable in plants representing the fully irrigated treatment, but CC declined rapidly in plants representing the drought treatments (Table 3.4). Although significant differences in CC were noted between different drought treatments, the NIBCs did not exhibit any significant differences in CC at any time point in either drought treatment (Table 3.4).

Table 3.4: Variation in CC of near isogenic sorghum lines with contrasting dhurrin biosynthesis characteristics in the Tx642 bmr6 genetic background under post-flowering drought and fully irrigated conditions in controlled environment trials.

		CC μmol m^{-2} Days after Drought													
Treatment	Genotype	0		3		5		7		9		11		13	
Drought	Tx642-BC1F2 *cyp79a1*	620	a	530	a	383	a	320	a	303	a	283	a	215	a
	Tx642-BC1F2	533	a	424	a	394	a	365	a	332	a	367	a	320	a
Full Irrigation	Tx642-BC1F2 *cyp79a1*	584	a	539	a	577	b	524	b	558	b	543	b	561	b
	Tx642-BC1F2	567	a	530	a	563	b	541	b	570	b	566	b	583	b

Analyses of NDVI demonstrated a very similar pattern of expression in the NIBC progeny (Table 3.5) Significant effects was days of drought, treatment x days of drought interaction, and genotype x days of drought interaction effects were detected for NDVI. At the beginning of the drought treatments, the NIBC progeny exhibited similar NDVI across genotypes and treatments (Table 3.5). After 13 days, the NIBC progeny exhibited similar NDVI within treatments but significant differences across drought treatments with the drought treatment being 0.15 NDVI value lower in comparison to the fully irrigated plants. The NDVI values of plants in the drought treatment decreased by nearly one-half by day 13 while NDVI values of plants in the fully-irrigated treatments were similar on day 0 and day 13 (Table 3.5). The moisture content and dry biomass was also significantly lower in the drought treated plants. Comparisons of Tx642 NIBC progeny within treatments indicated no significant differences in NDVI, moisture contents, and dry weights of near isogenic sorghum lines with contrasting dhurrin biosynthesis characteristics except the Tx642 wild-type plants had a higher dry biomass in comparison to Tx642 *cyp79a1* within the fully irrigated treatment (Table 3.5).

Table 3.5: Variation in NDVI, moisture contents, and dry weights of near isogenic sorghum lines with contrasting dhurrin biosynthesis characteristics in the Tx642 genetic background under post-flowering drought and fully irrigated conditions in controlled environment trials.

		NDVI				%Moisture		DW (g)	
		Days After Drought							
Treatment	Genotype	0		13		13		13	
Drought	Tx642-BC1F2 *cyp79a1*	0.71	a	0.41	a	0.58	a	192	a
	Tx642-BC1F2	0.69	a	0.55	ab	0.59	a	225	ab
Full Irrigation	Tx642-BC1F2 *cyp79a1*	0.72	a	0.42	b	0.71	b	246	b
	Tx642-BC1F2	0.69	a	0.72	b	0.71	b	312	c

3.4.3 **Near-Isogenic Hybrids**

Two sets of NIHs were evaluated for differences in CC under drought and fully irrigated conditions in the Ag Alumni Seed Phenotyping Facility. (ATx623 x Excel)-F1 *bmr6 cyp79a1* and (ATx623 x Excel)-F1 plants expressed similar CC values in the drought and fully irrigated treatments with the wild-type plants exhibiting slightly higher CC values than the dhurrin-free mutant plants on day 1 in the drought treatment (Table 3.6). The CC values declined rapidly over time in the drought stressed plants with significant differences between plants in the drought and fully irrigated treatments by day 7 (Table 3.6). These differences between treatments became less significant as the drought persisted with (ATx623 x Excel)-F1 *bmr6 cyp79a1* and (ATx623 x Excel)-F1 plants exhibiting similar CC values across treatments by day 35. Analysis of CC in the other set of NIHs demonstrated a similar pattern of expression (Table 3.6). CC declined rapidly over time in the drought treated plants. Comparisons of CC in (ATx623 x MR732)-F1 *cyp79a1* and (ATx623 x MR732)-F1 detected a numerical difference in CC at day 16 in the drought treated plants with the wild-type plants exhibiting higher CC scores than the dhurrin free mutant (Table 3.6). This difference would be significant when using a p-value < 0.10. This difference in CC between NIHs in the drought treatment declined on day 21 and even more on day 35.

Table 3.6: Variation in CC of two sets of near isogenic sorghum hybrids with contrasting dhurrin biosynthesis characteristics under post-flowering drought and fully irrigated conditions in controlled environment trials.

		CC µmol m^{-2} Days after Drought									
Treatment	Hybrids	1		7		14		21		35	
Drought	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	419	a	308	a	277	a	203	a	135	a
	(ATx623 x Excel)-F1	497	ab	234	a	196	a	148	a	100	a
Full Irrigation	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	433	ab	431	b	361	ab	337	b	213	a
	(ATx623 x Excel)-F1	507	b	484	b	458	b	388	b	295	a
Drought	(ATx623 x MR732)-F1 *cyp79a1*	582	a	404	a	350	a	342	a	235	a
	(ATx623 x MR732)-F1	575	a	470	a	442	ab	411	a	283	a
Full Irrigation	(ATx623 x MR732)-F1 *cyp79a1*	591	a	584	b	546	bc	473	ab	395	ab
	(ATx623 x MR732)-F1	592	a	591	b	596	c	525	b	458	b

NDVI was also used to quantify variation in greenness of NIHs in the controlled environment trials (Table 3.7). Comparisons of (ATx623 x Excel)-F1 *bmr6 cyp79a1* and (ATx623 x Excel)-F1 indicated significant differences in NDVI between plants in the drought and fully irrigated treatments at 5, and 12 days after drought but these differences disappeared at 26 days after drought. At five days there was a slight variation between the NIHs in the drought treatment with the dhurrin-free line remaining greener than the wild-type, but this variation dissipated by day 12.The NIHs (ATx623 x MR732)-F1 *cyp79a1* and (ATx623 x MR732)-F1 displayed a decline in NDVI within the drought plants in comparison to the fully irrigated treatments at 5 and 12 days after drought but these differences disappeared at 26 days after drought (Table 3.7).

Table 3.7: Variation in NDVI of two sets of near isogenic sorghum hybrids with contrasting dhurrin biosynthesis characteristics under post-flowering drought in controlled environment trials. Comparisons were made on each day between NIHs, across treatments.

		NDVI Days after Drought									
Treatment	Hybrids	1		5		12		26		35	
Drought	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	0.69	a	0.53	a	0.53	a	0.57	a	0.48	a
	(ATx623 x Excel)-F1	0.67	a	0.48	b	0.54	a	0.54	a	0.45	a
Full Irrigation	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	0.68	a	0.65	c	0.60	b	0.58	a	0.52	a
	(ATx623 x Excel)-F1	0.68	a	0.65	c	0.61	b	0.59	a	0.51	a
Drought	(ATx623 x MR732)-F1 *cyp79a1*	0.69	a	0.52	a	0.53	a	0.58	a	0.50	a
	(ATx623 x MR732)-F1	0.71	a	0.55	a	0.54	a	0.61	a	0.52	a
Full Irrigation	(ATx623 x MR732)-F1 *cyp79a1*	0.70	a	0.68	b	0.64	b	0.64	a	0.51	a
	(ATx623 x MR732)-F1	0.70	a	0.68	b	0.66	b	0.63	a	0.51	a

Destructive harvesting at the end of the controlled environment study indicated no difference in moisture content between drought treatments or NIHs (data not shown). Dry weights of (ATx623 x Excel)-F1 *bmr6 cyp79a1* and (ATx623 x Excel)-F1 were similar at 14 d after drought but the drought treated plants were smaller than plants in the fully irrigated treatment at 21 d and 35 d after drought (Table 3.8). Comparisons of (ATx623 x MR732)-F1 *cyp79a1* and (ATx623 x MR732)-F1 indicated a similar trend with similar dry weights at 14 d after drought but the drought treated plants accumulated less biomass than plants in the fully irrigated treatment at 21 d and 35 d after drought (Table 3.8). No variation in dry weight were detected between NIHs within the drought or fully irrigated treatments (Table 3.8).

Table 3.8: Dry weights of near isogenic sorghum hybrids with contrasting dhurrin biosynthesis characteristics under post-flowering drought and fully irrigated treatments in growth chamber studies. Comparisons were made on each day between NIHs, across treatments

		DW (g) Days after Drought					
Treatment	Hybrids	14		21		35	
Drought	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	228	a	248	a	250	a
	(ATx623 x Excel)-F1	239	a	226	a	262	ab
Full Irrigation	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	266	a	357	b	348	b
	(ATx623 x Excel)-F1	274	a	355	b	334	ab
Drought	(ATx623 x MR732)-F1 *cyp79a1*	214	ab	254	a	238	a
	(ATx623 x MR732)-F1	168	a	196	a	228	a
Full Irrigation	(ATx623 x MR732)-F1 *cyp79a1*	247	b	363	b	378	b
	(ATx623 x MR732)-F1	241	ab	331	b	339	b

Analyses of the NIHs in managed drought field trials at the KARE facility indicated significant pedigree, treatment, days of drought, pedigree x treatment, treatment x days of drought and the interaction between all three factors. The pairs of NIHs in the drought and fully irrigated treatments exhibited similar CC values at the onset of the drought treatment with some variation in CC among NIHs representing different genetic backgrounds (Table 3.9). (ATx623 x Excel)-F1 *bmr6 cyp79a1* had the highest CC value at 708 µmol m^{-2} and (ATx399 x MR732)-F1 *cyp79a1* had the lowest CC value at 592 µmol m^{-2}. After 33 days of drought, the CC values of plants in the drought treatments were significantly lower than in plants in the fully irrigated treatment for each set of NIHs (Table 3.9). The fully irrigated plants exhibited relatively high CC values and did not vary much between the hybrids. The CC values of plants representing the drought treatment were lower and showed more variation among the pedigrees (Table 3.9). Most the pedigree pairs exhibited similar CC values in the irrigated and drought treatments; however, (ATx623 x MR732)-F1 *cyp79a1* and (ATx623 x MR732)-F1 exhibited similar CC values at the onset of the drought treatment but were significantly different after 33 days of drought treatment (Table 3.9). Although both hybrids exhibited a lower CC values than in the fully irrigated treatment, the wild-type hybrid exhibited a significantly higher CC value than the dhurrin-free hybrid for this set of NIHs (Table 3.9). Analyses of variation in NDVI showed that pairs of NIHs in the drought and fully irrigated treatments exhibited similar CC values on day 1. On day 33, the (ATx623 x MR732)-F1 *cyp79a1* and (ATx623 x MR732)-F1, NIHs showed differences between each other with the wild-type having a higher NDVI value in the drought treatment. The (ATx623 x MR732)-F1 hybrid did not show differences to the fully irrigated NIHs. (Table 3.9). Most plants in the drought treatments did not show a significant reduction in NDVI compared to plants sampled from NIHs in the fully irrigated treatments (Table 3.9).

Table 3.9: Variation in CC and NDVI of near isogenic sorghum hybrids with contrasting dhurrin production characteristics in managed-stress field trials at KARE. Comparisons were made on each day between NIHs, across treatments.

		Days after Drought							
		CC (µmol m^{-2})				NDVI [1]			
Treatment	Hybrids	1		33		1		33	
Drought	(ATx399 x Excel)-F1 *bmr6 cyp79a1*	646	a	521	a	0.76	a	0.67	a
	(ATx399 x Excel)-F1	636	a	523	a	0.76	a	0.63	a
Full Irrigation	(ATx399 x Excel)-F1 *bmr6 cyp79a1*	615	a	676	b	0.73	a	0.65	a
	(ATx399 x Excel)-F1	622	a	670	b	0.75	a	0.67	a
Drought	(ATx399 x MR732)-F1 *cyp79a1*	611	a	583	a	0.79	a	0.73	a
	(ATx399 x MR732)-F1	587	a	606	a	0.76	a	0.70	a
Full Irrigation	(ATx399 x MR732)-F1 *cyp79a1*	592	a	704	b	0.79	a	0.74	a
	(ATx399 x MR732)-F1	613	a	717	b	0.82	a	0.76	a
Drought	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	686	a	601	a	0.77	a	0.63	a
	(ATx623 x Excel)-F1	705	a	569	a	0.76	a	0.66	a
Full Irrigation	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	692	a	691	b	0.76	a	0.69	a
	(ATx623 x Excel)-F1	699	a	686	b	0.74	a	0.68	a
Drought	(ATx623 x MR732)-F1 *cyp79a1*	644	a	489	a	0.78	a	0.62	a
	(ATx623 x MR732)-F1	649	a	556	b	0.77	a	0.72	b
Full Irrigation	(ATx623 x MR732)-F1 *cyp79a1*	661	a	706	c	0.78	a	0.73	b
	(ATx623 x MR732)-F1	620	a	726	c	0.81	a	0.72	b

[1] *Values are based on the non-transformed data*

Assessment of agronomic traits in the managed drought field trials at the KARE facility indicated no variation in flowering time between pairs of NIHs (not shown). Destructive harvesting of biomass indicated significant variability between pedigrees and the treatments (Table 3.10). Hybrids from the drought treatment exhibited lower and often significantly lower moisture at harvest compared to plants in the fully irrigated treatment (Table 3.10). Some variation in biomass among hybrids was detected but no consistent pattern was noted (Table 3.10).

Table 3.10: Variation in moisture content and biomass yields of near isogenic sorghum hybrids with contrasting dhurrin production characteristics in managed-stress field trials at KARE. Comparisons were made on each day between NIHs, across treatments.

Treatment	Hybrids	% Moisture[1]		Yield (tons h^{-1})	
Drought	(ATx399 x Excel)-F1 *bmr6 cyp79a1*	68.4	a	10.0	a
	(ATx399 x Excel)-F1	64.3	a	14.0	ab
Full Irrigation	(ATx399 x Excel)-F1 *bmr6 cyp79a1*	72.5	b	14.0	ab
	(ATx399 x Excel)-F1	69.7	b	17.9	b
Drought	(ATx399 x MR732)-F1 *cyp79a1*	75.9	ac	12.6	a
	(ATx399 x MR732)-F1	70	b	14.4	a
Full Irrigation	(ATx399 x MR732)-F1 *cyp79a1*	79	d	12.0	a
	(ATx399 x MR732)-F1	77.5	c	11.1	a
Drought	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	61.1	a	8.6	a
	(ATx623 x Excel)-F1	62.5	a	12.4	ab
Full Irrigation	(ATx623 x Excel)-F1 *bmr6 cyp79a1*	71.9	b	10.1	ab
	(ATx623 x Excel)-F1	70.3	b	12.5	b
Drought	(ATx623 x MR732)-F1 *cyp79a1*	54.4	a	11.3	a
	(ATx623 x MR732)-F1	64.2	b	11.5	a
Full Irrigation	(ATx623 x MR732)-F1 *cyp79a1*	72.4	c	10.6	a
	(ATx623 x MR732)-F1	74.7	c	10.0	a

[1] *Values are based on the non-transformed data.*

3.5 Discussion

Stay-green drought tolerance is one of the most important adaptation traits of sorghum (Rosenow et al., 1983; Borrell et al., 2000; Varoquaux et al., 2019). The BTx642 germplasm source of the stay-green trait was widely used in sorghum breeding programs has transformed sorghum agriculture in many semi-arid regions of the globe. Stay-green genotypes remain photosynthetically active longer under post-flowering drought conditions in comparison to senescent varieties leading to higher grain and forage yields (Borrell et al., 2000; van Oosterom et al., 2010; Thomas and Ougham, 2014). Previous studies have shown a correlation between dhurrin content at the seedling stage and the ability of sorghum to stay-green under post-flowering drought stress conditions (Burke et al. 2013; Hayes et al. 2016). Dhurrin metabolism also plays an important role in host-plant resistance to insect feeding and nitrogen metabolism; however, it is not clear how this metabolic hub can be optimized to improve environmental adaptation of the crop (Krothapalli et al., 2013; Neilson et al., 2015; Hayes et al., 2016; Adeyanju et al., 2016; Rosati et al., 2019).

The central goal of this project was to explore trade-offs for manipulating dhurrin metabolism under post-flowering drought stress conditions. Based on earlier studies, it was hypothesized that dhurrin contributes to stay-green drought tolerance by providing a source of nitrogen to sorghum plants under drought stress conditions when nitrogen is often limiting (Ding et al. 2018). A genetic mutation that disrupts dhurrin biosynthesis was used to test this hypothesis by evaluating sets of near isogenic lines, populations, and hybrids under post-flowering drought conditions. If dhurrin metabolism contributes to the expression of stay-green, disruption of dhurrin biosynthesis should result in more senescent plants that could be identified by decreases in greenness and CC.

One set of NILs in the Tx623 *bmr6* background were assessed for differences in stay-green drought tolerance in controlled environment studies. Although the drought treatments had a significant impact on plant greenness measured by CC or NDVI and plant growth measured by biomass, few differences in CC or NDVI were ever detected between Tx623 *bmr6* and Tx623 *bmr6 cyp79a1* with contrasting dhurrin accumulation patterns. This indicates that dhurrin does not directly contribute to differences in stay-green in this genetic background. If dhurrin were a direct contributor to stay-green, wild-type plants would exhibit higher CC and NDVI values under post-flowering drought stress.

Since Tx642 is one of the most important sources of the stay-green trait, NIBC progeny with contrasting dhurrin accumulation characteristics in the Tx642 genetic background were evaluated for differences in drought tolerance. Although the drought treatments had a significant impact on plant greenness measured by CC or NDVI and on plant growth measured by dry weight, no differences in CC or NDVI were detected between NIBC progeny with contrasting dhurrin accumulation patterns. Burke et al. (2013) observed that the expression of stay-green was strongly correlated with dhurrin accumulation in Tx642. Follow-up studies suggested that dhurrin may function as an osmoprotectant involved in stay-green expression (Burke et al., 2015). Comparisons of CC and NDVI in NIBC of Tx642 indicated that dhurrin accumulation does not directly contribute to differences in stay-green in this genetic background. It is known that genes for stay-green influence plant nitrogen status (Harris et al., 2006), leaf size and tiller number (Borrell et al., 2014), as well as root architecture (Mace et al., 2012) so it is possible that these genes also have a positive impact on dhurrin metabolism that might explain the correlation in phenotypes, and growth differences due to dhurrin metabolism have been identified previously (Gruss and Tuinstra 2021).

Since sorghum is normally produced using hybrid cultivars, four different sets of NIHs were evaluated for stay-green in controlled environment studies and field trials. In field trials post-flowering drought treatments resulted in significant reductions in CC and NDVI as well as biomass accumulation; however, only one set of NIHs, (ATx623 x MR732)-F1 and (ATx623 x MR732)-F1 *cyp79a1*, exhibited differences in CC and NDVI under drought where the wild-type hybrid exhibited higher CC and NDVI values than the wild-type hybrid. Controlled environment trials showed a similar result when using a p-vale of 0.1. These results suggesting that dhurrin biosynthesis may play a role in post-flowering drought tolerance (Burke et al. 2015; Hayes et al., 2016; Rosati et al. 2019). These studies indicate that dhurrin production may contribute to the expression of stay-green in some genetic backgrounds, but the effects are small and genotype specific.

Throughout the controlled environment and field trials, flowering, dry biomass, and moisture data varied between treatments but not necessarily between the sets of genotypes and hybrids examined. In a previous study, a totally cyanide deficient mutant described by Blomstedt et al. (2012), was examined for biomass and flowering (DAS). It was noted a decrease in growth and delayed flowering times for the mutant lines (Sohail, Blomstedt, and Gleadow 2020). Analyses of the

NILs, Tx623 *bmr6* and Tx623 *bmr6 cyp79a1*, indicated that the dhurrin free plants produced more biomass than wild-type plants in the fully irrigated treatments. In the segregating NIBC population, the wild-type plants produced more biomass compared to dhurrin-free plants. Again, when examining the flowering times, there was no consistent variation noted, and generally the pairs were statistically similar for flowering time. These results contradicted the results by Sohail et al (2020). This variation may be due to differences in the mutant causing the acyanogenic phenotype.

Taken together, these studies did not indicate a strong relationship between dhurrin biosynthesis and expression of the stay-green trait. The only one set of NIHs exhibited a reduction in CC in the dhurrin free hybrid compared to the wild type. Dhurrin biosynthesis could still play a role in pre-drought tolerance (Burke et al. 2013; Adeyanju et al. 2016; Varoquaux et al. 2019) and nitrogen use efficiency (Blomstedt et al. 2018; Rosati et al. 2019). It was determined to play a role in seedling growth and insect resistance when metabolic tradeoffs were made when inhibiting dhurrin biosynthesis (Gruss and Tuinstra 2021). Understanding the effects of dhurrin biosynthesis on primary and secondary metabolism of sorghum provides insight into the tradeoffs and interactions with more complex agronomic and physiological traits.

3.6 Conclusions

In conclusion, dhurrin metabolism has been hypothesized to play role in primary and secondary metabolism. This has been previously demonstrated when introducing sorghum to both abiotic and biotic stresses. The stay-green trait is a valuable trait of sorghum in grain and forage production. The disruption of the biosynthesis of dhurrin lead to a small effect within a specific genotypic background but was not noted within any of the other backgrounds tested including Tx642, one of the most important stay-green backgrounds. Dhurrin metabolism plays a role in the stay-green trait but is small effect and seems to be specific to certain genetic backgrounds.

CHAPTER 4. SAFE AND TASTY FORAGE SORGHUM

4.1 Abstract

Sorghum is an important crop in many semi-arid environments around the world and contributes both to grain and forage production systems. Sorghum produces a cyanogenic glucoside called dhurrin. The breakdown of dhurrin leads to the release of hydrogen cyanide (HCN), which can be detrimental to animals feeding on sorghum forages. Dhurrin concentrations are normally highest in young plant tissues or plants produced in stressful environments such as extreme drought and frost. Dhurrin-free sorghum genotypes have been developed through chemical mutagenesis by selection for a missense mutation in CYP79A1, the first enzyme in the dhurrin biosynthetic pathway. A C493Y mutation in *cyp79a1* was bred into sorghum lines to create an dhurrin-free experimental hybrid (sorghum x sudangrass *bmr6 cyp79a1*) that was compared to three commercial hybrids, Sweet 6 (sorghum x sudangrass *bmr6*), Sweet Bites (sorghum x sudangrass), and GreenTreat Rocket® (sudangrass x sudangrass *bmr6*) and a set of near-isogenic lines, Tx623 *bmr6* and Tx623 *bmr6 cyp79a1*, for palatability and preferential grazing of ewes. Biomass was measured before and after grazing to determine the amount of biomass grazed by the ewes. A drone with a multispectral sensor was flown each day at midday to quantify changes over time in NDVI. During grazing, trail cameras were used to track animal movement and time spent grazing within each plot. The dhurrin-free experimental hybrid was preferentially grazed at 35% in cycle 2 and 45% in cycle 3, followed by Sweet Bites at 21% for cycle 2 and Sweet 6 at 26% for cycle 3. The Tx623 *bmr6 cyp79a1* was grazed 20% more than Tx623 *bmr6* in each cycle. Remote sensing data from trials in 2019, showed an average drop in NDVI of 27% in the dhurrin-free experimental hybrid during the three grazing periods followed by 17% in Sweet 6. The analysis of RGB images from trail cameras showed that the ewes spent most of their time in either the dhurrin-free experimental hybrid or Sweet Bites during the grazing cycles. Overall, the ewes in these trials demonstrated preferential feeding of the dhurrin-free experimental hybrid and the dhurrin-free NIL compared to conventional sorghums.

4.2 Introduction

Warm season annuals have become an important component in forage rotations for livestock as silage, pasture, and hay. Commonly grown warm season annuals produced as forages include pearl millet, corn, sudangrass, and sorghum. These crops are high yielding and help during mid-summer heat when cool season perennials may be going through a "summer slump," or period of slow growth due to the heat (Bledsoe et al. 2016).

Sorghum has become widely used as a forage crop, especially as the benefits of the brown midrib (*bmr*) trait have been introduced into sorghum. The bmr trait helps with the ease of digestion for livestock due to the lower lignin content of *bmr* hybrids (Porter et al. 1978). The introduction of this trait has been a huge benefit for the quality of forage sorghum. An overall increase in digestibility led to an increase in milk production in mid-lactating cows that weas similar to corn forages (Grant et al. 1995; Marsalis et al. 2010). The bmr trait also leads to higher levels of palatability in forage sorghum in comparison to conventional forage sorghums (Grant et al. 1995; Porter et al. 1978). Sorghum also benefits from having adaptation to drought-prone environments (Rosenow et al. 1983; Tuinstra et al. 1997; Borrell et al. 2000; Sanchez et al. 2002; Borrell et al. 2014) and infertile soils (Borrell and Hammer 2000; Blomstedt et al. 2018).

Although sorghum has many benefits, it contains the CG dhurrin. CGs are found in many plant species and when broken down will release HCN, which is toxic to animals. HCN interacts with chytochrome c oxidase, a metalloenzyme, that is the final enzyme in the electron transport chain for respiration; thereby, inhibiting the use of oxygen in respiration leading to loss of consciousness and potentially death (Price 1985) in humans or livestock. Some cyanogenic species such as birdsfoot trefoil and white clover have naturally occurring varieties that are acyanogenic. Quantitative variation in dhurrin content has been discovered in sorghum but no dhurrin-free accessions have been discovered in the extent genetic variation of the crop (Blomstedt et al. 2012; Burke et al. 2013). Dhurrin content within sorghum can vary greatly depending on different factors, including plant age, variety, fertilizer applications, and environmental conditions (Harms and Tucker 1973; Burke et al. 2013; Gleadow and Møller 2014; Halkier and Moller 1989; Kojima et al. 1979; Harms and Tucker 1973; Wheeler et al. 1990).

Dhurrin concentration varies greatly in sorghum plants, but generally peaks within the first few days after germination and is highest in the youngest leaves (Kojima et al. 1979; Harms and Tucker 1973). As the plant ages, dhurrin concentration declines but can spike again depending on environmental concentrations such as drought, over-fertilization, and frost (Harms and Tucker 1973; Wheeler et al. 1990; Busk and Moller 2002; O'Donnell et al. 2013; Gleadow and Møller 2014; Neilson et al. 2015). Farmers need to be careful when using sorghum as a forage to avoid concentrations of HCN greater than 750 mg kg^{-1}, which can be very dangerous to cattle (Strickland et al. 2017). Management practices to avoid high HCN include utilization of sorghum as a silage to reduce HCN potential (HCNp) (Smith and Frederiksen 2000; Strickland et al. 2017).

Utilization of dhurrin-free sorghums could eliminate the risk of HCN poisoning; however, producers still need to manage nitrate potential of the crop. Blomstedt et al. (2012) described a P414L mutation in CYP79A1 that exhibited increased nitrate concentration in comparison to conventional sorghums (Blomstedt et al. 2018; Rosati et al. 2019). The relationship between nitrate accumulation and dhurrin accumulation is not clear; however, the benefits of no release of HCN may be negated by an increase in nitrates if the traits are linked. Also, the P414L mutants exhibited slower growth and extended flowering times (Sohail et al. 2020). Taking into consideration the tradeoffs of inhibiting dhurrin biosynthesis is important in understanding the overall efficacy and safety of dhurrin-free sorghums

This study looked at the preferential grazing of sorghum comparing three conventional hybrids and a dhurrin-free sorghum hybrid along with comparisons between NILs with contrasting dhurrin accumulation characteristics in sheep grazing trials. Understanding the palatability of dhurrin-free sorghum could be an important factor when using it as a forage. Palatability can be a significant factor influencing animal performance (Oregon State University 2021). Nutritional quality, nitrate content and HCN release were examined in each of the hybrids. It was hypothesized that the ewes would exhibit preferential grazing of the dhurrin-free hybrid, due to the acyanogenic phenotype. In a previous study the higher the release of HCN the more bitter almonds tasted (Arrázola et al. 2012), which may lead to favorable feeding on the dhurrin-free hybrids.

4.3 Material and Methods

4.3.1 Field Design

Pasture trials were conducted in 2019 and 2020 at the Animal Sciences Research and Education Center (ASREC) at Purdue University in West Lafayette, IN. The trial was planted with Greentreat Rocket® (sudangrass x sudangrass *bmr6*), Sweet 6 (sorghum x sudangrass *bmr6*), Sweet Bites (sorghum x sudangrass), and the dhurrin-free hybrid (sorghum x sudangrass *bmr6 cyp79a1*). Seeds for the commercial varieties were obtained through Cisco Seeds (Indianapolis, IN). Planting occurred on June 12, 2019 and on May 27, 2020 using a drill with nine rows and 19.05 cm row spacing for planting and calibrated to each seed size. Greentreat Rocket had 69,850 seeds kg^{-1}, Sweet Bite had 48,260 seeds kg^{-1}, Sweet 6 had 41,910 seeds kg^{-1}, and the dhurrin-free hybrid had 39,370 seeds kg^{-1}. The experiment was planted using a randomized complete block design (RCBD) with four replications for a total of 16 plots planted at a rate of 33.6 kg $hectare^{-1}$. Plots were 1.83 m wide and 7.62 m long with approximately 0.5 m between each of the plots. Each replication was surrounded by a border that consisted of a mixture of seed for all four hybrids, and there was an acclimation area to acclimate to sheep to sorghum forages before entering the trials (Figure 4.1). At planting there was 67.3 kg $hectare^{-1}$ of urea (46 – 0 – 0) applied and again after the first grazing cycle. Japanese beetle pheromone traps were set up around the field. Plants were over 40 cm tall before grazing occurred. Cycleup Power Max was applied to control Canadian thistle a month before planting and at planting in 2019. In 2020, the seed bed was tilled before planting to avoid any compaction from the previous season.

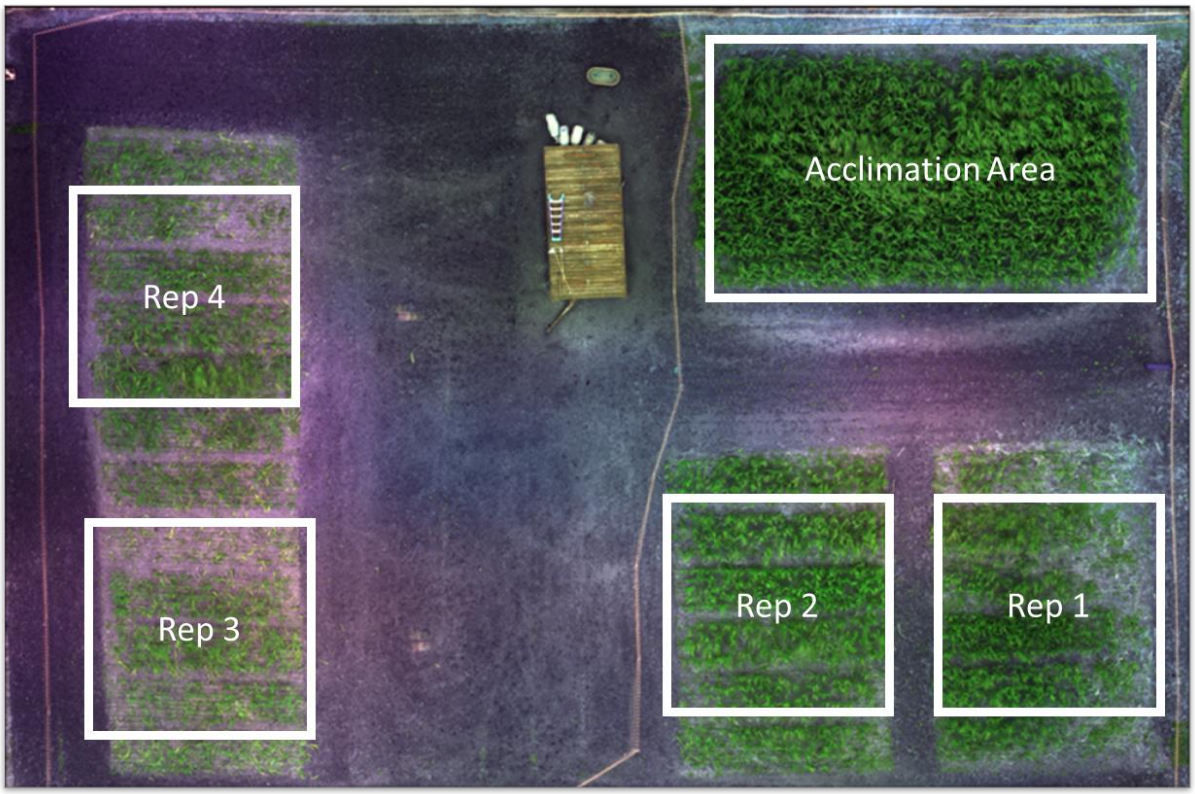


Figure 4.1: The design of the experiment for the hybrid grazing.

A set of near-isogenic lines (NILs), Tx623 *bmr6* and Tx623 *bmr6 cyp79a1*, were included in the preference trial in 2020. The NILs were evaluated using a RCBD with five replications with borders on each side of the ten plots. Borders for NILs consisted of a 1:1 mixture of the two NILs (Figure 4.2). The NILs were planted on the same date and rate as the hybrids with the same field preparation as the hybrids. Tx623 *bmr6* had 47,965 seeds kg^{-1} and Tx623 *bmr6 cyp79a1* had 41,628 seeds kg^{-1}.

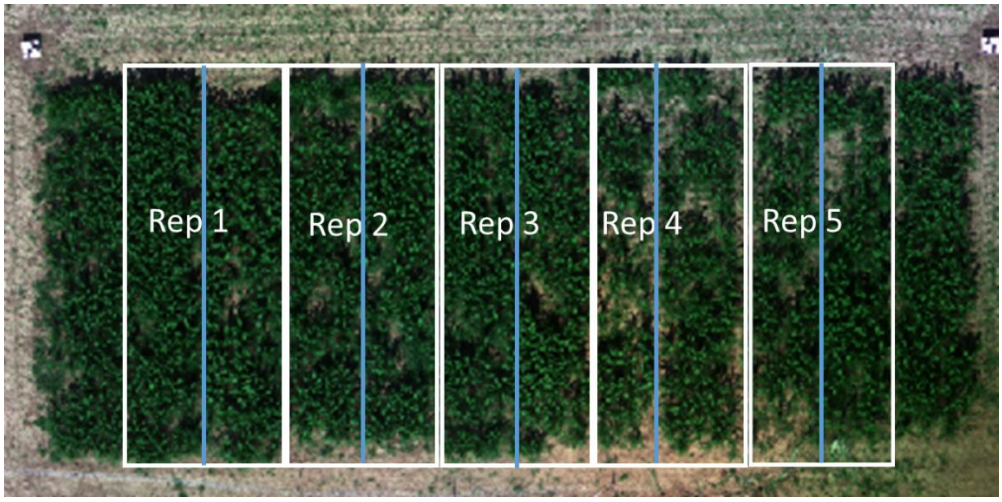


Figure 4.2: The experimental design of the NILs grazing trial. The blue bars represent the designation between plots.

4.3.2 **Grazing Cycles**

The hybrid experiment had six ewes per two replications in 2019 and four ewes per two replications in 2020. For the NILs, four ewes grazed on all five replications at a time. The length of the grazing cycle varied between 2 to 5 days. Differences among grazing times were caused by the different rates of grazing among the ewes. Variations in weather such as high heat or rain would slow grazing, while cool temperatures encouraged more active grazing. The amount of plant material and number of ewes within the field also influenced the length of the cycle. At the end of the grazing cycle, the plots were clipped with a mower to 15.2 cm for uniformity among the plots and allowed to regrow for the following cycle. There were three grazing cycles for the hybrids in both years. The NILs had two grazing cycles in 2020.

4.3.3 **Data Collection**

Pre-grazing samples were collected for fresh and dry biomass (g) from two meters of two interior rows from each plot in both the hybrids and NILs. Fresh weight was measured in the field directly after cutting, then placed in a brown paper bag and dried at 60 °C for at least one week then weighed for total dry biomass (g). The same procedure for biomass collection was used for sampling the post-grazing biomass samples. The post-grazing samples were taken immediately following the removal of the ewes from the plots. The hybrid biomass data collection on the first

cycle of grazing did not immediately happen before or following grazing, which allowed time for growth while the sheep were not on the plots. For this reason, the first grazing cycle in both years was omitted from the analysis.

Trail cameras were set up to track sheep movement in 2019. One trail camera was set to watch two plots during the grazing interval for the first two cycles. RGB data was collected for each plot during grazing. The trail camera was set to detect motion and if motion was detected the camera would take a photo every 20 seconds in cycle 1 and every 5 seconds in cycle 2. The images were analyzed by counting the number of sheep in each plot in every photo for the daylight hours for each day of the grazing cycle. The number of ewes per plot were totaled for the amount of time spent within the plots on a daily basis.

An unmanned aerial vehicle (UAV) flown at 25 m was used to collect remote sensing data at midday of grazing on each day of the grazing trial. A MicaSense RedEdge Multispectral Sensor (AgEagle Sensor Systems Inc., Seattle, WA) was used for data acquisition in 2019 and a HeadWall Nano-Hyperspec (Boston, MA) VNIR (400 – 1000 nm) Hyperspectral Imager was used in 2020. Images were processed using ENVI from Harris Geospatial Solutions, Inc. (Broomfield, CO). A region of interest (ROI) was determined using pixel-based count for each of the plots, and the ROI was the same size for each of the plots. The ROI was slightly smaller than the actual plots to avoid any edge effects. The average NDVI was calculated for each plot on each day of the grazing cycles. NDVI was calculated using equation 1.

$$equation\ 1 : NDVI = \frac{(NIR - Red)}{(NIR + Red)}$$

4.3.4 **HCN Test**

Before each grazing cycle, plants in each plot were tested for the release of HCN using the Feigl Anger (FA) Assay (Feigl and Anger, 1966). A small plant tissue sample from three to four plants within each plot was collected from the tip of a top collared leaf and placed in a 96 well plate. The samples were kept on ice in a cooler during sampling then transferred to a -80 °C freezer for storage overnight. The next day, tissue samples were removed from the freezer and covered with the FA paper for 30 minutes at room temperature. After the 30 min, the FA paper was removed and checked for blue coloring indicating the release of HCN.

4.3.5 **Quality Analysis**

The whole plants from the pre-grazing biomass samples from cycles two and three in 2019 were ground with a Udy cyclone-mill (UDY Corp., Fort Collins, CO) using a 1 mm sieve after the samples were weighed for dry weight. The dried and ground samples were sent to Cumberland Valley Analytical Services (Waynesboro, PA) for forage quality analysis. The analysis provided information of crude protein (CP), soluble protein (SP), ruminant digestible protein (RDP), acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrients (TDN) and NO_3.

4.3.6 **Statistical Analysis**

The statistical analyses was conducted using Rstudio software.

Data was first examined using boxplots to understand the trends and potential outliers in the data. To identify influential data points and outliers, four times cook's distance from the average was used in combination with boxplots. An example of an outlier is from the second cycle of the hybrid data collection. The outliers were shown to be 1,000 kg ha^{-1} larger than any of its other replications within their grazing time. The identified outliers were removed from the datasets.

Data was checked for normality and equal variances. The NDVI data for the first cycle in 2019 and for all the 2020 data was transformed using a square root transformation to fit a normal distribution.

A mixed effects model was used for the biomass data using total dry weight (g) of the samples as the dependent variable and the sampling time (pre- and post- grazing), hybrids or NILs, cycle, and their interaction as the fixed effects. Plots were nested within each year. Similar models were used to test changes in NDVI over days and the number of sheep per day using the trail cameras.

The forage quality data were analyzed by comparing the hybrids within each round. It was analyzed using a linear model using hybrids and cycle as the effects.

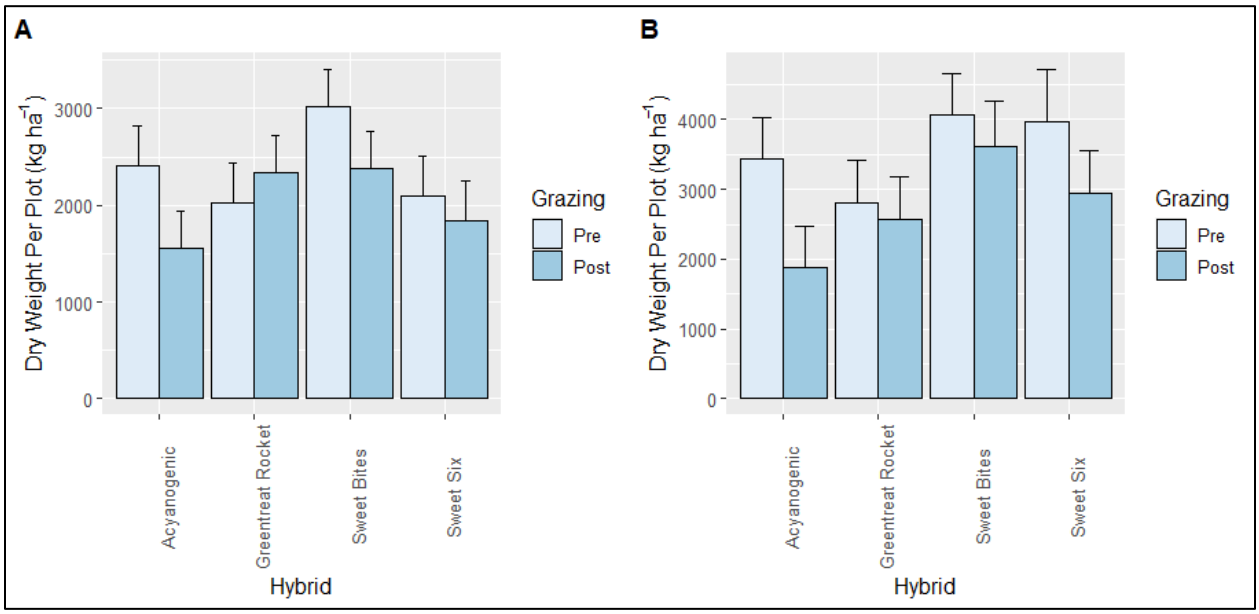
4.4 Results

4.4.1 Hybrids

4.4.1.1 Biomass Comparison

The first grazing cycle data from 2019 and 2020 were excluded from the analysis due to plant harvesting issues. The second cycle in 2019 and 2020 had similar patterns and were examined together. A similar pattern was noted with the third cycle and analyzed in the same manner. The pre-grazing biomass yields from cycle 2 and cycle 3 showed that the dhurrin-free hybrid was consistent with the other three hybrids with 2,407 kg ha^{-1} in cycle 2 and 3,432 kg ha^{-1} in cycle 3. Sweet Bites exhibited the highest yield at 3,019 kg ha^{-1} in cycle 2 and 4,065 kg ha^{-1} in cycle 3 but these were not significant compared to the other hybrids.

Comparisons of pre-grazing biomass with post-grazing biomass indicated the amount of grazing that occurred for each hybrid. An interaction was found between the hybrids and sampling time (pre- and post-grazing) showing there was variation between the hybrids and the sampling time in cycle 2 and cycle 3. Only the dhurrin-free hybrid exhibited a significant difference between pre-grazing biomass and post-grazing biomass in each cycle (p-value$\leq$ 0.05) (Fig. 4.3). The dhurrin-free hybrid exhibited the lowest biomass of all hybrids in its post-grazing sample at 1,562 kg ha^{-1} in cycle 2 and 1,880 kg ha^{-1} in cycle 3. These comparisons show that the dhurrin-free hybrid was grazed at 35% in cycle 2 and 45% in cycle 3 (Fig. 4.3C).



C

Percent Grazed

Hybrids	Cycle 2	Cycle 3
Dhurrin-free	35*	45*
Greentreat Rocket	-15	11
Sweet Bites	21	5
Sweet Six	12	26

*Significance between pre- and post- grazing samples within the hybrid

Figure 4.3: Comparisons of pre- and post-grazing biomass of hybrids from (A) cycle 2 and (B) cycle 3 from 2019 and 2020. The hybrids were compared for total dry weight per plot (kg ha^{-1}). The bars show the hybrid mean values with the error bars representing the upper confidence limits. (C) Comparisons of hybrids for percent grazed cycle 2 and cycle 3.

4.4.1.2 Changes in NDVI

Remote sensing data were collected each cycle to quantify changes in NDVI associated with grazing. In 2019, the hybrids responded differently over the three cycles, so each cycle was analyzed separately. All four hybrids exhibited similar NDVI values on Day 0 for the grazing trials in July, August, and September (Table 4.1). Significant differences in NDVI among hybrids were

already noted by Day 1 with the dhurrin-free hybrid exhibiting the lowest NDVI value among hybrids in each cycle. The hybrids exhibited even greater differences in NDVI on Day 2 with the dhurrin-free hybrid exhibiting the lowest NDVI value among all the hybrids for the grazing cycle in September (Table 4.1). On Day 3, the dhurrin-free hybrid had the lowest NDVI value of any hybrids for the grazing cycle in July and August (Table 4.1). No remote sensing data were collected due to weather limitations on Day 2 of the grazing cycle in August. Cycle 3 grazing period ended on Day 2 due to favorable grazing temperatures and weather.

Table 4.1: Comparisons of NDVI values of sorghum hybrids on Days 0, 1, 2, and 3 of grazing for the three cycles in 2019. Comparisons were made using Tukey's comparisons across hybrids, within days, and within cycles.

2019 Cycles	Hybrids	Days of Grazing (NDVI)							
		0		1		2		3	
July	Dhurrin-free	0.77	a	0.76	a	0.63	a	0.45	a
	Greentreat Rocket	0.78	a	0.83	ab	0.82	b	0.59	b
	Sweet Six	0.77	a	0.83	ab	0.78	b	0.63	b
	Sweet Bites	0.77	a	0.85	b	0.72	ab	0.69	b
August	Dhurrin-free	0.88	a	0.85	a	-	-	0.65	a
	Greentreat Rocket	0.91	a	0.92	ab	-	-	0.79	b
	Sweet Six	0.9	a	0.9	ab	-	-	0.74	b
	Sweet Bites	0.91	a	0.93	b	-	-	0.77	b
September	Dhurrin-free	0.89	a	0.8	a	0.73	a	-	-
	Greentreat Rocket	0.9	a	0.88	b	0.86	b	-	-
	Sweet Six	0.9	a	0.86	b	0.84	b	-	-
	Sweet Bites	0.91	a	0.88	b	0.86	b	-	-

The grazing trials in 2020 produced similar results and were analyzed in a combined analysis. NDVI remained high for the first four days of grazing and began to decrease on the fifth day (Table 4.2). Greentreat Rocket had the lowest NDVI and was similar to the dhurrin-free hybrid and Sweet Bites. Sweet 6 had the highest NDVI at 0.75 (Table 4.2). NDVI values did not decrease as quickly as observed in 2019 with less variation in NDVI between hybrids.

Table 4.2: Comparison of NDVI values for hybrids from a combined analysis of the three grazing cycles in 2020.

2020 Cycles	Days of Grazing (NDVI)											
Hybrids	0		1		2		3		4		5	
Dhurrin-free	0.9	a	0.88	a	0.85	a	0.83	a	0.82	a	0.67	ab
Greentreat Rocket	0.9	a	0.89	a	0.85	a	0.86	a	0.8	a	0.65	a
Sweet Six	0.9	a	0.9	a	0.85	a	0.85	a	0.85	a	0.75	b
Sweet Bites	0.91	a	0.89	a	0.85	a	0.86	a	0.84	a	0.7	ab

4.4.1.3 Comparison in Time Spent within Plots

RGB data from trail cameras were collected in 2019 for all four replications in the first two grazing cycles. Significant differences among hybrids were detected in July but there was not a significant effect due to days of grazing or the interaction between hybrids x days of grazing. Significant differences among hybrids were only noted on Day 1 with less time spent in Greentreat Rocket than in the dhurrin-free hybrid or Sweet Bites (Table 4.3). Comparing the hybrids over the whole grazing cycle showed that the ewes spent the most time in the dhurrin-free hybrid, (0.50 h) but this was not higher than Sweet 6 at 0.42 h and Greentreat Rocket at 0.36 h (Table 4.4).

Table 4.3: Comparison of daylight hours ewes spent in each of the hybrids for the two cycles in 2019 by days of grazing. Comparisons were made using Tukey's comparisons across hybrids, within days and cycles.

	Days of Grazing (h)						
2019 Cycle	Hybrids	1		2		3	
July (Cycle 1)	Dhurrin-free	0.59	a	0.41	a	0.49	a
	Greentreat Rocket	0.22	b	0.48	a	0.39	a
	Sweet Six	0.29	ab	0.49	a	0.49	a
	Sweet Bites	0.29	a	0.34	a	0.38	a

Table 4.4: Comparing the average daylight hours spent within the hybrids for all of Cycle 1.

Average Grazing (h)			
2019 Cycle	Hybrids		
July (Cycle 1)	Dhurrin-free	0.50	a
	Greentreat Rocket	0.36	ab
	Sweet Six	0.42	ab
	Sweet Bites	0.33	b

4.4.1.4 Nutritional Quality

The four hybrids performed similarly when examining nitrates, CP, SP, and RDP within both grazing cycles. Significant differences in TDN and ADF were noted among hybrids in August (Table 4.5). All nutritional quality values were expressed on a dry matter. The dhurrin-free experimental hybrid had the highest TDN at 62.8 mg g^{-1}, and this was significantly higher than Sweet Bites at 59.9 mg g^{-1} and Greentreat Rocket at 59.7 mg g^{-1}. Sweet Bites had the highest ADF at 38.4 mg g^{-1} which is significantly higher than the dhurrin-free experimental hybrid at 33.1 mg g^{-1}, Greentreat Rocket at 33.6 mg g^{-1}, and Sweet 6 at 34.8 mg g^{-1}. The other nutrients, NO_3 ion, CP, SP, and RDP were similar across hybrids in both grazing cycles.

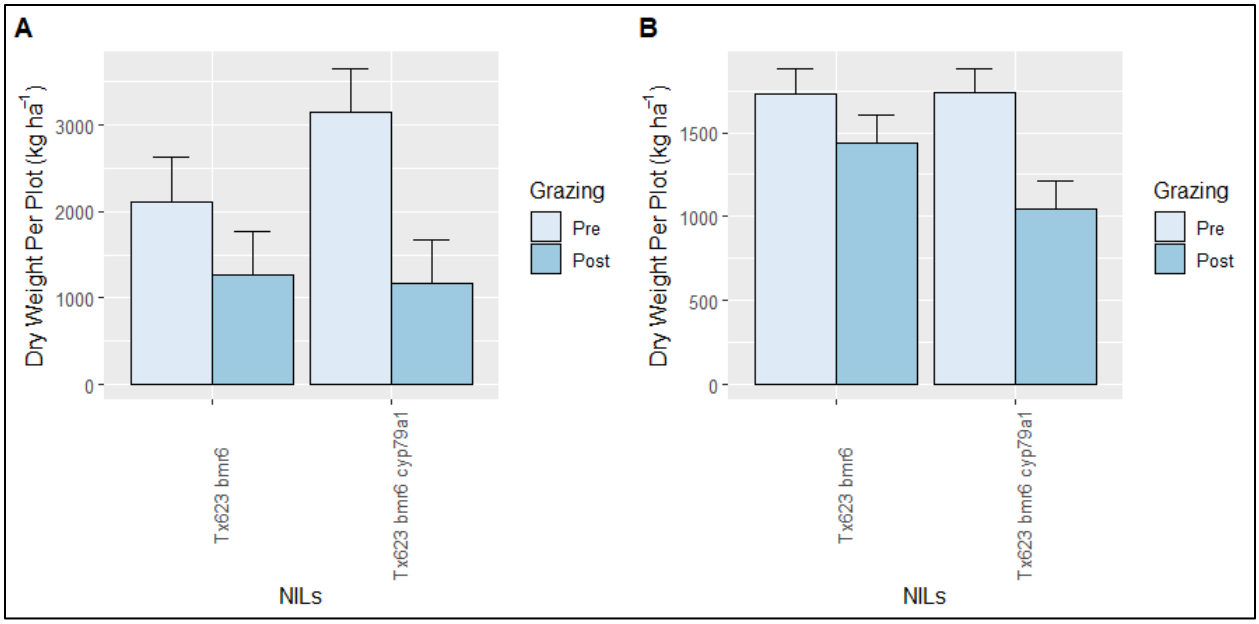
Table 4.5: Comparisons of nutrient composition of hybrids harvested in June and July of 2019. Comparisons were made when significant differences between the four hybrids were noted.

Nutritional Quality (mg $g^{-1)}$									
2019 Cycle	Hybrid	NO_3	CP	SP	RDP	TDN		ADF	
August	Dhurrin-free	6.6	193	58	126	628	a	331	a
	Greentreat Rocket	8.5	194	58	126	597	b	336	a
	Sweet Six	8.7	187	53	120	612	ab	348	a
	Sweet Bites	10.0	163	54	108	599	b	384	b
September	Dhurrin-free	5.5	169	56	115	618	a	356	a
	Greentreat Rocket	4.6	157	53	104	608	a	345	a
	Sweet Six	2.0	161	53	108	616	a	355	a
	Sweet Bites	2.3	153	52	102	606	a	375	a

4.4.2 **Near-isogenic Lines**

4.4.2.1 Biomass Comparison

Pre- and post-grazing biomass yields of the NILs were determined in each of the two grazing cycles completed in 2020. Cycle 1 exhibited significant differences in sampling time and the interaction between NILs x sampling time. Cycle 2 exhibited significant differences in NILs, sampling time, and their interaction. Tx623 *bmr6 cyp79a1* had a higher pre-grazing biomass yields in comparison to Tx623 *bmr6* (p-value ≤0.05) for Cycle 1 but the NILs had similar pre-grazing biomass yield in Cycle 2 (Fig. 4.4). A significant interaction (p-value ≤ 0.05) between NILs and sampling times were found in Cycle 1 and Cycle 2. This interaction indicated a greater difference between pre- and post- grazing yield for Tx623 *bmr6 cyp79a1* compared to Tx623 *bmr6*. This difference represents the amount grazed by the ewes (Fig. 4.4.C). Tx623 *bmr6 cyp79a1* was grazed more than Tx623 *bmr6* in both cycles.



C

Percent Grazed

NILs	Cycle 2	Cycle 3
Tx623 *bmr6 cyp79a1*	63*	40*
Tx623 *bmr6*	40*	17

*Significance between pre- and post- grazing for the NILs within the cycle.

Figure 4.4: Comparison biomass pre- and post-grazing biomass of NILs from (A) cycle 1 and (B) cycle 2 trials in 2020. The NILs are compared for total dry weight per plot (kg ha^{-1}) for pre- and post- grazing. The bars are the mean values from the mixed effects models with the error bars representing the upper confidence limits. C) Comparison of NILs for percent grazed cycle 1 and cycle 2.

4.4.3 **HCN Release**

FA testing indicated no HCN was released from samples of the dhurrin-free hybrid (Table 4.5) or Tx623 *bmr cyp79a1* near-isogenic pair at any time point in 2019 or 2020. However, each of the conventional hybrids and Tx623 *bmr* tested positive for HCN in at least one of the replications from each cycle (Fig. 4.5).

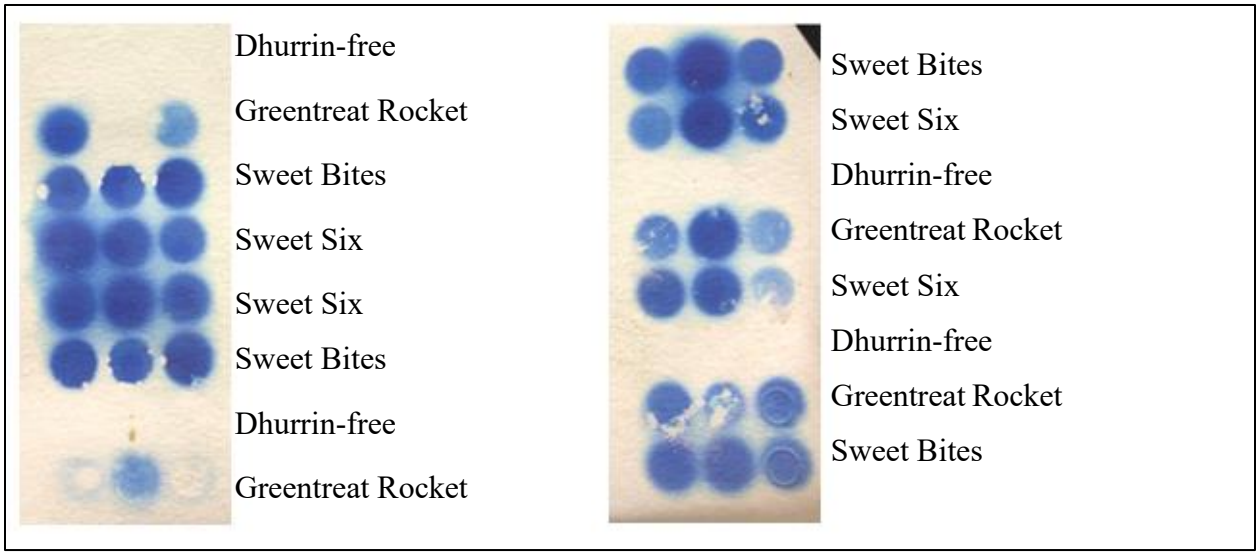


Figure 4.5: FA test of the sorghum hybrids before cycle 3 in 2020 with three samples per replication. Blue coloring identifies the evolution of HCN.

4.5 Discussion

Sorghum x sudangrasses and sudangrasses are good options for forage because they are high yielding, exhibit good nutritional quality, especially with the *bmr* trait (Grant et al. 1995; Miller and Stroup 2003), and productive in diverse environments (Saeed and El-Nadi 1998). However, producers need to practice careful management when using forage sorghum, sorghum x sudangrass, and sudangrass x sudangrass to minimize dhurrin production and avoid HCN toxicity to animals caused by the breakdown of dhurrin. Recently developed dhurrin-free sorghum (Blomstedt et al. 2012; Tuinstra et al. 2013) could eliminate this problem, because there is no release of HCN from these genotypes. This study looked at the palatability of dhurrin-free forage sorghum x sudangrass hybrids using ewes as the representative ruminant animal.

Sheep grazing trials demonstrated strong preference for the dhurrin-free hybrid over conventional forage sorghum hybrids. Changes in biomass yields, NDVI from remote sensing studies, and animal tracking from RGB images were used to quantify the feeding preferences and grazing habits of the ewes in field trials. Prior studies have shown that the brown midrib forage sorghums are more palatable than conventional types (Miller and Stroup 2003). This trial demonstrated a similar feeding preference for acyanogenic, dhurrin-free sorghums. In both years, the dhurrin-free hybrid exhibited similar pre-grazing yields to the three conventional hybrids but was grazed at a much higher rate in comparison to the conventional hybrids. A large decrease in post grazing biomass

was measured in the dhurrin-free hybrid in both the second and third cycle with the dhurrin-free hybrid being grazed almost 15% more than the next best conventional hybrid.

These differences in biomass consumptions were supported with remote sensing data showing a sharp decrease in NDVI for the dhurrin-free hybrid over the course of the grazing cycles in 2019. The dhurrin-free hybrid had the greatest decrease in NDVI due to more plant material being grazed from each plot. Although this indicates that the dhurrin-free hybrid was more palatable to the ewes than the other hybrids, it was not clear whether this was due to differences in dhurrin content or some other factor that might be unique to the dhurrin-free hybrid. To more directly test the effects of dhurrin and the *cyp79a1* mutation on grazing preference in sorghum, the NILs Tx623 *bmr6* and Tx623 *bmr6 cyp79a1* were grazed in two cycles in 2020. The dhurrin-free NIL exhibited higher grazing preference and was more palatable for the ewes. Given the genetic similarity of these genotypes, this preference suggests the sheep can detect the presence of dhurrin or HCN through smell or taste. Almonds contain the CG, amyglobin. Arrázola et al. (2012) showed that almonds with high amyglobin content were more bitter in taste in comparison to almonds with low amyglobin. The bitter taste is believed to be from the release of HCN. A similar phenomena may be occurring in dhurrin-free sorghum. As the ewes grazed on the conventional hybrids or wild-type NIL, HCN would be released leading a potential bitter taste or smell that may discourage feeding. Dhurrin-free sorghum lines and hybrids may not have a bitter taste because there is no release of HCN.

Tx623 *bmr6 cyp79a1* had a higher biomass yield in the first grazing cycle in comparison to its near isogenic pair. A similar pattern in growth was observed in dhurrin-free sorghum seedlings (Gruss and Tuinstra 2021). Disruption of dhurrin biosynthesis may increase the availability of nitrogen and carbon stores leading to increased plant growth.

Nutrient analyses of conventional and dhurrin-free sorghum hybrids showed that the feed quality characteristics were generally similar. The dhurrin-free hybrid exhibited higher TDN and lower ADF in comparison to Sweet Bites in one of the cycles. It is worth noting that this difference may not be due to the *cyp79a1* mutation but to differences in the *bmr6* trait for these hybrids. As previously noted the *bmr* trait is beneficial in forage quality by decreasing the amount of lignin, overall decreasing the ADF and increasing TDN (Porter et al. 1978). This is supported because the

dhurrin-free hybrid had similar ADF and TDN in comparison the Sweet Six and GreenTreat Rocket, which both contain the *bmr* trait.

Differences in nitrate content between dhurrin-free and conventional sorghums were of particular interest because recent studies have suggested the two traits might interact in forage sorghums (Blomstedt et al. 2018; Rosati et al. 2019). These studies observed an increase in nitrate content when removing dhurrin, using a different mutation within the CYP79A1 enzyme. In two rounds of replicated testing, the expression of nitrate as mg g^{-1} dry weight showed no differences between the dhurrin-free hybrid and the conventional hybrids. This increases the overall safety of the crop with no evolution of HCN throughout the trials and no increase in nitrate production in the dhurrin-free hybrid compared to the conventional hybrids.

4.6 Conclusions

A dhurrin-free hybrid was more palatable to ewes than conventional sorghum hybrids. Analyses of NILs contrasting for dhurrin production suggested this preference is directly related to absence of dhurrin or HCN. Grazing ewes were able to detect the dhurrin-free plants and selectively feed on these plants. Dhurrin-free hybrids were shown to perform as well as the conventional hybrids with high biomass production and similar nutritional qualities.

CHAPTER 5. STABILITY OF DHURRIN AND HYDROGEN CYANIDE RELEASE IN DRIED SORGHUM SAMPLES

5.1 Abstract

Sorghum is an important forage crop but contains the cyanogenic glucoside (CG) dhurrin, which can be detrimental to livestock when released as hydrogen cyanide (HCN). The risks of HCN poisoning may be reduced by proper production management and handling practices including drying for a hay product. Previous reports suggested that haymaking can decrease dhurrin content by 50% but the relationships between hay production, processing, and dhurrin content are not well understood. In this study, quantitative and qualitative methods were used to study changes in dhurrin content and HCN production in sorghum hybrids harvested and stored as dry tissue samples and dry samples rehydrated under varying conditions. It was hypothesized that dhurrin would quickly decompose over time; however, quantitative analyses showed that dry plant samples maintained similar dhurrin content throughout a two month long drying process. The analysis of HCN release in genetic mutants varying in dhurrin biosynthesis and dhurrin catabolism suggested that the drying process inactivated the enzyme dhurrinase2 leading to a slower release of HCN in the dry tissue. Rehydration of dry sorghum samples in rumen fluid resulted in rapid release of HCN, even in dhurrinase2 inactive genotypes. These studies demonstrated that dhurrin was stable in dry sorghum plant material for at least two months contrary to conventional wisdom. Furthermore, the dhurrin maintained in dry sorghum tissues was readily released as HCN following rehydration in rumen fluid.

5.2 Introduction

Sorghum is an important grain crop worldwide with over 40 million hectares harvested in 2019 ("FAOSTAT" 2019). Sorghum is also produced as a forage crop using forage sorghums, sorghum x sudangrass, and sudangrass cultivars. Sorghum forages are grown as summer annuals with many valuable characteristics including high biomass production (Marsalis et al. 2010), brown midrib (*bmr*) traits that improve digestibility (Porter et al. 1978; Grant et al. 1995), drought tolerance (Rosenow et al., 1983; Smith and Frederiksen 2000), and high nitrogen use efficiency (Blomstedt

et al. 2018; Rosati et al. 2019) making it an ideal crop for use in drought-prone and low-fertility environments (Smith and Frederiksen 2000).

Dhurrin is a specialized metabolite of sorghum (Nielsen et al. 2016) that plays a role in plant responses to insect feeding (Krothapalli et al. 2013; Gruss and Tuinstra 2021), drought tolerance (Burke et al. 2013), and nitrogen metabolism (Blomstedt et al., 2018; Rosati et al., 2019). Dhurrin accumulates in both roots and shoots with the highest concentrations observed in the leaves of young seedlings (Akazawa et al. 1960; Conn 1994; Gleadow and Møller 2014; Emendack et al. 2017). Dhurrin concentrations as high as 10% dry weight have been reported in sorghum seedlings with tissue specific accumulation patterns at later stages depending on environmental conditions and stage of development (Nielsen et al, 2016).

Over 2500 plant species contain CGs (Conn 1981; Gleadow and Møller 2014); however, dhurrin is specific to sorghum. Dhurrin hydrolysis leads to the release of hydrogen cyanide (HCN) which in concentrations greater than 750 mg kg^{-1} can be very dangerous to cattle, leading to decreased performance and potentially death (Strickland et al. 2017). HCN interacts with an animal's ability to utilize oxygen by disrupting the respiratory pathway (Price 1985). Death from cyanosis can occur quickly, within 15 minutes in severe cases (Smith and Frederiksen 2000).

Dhurrin production varies greatly depending on sorghum cultivar (Burke et al. 2013), plant age (Kojima et al. 1979; Halkier and Moller 1989; Adewusi 1990), plant organ (Loyd and Gray 1970; Kojima et al. 1979;), and environmental conditions (Wheeler 1994; Busk and Moller 2002; Marsalis et al. 2010; O'Donnell et al. 2013). Management practices are important to keep HCN potential (HCNp) low. Sorghum plants under drought stress (O'Donnell et al. 2013; Rosati et al. 2019) or exposed to freezing temperatures (Stochmal and Oleszek 1997; Gleadow and Møller 2014) may accumulate dhurrin thereby increasing HCNp.

It is often reported that processing and feeding sorghum as silage can reduce HCNp by 70% and or haymaking can reduce HCNp by 50% . These options are listed as best management practices if HCNp is a concern (Smith and Frederiksen 2000; Strickland et al. 2017). When sorghum samples are dried at 60 ºC to 70 ºC and stored at low moisture levels, dhurrin content was stable (Gleadow et al. 2012). When making hay, sorghum is normally dried at ambient temperatures and stored dry under variable humidity conditions. The impacts of these drying and storage conditions on dhurrin stability are not well documented or understood.

Dhurrin production in sorghum can also be altered by using genetic mutations that disrupt the metabolic pathway for this secondary metabolite (Blomstedt et al. 2012; Krothapalli et al. 2013; Tuinstra et al., 2016). One mutation has been discovered that disrupts dhurrinase2 (*dhr2-1*), the enzyme that catalyzes the breakdown of dhurrin into HCN and p-hydroxybenzaldehyde (Krothapalli et al. 2013). Blomstedt et al. (2012) and Tuinstra et al. (2016) describe two additional mutations that disrupt the first enzyme, CYP79A1, in the biosynthetic pathway creating dhurrin-free sorghum (Blomstedt et al. 2012; Tuinstra et al. 2016).

In this study, quantitative and qualitative methods were used to study changes in dhurrin content and HCN release in sorghum harvested, processed, and stored as dry samples. Specific objectives for this study included analysis of dhurrin content and HCN release characteristics of (1) commercial hybrids and experimental sorghum hybrids produced in Indiana and Kansas, (2) sorghum genotypes with mutations in the dhurrin biosynthetic and catabolic pathways, and (3) dry sorghum samples rehydrated under varying conditions.

5.3 Materials and Methods

5.3.1 Genetic Materials

Eight hybrids were used to test the stability of dhurrin in dried tissues. Four hybrids were produced in West Lafayette, Indiana, and Colby, Kansas: Sweet Bites, a sorghum x sudangrass; Sweet Six, a sorghum x sudangrass bmr; GreenTreat Rocket®, sudangrass x sudangrass bmr; and (ATx623 x Excel S235)-F1 *bmr6 cyp79a1*, sorghum x sudangrass bmr *cyp79a1*. The three commercial hybrids were obtained from Cisco Seeds (Indianapolis, IN). Four additional hybrids were grown in Hays, Kansas, and Garden City, Kansas: Magnum Ultra BMR (Star Seed Inc, Osborne, KS), forage sorghum bmr; Super Sweet 10 (Dyna-Gro, Geneseo, IL), sorghum x sudangrass; Fullgraze II (Dyna-Gro Seed, Geneseo, IL), sorghum x sudangrass, and ADV S6504 (Alta Seeds, Amarillo, TX), sorghum x sudangrass bmr.

Sorghum EMS mutants and near-isogenic lines (NILs) with contrasting dhurrin production characteristics were used to assess the impact of dhurrin metabolism on dhurrin stability and HCN release over time (Table 5.1). These lines included Tx623, the reference genome (Paterson 2008), SbEMS932 *dhr2-1* described by Krothapalli et al. (2013), and SbEMS2447 *cyp79a1* described by Tuinstra et al. (2016) (Table 5.1). The near isogenic lines (NILs) included Tx623 *bmr6* (Oliver et

al. 2005b; Pedersen et al. 2006) and Tx623 *bmr6 cyp79a1*. Tx623 *bmr6 cyp79a1* was developed by crossing and backcrossing Tx623 *bmr6* to B Tx623 *cyp79a1* with selection for acyanogenic plants using the Feigl Anger assay (Feigl and Anger 1966).

Table 5.1: Sorghum EMS mutants and near-isogenic lines (NILs) with contrasting dhurrin production characteristics used to assess impacts of metabolism on dhurrin stability and HCN release over time. The genotypes are classified based on potential for dhurrin production (cyp79a1) and HCN release (dhr2).

Genotype	Dhurrin	Dhurrinase2
Tx623	+	+
Tx623 *bmr6*	+	+
SbEMS932 *dhr2-1*	+	-
SbEMS2447 *cyp79a1*	-	+
Tx623 *bmr6 cyp79a1*	-	+

5.3.2 Dhurrin Content in Dry and Fresh Samples

Comparisons of dhurrin extraction efficiencies from dry and fresh leaf samples utilized field grown seedlings of Tx623 at the V3 stage. The top collared leaf was sampled and split longitudinally down the midrib. Each half of the leaf was weighed then either (1) air dried at 24º to 35º C in a greenhouse, or (2) frozen in liquid nitrogen and stored at -80°C. One week after sampling, the dried and frozen leaf samples were ground and the dhurrin extracted and analyzed with ultra-high performance liquid chromatography (UHPLC) using the procedure described in sections 5.3.5 and 5.3.6. The results for dhurrin content in the two halves of the leaf blade were expressed on a fresh weight basis to compare extraction efficiencies of the two methods.

5.3.3 Stability of Dhurrin in Dried Samples

The three commercial hybrids, Sweet Bites, Sweet Six, GreenTreat Rocket®, and the dhurrin-free experimental hybrid (ATx623 x Excel S235)-F1 *bmr6 cyp79a1*, were evaluated in field trials at the Kansas State Research-Extension Center at Colby, KS and the Animal Sciences Research and Education Center (ASREC) at Purdue University, West Lafayette, IN using a randomized complete block design with four replications (Figure 5.1). The ASREC trial in West Lafayette, IN had 37.2 kg ha^{-1} of nitrogen (N) added pre-plant and 37.2 kg ha^{-1} was added again after the first

biomass harvest. The Colby, KS field site had sprinkler irrigation that was used to establish the stand, by supplementing 2 cm of water. A pre-plant fertilizer was applied at a rate of 168 kg ha^{-1} of N, and 34 kg ha^{-1} for phosphorous. The plots were 1.5 m by 6.1 m at Colby. ASREC had slightly larger plots at 2.1 m by 7.62 m.

Four additional commercial hybrids, Magnum Ultra BMR, Super Sweet 10, Fullgraze II, and ADV S6504, were evaluated in trials at Kansas State Research-Extension Centers at Garden City, KS and Hays, KS using a randomized complete block design with three replications. There was 179 kg ha^{-1} of N at the Garden City location, and 56 kg ha^{-1} of N at the Hays location applied as a pre-plant fertilizer. Garden City had sprinkler irrigation that supplemented a total of 32 cm of water to the plots. The plots were laid out in a RCBD with three replications.

Figure 5.1: An example of the layout of the field for data collection at the West Lafayette, IN location. There are four plots within each replication with each hybrid represented in each replication.

Each trial was harvested for biomass yields and plant samples for dhurrin extraction were harvested from the regrowth. At the ASREC location, plant samples were collected after the second biomass harvest in early August and after the third biomass harvest in early September. While all the Kansas locations had one date for collection following a late summer biomass harvest. Eight plants smaller than 31 cm in height were harvested from each plot on each sampling date. The plant samples were dried in either a greenhouse or oven at 32.2° C for two days. The

dried samples were sent from Kansas to Purdue University for long term storage and analysis. Two plants per plot were assigned to represent drying treatments of one week, two weeks, one month and two months.

Sorghum plants were stored in brown paper bags in a greenhouse at Purdue University until the day of analysis. The whole plant samples were ground to 1 mm with a Udy cyclone-mill (UDY Corp., Fort Collins, CO) at one week, two weeks, one month, and two months after being sampled from the field and analyzed for dhurrin. The ground plant samples were weighed and put into a 5-ml vial with a screw top lid. A plant from each replication was analyzed with UHPLC for dhurrin content as described in sections 5.3.5 and 5.3.6, and the second plant was tested for HCN release using the Feigl and Anger (1996) test as described below, in section 5.3.4. Dried maize leaf samples were included in the UHPLC analysis as a negative control.

5.3.4 **HCN Release**

The potential for release of HCN was determined using the Feigl Anger assay (Feigl and Anger, 1966). Dried ground plant samples (20 mg) were transferred to the cells of a 96-cell deep well plate, ~250 µl of deionized water was added to the cells. Feigl and Anger (FA) paper was used to cover the plate followed by the plate lid to form a tight seal between the treated paper and the plate. The plates were stored at room temperature for 20 hours and then the FA papers were photographed to record blue coloring to indicates HCN release.

5.3.5 **Dhurrin Extraction**

The ground plant samples were extracted using 1:1 MeOH:H_2O containing 0.05 mg/mL *p*-hydroxybenzaldehyde as an internal standard. The extraction was performed by adding 3 ml of extraction solvent to each 5 mL tube containing ground sample tissue. Tubes were incubated in a water bath at 75 °C for 15 min, then removed, vortexed, and placed at 4 °C for 16-24 hr. The next day, the tubes were vortexed and centrifuged and 1 mL of extract was collected and filtered through a 0.2 µm filter into a labeled 1.5 mL microcentrifuge tube. 200 µL of the filtered extract was transferred into HPLC vials for UHPLC analysis.

5.3.6 **UHPLC Analysis**

UHPLC analysis was performed by using a 1290 Infinity II system with a diode array detector (Agilent Technologies, Santa Clara, CA, USA). The separation of compounds was achieved on the Zorbax SB-C18 column (1.8 µm, 2.1 × 50 mm; Agilent) with column temperature maintained at 30 °C. Water (A) and ACN (B) were used as mobile phase solvents with 0.1% formic acid (*v*/*v*) at a flow rate of 0.3 mL min^{-1}. The solvent gradient program was set as shown in Table 5.2 with a total run time of 7 min. 5 µL of injection volume was used both for samples and standards.

Table 5.2: HPLC solvent gradient program

Time (min)	**A [%]**	**B [%]**	**Flow [ml min^{-1}]**
0.00	90.00	10.00	0.300
1.00	90.00	10.00	0.300
3.00	75.00	25.00	0.300
4.00	75.00	25.00	0.300
5.00	5.00	95.00	0.300
6.00	5.00	95.00	0.300
7.00	90	10.00	0.300

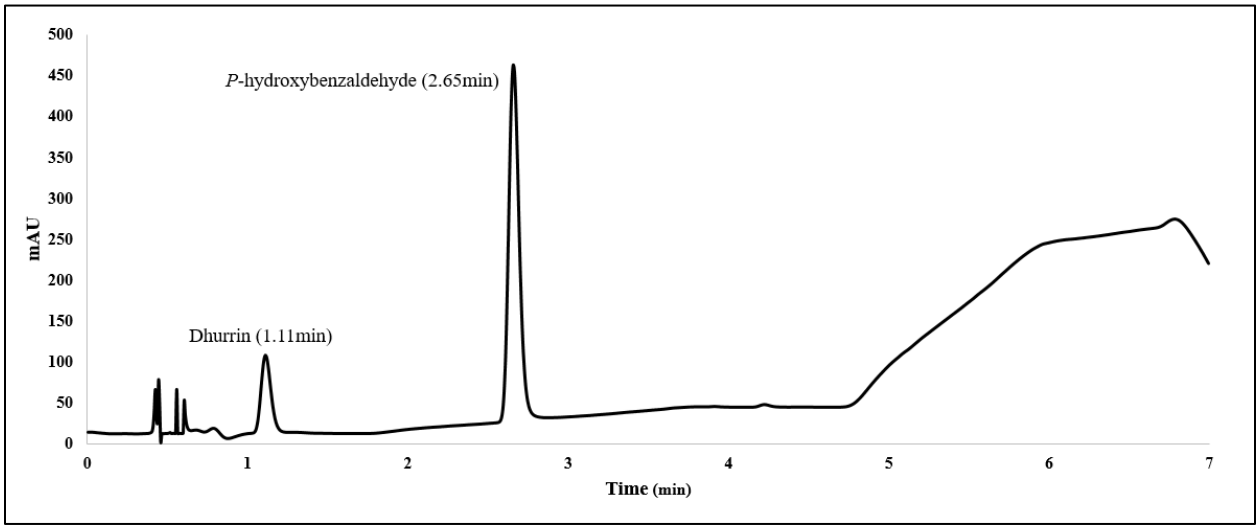


Figure 5.2. DAD chromatogram (232 nm) of dhurrin (1.11 min) and p-hydroxybenzaldehyde (2.65) at the concentrations of 0.01 and 0.05 mg mL-1, respectively.

Dhurrin and *p*-hydroxybenzaldehyde were eluted at 1.11 and 2.65 min, respectively (Fig. 5.1) and absorbance for both compounds was recorded at 232nm. Dhurrin quantitation was performed by analyzing the linear range of 0.0025, 0.0050, 0.0100, 0.0250, 0.0500 mg mL^{-1}standards each containing 0.05 mg mL^{-1} *p*-hydroxybenzaldehyde as an internal standard. Instrument operation and data analyses were performed using OpenLAB CDS ChemStation software version C.01.09.

5.3.7 **Dhurrinase Activity**

Next the release of HCN within the dried samples was tested comparing dhurrinase2 activity within the plants. Sorghum EMS mutants; SbEMS 932 *dhr2-1* and SbEMS2447 *cyp79a1*, Tx623 and NILs; Tx623 *bmr6* and Tx623 *bmr6 cyp79a1* were sampled by taking a 2.5 cm sample from the top collared leaf, weighed, and dried in the greenhouse at 24 °C to 35 °C for one week (Table 5.1). Dried leaf tissue was ground with a mortar and pestle and randomly placed into the 96-cell deep well plate with 250 µl of deionized water. The FA assay was conducted over 20 h to assess HCN release. Fresh samples were collected using the same methods except were frozen in -80 °C overnight and allowed to thaw the next day with a piece of FA paper on top of the plate. Fresh and dry samples were evaluated for differences in HCN release that could be due to the differences in dhurrinase2 activity. Photographs were taken to record the appearance of blue coloring.

5.3.8 ***In vitro* Dry Matter Digestibility Procedure**

Sorghum EMS mutants and near-isogenic lines (NILs) were examined for potential HCN release using a modified *in vitro* dry matter digestibility (IVDMD) assay. The procedure was replicated on three separate days. In the first run, approximately 0.008 g dried and ground leaf tissue from the top collared leaf of V3 plants was used to test IVDMD. In the second run, approximately 0.008 g dried and ground tissue from whole dried plants at the V5 growth stage were used to determine IVDMD. In the third run, approximately 0.008 g dried and ground tissue from whole dried plants at the V5 growth stage were used to determine HCN release when only using rumen fluid, rehydrating with water, and rehydrating with solution A. Tissue samples were ground with an Udy cyclone-mill using a 1 mm sieve. Samples were weighed and stored in a 50-ml polypropylene tubes with screw top lids.

Samples were evaluated for IVDMD using a modified procedure described by Twidwell et al. (1988). Solution A, a synthetic saliva, was made prior to the day of rumen fluid extraction. Solution A consists of 10g of KH_2PO_4, 0.5g of $MgSO_4$- 7 H_2O, 0.5g of NaCl, and 0.1g of $CaCl_2$ - H_2O in 1 liter of deionized water. On the day of rumen fluid extraction, solution A was heated to 39º C, and the pH was tested. To reach a pH of 6.9, a solution of 15g of Na_2CO_3 in 100 ml of deionized water was slowly added to solution A until the desired pH was reached.

The IVDMD assay was initiated by addition of 640 µl of solution A to each of the samples. The fluid was added 160 µl at a time. Then tubes were swirled, and the samples were covered with rubber stoppers with one-way valves. A piece of FA paper was attached to the bottom of the stopper with a push pin. After the tissue samples were suspended in solution A and the samples were placed back in the incubator at 39 °C, rumen fluid was extracted from a fistulated dairy cow on a diet that excluded sorghum. Solid material was removed from the rumen and rumen fluid was strained through a funnel lined with a cheese cloth into a jar. The jar was incubated at 39 °C and CO_2 was bubbled through the rumen fluid for 10 min.

Next, 1.4 ml of rumen fluid was added to each sorghum tissue sample suspended in solution A. The tubes were immediately closed using the stoppers with the FA paper attached and sealed using parafilm. Samples were gently swirled and were placed in the incubator at 39 °C. Each sample was periodically checked for HCN release based on the changing color of the FA paper without removing the stopper. Samples were removed from the incubator when blue coloring was noted. Samples that did not turn blue remained in the incubator overnight and were checked for blue coloring of the FA paper for a final time after 20 hours.

The modified IVDMD procedure was conducted in both the first and second run. The third run consisted of three full sets of samples with varying rehydration methods to compare HCN release to the IVDMD. The first excluded the addition of solution A, and only had rumen fluid added as described above to determine if HCN release was being caused by solution A or the rumen fluid. The second set of samples was rehydrated with solution A only to assess if solution A would release HCN and how long it would take. The third set of samples was rehydrated with deionized water and incubated at 39 °C. This last set was to determine the effects of incubating the samples compared to the timing of HCN release.

5.3.9 Statistical Analysis

To compare extraction efficiencies for the fresh and dry samples from the two halves a leaf, a paired t-test was conducted within Microsoft Excel.

Data from each of the field trials of the sorghum hybrids were screened for outliers using Cook's Distance. An outlier was declared if the value was four times or greater from the mean based on Cook's Distance. For example, within the Garden City, KS samples there was one point identified and it was three times larger than the other points for that hybrid at that time. Additionally, one other outlier was removed from the trial at the West Lafayette location because it was 10 times higher than any other samples from the same hybrid at either the West Lafayette or Colby location. The outliers were removed from the dataset, because they may be from a sampling error.

A mixed effects model was used to evaluate dhurrin content across the hybrids and storage time. Replication was treated as a random effect. This model was used for all the Kansas locations.

The West Lafayette, IN location was analyzed first to determine if there was an effect between the two sampling dates, but there was not, and the data from the two-sampling dates was run in a combined analysis using the hybrids and storage time as the effects.

Tukey's post-hoc comparison was used to determine similarities and differences among hybrids across time. Significance was determined using p-value of 0.05.

Data from the trial at Colby, KS was transformed using a log transformation to fit a normal distribution. The transformed data was used for analyses and comparisons.

Dried maize samples were included in UHLPC analysis as a negative dhurrin control. Comparisons with the dhurrin-free experimental hybrid were made using a t-test.

5.4 Results

5.4.1 Dhurrin Extraction from Dry and Fresh Tissue Samples

Dry and fresh tissue samples from individual leaves from Tx623 were extracted for dhurrin content to compare extraction efficiencies (Table 5.3). Dhurrin extraction efficiencies were similar using fresh and dried tissue samples (p-value < 0.05).

Table 5.3: The effects of one week of drying on dhurrin content within sorghum leaf samples.

Leaf Sample	Dhurrin (mg fwg^{-1})	Type of sample
1	4.46	Fresh Sample
	6.92	Dry Sample
2	3.66	Fresh Sample
	3.88	Dry Sample
3	11.69	Fresh Sample
	16.89	Dry Sample
4	5.12	Fresh Sample
	7.05	Dry Sample

5.4.2 **Stability of dhurrin in dried tissue**

Dhurrin was found in all the dried samples from each conventional sorghum hybrid (Table 5.4). In West Lafayette, sorghum samples were collected after the 1st and 2nd cutting. Cutting date did not have a significant effect on dhurrin content over time. Analysis of variance (ANOVA) of hybrids and storage times indicated significant differences among hybrids at every location, but storage time was only significant in West Lafayette (Table 5.4). The dhurrin-free experimental hybrid exhibited the lowest dhurrin content in trials at the West Lafayette, IN and Colby, KS locations. Comparisons of conventional sorghum hybrids at Garden City, KS and Hays, KS indicated that Super Sweet 10 had the lowest dhurrin content of the samples harvested from trials at these locations (Table 5.4). Storage time was only significant in the West Lafayette trials with samples showing a reduction in dhurrin content over time.

Maize tissue samples were used as a negative control for comparisons of dhurrin content in sorghum, because maize does not contain dhurrin. UHPLC analyses of maize indicated value of 0.602 mg g^{-1} dhurrin with no differences in dhurrin content between maize and the dhurrin-free experimental hybrid. Using the maize as our dhurrin baseline for no dhurrin content, it was determined there was no dhurrin in the dhurrin-free hybrid.

Table 5.4: Effects of storage time on concentration of dhurrin in dried tissue samples.

West Lafayette					Colby[2]			
Hybrids	Week 1	Week 2	Month 1	Month 2	Week 1	Week 2	Month 1	Month 2
Dhurrin-free	0.9	0.5	0.5	0.2	1.3	1.3	0.6	1.4
Sweet Bites	10.3	7.3	11.9	7.3	16.8	17.3	16.6	14.3
Sweet Six	11.0	11.7	11.8	9.9	20.0	14.3	18.7	17.4
GreenTreat Rocket	8.4	10.6	8.9	6.1	16.1	11.4	14.4	11.3
Significance[1]	Hybrids		***		***			
	Treatment		**					
	Hybrids*Treatment							
Garden City					Hays			
Hybrids	Week 1	Week 2	Month 1	Month 2	Week 1	Week 2	Month 1	Month 2
ADV S6504	7.4	7.8	6.4	5.0	7.2	4.3	7.7	4.7
Fullgraze II	7.0	5.9	9.9	7.9	4.4	12.9	8.6	9.5
Magnum Ultra BMR	10.3	11.2	10.5	13.1	11.5	6.4	14.7	15.4
Super Sweet 10	4.9	4.1	3.1	2.9	2.3	2.0	4.3	1.9
Significance	Hybrids		***		***			
	Treatment							
	Hybrids*Treatment							

[1] **=0.05, **=0.01, ***=0.001*

5.4.3 **HCN Release**

The FA assay was used to evaluate the potential for HCN release from dried sorghum plant tissues rehydrated in water. HCN was never detected in fresh or dried samples of the dhurrin-free hybrid; however, HCN was detected in the conventional hybrids after dry storage for one week, two weeks, one month, and two months (Fig. 5.3). Based on changes in color intensity, the amount of HCN released decreased over time for the dry samples. Color intensity is an identifier of quantity of HCN release (Reddy et al. 2016).

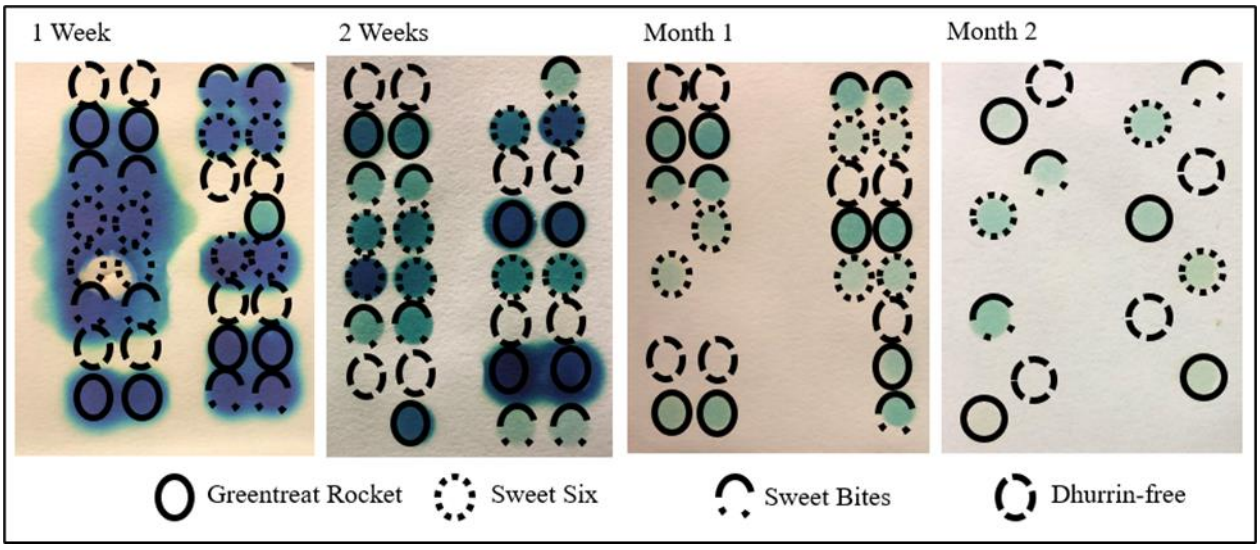


Figure 5.3: The release of HCN across the dried sorghum hybrid tissue samples after 1 week, 2 weeks, 1 month, 2 months.

5.4.4 **Dhurrinase Activity in Dried vs Fresh Samples**

The comparisons of HCN release in dried and fresh tissue was to help assess dhurrinase2 activity within the dried samples (Figure 5.3). EMS mutants and NILs varying in dhurrinase activity and dhurrin content were compared for HCN release from fresh and dried tissue samples (Table 5.1). HCN release was much quicker in fresh samples (Fig. 5.4) as compared to dried plant samples (Fig. 5.5) that contained dhurrin. Tx623 and Tx623 *bmr6* fresh tissue started showing blue coloring within 15 minutes; these genotypes contained dhurrin and an active dhurrinase2. SbEMS932 *dhr2-1* does not have an active dhurrinase2 and it took 90 minutes for fresh tissue samples to produce blue coloring (Fig 5.4) showing an active dhurrinase2 will increase the speed of HCN release. Fresh tissue samples for SbEMS2447 *cyp79a1* or Tx623 *bmr6 cyp79a1* never produced blue

coloring in the FA assay. In the dried plant tissue rehydrated with water, blue coloring began to appear at the same time at 20h for Tx623, SbEMS932 *dhr2-1*, and Tx623 *bmr*; these genotypes contain dhurrin, but vary in initial dhurrinas2 activity (Fig. 5.5). No blue coloring was detected in SbEMS2447 *cyp79a1* or Tx623 *bmr6 cyp79a1*.

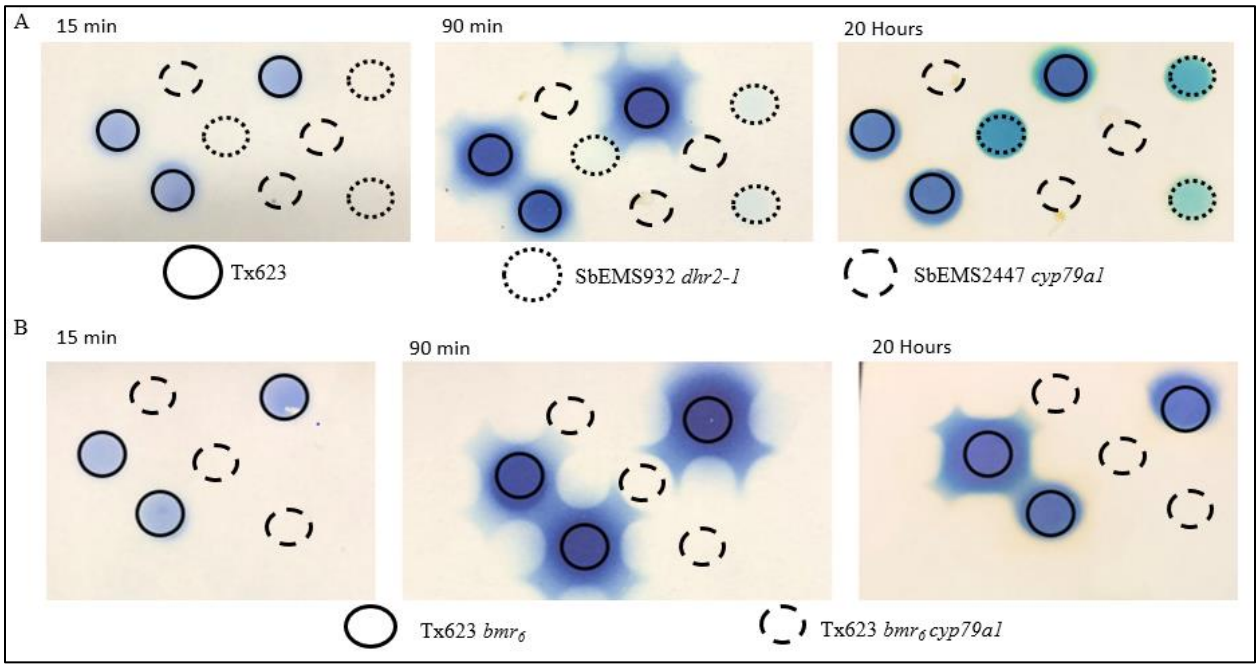


Figure 5.4: Comparison of time for HCN release from fresh tissue, varying in dhurrin content and dhurrinase2activity. A) Tx623, SbEMS932, and SbEMS2447. B) Tx623 *bmr6* and Tx623 *bmr6 cyp79a1*.

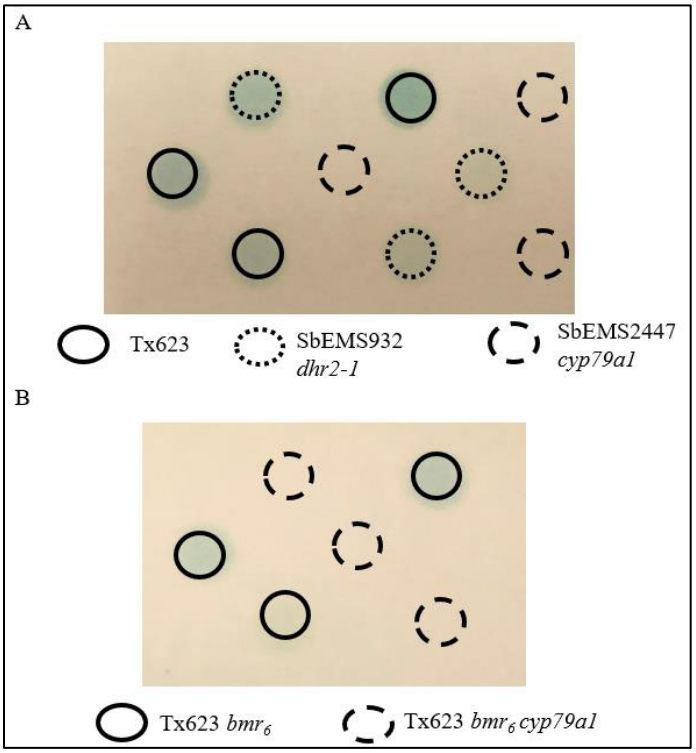


Figure 5.5: Comparison of HCN release from dried tissue that vary in dhurrin content and dhurrinase2 activity after 20 hours. A) Tx623, SbEMS932, and SbEMS2447. B) NILs, Tx623 bmr6, and Tx623 bmr6 cyp79a1.

5.4.5 **Use of In vitro Dry Matter Digestibility Procedure for Testing HCN release**

Dried top-collared leaf tissues were examined first using the IVDMD modified protocol. When the samples were introduced to the rumen fluid it took approximately fifteen minutes for the dhurrin containing genotypes, Tx623, SbEM932 *dhr2-1*, and Tx632 *bmr6*, to turn blue demonstrating the release of HCN (Fig 5.6). This blue coloring appeared whether the genotypes had an active or inactive dhurrinase2 enzyme in the fresh tissue. The negative control and the two genotypes without dhurrin, SbEMS2447 *cyp79a1* and Tx632 *bmr6 cyp79a1*, did not show any blue coloring after 20 hours exposure in the rumen fluid, indicating no release of HCN (Fig 5.6). The dark blue coloring of the FA paper indicates high levels of HCN release.

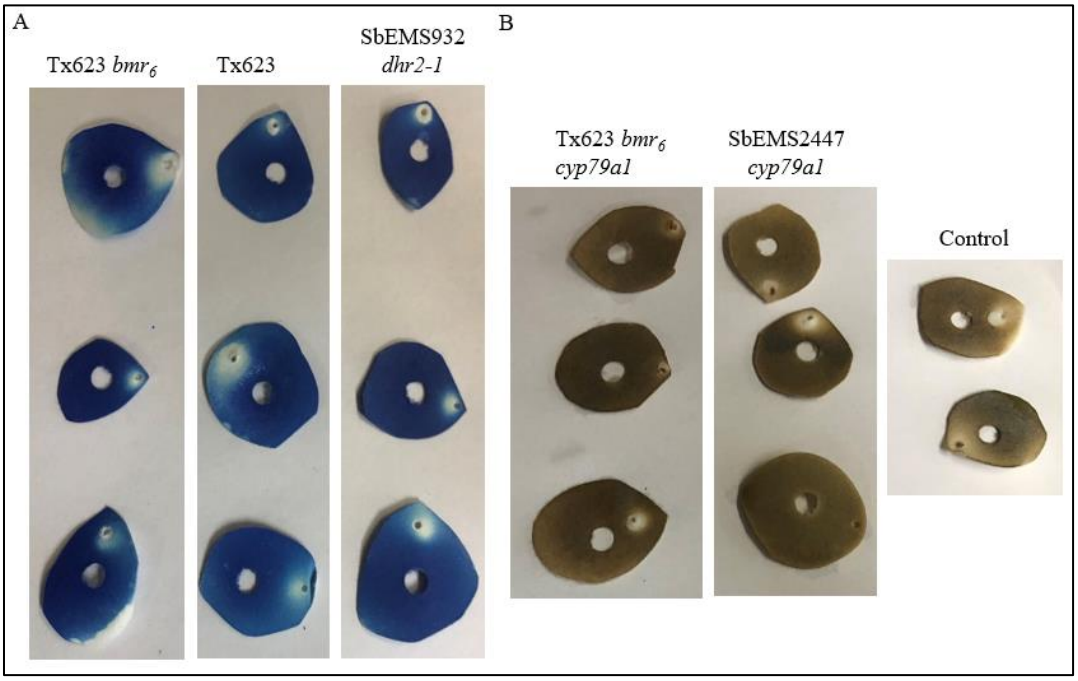


Figure 5.6: Feigl Anger Assay for HCN release from dried and ground plant material after addition of rumen fluid and solution A to (A) Tx623 *bmr6*, Tx623, and SbEMS932 *dhr2-1* after 15 min and B) Tx623 *bmr6 cyp79a1*, and SbEMS2447 *cyp79a1* and control without any plant material after 20 hours

Whole dried plants were also evaluated for HCN release using the modified IVDMD (Fig. 5.7), after rehydrating the dry tissue with Solution A (Fig. 5.8), and after rehydrating the dry samples with water (Fig. 5.9). Dry samples tested using the modified IVDMD assay released HCN much more quickly than the samples rehydrated with water or Solution A. All three genotypes containing dhurrin produced dark blue coloring within 15 min of the addition of rumen fluid (Fig. 5.7). The dhurrin containing samples rehydrated with water took approximately 20 h for blue coloring to appear (Fig. 5.8) and dry samples rehydrated with Solution A developed blue coloring took approximately 45 min for blue coloring to appear (Fig. 5.8). When rehydrated with water, there was no blue coloring for any of the hybrids at 45 mins (Fig. 5.9). No blue coloring in the FA assay was detected for Tx623 *bmr6 cyp79a1* and SbEMS2447 *cyp79a1* after the modified IVDMD procedure, water or rehydration with water, solution A, or rumen fluid only.

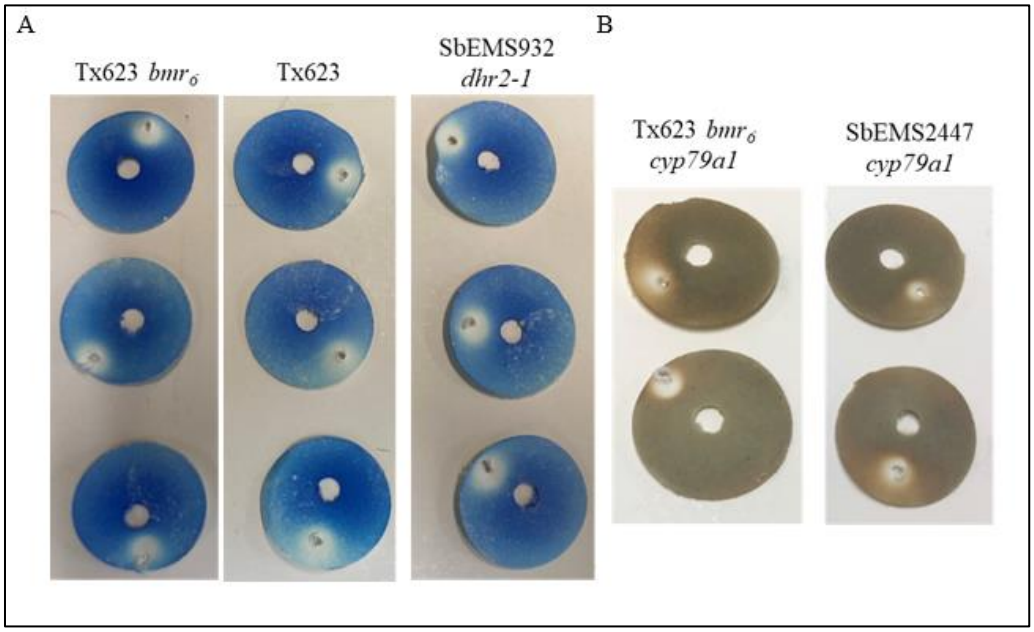


Figure 5.7: Feigl Anger Assay for HCN release from dried, ground plant tissue of (A) Tx623 *bmr*$_6$, Tx623, and SbEMS932 *dhr2-1* after 15 minutes of introducing Solution A and rumen fluid from a lactating cow, and (B) Tx623 *bmr6 cyp79a1*, and and SbEMS2447 *cyp79a1* after 20 hours of introducing Solution A and rumen fluid from a lactating cow.

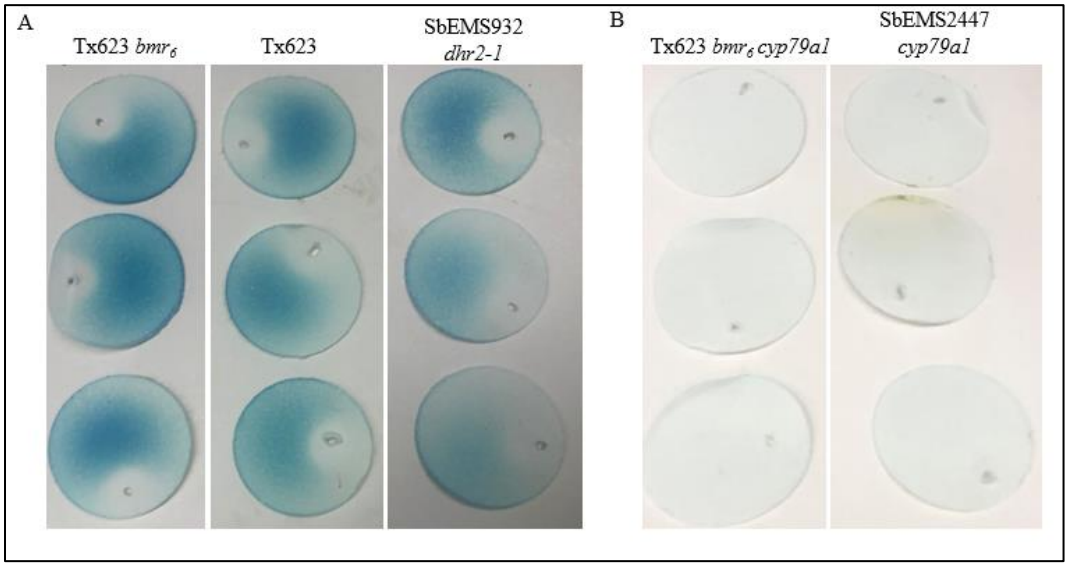


Figure 5.8: Feigl Anger Assay for HCN release from dried, ground plant tissue of (A) Tx623 *bmr6*, Tx623, and SbEMS932 *dhr2-1* after 45 minutes of introducing Solution A, and (B) Tx623 *bmr6 cyp79a1* and SbEMS2447 *cyp79a1* after 20 hours of introducing Solution A.

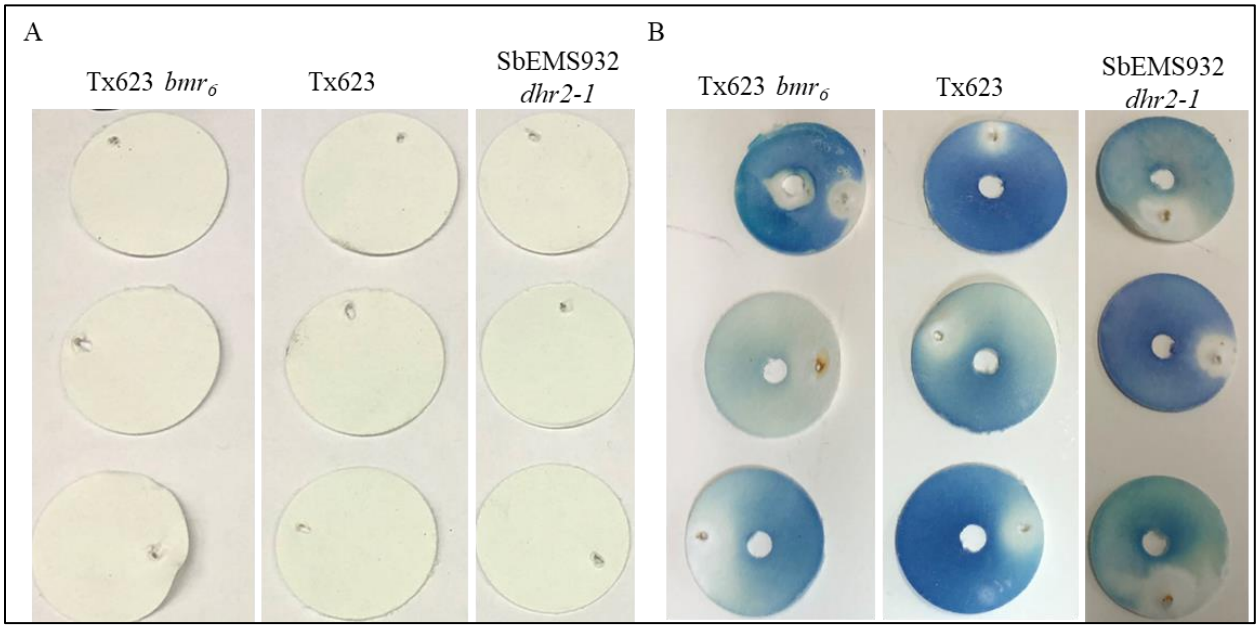


Figure 5.9: HCN release from dried, ground plant tissue of Tx623 *bmr6*, Tx623, and SbEMS932 *dhr2-1* rehydrated in water after (A) 45 min and (B) 20 hours.

Whole dried plants were also evaluated for HCN release with rumen fluid only to ensure that the rumen fluid was causing the release of HCN and not Solution A, the synthetic saliva. All of the dhurrin-containing samples produced blue color with the FA assay within 15 min after the addition of rumen fluid to the tissues (Fig. 5.10). There was no detectable HCN released in any of the samples carrying the *cyp79a1* mutation, even after 20 h (Fig. 5.10).

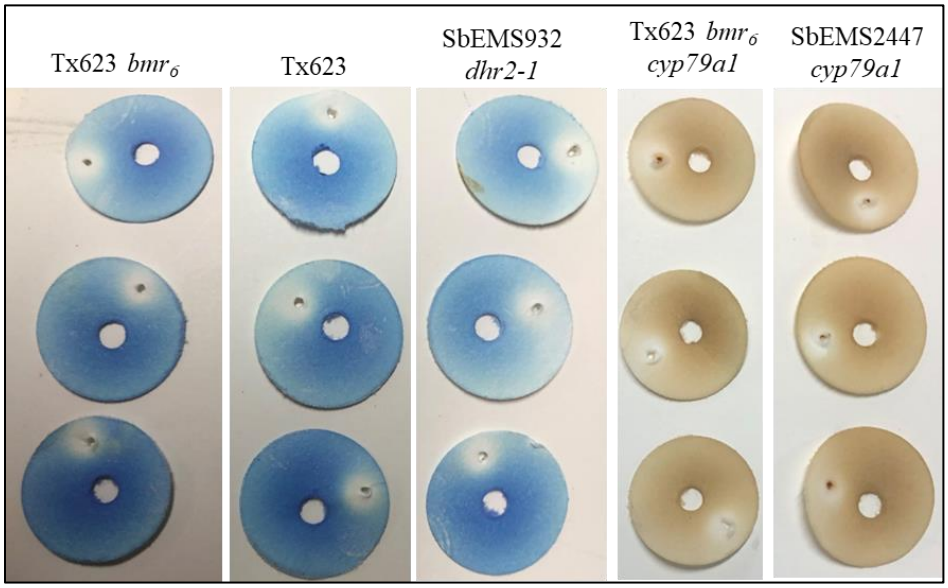


Figure 5.10: Feigl Anger Assay for HCN release from dried, ground plant tissue after 15 min for Tx623 *bmr6*, SbEMS932 *dhr2-1*, Tx623 *bmr6 cyp79a1*, and SbEMS2447 *cyp79a1* within only rumen fluid.

5.5 Discussion

Dhurrin content in sorghum has previously reported to be reduced by up to 50 percent during the hay curing process (Smith and Frederiksen 2000; Strickland et al. 2017). The experimental evidence supporting this relationship is not clear. In this study, dhurrin content of dried and fresh leaf samples were similar after one week of drying with no indication that dhurrin was broken down during the drying process. Follow up studies on the stability of dhurrin in seven commercial sorghum hybrids and a dhurrin-free experimental hybrid over a two-month timeframe showed similar results. Dhurrin content varied among hybrids but did not vary within a hybrid over time at three of the four locations. This showed that dhurrin was generally stable in the dried samples for at least two months. This supports a report by Gleadow et al. (2011) that dhurrin was stable for over a year in tissues stored at low moisture levels and in the dark. In this study, moisture was not controlled and the samples were exposed to indirect and direct sunlight but dhurrin content was quite stable over time in samples produced at three of the four locations. The dhurrin-free hybrid did not contain any dhurrin.

HCN was released when dry tissue samples stored for one week, two weeks, one month, and two months were rehydrated with water. HCN release was considerably slower with the dried samples in comparison to fresh samples. Release of HCN seemed to decrease over time of tissue storage

with the darkest blue color showing at week one and a considerably lighter blue by two months. Dhurrin content was similar over time for the eight hybrids, showing the variation in HCN release may be due to another factor, such as dhurrinase2 activity. Dhurrinase2 is the enzyme that catalyzes the hydrolysis of dhurrin and leads to the quick release of HCN (Krothapalli et al. 2013). The role of dhurrinase2 in release of HCN from dry tissue samples was investigated using Tx623 and SbEMS932 *dhr2-1*. Dhurrinase2 increases the rate of hydrolysis of dhurrin and the release of HCN (Krothapalli et al., 2013). In fresh tissue samples, Tx623 released HCN in 15 min while SbEMS932 *dhr2-1* released HCN after 90 min. In the dried tissue samples, HCN was released after 20 hr for both genotypes. This suggests that dhurrinase2 is inactivated during the drying process. If it were active, it would be expected that Tx623 would release HCN more quickly in comparison to the SbEMS932 *dhr2-1* in the dry material, similar to what was found in the fresh tissue.

The amount of time required for release of HCN from dried plant tissues of dhurrin-containing plants was dramatically reduced when using the modified IVDMD procedure. HCN was released from dried tissues of dhurrin containing plants as quickly as fresh plant tissues. This supports the hypothesis that enzymes such as non-specific β-glucosidases within the stomachs of mammals can either catalyze the hydrolysis of dhurrin or make dhurrin more available for catalysis (Singh et al., 2016). Dry plant tissues exhibited high levels of HCN release within 15 minutes of being introduced to rumen fluid. No HCN release was observed in dhurrin-free sorghum tissues.

Drying sorghum tissue samples for one week, two weeks, one month, or two months had an impact on HCN release with reduced blue coloring in the FA assay observed in samples dried for longer periods of time. The blue color was much lighter after two months when the tissues were rehydrated for 20 h. The capacity to release HCN was restored in dried tissue samples containing dhurrin when conducting the IVDMD modified procedure (solution A and rumen fluid) and with addition of only rumen fluid. Solution A, which is a synthetic saliva, also caused HCN release within an hour suggesting that initial HCN release can occur in the ruminant livestock's mouth when masticating. Once the masticating tissue reaches the rumen, the rumen fluid has the ability to release HCN very quickly highlighting the importance of managing dhurrin content in sorghum hay.

5.6 Conclusions

Drying and long-term storage of dry sorghum samples did not decrease dhurrin content levels in sorghum. The dhurrin in these tissues could be catalyzed to release HCN over extended time periods although the rate of HCN release was slower when rehydrating samples with water. These results highlight the importance of proper management of sorghum hay to minimize dhurrin content; especially because ruminant livestock can process dhurrin to release HCN within the rumen. This danger can be avoided with the use of a dhurrin-free forage sorghums which did not release HCN from fresh or dry tissue samples.

CHAPTER 6. THE EFFECTS OF FROST ON DHURRIN ACCUMULATION AND PERSISTANCE IN SORGHUM FORAGES

6.1 Abstract

Sorghum is an important forage crop that requires careful management to avoid hydrogen cyanide (HCN) toxicity in animals. Management practices are important to ensure livestock safety. Key practices include avoid grazing sorghum that is undergoing extreme stress including drought conditions and frost events that may cause dhurrin accumulation. Understanding dhurrin accumulation and persistence following frost conditions is important in avoiding HCN. This study evaluated dhurrin content (mg fresh weight g^{-1}) of eight hybrids including seven dhurrin producing and one dhurrin-free hybrid before and following a frost. The hybrids were planted in controlled environment trials and at three field locations. Frost events caused dhurrin accumulation in the conventional hybrids for three days following the frost event. The dhurrin content of sorghum increased by 52% after the frost at Colby, KS, and by 49% at the West Lafayette, IN location. Sorghum trials at Garden City, KS also exhibited increased dhurrin content but not until seven days following the frost. Depending on the weather events following the first frost, dhurrin content returned to low levels at the West Lafayette, IN location, or remained high at the Colby, KS location. Indiana had warmer weather following the first frost and remained warm for several days while the two KS locations remained cold and had multiple frost events in succession. The dhurrin-free hybrid did not produce any detectable dhurrin at any location or time point.

6.2 Introduction

Farmers planted 2.3 million hectares of sorghum in the United States (U.S.) in 2018 (USDA and NASS 2019). Sorghum is an annual warm season grass commonly used in forage production. Forage sorghum can be utilized as silage, hay, green chop, and as a pasture crop. Forage sorghum is commonly grown in the south-central region of the U.S. in semiarid to arid environments (Undersander et al. 1990) because of its natural adaptability to drought and poorly fertilized environments (Borrell and Hammer 2000; Sanchez et al. 2002). Brown midrib (bmr) sorghum hybrids are valued for having improved nutritional characteristics (Porter et al. 1978), increasing

the digestibility of the plant by decreasing the lignin content (Grant et al. 1995; Marsalis et al. 2010)—thereby increasing its quality and value as a forage crop.

Sorghum produces the cyanogenic glucoside (CG) dhurrin. The hydrolysis of dhurrin leads to the quick release of HCN. HCN is toxic to animals at high concentrations; HCN greater than 750 mg kg^{-1} on a dry matter basis is considered dangerous for animal consumption (Strickland et al. 2017). HCN interacts with the animals' ability to utilize oxygen during respiration by inhibiting the final enzyme in the electron transport chain (Price 1985). Acute HCN poisoning can lead to death within minutes (Smith and Frederiksen 2000), and symptoms include labored breathing, convulsions, and bright red blood.

Dhurrin content can vary significantly based on variety, age, plant part, and environmental factors such as extreme drought or frost (Wheeler et al. 1990; Busk and Moller 2002; Burke et al. 2013; Gleadow and Møller 2014; O'Donnell 2016). To avoid HCN toxicity, management practices are implemented by avoiding plants that could have high HCN potential (HCNp); plants under 45 cm in height (Strickland et al. 2017), highly fertilized plants (Wheeler et al.1990; Busk and Moller 2002), feeding young regrowth, or plants that have been under stress such as drought (Rosati et al. 2019) or frost (Wheeler et al.1990; Strickland et al. 2017). Frost concerns start later in the growing season when night temperatures begin to drop.

It is commonly advised to keep animals off frost-damaged plants for seven days to avoid high dhurrin accumulation within the plant (Strickland et al. 2017). Temperature has been shown to influence other cyanogenic plant species (Gleadow and Møller 2014). In a white clover study, low temperatures led to a 15-24% increase in HCNp within the plant tissues (Vickery et al.1987; Stochmal and Oleszek 1997; Gleadow and Møller 2014). In the study conducted by Stochmal and Oleszek (1997), increased CG in white clover occurred at temperatures below 15°C. Other studies in forage sorghum did not observe an effect of temperature on HCNp (Wheeler et al. 1990). Understanding the interaction between dhurrin accumulation and cold stress is essential in avoiding HCN toxicity.

Genetic technologies are being developed to modify dhurrin metabolism in sorghum (Blomstedt et al. 2012; Tuinstra et al. 2013; Krothapalli et al. 2013). Krothapolli et al. (2013) found a mutation that affects the catabolic pathway, leading to the production of dhurrin but without an active dhurrinase-2 enzyme (*dhr2-1*). The *dhr2-1* mutants release HCN more slowly but may not be

beneficial in forage sorghum because many livestock animals produce non-specific glucosidase enzymes in the gut (Singh et al. 2016), which may hydrolyze dhurrin after consumption. Dhurrin-free sorghum forages would be a better option to avoid HCN production. Blomstedt et al. (2012) and Tuinstra et al. (2013) identified dhurrin-free sorghum mutants by disrupting CYP79A1, the first enzyme in the biosynthetic pathway, through EMS mutagenesis. These mutants produce very little or no dhurrin.

In this study, conventional forage sorghum hybrids and a dhurrin-free hybrid were examined for dhurrin content in response to frost events to better understand dhurrin production and stability over time. Overall, we expect to see an increase in dhurrin accumulation within the conventional hybrids within a few days following the frost, but dhurrin content to decrease to lower levels over time.

6.3 Materials and Methods

6.3.1 Plant Materials and Testing Environments

Eight sorghum hybrids were evaluated in field and greenhouse trials. Sweet Six (sorghum x sudangrass *bmr;* Cisco Seeds, Indianapolis, IN), Sweet Bites (sorghum x sudangrass*;* Cisco Seeds, Indianapolis, IN), Greentreat® Rocket (sudangrass x sudangrass *bmr;* Cisco Seeds, Indianapolis, IN), and an dhurrin-free experimental hybrid (sorghum x sudangrass *bmr6 cyp79a1*) were planted in greenhouse trials at Purdue University in West Lafayette, IN and field trials at the Animal Science Research and Education Center (ASREC) at Purdue University in West Lafayette, IN and at Kansas State Research and Extension Station in Colby, Kansas. Alta Seeds ADV S6504 (sorghum x sudangrass; Alta Seeds, Amarillo, TX), Dyna-Gro Seed Fullgraze II (sorghum x sudangrass; Dyna-Gro Seed, Geneseo, IL), Star Seed Magnum Ultra BMR (forage sorghum bmr; Star Seed Inc, Osborne, KS), and Super Sweet 10 (sorghum x sudangrass bmr; Dyna-Gro Seed, Geneseo, IL) were grown in field trials at Kansas State Research-Extension Centers at Hays, KS, and Garden City, KS.

ASREC had 37.2 kg ha^{-1} of nitrogen (N) added pre-plant and 37.2 kg ha^{-1} was added again after the first biomass harvest. The Colby field site had sprinkler irrigation that was used to establish the stand by supplementing 2 cm of water, and a pre-plant fertilizer was applied at a rate of 168 kg ha^{-1} of N, and 34 kg ha^{-1} for phosphorous. The plots at both locations were laid out in a randomized

complete block design (RCBD) with four replications (Fig. 6.1). There was 179 kg ha^{-1} of N at the Garden City, and 56 kg ha^{-1} of N at the Hays location applied as a pre-plant fertilizer. Garden City had sprinkler irrigation that supplemented a total of 32 cm of water to the plots throughout the season. The plots were laid out in a RCBD with three replications.

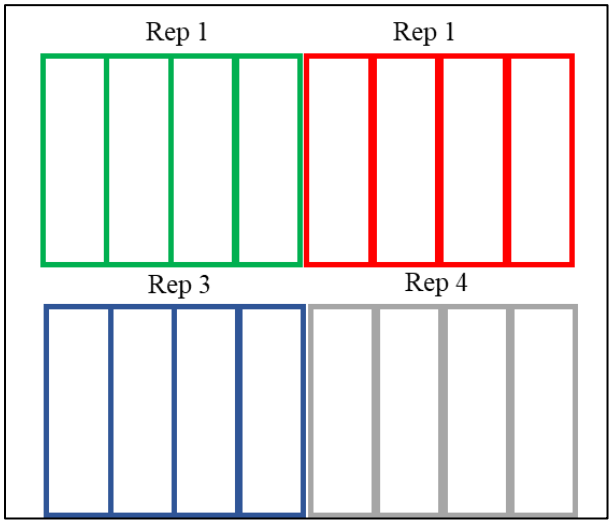


Figure 6.1: Design for the field trial at the Colby, KS location. The four hybrids were in planted in plots represented by the rectangles within each replication, and the replications are separated by color.

6.3.2 **Field Sites and Sampling**

Four hybrids were planted in trials in West Lafayette, IN and Colby, KS using a randomized complete block design with four replications (Fig. 6.1). Four hybrids were planted in trials in Hays, KS and Garden City, KS using a randomized complete block design with three replications. Plants were harvested for forage production in the late summer and allowed to regrow. At the West Lafayette, IN location, plant tissue samples were collected on day 0 before the frost and on days 1, 2, 3, and 7 after the frost. At the Kansas locations, plant tissue samples were collected on day 0 before the frost and on days 3 and 7 after the frost. A frost was considered weather below 0 °C for any time frame throughout the night or if frost was visible on the plants the following morning.

On sampling days, plants were randomly selected based on uniformity with the rest of the plot. The top collared leaf was harvested and a two-inch sample of the green tissue from the mid-section was collected. The sample was placed in a 5-mL vial with a screw top lid then placed on ice and then frozen in LN. Frozen samples from ASREC were transported to the lab. Tissue samples from

Kansas locations were mailed to Purdue University on dry ice. The samples were kept in a -80 °C freezer until processed for dhurrin analysis using ultra high-performance liquid chromatography (UHPLC) as described on section 6.3.5 and 6.3.6. Each sample was weighed for fresh weight grams (fwg) using a microbalance scale accurate to 0.0001 within the laboratory. This was done by weighing the weight of the 5-mL vials before and after tissue sample collection and taking the difference in weights for fwg of the tissue sample.

6.3.3 Controlled Environment

Four hybrids were planted in pots using a randomized complete block design with four replications and four sampling dates. Plants were grown for a month with the with ~10 g of a slow-release pearl fertilizer (14-14-14 by Harrel's LLC Lakeland, FL) added at planting. A month after planting, plants were placed in a vernalization chamber set to -0.6 °C ± 2 °C from 8 PM to 8 AM. Temperatures were measured throughout the night. Plants were sampled for dhurrin content on day 0 prior to freezing treatment and on day 1, 3, and 7 after the freezing treatment. To avoid any effects due sampling, new plants were selected for each set of tissue samples. Sampling, weighing, and storage procedures were the same as the field sites, in section 6.3.2.

6.3.4 Maize Control

Fresh maize leaf tissue samples were collected as a negative control for UHPLC since maize does not contain dhurrin. Leaves were sampled in similar manner as the sorghum hybrids. They were frozen in LN and stored at -80 °C until extraction.

6.3.5 Dhurrin Extraction

On the day of extraction, the extraction buffer; 1:1, MeOH: H2O with 0.05 mg/ml p-hydroxybenzaldehyde was prepared. The samples were kept frozen by placing them in liquid nitrogen (LN). Samples were removed one at a time and crushed using micro-pestle in LN. Then 3 mL of the extraction buffer were pipetted into the crushed sample. Each sample was vortexed for 10 seconds and then placed into a 75 °C water bath for 15 minutes. The samples were stored at 4 °C overnight. The next day, the samples were vortexed, and 1 mL of the liquid was removed with a disposable syringe and filtered through 0.2 µm nylon filter into a 1.5 ml microcentrifuge

tube. Liquid samples were stored at -20 °C until UHPLC analysis. On the day of analysis, samples were warmed to room temperature and vortexed. 200 µL of the vortexed sample was pipetted into a HPLC vial.

6.3.6 **UHPLC Analysis**

UHPLC analysis was performed by using a 1290 Infinity II UHPLC system with a diode array detector (Agilent Technologies, Santa Clara, CA, USA). The separation of compounds was achieved on a Zorbax SB-C18 column (1.8 µm, 2.1 × 50 mm; Agilent) with column temperature maintained at 30 °C. Water (A) and ACN (B) were used as mobile phase solvents with 0.1% formic acid (*v*/*v*) at a flow rate of 0.3 mL min^{-1}. The solvent gradient program was set as shown in Table 6.1 with a total runtime of 7 min. 5 µL of injection volume was used both for samples and standards.

Table 6.1 HPLC solvent gradient program

Time (min)	**A [%]**	**B [%]**	**Flow [ml/min]**
0.00	90.00	10.00	0.300
1.00	90.00	10.00	0.300
3.00	75.00	25.00	0.300
4.00	75.00	25.00	0.300
5.00	5.00	95.00	0.300
6.00	5.00	95.00	0.300
7.00	90	10.00	0.300

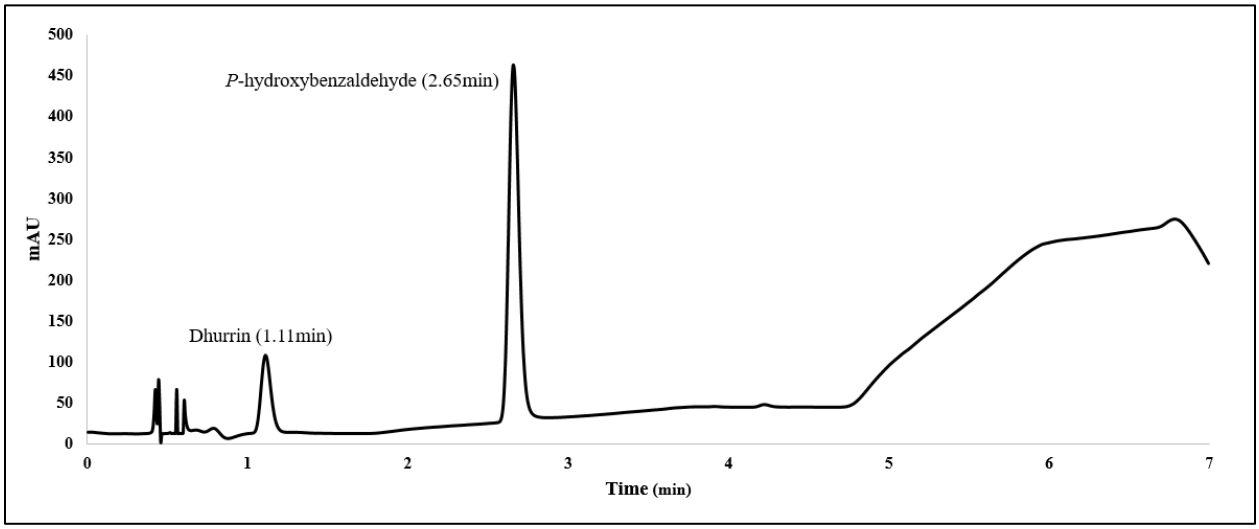


Figure 6.2 DAD chromatogram (232 nm) of dhurrin (1.11 min) and p-hydroxybenzaldehyde (2.65) at the concentrations of 0.01 and 0.05 mg/mL respectively.

Dhurrin and *p*-hydroxybenzaldehyde were eluted at 1.11 and 2.65 min, respectively (Fig. 6.1) with absorbance for both compounds recorded at 232nm. Dhurrin quantitation was performed by analyzing the linear range of 0.0025, 0.0050, 0.0100, 0.0250, 0.0500 mg/mL standards each containing 0.05 mg/mL *p*-hydroxybenzaldehyde as an internal standard. Instrument operation and data analyses were performed using OpenLAB CDS ChemStation software version C.01.09.

6.3.7 **Statistical Analysis**

Each location was analyzed separately. The data was screened for influential points using boxplots and Cook's distance. The influential points were screened for outliers that may have been due to sampling error or from the field. For example, one data point was removed from the Hays dataset because it was over three times higher in comparison to any other reading, and another outlier was removed in the Colby location with a single value within Greentreat rocket being double on that date than any other data point.

The West Lafayette, IN location was analyzed by first looking at each day that was sampled using a mixed effects model. The models developed were mixed effects model with the dhurrin content (mg fwg^{-1}) as the response and hybrids, the days after frost, and their interaction were the fixed factors. Tukey's post hoc test was used to determine significant effects. A similar model was

developed to determine the effects of the frost with the other four hybrids at the Garden City, KS and Hays, KS locations.

To understand the changes occurring in the conventional hybrids, the dhurrin-free hybrid was removed from the data set at the West Lafayette, IN and Colby, KS locations. The ASREC, Colby, KS, and Garden City, KS locations were examined for the response of the conventional hybrids as one group. First, it was determined that there were no significant differences among the hybrid's reaction to the frost at all three locations. Then the model was built to see how all the conventional hybrids reacted to the frost as a group at the three locations. The model had dhurrin content as the response and days after frost as its only factor. The three locations were run separately.

The Colby, Kansas conventional model was transformed using a reciprocal transformation to fit the assumption of normality.

Maize samples were used as a negative control for dhurrin in sorghum. Comparisons between maize and dhurrin-free hybrids were made using a two-tailed t-test with heterodastic variation.

All analyses were conducted in Rstudio.

6.4 Results

6.4.1 Controlled Environment

The sorghum hybrids in the greenhouse trials did not respond to the freezing treatment. There was not visible frost damage or increase in dhurrin content following the frost. Analysis of variance showed a significant effect due to hybrids but no effect due to days after the frost or the interaction between hybrids and days after the frost (data not shown). The differences among the hybrids were expected with dhurrin content of the dhurrin-free hybrid being considerably lower than the conventional hybrids.

6.4.2 Field Trials

The field trials at West Lafayette, IN and Colby, KS exhibited significant differences in dhurrin content for days following the frost, hybrids, and the interaction between the days following the frost and hybrids (Table 6.2). The conventional hybrids at the West Lafayette, IN location

exhibited an increase in dhurrin content on days 1, 2, and 3 after the frost and then it decreased to the original levels by day 7 (Table 6.2). The dhurrin-free hybrid at the West Lafayette, IN location did not exhibit any dhurrin accumulation, this was determined from the maize baseline. The conventional hybrids at the Colby, KS location showed similar pattern as West Lafayette, IN with an increase in the dhurrin content of the conventional hybrids on day 3 following the frost but with elevated levels of dhurrin persisting through day 7 after the frost (Table 6.2).

The Garden City, KS location exhibited a significant difference in dhurrin content for days following the frost but not for hybrids or the interaction between the days following the frost and hybrids (Table 6.2). Significant increases in dhurrin were noted in the hybrids on day 7 after the frost (Table 6.2). An example is Fullgraze II increased 0.4 mg fwg^{-1} three days following the frost and continued to increase by 0.1 mg fwg^{-1} by day 7. The field trials at Hays, KS did not exhibit significant differences in dhurrin content for days following the frost, hybrids, or the interaction between the days following the frost and hybrids (data not shown).

Analyses of dhurrin content in the conventional hybrids as a group indicated increased dhurrin content by day 3 after the frost at the West Lafayette, IN location with levels returning to pre-frost levels by day 7 (Fig. 6.3). The conventional hybrids at the Colby and Garden City locations exhibited increased dhurrin content by day 3, by day 7 dhurrin content had almost doubled in both locations in comparison to the pre-frost dhurrin levels (Fig. 6.3).

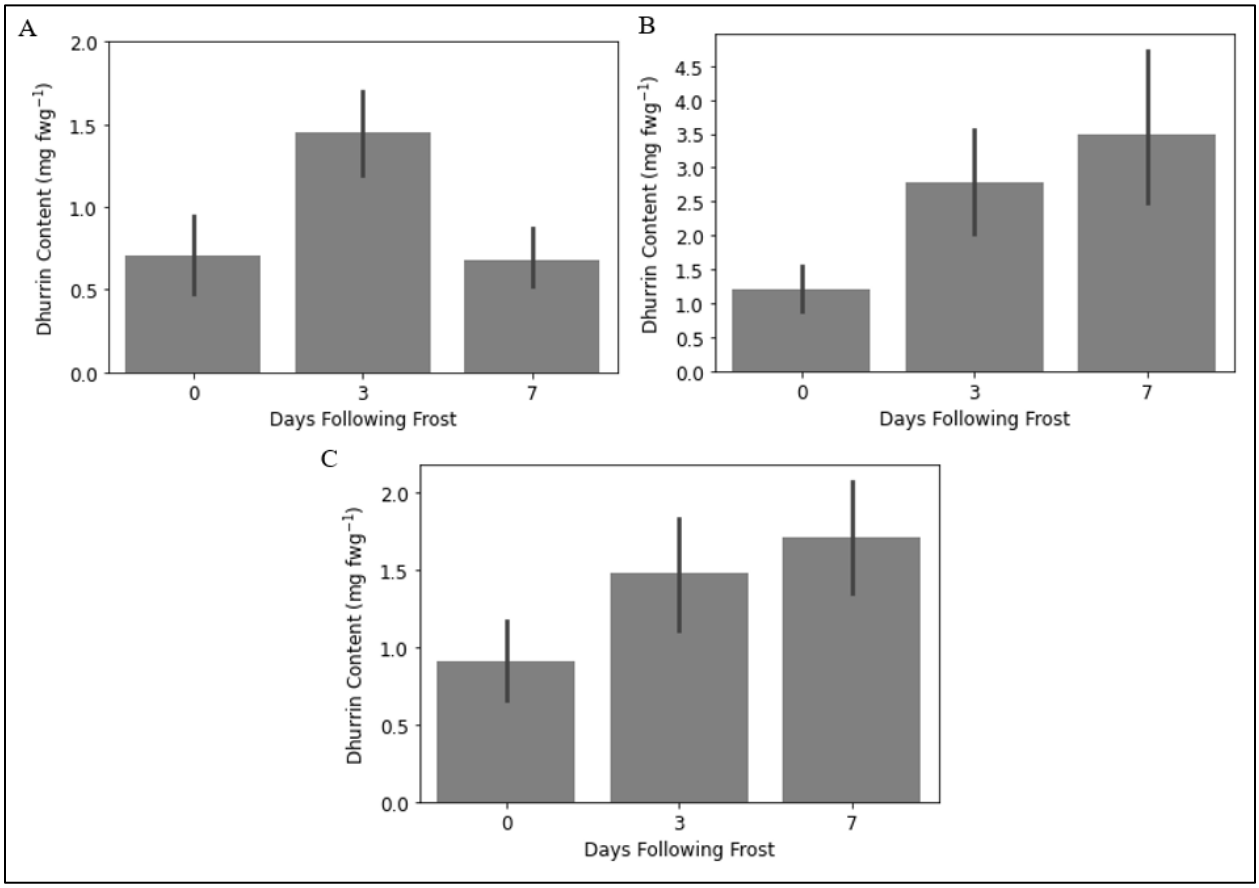
Maize tissue samples were collected as negative control for dhurrin content. Maize registered 0.1 mg fwg^{-1} as a baseline for no dhurrin content. This was similar in comparison to the dhurrin-free experimental hybrid, concluding there was no dhurrin within the dhurrin-free hybrid.

Table 6.2: Comparisons of dhurrin content (mg fwg^{-1}) of sorghum hybrids collected before and after a frost in West Lafayette, IN, Colby, KS, and Garden City, KS.

Dhurrin Content (mg fwg^{-1}) following a Frost Event								
West Lafayette, IN[1]								
Hybrid	Day 0	Day 1	Day 2	Day 3	Day 7			
Dhurrin-free	0.0	0.1	0.1	0.0	0.0			
GreenTreat Rocket	0.6	0.9	1.1	1.5	0.8			
Sweet Bites	0.8	1.8	1.8	1.4	0.6			
Sweet Six	0.7	0.6	1.5	1.2	0.6			
Significance[2]	Hybrid		*					
	Days		*					
	Hybrid*Days		*					
Colby, KS				Garden City, KS				
Hybrid	Day 0	Day 3	Day 7	Hybrid	Day 0	Day 3	Day 7	
Dhurrin-free	0.0	0.0	0.0	ADV S6504	0.7	0.6	2.3	
GreenTreat Rocket	0.6	3.0	5.8	Fullgraze II	0.9	1.3	1.4	
Sweet Bites	1.2	3.1	2.2	Magnum Ultra BMR	1.4	2.0	1.8	
Sweet Six	1.6	2.2	2.5	Super Sweet 10	0.7	2.0	1.5	
Significance[2]	Hybrid		*		Hybrid			
	Days		*		Days		*	
	Hybrid*Days		*		Hybrid*Days			

[1] The Hays, KS values were excluded because there were no significant effects.

[2] ·=0.10,*=*0.05, **=0.01, ***=0.00*\



*The data is displayed on the non-transformed values.

Figure 6.3: Comparisons of dhurrin content in conventional sorghum hybrids (mg fwg^{-1}) measured before and after a frost at A) West Lafayette, IN, B) Colby, KS, and C) Garden City, KS.

6.5 Discussion

Sorghum is commonly used a forage crop. Understanding the factors that influence dhurrin accumulation is very important in utilizing forage sorghum to avoid HCN toxicity. The CG content of other cyanogenic plant species can be affected by cold temperatures (Vickery et al. 1987; Stochmal and Oleszek 1997; Gleadow and Møller 2014), but the impacts of cold and freezing temperatures in sorghum are not well understood. In this study, conventional sorghum hybrids exhibited increased dhurrin content on the days following frost events in West Lafayette, IN; Colby, KS and Garden City, KS. Both West Lafayette, IN and Colby, KS locations responded with dhurrin accumulation within the first three days following a frost but the locations varied in persistence of dhurrin after 7 days. The hybrids at the Garden City, KS location exhibited increased

dhurrin content as well but not as quickly as the conventional hybrids at other locations. It was not until seven days after the frost that dhurrin content was significantly greater than at time zero. These increases in dhurrin after frost events align with what was reported for CG accumulation in white clover after exposure to cold temperatures (Vickery et al. 1987). The differences in persistence of dhurrin after a week may have been caused by subsequent frost events occurring at Colby and Garden City locations. No differences in dhurrin content were noted after freezing temperature treatments in greenhouse trials in West Lafayette, IN or field studies at Hays, KS. It is possible that the cold treatment or frost event were too mild to cause enough stress on the plant to respond with dhurrin accumulation. Within the greenhouse there was no visible tissue damage on the tips of the leaves as was noted in the field trials.

The dhurrin-free experimental hybrid consistently had the lowest dhurrin content in pre-frost samples and did not accumulate after frost events at any location. No differences were detected in comparison to maize samples that were used as a negative control for dhurrin.

Overall, the dhurrin-free experimental hybrid did not show signs of dhurrin accumulation before or after frost events. The dhurrin content of the conventional hybrids generally increased after frost events with significant increases observed between 3 and 7 days after a frost. In some cases, dhurrin content returned to pre-frost levels by day 7 after a frost while in other cases these differences persisted for at least seven days. These observations align with the best management practices described by Strickland (2017) recommending that farmers should (1) take precautions when trying to feed frosted samples to livestock and (2) use the crop as silage to reduce the risk of HCN toxicity from frost-damaged sorghum forages. Silage production has been reported to reduce dhurrin content up to 70 % (Smith and Frederiksen 2000; Strickland et al. 2017). The development and production of dhurrin-free sorghum hybrids may represent a new best management practice for forage production in environments at risk of frost during production.

6.6 Conclusions

Frost events caused an increase in dhurrin accumulation in conventional sorghum hybrids, while the dhurrin-free experimental hybrid carrying the *cyp79a1* mutation did not accumulate dhurrin at any time before or after freezing conditions. Production of dhurrin-free hybrids could be a useful

management strategy for sorghum producers, especially for double cropping systems where sorghum could be grown into the fall when frost conditions become a concern.

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APPENDIX A. EXAMPLES FOR R CODE

R-Code analysis for Chapter 2

```
#Packages generally needed in each chapter
library(lme4)
library(ggplot2)
library(plyr)
library(dplyr)
library(emmeans)
library("car")
library("languageR")
library("multcomp")
#library("lmerTest")
library(ggplot2)
library(readxl)
library(ggpubr)
library(readr)

#Example of the field trial in 2019 for the first round
WL1_la <- read_excel("WL19_Field_IH_data_round1.xlsx",sheet= 2)
#Selecting ten days for analysis
select<-c("10")
WL1_la<-WL1_la[ WL1_la$Days_infested  %in% select,]
WL1_la<-na.omit(WL1_la)
#Removing outliers from the data
influential<-as.numeric(Boxplot(Total_Leaf_Area~ Entry*Insect_Treatment, data=WL1_la))
print(influential)
WL1_las<-WL1_la[-influential,]
anova_wl1_la<-lmer(Total_Leaf_Area~Entry*Insect_Treatment +(1|Rep), data= WL1_la)
anova(anova_wl1_la)
```

```
summary(anova_wl1_la)
plot(residuals(anova_wl1_la))
qqPlot(residuals(anova_wl1_la))
#making comparisons using Tukey's Post Hoc Test
lsmeans(anova_wl1_la, pairwise~Entry*Insect_Treatment)
lsmip(anova_wl1_la, Entry~Insect_Treatment )

#Greenhouse Trial example of dry weight and green plant area for the greenhouse in 2018
aris_dry<- read_excel("Gh18_aris_comparison.xlsx", sheet="arisvsdry")
aris_image<-read_excel("Gh18_aris_comparison.xlsx", sheet="imageJ")
gh18_aris <- read_excel("GH18_insect_data.xlsx", sheet="aris")
gh18_d<-read_excel("GH18_insect_data.xlsx", sheet="des_methods")
gh18_aris<-na.omit(gh18_aris)
#Changing the order of the factor levels in Treatment
gh18_d$Treatment<-factor(gh18_d$Treatment,levels=c("non-infested", "infested"))
#Removing the outliers
influential<-as.numeric(Boxplot(DryWeight~Genotype*Treatment, data=gh18_d))
print(influential)
gh18_ds<-gh18_d[-influential,]
#building the model from within the greenhouse in 2018
ANOVAdw=lmer(DryWeight~Genotype*Treatment +(1|Box),data=gh18_d)
anova(ANOVAdw)
summary(ANOVAdw)
#Checking assumption of normality and equal variances
plot(residuals(ANOVAdw))
qqPlot(residuals(ANOVAdw))
#Making comparison using Tukey's test
lsmeans(ANOVAdw, pairwise~Genotype*Treatment)
x=lsmeans(ANOVAdw, pairwise~Treatment*Genotype)
lsmip(ANOVAdw, Genotype~Treatment)
#building model explaining GPA(ARIS)
```

```
anova_gh3<-lmer(ARIS~ Pedigree*Hours*Treatment
                + (1|Box:Hours), data= GH20s)
anova(anova_gh3)
summary(anova_gh3)
#Checking normality and equal variances
plot(residuals(anova_gh3))
hist(residuals(anova_gh3))
plot(residuals(ANOVAaris))
qqPlot(residuals(ANOVAaris))
#Making comparison using Tukey's test
lsmeans(ANOVAaris, pairwise~Genotype*Treatment*Hour)
x=lsmeans(ANOVAaris, pairwise~Genotype*Treatment*Hour)
lsmip(ANOVAaris, Genotype~Hour|Treatment)
#Corelation Test between GPA and total leaf area with using Image J software and building
graphs
par(mfrow=c(1,1))
plot(ARIS~ImageJ,data=aris_image, pch=20,
    xlab=expression("Total Leaf Area" ~(mm^{2})),
    ylab=expression("Total Plant Area"~(mm^{2})),
    cex.main=1,
    cex.sub=.9,
    cex.lab=.9)
mtext(expression("y = 102.68x +(-169.68);" ~R^{2}~"= 0.86"),
     cex=0.8)
summary(lm(ARIS~ImageJ,data=aris_image))
print(abline(lm(ARIS~ImageJ, data=aris_image), col="black"))
cor.test(aris_image$ARIS,aris_image$ImageJ)
p=ggscatter(aris_image, x = "ImageJ", y = "ARIS", add = "reg.line",ylab=expression("Total
Plant Area"~(mm^{2})),xlab=expression("Total Leaf Area" ~(mm^{2}))) +
  stat_cor(label.x = 4, label.y = 7500) +
  stat_regline_equation(label.x = 3, label.y = 7000)
```

#similar model was used to explain the comparison with dry weight

R Code Examples for explaining stay-green patterns in Chapter 3

```
#Uploading information for CC within the greenhouse for NILs
Tx642s<-read_excel("GH19_CC.xlsx", sheet="Tx642")
#Examing the data and remove the outliers
Boxplot(CC~Phenotype*Treatment*days_drought, data=Tx642s)
model<-lm(CC~Phenotype*Treatment*days_drought, data=Tx642s)
plot(model)
cooksD <- cooks.distance(model)
influential <- cooksD[(cooksD > (4 * mean(cooksD, na.rm = TRUE)))]
print(influential)
influential<-as.numeric(names(influential))
Tx642ss<-Tx642s[-influential,]
#Building the model to account for unequal variances
model_drought<-lme(CC~Phenotype*Treatment*days_drought, random=~1|POT_BARCODE,
weights=varIdent(form=~1|Phenotype), data=Tx642ss, na.action=na.omit)
anova(model_drought)
summary(model_drought)
#Checking assumptions of normality and equal variances
plot(model_drought)
plot(residuals(model_drought))
hist(residuals(model_drought))
#Comparing factors using Tukey's Test and graphical analysis of comparisons
lsmip(model_drought, Phenotype~days_drought|Treatment)
lsmeans(model_drought, pairwise~Phenotype*Treatment*days_drought)
#Looking at the Dry tons per acre data from CA
# Building the model to account for the unequal variances
model_CAb<-lme(DTh~Pedigree*Treatment,random=~1|Rep,
weights=varIdent(form=~1|Pedigree), CA_b)
anova(model_CAb)
```

```
summary(model_CAb)
#Checking assumptionsor normality and equal variances
qqPlot(residuals(model_CAb))
plot(residuals(model_CAb))
plot(model_CAb)
#Making comparison using Tukey's test
lsmeans(model_CAb, pairwise~Pedigree*Treatment)
# Similar models were built for the field and other greenhouse trials for CC and NDVI
# If there were equal variances the model was modified
model_cepf2<-lmer(Moisture~Phenotype*Treatment*Date +  (1|Rep), data=cepf2_m)
```

R Code Preference trial of Ewes in Chapter 4

```
# Similar models from chapter 2 and 3
#Uploading data from and separating out information from round2 in the experiments
all_hyb<- read_excel("WL19_20_pref_biomass.xlsx",
    sheet = "Hybrids")
all_hyb2<-all_hyb[c(1:31,64:95),]
select<-c(2)
hyb2<-all_hyb[ all_hyb$Round  %in% select,]
#Remove outliers
model<-lm(DW~Hybrid*Grazing*Year, data= hyb2 )
cooksD <- cooks.distance(model)
influential1<- cooksD[(cooksD > (4 * mean(cooksD, na.rm = TRUE)))]
influential1<-as.numeric(names(influential1))
influential1<-na.omit(influential1)
print(influential1)
hyb2s<-hyb2[-influential1,]
#building the model
all_hyb2s$Grazing<-factor(all_hyb2s$Grazing, levels= c("Pre", "Post"))
model_all_hyb<-lmer(DW~Hybrid*Grazing*Year +(1|Plots:Year), data=all_hyb2s)
anova(model_all_hyb)
```

```
summary(model_all_hyb)
#checking assumptions of normality and equal variances
qqPlot(residuals(model_all_hyb))
plot(residuals(model_all_hyb))
hist(residuals(model_all_hyb))
#making comparisons using Tukey's Test
x=lsmeans(model_all_hyb, pairwise~Grazing*Hybrid)
print(x)
y0=x[['lsmeans']]
write.csv(y0, "y0.csv")
#Graphs for hybrids pre and post grazing (y0=round 2 and y1=round 3)
y0$Grazing<-factor(y0$Grazing, levels= c("Pre", "Post"))
y1$Grazing<-factor(y1$Grazing, levels= c("Pre", "Post"))
p1 <- ggplot(data=y0, aes(x=Hybrid, y=lsmean, fill=Grazing))+
geom_errorbar(aes(ymin=lower.CL,ymax=upper.CL),position=position_dodge(0.9),width=0.2,si
ze=0.3, data=y0)+
  geom_bar(stat="identity", color="black",
position=position_dodge())+theme(axis.text.x=element_text(angle=90))+
  xlab("Hybrid")+
  ylab("DW/Plot (g/2m)")+
  ggtitle("")+scale_fill_brewer()+theme(text = element_text(size = 10))
p2 <- ggplot(data=y1, aes(x=Hybrid, y=lsmean, fill=Grazing))+
geom_errorbar(aes(ymin=lower.CL,ymax=upper.CL),position=position_dodge(0.9),width=0.4,si
ze=0.3, data=y1)+
  geom_bar(stat="identity", color="black",
position=position_dodge())+theme(axis.text.x=element_text(angle=90))+
  xlab("Hybrid")+
  ylab("")+
  ggtitle("")+scale_fill_brewer()+theme(text = element_text(size = 10))
ggarrange(p1, p2, labels= c("A", "B"))
```

R Code for Dhurrin Content in Dry Matter in Chapter 5

```
# Similar models from chapter 2 ,3 and 4
haycx<-read_excel("WL_KS20_hybrid_dhurrin.xlsx", sheet="ASRECColby")
#locations separated
#Transforming the data
#Dropping outliers
select<-c("Colby")
hayc<-haycx[ haycx$Location  %in% select,]
haycs<-na.omit(hayc)
hayc$mgx<-log(hayc$`mg/g`)
model<-lm(mgx~ Hybrid*Weeks, data=hayc)
cooksD <- cooks.distance(model)
influential1 <- cooksD[(cooksD > (4 * mean(cooksD, na.rm = TRUE)))]
print(influential1)
influential1<-as.numeric(names(influential1))
influential1<-na.omit(influential1)
haycs<-hayc[-influential1,]
#Building model to account for unequal variances
lm_hayc<-lme(mgx~Hybrid*Weeks, random=~1|Rep, weights=varIdent(form=~1|Hybrid),
data=haycs, na.action=na.omit)
anova(lm_hayc)
summary(lm_hayc)
#Checking assumptions of normality and variances
plot(residuals(lm_hayc))
plot(lm_hayc)
qqPlot(residuals(lm_hayc))
hist(residuals(lm_hayc))
#Making comparison using Tukey's test and graphical comparison
lsmip(lm_hayc, Hybrid~Weeks)
lsmeans(lm_hayc, pairwise~Hybrid*Weeks)
lm_haycx<-lm(`mg/g`~Hybrid*Weeks, data=haycs)
```

```
lsmeans(lm_haycx, ~Hybrid*Weeks)
lsmip(lm_haycx, Hybrid~Weeks)
#Data was transformed when needed and models were built based on equal variances or not
```

R Code for Dhurrin Content in Frosted Samples in Chapter 6

```
#uploading the data from the Colby and ASREC locations
frostwlc<-read_excel("WL_KS20_frost_dhurrin.xlsx", sheet="ASRECColby")
#Selecting the Colby location
select<-c("Colby")
frostc<-frostwlc[ frostwlc$Location  %in% select,]
#Removing the outliers
model<-lm(`mg/fwg`~Hybrid*Day_frost, data=frostc)
cooksD <- cooks.distance(model)
influential <- cooksD[(cooksD > (4 * mean(cooksD, na.rm = TRUE)))]
print(influential)
influential<-as.numeric(names(influential))
frostcs<-frostc[-influential,]
#Builing the model describing changes in mg/fwg
lm_frostwlcs<-lmer(`mg/fwg`~Hybrid*Day_frost+(1|Rep), data=frostcs)
anova(lm_frostwlcs)
summary(lm_frostwlcs)
#Checking assumptions of normality and equal variances
plot(residuals(lm_frostwlcs))
qqPlot(residuals(lm_frostwlcs))
#Making comparison using Tukey's test and graphical comparison
lsmip(lm_frostwlcs, Hybrid~Day_frost)
lsmeans(lm_frostwlcs, ~Hybrid*Day_frost)
#Similar models were made for the other locations
# Model for the Conventional hybrids did not have the factor hybrid
```

APPENDIX B. PYTHON CODE

Python code for creating graphs in Chapter 6.

```
import pandas as pd
import numpy as np
import matplotlib.pyplot as plt
import seaborn as sns
import matplotlib.ticker as ticker

asrec=pd.read_csv("WL_frostacs.csv")
colby=pd.read_csv("WL_frostccs.csv")
gc=pd.read_csv("WL_frostgcs.csv")


#making a barplot for the asrec west lafayette conventional hybrids
sns.barplot(x="Day_frost", y="mg/fwg", data= asrec, ci=95, facecolor='grey')
plt.ylabel("Dhurrin Content (mg $fwg^{-1}$)",  fontsize=12)
plt.xlabel("Days Following Frost", fontsize=12)
plt.title("ASREC")
plt.xticks( rotation=70, fontsize=12)
plt.yticks( fontsize=12)
plt.show()


#making a barplot for the Colby conventional hybrids
sns.barplot(x="Day_frost", y="mg/fwg", data= colby, ci=95, facecolor='grey')
plt.ylabel("Dhurrin Content (mg $fwg^{-1}$)",  fontsize=12)
plt.xlabel("Days Following Frost", fontsize=12)
plt.title("Colby, KS")
plt.xticks( rotation=70, fontsize=12)
```

```
plt.yticks( fontsize=12)
plt.show()

#making a barplot for the garden city conventional hybrids
sns.barplot(x="Day_frost", y="mg/fwg", data= gc, ci=95, facecolor='grey')
plt.ylabel("Dhurrin Content (mg $fwg^{-1}$)",  fontsize=12)
plt.xlabel("Days Following Frost", fontsize=12)
plt.title("Garden City, KS")
plt.xticks( rotation=70, fontsize=12)
plt.yticks( fontsize=12)
plt.show()
```

APPENDIX C. DHURRIN-FREE LINES WITHIN WATER

The two samples were two dhurrin-free lines within water after twenty h.

VITA

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Education

Purdue University
Ph.D. Agronomy
Specialization: Plant Breeding and Genetics — Expected 2021
University of Illinois at Urbana Champaign
M.S. Professional Science Masters in Agricultural Production — 2015-2017
B.S. in Crop Science Concentration: Biotechnology and Molecular Biology — 2013-2015

Professional Experience

Agronomy Graduate Research Assistant — 2018-2021
Department of Agronomy Purdue University

- Assisted with field preparation and harvest, from preparing seeds, pollination, to harvest and threshing of corn and sorghum
- Conducted multiple research projects from developing a hypothesis and design of the experiment to data analysis
- Gathered phenotypic data, leaf area, plant area, fresh weight, dry weight, and height
- Worked with high throughput phenotyping technologies, specifically the hyperspectral data from drone imagery to data collected by Purdue's Ag Alumni Environmental Phenotyping Facility

Sustainability Food Energy Water System (SFEWS) National Science foundation Research Trainee
Purdue University — 2018-2021

- Conducted trials under an agro-photovoltaics system to understand the impacts on crop growth and development from a solar array
- Organized and taught graduate students across multiple disciplines to set up plots, and collect crop field data, including flowering notes, height measurements, and yield
- Helped conduct analysis in conjunction with other graduate students to understand the impacts of reduced irradiance in sections of the field

- Participated in weekly meetings to discuss the development of the project

Image Processing Intern
Ag Alumni Seed 2020-2021

- Process lidar, RGB, and VNIR drone images for multiple field and flights throughout the summer of 2020
- Coordinate between GRYFN and Ag Alumni Seed with processing and goals

Graduate Teaching Assistant - Food and Energy Farms
Department of Chemical Engineering Purdue University Fall 2019

- Assisted with the Food and Energy Farms course
- Taught lectures covering origins of agriculture and the basis of photosynthesis
- Graded homework and test scores

Diagnostician Assistant
University of Illinois Plant Clinic May 2017-December 2017
Graduate Intern
University of Illinois Plant Clinic Fall 2016

- Ran Nanodrop, diluted samples, and set up qPCR plates to test for glyphosate resistance and PPO inhibitor resistance in *Amaranth* species
- Diagnosed plant diseases and identified plant species from samples submitted to the Plant Clinic
- Wrote newsletters for prevalent diseases in the area or new diseases that have been identified
- Checked in samples, and filed paperwork

Urban Ag Research Undergraduate Research Position
University of Illinois Summer 2014

- Pruned, harvested, and maintained pH balance for hydroponic strawberries
- Set up, weeded, and gathered yield, weed, and degradation data on biodegradable mulch site
- Built, planted, gathered weed and water tension data, and set up an irrigation system on the community gardens

Honors and Awards

1st Place AFGC Emerging Scientist Oral Competition, Sponsored by Corteva Agriscience at American Forage and Grassland Annual Council 2020
College of Agriculture Bayer AG and Bayer Crop Science Plant Breeding Travel Award 2019

Extra-Curricular Activities

RIISE Student Organization, Graduate Mentor 2020-2021

- Contribute with ideas for projects and events
- Participate in events and podcast

Wheel Rise Inaugural Event, Co-Founder Fall 2019

- Organize and develop the inaugural Wheel Rise event to bring awareness to para-sports
- Coordinate travel for the University of Illinois Wheelchair Basketball teams
- Co-host and organize guest speakers

Women's Wheelchair Basketball, United States National Team, Captain Jan. 2017-Dec. 2018

- Represent the United States of America in International Competitions
- Attend training camps, where skills are evaluated and selections to continue with the team
- Keep a training schedule with shooting and strength and conditioning workouts multiple times a week
- Represent and lead the team in multiple settings, and relay information between players and the coaches

Women's Wheelchair Basketball for University of Illinois, Captain Aug. 2013-March 2017

- Participate and be a leader in training, game situations, and in life if necessary

Non-Scientific Publication

Gruss, Shelby. 2020. "Safe and Tasty Forage Sorghum." *Hay & Forage Grower*, May 2020

Submitted Publication

Gruss, Shelby M., and Mitchell R. Tuinstra. 2021. "Seedling Growth and Fall Armyworm Feeding Preference Influenced by Dhurrin Production in Sorghum." Paper submitted for publication.