THESIS

# PHENOTYPING TOOLS AND GENETIC KNOWLEDGE TO FACILIATE BREEDING OF DHURRIN CONTENT AND CYANOGENIC POTENTIAL IN SORGHUM

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# ABSTRACT

## PHENOTYPING TOOLS AND GENETIC KNOWLEDGE TO FACILIATE BREEDING OF DHURRIN CONTENT AND CYANOGENIC POTENTIAL IN SORGHUM

Cyanogenic glucosides are important secondary compounds found in plants serving roles such as plant defense, pollinator attraction, nitrogen (N) sources, and drought tolerance. Sorghum (*S. bicolor* [L.] Moench), an important grain crop predominantly grown in drought-prone environments, contains a cyanogenic glucoside known as dhurrin where it functions as a source of hydrogen cyanide (HCN) after the leaf tissue is disrupted. Dhurrin has been hypothesized to serve as an osmoprotectant, N turnover source, and sorghum aphid resistance mechanism. In addition, dhurrin concentrations can vary due to growth stage, environment, and genotype, and this variability can cause limitations for effective dhurrin phenotyping. To facilitate the breeding of dhurrin and HCNp, we developed a semi-quantitative phenotyping method to detect HCNp and investigated the genetics of dhurrin and HCN variation in global sorghum germplasm.

In the first study, we developed a simple, semi-quantitative, high-throughput phenotyping method to detect HCNp in sorghum leaf tissue. Biochemical methods have been used to determine dhurrin content quantitatively, however these methods are laborious and costly. As a result, we developed a semi-quantitative phenotypic assay using commercial test strip paper to measure HCNp utilizing a F13 *Stg* Recombinant Inbred Line (RIL) population with previously reported dhurrin concentrations. We found that later sampling time improved the detection of HCNp variation with broad-sense heritability ($H^2$) values highest at flowering. In addition, we found that other covariates such as leaf number may play a role in effective phenotyping.

Altogether this assay can be used to screen a sorghum breeding population in both a greenhouse and field setting for smallholder breeding programs looking to advance their breeding generations more efficiently.

In the second study we sought to understand the genetics underlying HCN and dhurrin variability, as well as investigate the relationship between drought and dhurrin using diverse sorghum landraces. We found no direct correlation between dhurrin and drought, but the slight positive correlation could suggest other environmental factors, such as pest pressures, are driving HCN and dhurrin variation. To further understand the biological relationship between dhurrin and HCN, we conducted a genome-wide association study (GWAS) for HCNp and dhurrin. We identified several significant associations between HCNp and known dhurrin biosynthetic and catabolic genetic markers, but major biosynthesis loci were not all significantly associated with HCNp. In addition, we performed a GWAS on dhurrin and found peaks associated with the dhurrin biosynthetic gene cluster, as well as other unknown loci that could contribute to dhurrin variation. This suggests that genetic variation for genes in the dhurrin biosynthesis, catabolism, and recycling pathway contributes to HCNp variability, and they are not direct proxies for each other. As a result, breeders should de-couple phenotyping methods for dhurrin and HCNp depending on the trait of interest.

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# CHAPTER I: THE ROLE OF DHURRIN DURING SORGHUM GROWTH AND DEVELOPMENT

## Introduction

Climate change is impacting agricultural production, leading to a decrease in crop yields due to limitations on water and nutrient availability (Mueller et al., 2012). *Sorghum bicolor* (L. Moench) *is* a drought resilient crop grown in the semiarid regions of Africa and Asia used for food, forage, fiber, and fuel (Paterson et al., 2009). Furthermore, it is closely related to vital cereal crops including maize, sugarcane, and switchgrass. Thus it has high potential as a model system to improve breeding programs against the threat of food insecurity.

Physiological traits such as the production of specialized secondary metabolites help plants adapt to various biotic and abiotic stresses. Cyanogenic glucosides (CGs) are secondary metabolites that aid in plant defense, pollinator attraction and other advantageous purposes (Conn, 1979). CGs are phytoanticipins where they are constitutively present regardless of outside abiotic and biotic factors. Plants containing CGs release hydrogen cyanide (HCN), which serves as a plant defense mechanism upon tissue degradation. Unlike its close relatives, sorghum contains dhurrin, a CG naturally produced in all plant tissue types (Busk & Møller, 2002; Cowan et al., 2022). Dhurrin content can vary by genotype, growth stage, and environment with many beneficial functions including pest resistance (Gruss et al., 2022; Krothapalli et al., 2013), drought tolerance (Burke et al., 2013; Busk & Møller, 2002; Gruss, Souza, et al., 2023; Hayes et al., 2016), and as a N turnover source (Blomstedt et al., 2018; Rosati et al., 2019). However, forage quality can be negatively affected by high dhurrin content due to toxic levels of HCN upon feeding livestock (Emendack et al., 2018; Gleadow & Møller, 2014; Halkier & Møller, 1989; Nakasagga et al., 2022; Wheeler et al., 1990).Understanding the role of dhurrin in

sorghum will further the advancement of sorghum varieties and inform hypotheses on the mechanisms underlying phenotypes associated with dhurrin content. The present review aims to address CGs but more specifically, dhurrin, and its known biological roles to improve the overall understanding of dhurrin in sorghum to inform future plant breeding decisions.

**Biosynthesis of CGs in Plants**

CGs are present in over 2500 different species across the fern, gymnosperm, and angiosperm classes (Conn, 1979). Generally, all cyanogenic glucosides share similarities in synthesis and overall function, as they have the ability to release HCN (Bak et al., 2006; Conn, 1979). The term cyanogenic refers to the release of HCN which can deter herbivores from feeding on cyanogenic parts of a plant (Gruss et al., 2022; Krothapalli et al., 2013). The exposure to high amounts of HCN can prevent respiration, constricting oxygen supplies (Way, 1984). HCN is also known to have a bitter flavor, deterring pests from feeding (Arrázola, 2012; Gleadow & Møller, 2014).

Sorghum bicolor can be utilized as a model system to understand the biosynthesis, catabolism, and detoxification processes of cyanogenic glucosides. Sorghum bicolor contains a CG known as dhurrin where many of the processes are known (Bak et al., 1998; Nielsen et al., 2016); Jones et al., 1999; Sibbesen et al., 1994). In addition to the known dhurrin pathway, the biosynthesis of CGs share similarities among different plant species. The first step in CG synthesis starts with a cytochrome P450 complex (Sibbesen et al., 1994). This complex catalyzes a hydroxylation of the parent amino acid contributing to decarboxylation and carboxylation reactions from two cytochrome P450s, CYP79A1 and CYP71E1, resulting in the formation of α-hydroxynitrile (cyanohydrin). Cyanohydrin is glycosylated by a UDPG-dependent

glycolsyltransferase, UGT85B1, resulting in the release of HCN (Jones et al., 1999; Gleadow & Møller, 2014; Vogt & Jones, 2000).

In plants, CG concentration can vary due to external abiotic and biotic factors such as leaf age and environmental conditions (Fürstenberg-Hägg et al., 2013; Gleadow & Woodrow, 2002). As sorghum matures and overall biomass increases, biosynthesis occurs at a lower rate due to a decrease in catabolic turnover of dhurrin (Busk & Møller, 2002). This signifies that cyanogenic potential (HCNp) in plants decreases over time. For example, in sorghum seedlings, dhurrin content is very high and has the potential to kill grazing livestock, however once the plant matures, it becomes suitable for livestock to graze. This highlights the importance of understanding the biosynthetic pathway of cyanogenic glucosides because each step may alter the HCNp found in plants, affecting their potential commercial uses and ecosystem roles.

**Sorghum Bicolor Cyanogenic Glucoside Biosynthesis**

As sorghum serves as the model system for cyanogenic glucoside synthesis in plants, the present section will describe and illustrate the processes involved in dhurrin synthesis. Dhurrin is a cyanogenic glucoside that releases HCN, otherwise known as hydrogen cyanide (HCN). In sorghum, dhurrin is the cyanogenic glucoside derived from the aromatic amino acid tyrosine (Bak et al., 1998; L. J. Nielsen et al., 2016; Sibbesen et al., 1994). Dhurrin is synthesized with a NADPH-dependent cytochrome P450 oxidoreductase and two cytochrome P450 enzymes. The last step is conducted by a soluble UDP-glucosyl transferase. All these steps are catalyzed by enzymes CYP79A1, CYP71E1, and UGT85B1 which are clustered on the distal arm of chromosome 1 (Krothapalli et al., 2013; Hayes et al., 2016). CYP79A1 catalyzes the rate-limiting step in the dhurrin biosynthetic pathway, creating (Z)-ρ-hydroxymandelonitrile (Figure

1) (Busk & Møller, 2002). Consequently, CYP79A1 is widely used as a molecular marker for identifying acyanogenic mutants (Busk & Møller, 2002).

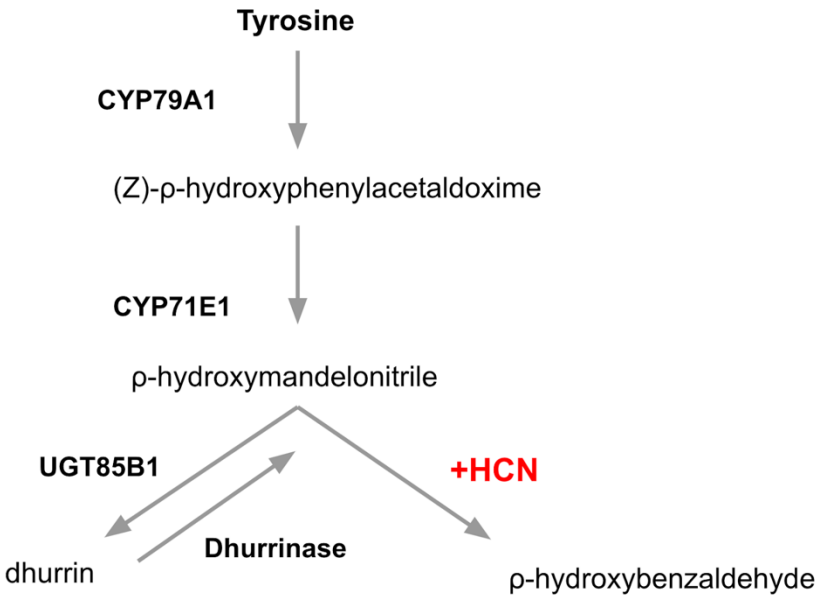


**Figure 1.1: The dhurrin biosynthetic and catabolic pathway produces HCN upon tissue disruption.** A simplified figure of the dhurrin biosynthetic pathway found in sorghum. Many of these enzymes are conserved across plant species, specifically CYP79 sequences (Bak et al., 2006; Busk & Møller, 2002; Werck-Reichhart & Feyereisen, 2000). Adapted from (Busk & Møller, 2002).

When leaf tissue is disrupted, dhurrin and dhurrinase come into contact to create HCN and the byproduct, ρ-hydroxybenzaldehyde. There are four types of dhurrinases in sorghum: *Dhr* 1, 2, like-3, and like-4, each distinguished by their plant tissue specificity (Cicek & Esen, 1998; Gleadow et al., 2021). *Dhr* 1 is expressed in coleoptiles and hypocotyls, while *Dhr* 2 is expressed in leaves (Kojima et al., 1979) and has highest expression level among the four dhurrinases (Choi et al., 2020). Though expressed at a lower level, *Dhr* 1 contributes to the high percentage of variation in dhurrin content (Hayes et al., 2016).

The biosynthesis of dhurrin is a highly channeled system with enzymes CYP791A, CYP71E1, and UGT85B1 forming a metabolon to increase catalytic efficiency while decreasing the amount of toxic intermediates formed from dhurrin production (Møller & Conn, 1980; Laursen et al., 2016). The consumption of HCN can deter herbivores from feeding, but the amount of dhurrin present in the plant tissues prevents (Nahrstedt, 1985; Gleadow & Møller, 2014). The entire pathway of dhurrin has been transferred in *Arabidposis thaliana.* The three dhurrin biosynthetic genes (CYP79A1, CYP71E1, and UGT85B1) were transformed into *A. thaliana* to assess dhurrin production and effects on herbivore feeding preferences (Tattersall et al., 2001). Overall, this prevented the feeding of *Phyllotreta nemorum* (Coleoptera), indicating that the dhurrin biosynthetic pathway could be successfully transferred into different species. As a result, new studies regarding cyanogenic glucoside biosynthesis could use similar gene editing technology to further understand herbivore resistance in other plant species.

**Abiotic and Biotic Factors Affecting Dhurrin Content**

Understanding the different factors that can affect dhurrin concentrations can provide deeper insight into dhurrin synthesis and catabolism. Dhurrin content may vary among different growth stages, environmental factors, plant organ, and genetic backgrounds, therefore affecting HCNp concentrations (Burke et al., 2013; Busk & Møller, 2002; Emendack et al., 2018; Gleadow et al., 2021; Halkier & Møller, 1989; Hayes et al., 2015; Kojima et al., 1979; Wheeler et al., 1990). Environmental factors such as severe drought or freezing can increase dhurrin content, thus increasing HCN concentrations (Gleadow & Møller, 2014; O'Donnell et al., 2013; Rosati et al., 2019). Although this is reported, other literature has found no increase after sudden environmental stressors. This discrepancy could be due to genotypic differences and their ability to metabolize and catabolize dhurrin (Busk & Møller, 2002; Hayes et al., 2015). The amount of

dhurrin is up-regulated at early growth stages where the transcriptional regulation of the dhurrin biosynthesis enzymes, CYP79A1 and CYP71E1, play a large role in dhurrin concentrations (Busk & Møller, 2002; Halkier & Møller, 1989). Nitrogen fertilization also up-regulates the dhurrin biosynthesis enzymes during later growth stages (Busk & Møller, 2002). The dhurrin biosynthesis genes have been found to have decreased expression at maturity (Gleadow et al., 2021) but the down-regulation of dhurrin has not been reported, although decreased levels of HCN were found in transgenic sorghum plants with the down-regulation of CYP79A1 (Pandey et al., 2019). The down-regulation of dhurrin biosynthetic enzymes during maturity could increase available N during grain fill (Gleadow et al., 2021).

Yet the genetic makeup of sorghum lines contribute to the largest source of dhurrin variation (Burke et al., 2013; Gruss, Souza, et al., 2023; Hayes et al., 2015). Although there are poisoning concerns, grazing management strategies such as preventing livestock from feeding on young or severely stressed plants and practicing rotational grazing reduces the risk (Strickland et al., 2017; Gruss et al., 2023). Other strategies have found that feeding livestock sorghum hay or silage can prevent high amounts of HCN ingestion (Strickland et al., 2017; Vough & Cassel, 2006), yet dhurrin is still present in the dry sorghum leaf material for at least two months (Gruss et al., 2023).

Acyanogenic mutants have been utilized to study the effects of dhurrin and the subsequent release of HCN in sorghum forages, yet positive trade-offs, such as drought tolerance, pest resistance, and nitrogen turnover have been found to exist (Blomstedt et al., 2018; Gruss et al., 2022). The relationship between dhurrin and nitrate accumulation under drought-stress was investigated. This study found that acyanogenic mutants accumulated more nitrates under drought conditions, but the dhurrin-containing genotypes had normal nitrate

concentrations (Rosati et al., 2019). This trade-off for forage quality is important to consider as breeders continue to select for a decrease in leaf dhurrin concentration.

**Dhurrin Biosynthetic Gene Cluster (BGC) and Catabolic Gene Cluster (CGC)**

The genes associated with dhurrin biosynthesis are well understood. The genome of sorghum accession BTx623 has been fully sequenced and genes in the dhurrin biosynthetic, catabolic, and recycling pathways have been mapped and characterized (Goodstein et al., 2012). In addition, The dhurrin biosynthetic gene cluster (BGC) is highly conserved across sorghum genotypes and can be utilized to make evolutionary inferences (Bak et al., 2006; Møller & Laursen, 2021). Firstly, L-Tyrosine is converted to dhurrin with the biosynthetic gene cluster (BGC), consisting of the cytochrome P450 enzymes, *CYP79A1* & *CYP71E1*, and a uridine diphosphate-glucosyltransferase, otherwise known as *UGT85B1* located on chromosome 1 (Hayes et al., 2015; Nielsen et al., 2016). Dhurrin biosynthesis is most active in young leaf tissue (Busk & Møller, 2002), where the expression of the BGC is highest in the meristem and developing tissue. Diel cycles can also influence biosynthetic expression levels (Gleadow et al., 2021).

The biosynthetic pathway genes also form a metabolon which enables the efficient production of dhurrin and prevents toxic intermediates from accumulating in the plant tissue (Halkier & Møller, 1989; Laursen et al., 2016; K. A. Nielsen et al., 2008). The use of label-free Raman hyperspectral imaging analysis revealed the absence of dhurrin in the central vacuole, allowing for the potential storage of dhurrin formed into dense bio-condensates (Heraud et al., 2018; Laursen et al., 2016; Møller & Laursen, 2021).

When the tissue is disrupted, HCN is released due to the CGC. The CGC consists of *dhurrinase-1* (*Dhr1*), *dhurrinase-2* (*Dhr2*), *dhurrinase-like3* (*Dhr-like3*), and *dhurrinase-like4*

(*Dhr-like4*) where they are all colocalized on chromosome 8 (Cicek & Esen, 1998; Hayes et al., 2015; Kojima et al., 1979; Krothapalli et al., 2013). *Dhr1* and *Dhr2* are expressed in different leaf tissues (Kojima et al., 1979) as well as the additional dhurrinase-like genes, *Dhr-like3* and *Dhr-like4* (Gleadow et al., 2021). The CGC aids in the release of HCN, thus affecting the biotic factors in a given environment.

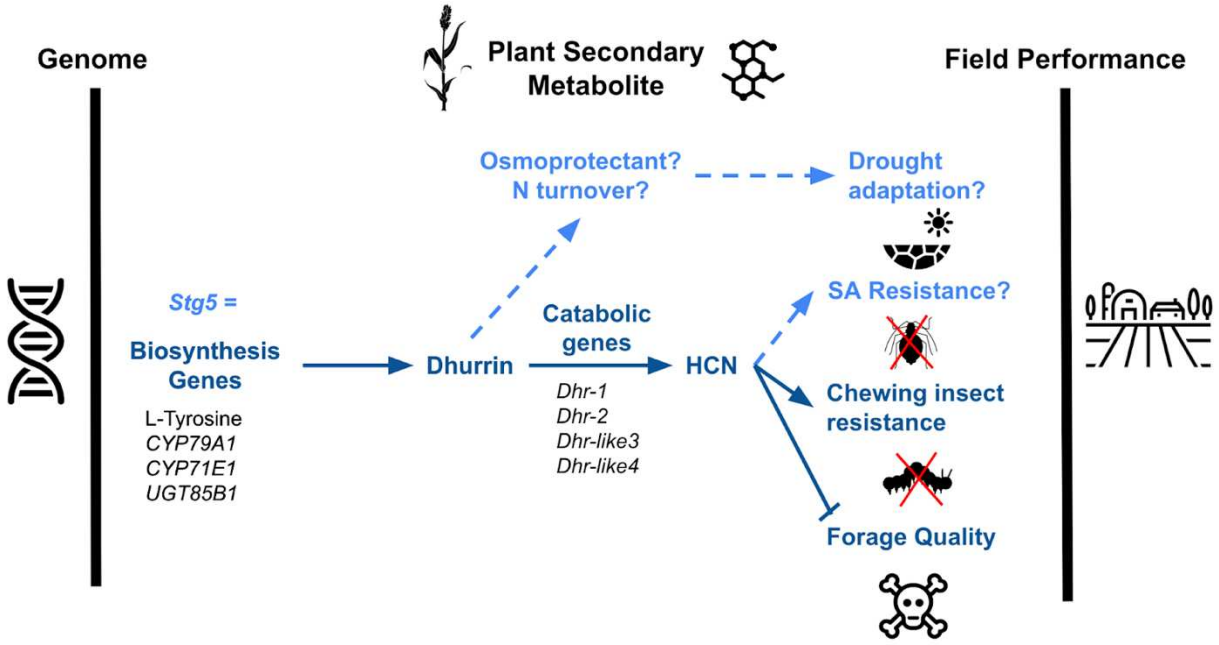


**Figure 1.2: From genome to field performance as a plant secondary metabolite.** A conceptual figure illustrating the role of dhurrin and HCN in mitigating abiotic and biotic stressors affecting overall field performance.

**Osmoprotectant**

Dhurrin has been hypothesized to have multiple functions such as sorghum aphid resistance, a mobilizable N storage source, and as an osmoprotectant under stress (Busk & Møller, 2002; Gleadow & Møller, 2014; Hayes et al., 2015; Heraud et al., 2018; Scott Armstrong

et al., 2015). Osmoprotectants regulate the osmotic balance in plants therefore improving stress tolerance (Zulfiqar et al., 2020). Osmoprotectants are found in many forms including amino acids, quaternary amines (glycine betaine), sugars (trehalose), sugar alcohols (inositol & mannitol), butyric acid (GABA), and an ammonium compound group (polyamines). High accumulation of osmoprotectants contributes to plant defense mechanisms under high stress environments like drought (Zulfiqar et al., 2020). In sorghum, the stay green (*Stg*) trait exhibits late-season drought tolerance traits such as extended leaf greeness during grain fill during post-anthesis drought stress (Borrell et al., 2001). Grain yield and the *Stg* trait are both positively correlated under terminal drought (Borrell et al., 1999, 2000; Jordan et al., 2003, 2012).

Burke et al. found a correlation between dhurrin content and post flowering drought tolerance (Burke et al., 2013). The *Stg* line, BTx642, had higher dhurrin levels than the senescent line, Tx7000, during a period of drought stress (Burke et al., 2013). High levels of dhurrin in grain sorghum genotypes exhibit *Stg* qualities during post-anthesis drought stress (Hayes et al., 2016). Hayes et al identified QTL associated with the *Stg* trait, and what the authors found was Dhu QTL aligned with a novel *Stg* QTL known as *Stg*5. The relationship between *Stg* and drought tolerance has been investigated and it was found that this relationship is influenced most by genotype (Gruss, Souza, et al., 2023).

Dhurrin has hypothesized to act as a bio-condensate due to the highly channeled biosynthetic gene cluster functioning as a metabolon (Laursen et al., 2016). When bio-condensates form, higher availability of dhurrin is present, thus allowing for the delivery of reduced N to the growth tissues found in sorghum, overall increasing growth efficiency (Bassard et al., 2017; Choi et al., 2020; Laursen et al., 2016). Dhurrin osmolyte functions are still being

investigated, but understanding this relationship can help us understand the physiological mechanisms underlying drought-stress in sorghum.

**Melanaphis sacchari (sorghum aphid) Resistance**

Sorghum aphid (SA) is a worldwide concern for sorghum yield loss, affecting the southernmost part of the United States, Africa, Asia, Australia, and Central and South America (Muleta et al., 2021; Scott Armstrong et al., 2015). Cyanogenic glucosides play a role in defense from insect feeding, yet aphid feeding in sorghum is not well understood due to different feeding mechanisms. Although SA was first found on sugarcane, it has spread rapidly to sorghum (Bowling et al., 2016; Muleta et al., 2021). SA is a pest that feeds on the sap of the sorghum plant. Sorghum plants contain dhurrin, a cyanogenic glucoside, as a defense mechanism. When the sorghum aphid feeds on the plant, it breaks down the dhurrin into hydrogen cyanide (HCN), which is toxic to the pest. The release of HCN can deter feeding and reduce the damage caused by the aphid. This highlights the dynamic relationship between plants, pests, and chemical defenses. Thus, there is a need to breed resistant lines to prevent further sorghum damage from SCA infestations.SA attacks the plant by sucking the phloem from leaves and stems, primarily underneath the leaf, progressing up to the sorghum head as the plant matures (Bowling et al., 2016; Singh et al., 2004). Infestation during the flowering stage can result in reduced seed production due to heavily infested heads, and in severe cases, it can lead to stunted plant growth and delayed maturation due to honeydew excretion.

Cyanogenic glucosides are associated with herbivory resistance, reducing insect feeding and improving overall plant health (Gruss et al., 2022; Krothapalli et al., 2013). Herbivory resistance with cyanogenic glucosides are affected by HCN concentration, rate of HCN release, and how the plant responds to different herbivore feeding mechanisms (Gleadow & Møller,

2014). SA uses their stylet to penetrate the leaf surface and ingest the phloem sap (Burd, 2002; Miles, 1999). As a result, this may cause minimal leaf damage, preventing the catabolism of dhurrin to HCN (Gleadow & Woodrow, 2002; Poulton, 1990). When an aphid feeds on phloem sap, it penetrates intracellularly but occasionally it can puncture the host cells, with the possibility of a release of HCN (Louis, Mondal, et al., 2012; Louis, Singh, et al., 2012). Although plant tissue disruption is necessary for HCN release, Dritschilo et al. found that aphids preferred the acyanogenic white clover genotypes over the cyanogenic white clovers genotypes used in the study (Dritschilo et al., 1979). Another study by Krothapalli et al. (2013) exhibited that dhurrinase2, one of the known four β-glucosidases able to catalyze HCN production, contributed to decreased insect feeding when exposed to other sorghum wild type accessions containing cyanogenic glucosides. With these findings, activation of the dhurrin biosynthetic pathway via aphid feeding could contribute as a defense mechanism via HCN release.

Developing molecular markers underlying SA resistance allows for the development of improved sorghum varieties that are adapted to a given environment, improving overall food security. *RMES1*, a dominant gene located on chromosome 6 in sorghum, has been identified as a resistance marker for SA. *RMES1* was found in a Chinese grain sorghum variety known as Henong 16 (HN16), and has been found to contribute to SA resistance (Chang et al., 2012; Muleta et al., 2021). Chang et al. searched for populations segregating with the *RMES1* locus using HN16 and BTx623 as parental lines to map the location of *RMES1* and found 11 molecular markers on chromosome 6 (Chang et al., 2012).

**Nitrogen Turnover**

Cyanogenic glucosides may have nitrogen storage and recycling functions throughout plant development. Previous studies have demonstrated that cyanogenic glucosides serve as

storage compounds for reduced nitrogen, which can be recycled for synthesis of other metabolites (Blomstedt et al., 2018; Busk & Møller, 2002). The detoxification and recycling pathways of dhurrin produces ammonia, which could be used as an additional N source (Blomstedt et al., 2018). As a result, dhurrin may function as a N source during grain fill, overall delaying leaf senescence (Busk & Møller, 2002). A study found that N fertilization leads to an increase in the activity of CYP79A1 & CYP71E1, thus increasing dhurrin content (Busk & Møller, 2002). This was confirmed when dhurrin content and the biosynthetic gene cluster on chromosome 1 was associated with N-fertilized environments and the catabolic gene cluster was associated with no additional N application (Hayes et al., 2015). Additional research is needed to determine the effect of N fertilization and dhurrin concentrations on sorghum field performance.

**Methods to Phenotype HCNp**

Different phenotyping methods have been used to evaluate HCNp in sorghum (De Nicola et al., 2011; Feigl & Anger, 1966). A phenotypic measurement produced by Feigl & Anger is known as the Feigl Anger (FA) test (Feigl & Anger, 1966). The FA test is a qualitative measurement of HCN content by displaying the color blue for a sample. A blue dot indicates HCN presence while no blue dot indicates no HCN presence. Another qualitative measure of measuring cyanide potential are commercial test strips. Commercial test strips detect HCN release through detection of a blue dot, although the color may vary depending on the brand of test strips used. The blue intensity of the sample also represents HCN concentrations. Colorimetric methods to semi-quantitatively measure HCNp in sorghum have been developed (Nakasagga et al., 2022; Reddy et al., 2016).

Although there are qualitative methods to detect HCN presence, quantitative methods can be used to determine the total amount of dhurrin present in a tissue sample. High Performance

Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) can quantitatively determine dhurrin content found in different sorghum tissue. Dhurrin has been quantified using both methods, yet extraction protocols can differ (Gleadow et al., 2012). Dried leaf tissue and fresh leaf tissue contained similar dhurrin concentrations post-harvest but dhurrin concentrations in freeze-dried tissues contained half the amount of dhurrin (Gleadow et al., 2012). These considerations must be considered when performing metabolomics analyses for dhurrin.

**Conclusion**

Understanding the role of dhurrin and HCNp in sorghum can further the improvement of global food security due its role in mitigating abiotic and biotic factors. The biosynthetic gene cluster and enzymatic pathways are well understood, and can be utilized to understand the evolutionary adaptation of dhurrin in sorghum. As the climate changes, it is important to study mechanisms to improve drought tolerance in sorghum. More research is needed to study the role of dhurrin as an osmoprotectant, SA resistant mechanism, and as an N storage compound to further improve stress tolerance in sorghum germplasm. These new hypotheses will improve our understanding of dhurrin's role in plant development and stress response. Testing these hypotheses could support and accelerate the introgression of high dhurrin genotypes into sorghum breeding programs.

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# CHAPTER II: A SIMPLE, HIGH-THROUGHPUT, SEMI-QUANTITATIVE METHOD FOR BREEDERS TO PHENOTYPE CYANOGENIC POTENTIAL IN SORGHUM

## Introduction

Plant breeding requires high-throughput phenotyping techniques to improve overall efficiency to select progeny in a plant breeding population. Smallholder breeding programs play a role in improving worldwide food security by enabling breeders to introduce locally adapted varieties. Sorghum (*Sorghum bicolor* (L) Moench) is a staple crop in smallholder plant breeding systems, where high-throughput phenotyping techniques are important to advance breeding generations to improve field performance.

Plant secondary metabolites (PSM) play an important role in mitigating abiotic and biotic stressors such as pest pressures. Cyanogenic glucosides are a PSM found in more than 2500 plant species (Conn, 1979) where they all share the ability to release cyanide (HCN) upon tissue disruption, a process known as cyanogenesis. HCN production in cyanogenic glucoside-producing plants is a heritable and stable trait (Zagrobelny et al., 2008). Sorghum contains a cyanogenic glucoside known as dhurrin. Dhurrin is derived from L-Tyrosine, two cytochrome P450 enzymes, CYP79A1 and CYP71E1, and a UDP-glucosyltransferase (UGT85B1). The membrane-bound cytochrome P450 enzymes and UGT85B1 found on chromosome 1 form a metabolon near the ER membrane which helps to prevent accumulation of toxic intermediates and inhibits substrates other than CYP79A1 from binding to the active site (Laursen et al., 2016; Nielsen et al., 2008).

The conversion of dhurrin to HCN occurs when cell disruption occurs, such as leaf chewing. β–glucosidases (dhurrinases) and *a*-hydroxynitrilases (HNLs) catalyze the release of

HCN from dhurrin. Dhurrinase (Dhu) 1 and 2 and dhu-like 3 and 4 are colocalized on chromosome 8 (Hayes et al., 2015, 2016), where they are expressed in different plant tissues (Cicek & Esen, 1998; Hösel et al., 1986; Gleadow et al., 2021). Not all dhurrin is converted to HCN. Dhurrin recycling pathway reduces the build-up of toxic intermediates where dhurrin is converted into *p*-hydroxyphenylacetic acid and ammonium. This process provides a source of additional nitrogen (N) during later developmental growth stages (Bjarnholt et al., 2018; Pičmanová et al., 2015), which may improve drought resilience.

Dhurrin concentrations are influenced by genotype (Burke et al., 2013; Emendack et al., 2018; Gruss et al., 2023; Hayes et al., 2015), growth stage (Busk & Møller, 2002; Gorz et al., 1986), environment (Gleadow & Møller, 2014; O'Donnell et al., 2013; Rosati et al., 2019), and tissue type (Gleadow et al., 2021; O'Donnell et al., 2013). After leaf tissue maceration HCN is produced. HCN is a toxic compound that can eventually poison and even kill livestock foraging on young sorghum plants. Although there are poisoning risks, HCNp, the amount of HCN that can be released from a plant tissue, aids in pest defense, particularly in the vegetative growth stage (Gruss et al., 2022; Krothapalli et al., 2013; Busk & Møller, 2002; Gleadow et al., 2021). Additionally, dhurrin has been hypothesized to contribute to post-flowering drought tolerance, sorghum aphid (SA) resistance, and as an additional N source during grain fill (Burke et al., 2013; Busk & Møller, 2002; Hayes et al., 2016; Krothapalli et al., 2013). Another *Stg* QTL, *Stg5,* was found to colocalize with the dhurrin biosynthesis genes on chromosome 1, generating the hypothesis that higher dhurrin levels are correlated with the *Stg* phenotype (Hayes et al., 2016), although this was proven to be more genotype specific rather than a straightforward correlation between the two (Gruss et al., 2023). The relationship between dhurrin/HCNp and its role in mitigating abiotic and biotic stressors have been studied (Burke et al., 2015; Emendack et al.,

2018; Fang et al., 2021; Gruss et al., 2023; O'Donnell et al., 2013), yet many tradeoffs exist. Therefore, biological hypotheses can be tested in breeding programs to improve field performance.

The HCN synthesized from dhurrin serves a role as a pest defense mechanism, yet aphid resistance associated with HCNp has not been studied. Sorghum aphid (SA) has threatened worldwide sorghum production, particularly in developing countries where food security is at risk. *RMES1*, a SA resistant marker, was found in resistant parent IRAT204 and introgressed into a susceptible parent background, RTx430, to develop near isogenic lines (NILs) (Muleta et al., 2021). This encouraged the development of a high-throughput, semi-quantitative phenotyping method to test the hypothesis that HCNp confers SA resistance.

Breeders in smallholder breeding programs are in need of simple, affordable, and high-throughput phenotyping methods to select for improved genotypes in a screening population. Biochemical methods such as HPLC and LC-MS have been used to quantify dhurrin content but are expensive and not high-throughput (Akazawa et al., 1960; Reddy et al., 2016). As a result, the goal of this study was to successfully develop a phenotyping method using commercially available test strips to semi-quantitatively measure HCN concentrations in sorghum leaf tissue. To do this, stay-green (*Stg*) recombinant inbred lines (RILs) with known variation in dhurrin concentrations were phenotyped with the HCNp assay. CIE Color lab space was used to semi-quantitatively measure HCNp and validated with broad-sense heritability and repeatability estimates. Overall, the semi-quantitative measurements using the HCNp assay can inform future breeding decisions in sorghum.

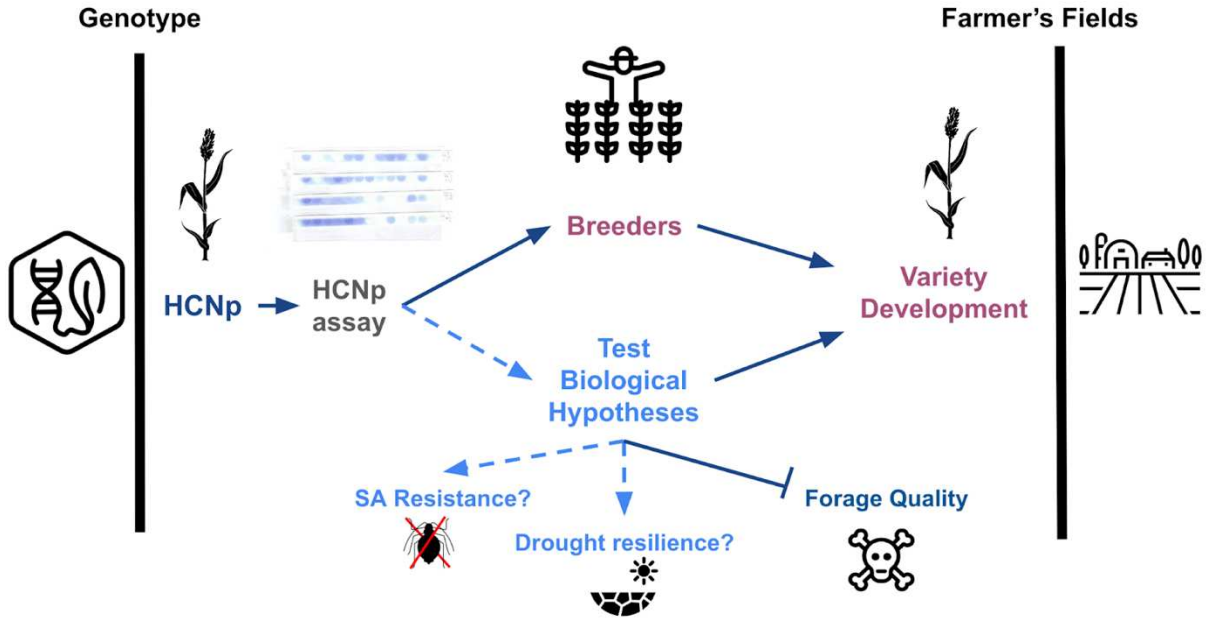


**Figure 2.1: The HCNp assay provides breeders with a phenotyping tool to develop improved sorghum lines.** A conceptual illustration of the HCNp assay and the deployment to breeders to improve overall field performance.

## Materials & Methods

### *Plant materials*

The sorghum germplasm was sourced from the Germplasm Resources Information Network (GRIN) determined by known dhurrin levels found in published literature. The genotypes were selected based on corresponding dhurrin content referenced from Hayes et al., 2016 and Burke et al., 2013. Seven genotypes were chosen based on the range of previously reported dhurrin content for initial HCNp assay development. These genotypes include R9188 (PI 656007), P898012 (PI656057), BTx642 (PI 656029), RTx436 (PI561071), Tx7000 (sourced from Kansas State University), and maize (PI 601270). Maize does not contain dhurrin so it served as our negative control.

Near isogenic lines (NILs) were developed with IRAT204 (*M. sorghi* resistant, donor parent) and backcrossed with RTx430 (*M. sorghi* susceptible) until the $BC_2F_3$ stage. The

population was developed at Kansas State University using a KASP marker for *RMES1* (Muleta et al., 2021). Both parents were phenotyped using the HCNp assay.

***BTx642/Tx7000 StgRIL population***

To confirm that the HCNp assay is able to detect HCN content in a diverse sorghum population, we used a recombinant inbred line (RIL) mapping population derived from parents BTx642 (B35), the *Stg* female parent and Tx7000, the non-*Stg* male parent, selfed single seed descent to the F13 generation. This germplasm was obtained from Chad Hayes, USDA (Hayes et al., 2016). The population was developed in the 1990s by Texas A&M University where Xu et al., 2000 mapped QTLs corresponding to the *Stg* regions.

High Performance Liquid Chromatography (HPLC) was performed on the StgRIL population to determine leaf dhurrin content at flowering by Hayes et al., 2016. The protocol followed is included in Hayes et al., 2016. This reported data was used to determine ten "high" dhurrin RILs and ten "low" dhurrin RILs for HCNp assay validation in the greenhouse (Hayes et al., 2016; Supplemental data).

***Plant growth facilities***

The experiments were grown in the Plant Growth Facilities (PGF) located at Colorado State University in Fort Collins, Colorado. The daytime temperatures were set to heat at 70℉ and cool at 74℉. Nighttime temperatures were set to 61℉ and cooled at 65℉. Relative humidity was ambient and fluctuated as humidity was not controlled. Lighting was set for a 16 hour photoperiod. All germplasm, in addition to maize, were planted in 5 gallon pots. Each 5 gallon pot was filled with Pro-Mix HP Biofungicide + Mycorrhizae, a tablespoon of osmocote slow-release fertilizer, and well-watered throughout plant growth.

The seven genotypes chosen for assay establishment were sowed with 4 technical replicates in the same pot, including. The StgRIL population consisted of 10 biological replicates per high and low known dhurrin RILs in a randomized block design. The parents, BTx642 and Tx7000, were both planted in each pot, totaling 200 biological replicates. Maize (PI 601270) was included in both experiments. The StgRIL population and SA resistant (IRAT204) and susceptible (RTx430) parents were sown in the PGF with the same environmental conditions. Corn leaf aphid (CLA) and spider mites were present throughout population growth. Biological controls as well as weekly pesticide applications were applied to mitigate the effects of pests.

***StgRIL field phenotyping***

The same F13 StgRIL population developed by (Hayes et al., 2016) was grown in Akron, Colorado under rainfed conditions. The site was long-term no till and the previous crop was wheat. The residue of wheat was still present during planting where the population was treated with Concep III before planting. 89 RILs and two inbreds (BTx2752 and Tx7000) were planted in two row plots per genotype totaling 17 ranges and 76 rows in a randomized complete block design. The population was phenotyped once in late August (8/30/2022) around the booting stage.

***HCNp assay materials and methods***

The method was adapted from the Feigl & Anger (FA) method to detect HCNp with the preparation of filter paper (Feigl & Anger, 1966). The test strips used in this study was Cyantesmo, with the ability to detect HCN gas. At the 5-8 week growth stage, the youngest leaf tissue per genotype was taken with a 6mm hole punch. The punches were taken on both sides of the leaf, excluding the midrib, totaling two punches per sample in the corresponding well. The punches were transferred into a polystyrene clear 96 well plate in a randomized experimental

design. Corn and blank spaces were used as negative controls to ensure the paper was working properly. After the leaf tissue was placed in polystyrene plates, they were immediately transferred into a styrofoam container filled with ice packs. The completed plates were placed in a -20 ℃ freezer overnight (~12 hours).

The Cyantesmo test strips were cut to fit the width of the 96 well plate. The plates were taken out of the freezer and the cut Cyantesmo test strips were placed over rows A, C, E, and G then taped down on the edge to keep them in place. Axygen microplate sealing film was placed above the spaced out strips on the plate. A tofu press was used to keep the strips as close as possible to the wells, preventing HCN gas leakage from one sample to another. Two plates were able to fit in each tofu press. The tofu press with both plates were placed in an incubator set at -35 ℃ for 20 minutes. After 20 minutes the plates were assigned visual scores and scanned with an Epson Perfection V39 for the semi-quantitative measurement.

***HCNp visual phenotypic measurement***

The Cyantesmo test strips were used to semi-quantitatively measure HCNp in different sorghum genotypes with varied levels of known dhurrin content. When HCN gas is released from the sampled leaf tissue, the Cyantesmo test strips turn blue, where the intensity of the blue-colored spots indicate high or low HCN content found in the corresponding well. The images were initially taken immediately after 20 min of incubation using a normal iPhone camera (12 megapixel) but after assay preparation an Epson Perfection V39 scanner was used. To validate the high, medium, and low cyanogenic genotypes visual scores were assigned to each cell in the 96 well plate (Figure 2.2). The visual scores given had a range of 0-5, with 0 indicating no HCN content, 1 indicating little HCN, and 5 indicating high HCN content with an intense blue dot on the Cyantesmo strips. The visual score was recorded according to the experimental design. These

visual scores were analyzed via boxplots to distinguish the high, medium, and low genotypes. A pairwise t test was performed to determine significance (p=0.05) between the means of each group in R (R Core Team, 2022). Blue spot development on the Cyantesmo strips were used to compare and validate the visual scores used for semi-quantitative analysis.

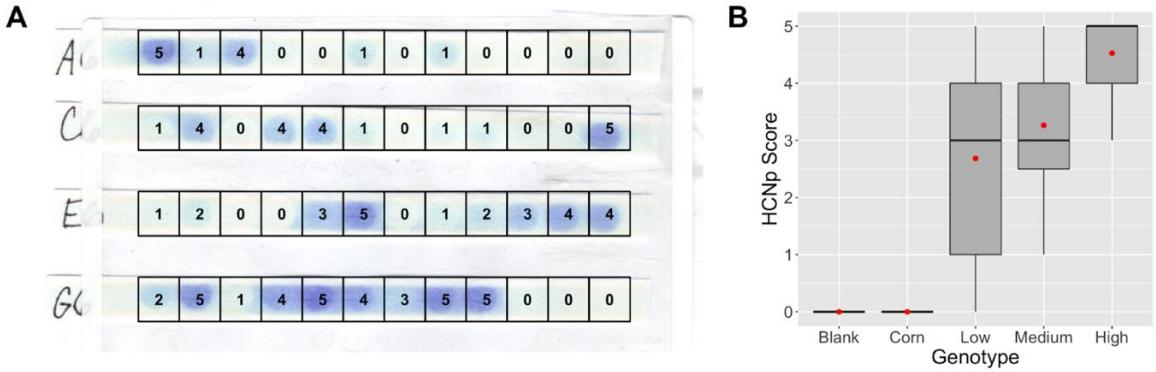


**Figure 2.2**: **A) The visual scores assigned to each sample utilizing the Cyantesmo test strips.** The phenotypic result and the assigned visual score (0-5) to each well. Each blue dot represents HCN presence and its abundance after the subsequent freeze to thaw process. **B) Variation of known low, medium, and high dhurrin genotypes differentiated by HCNp visual scores.** representing the negative controls (blank, corn) and the corresponding low, medium, and high genotypes plotted against the HCNp visual scores.

### *StgRIL phenotyping*

The F13 StgRIL mapping population was used to validate the HCNp phenotyping method. Three different time points were used to phenotype for HCNp using the HCNp assay detailed above in the PGF. The phenotyping growth stages for the StgRIL population were at the 6-7 leaf stage, 14-15 leaf stage, and flowering. Flowering time was determined when more than half of the population and half of the panicle was flowering.

In addition, the same StgRIL population was grown in the PGF was grown in Akron, CO under rainfed conditions. Total 89 RILs and two inbreds (BTx2752 and Tx7000) were planted in

two row plots per genotype totaling 17 ranges and 76 rows in a randomized complete block design. The population was sampled once in late August, 2022 around the booting growth stage for in-field assay validation.

***CIE LAB color space***

To validate the visual rankings, an algorithm was developed to semi-quantitatively distinguish the cyanide content associated with the blue intensity found on the test paper. International Commission on Illumination colorspace for perceptual lightness on red, green, blue and yellow, also known as CIE L*A*B space, (CIE LAB space) quantifies the blue intensity of each plant tissue sample using red-green-blue (RGB) images as the input and quantitative values based on blue intensity as the output. The algorithm included three steps: calibration, pre-processing, and measuring. Calibration involved locating the center of each well and well spacing was determined. The pre-processing stage converted the input image into the CIE LAB color space. The measurement step aligned the user-interface with the grid, and processed each well to output an array of blueness scores.

We utilized a 2560x1440 monitor, which required down-scaling for most images taken from a flatbed scanner. The images were scaled to roughly HD 1920 x 1080, which retained the aspect ratio, although additional steps such as cropping and rotation of the image occurred, depending on image differences. Additionally, some images varied so steps were taken to account for different grid spacing, camera angles, and distances. The captured images via a scanner can be used across different experiments if the images are consistently captured.

The RGB image was converted to a CIE LAB image. Iteratively, for each of the sample wells the algorithm did the following:

1. Generated a region of interest (ROI), using the coordinates (x,y) and dimensions (w,h) of the selection region. A sub image mask (I') was created from the contents of the selection ROI. The ROI included a small amount of white space (Fig 7). This white space is consistent to prevent sample variation.
2. Thresholding refers to the process of selecting certain pixels from an image based on a set of conditions. The thresholding process involved filtering pixels by the intersection of these two conditionals:
    a. Pixels that were considered blue from the range [0, 128] in the b* channel of the Lab* color space. The b* channel represents the blue and yellow color values.
    b. Pixels that were in the luminosity range [128, 255] which captures light to dark pixels. This used the L* channel of the Lab* color space and represents the lightness or darkness of a color.
3. A summation of all pixels after thresholding was calculated using Eq. 1

$f(P) = (255 - P[0])$ **(Eq 1.)**

A numeric value of 255 represents white luminosity. The darker blue pixels had a higher score due to a lower P[0] value.

4. Eq. 2 was the formula used after the score was added for each pixel which created a cumulative score within each ROI.

$A = \sum f(P)$ **(Eq 2.)**

5. Eq. 3 was the cumulative score divided by the area of the ROI and converted to an integer value (S). This integer value was assigned to the 96 well plate samples, where

dark blue pixels were assigned higher quantitative values, and light blue pixels were assigned lower quantitative values.

S = A / Area(ROI) **(Eq. 3)**


**Figure 2.3: The region of interest (ROI) and the corresponding integer value.** Examples of the region of interest (ROI) indicated by the green box, and integer values (S) assigned to the range of blue pixels on the Cyantesmo paper.

***Broad-sense heritability calculations***

Broad-sense heritability ($H^2$) was calculated for blue pixel score using the package lmer4 in R to generate a mixed linear model (MLM) to utilize the variance components of each variable. In Eq 4, BPS (blue pixel score) had a fixed effect while Genotype, Pot/Coordinate, Leaf, Plate and People were treated as random effects. $H^2$ was calculated using the variance components at each sampling date (6-7, 14-15 leaf stage, flowering, field) using Eq 5 where $\sigma_G^2, \sigma_\epsilon^2$, and $n^r$ represent variance components of Genotype ($G$), error, and number of replicates, respectively. The leaf number was excluded from leaf stage 6-7 due to a singular boundary as well as plate number. Additionally, the plate variable during the flowering stage was singular. We chose not to include the covariates recorded in the model for the $H^2$ calculation due to non-significant effects.

$lmer(BPS \sim (1|Genotype) + (1|Pot/Coord) + (1|Leaf) + (1|Plate) + (1|People))$ **(Eq 4.)**

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_\epsilon^2}{n_r}}$$ **(Eq. 5)**

## Results

### *Use of CIE LAB color space allows semi-quantitative measurement of HCNp*

To validate the visual rankings, an algorithm was developed to semi-quantitatively distinguish the cyanide content associated with the blue intensity found on the test strips. CIE lab space was able to quantify the blue intensity of each plant tissue sample using RGB images as the input and quantitative values based on blue intensity as the output. To test the hypothesis that the HCNp assay is able to detect HCN content in a diverse sorghum population, we used a recombinant inbred mapping (RIL) population derived from BTx642 (high dhurrin) and Tx7000 (low dhurrin). The CIE LAB color space algorithm was used to semi-quantitatively measure the blue phenotype representing HCNp in a given leaf. These values were the blue pixel scores used for assay validation.

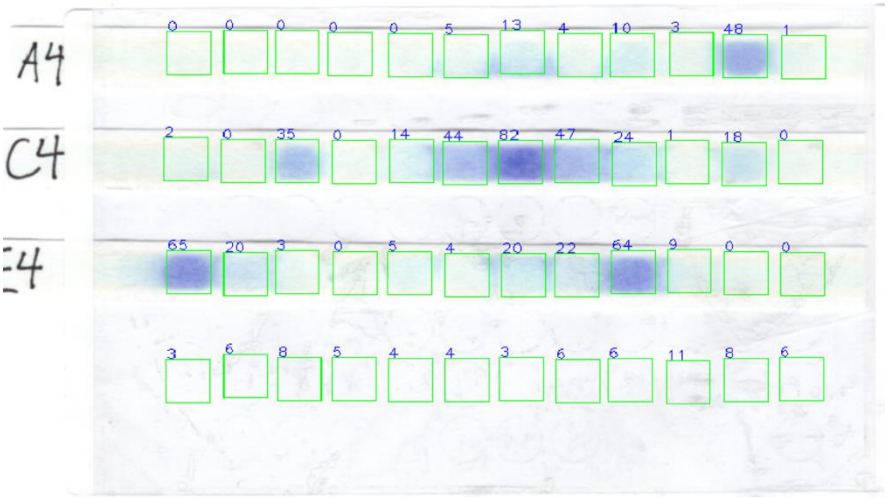


**Figure 2.4: The output after calibration, pre-processing and measuring steps using CIE LAB color space.** Each grid or ROI is assigned a numeric value corresponding to the sample in the 96-well plate.

***Visual scores are a good proxy for semi-quantitative measurements***

To test the hypothesis that the visual scores used in the HCNp assay is a good proxy for blue pixel score, a correlation analysis and a pairwise *t*-test between sampling times were evaluated (Fig. 2.5). Each group had *p*-values less than 0.005, indicated by three asterisks, and a Pearson's correlation coefficient of $r = 0.9$. A Pearson's correlation coefficient for each group was calculated but could not run due to a standard deviation of 0 for each group. We can conclude the visual scores represent the blue pixel score assigned to each sample.

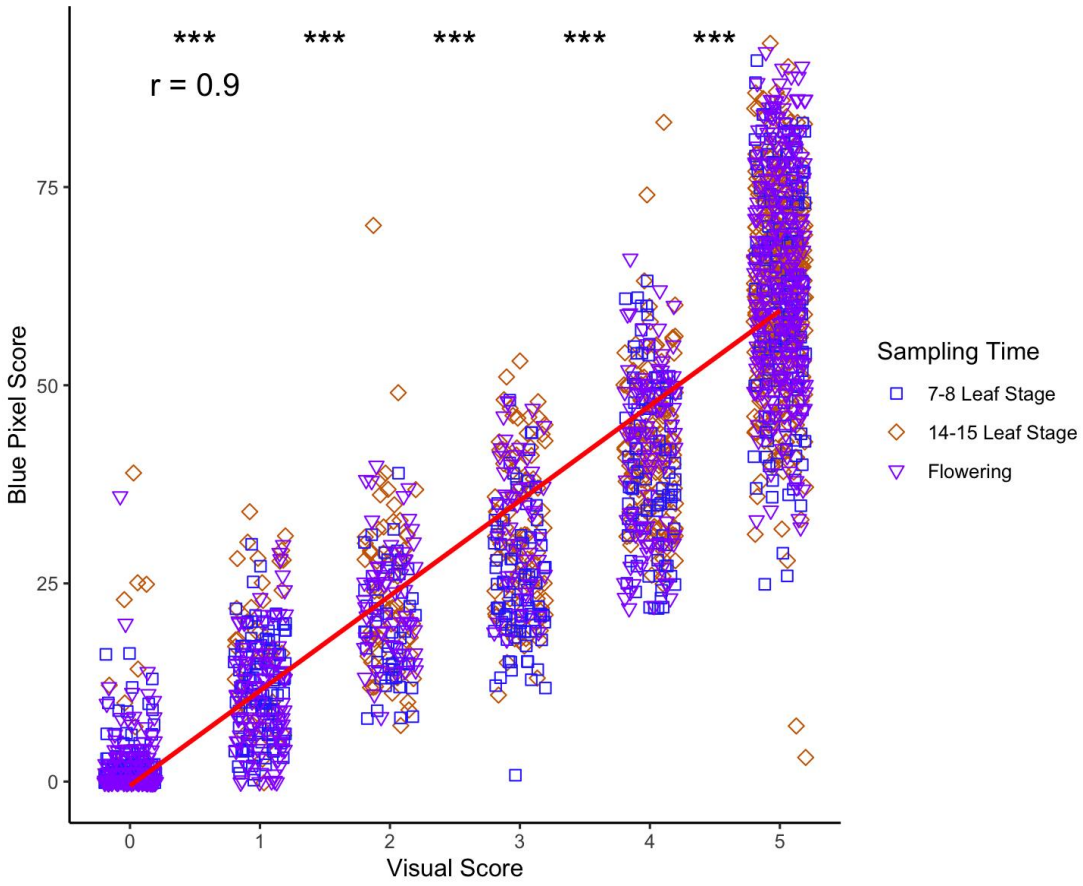


**Figure 5: Visual score plotted against blue pixel score including all three sampling times indicated by different shape and colors of points**. The Pearson's correlation coefficient is $r$=0.9. A pairwise *t*-test was performed between each group and three asterisks representing $p$<0.005.

***HCNp assay confirms previously reported HPLC data***

To test the hypothesis that the StgRIL population is able to detect known high and low genotypes, a pairwise t-test was calculated to determine significance between groups of high and low known dhurrin genotypes and parents, BTx642 and Tx7000 (Figure 2.6). We found that each group had *p*-values <0.005 indicated by three asterisks between each group. The groups for each *t*-test are grouped by neighboring group ranking scores. For example comparing groups 0 to 1, 1 to 2, 2 to 3 etc. We can conclude that the HCNp assay is able to distinguish high and low genotypes in a sorghum breeding population.

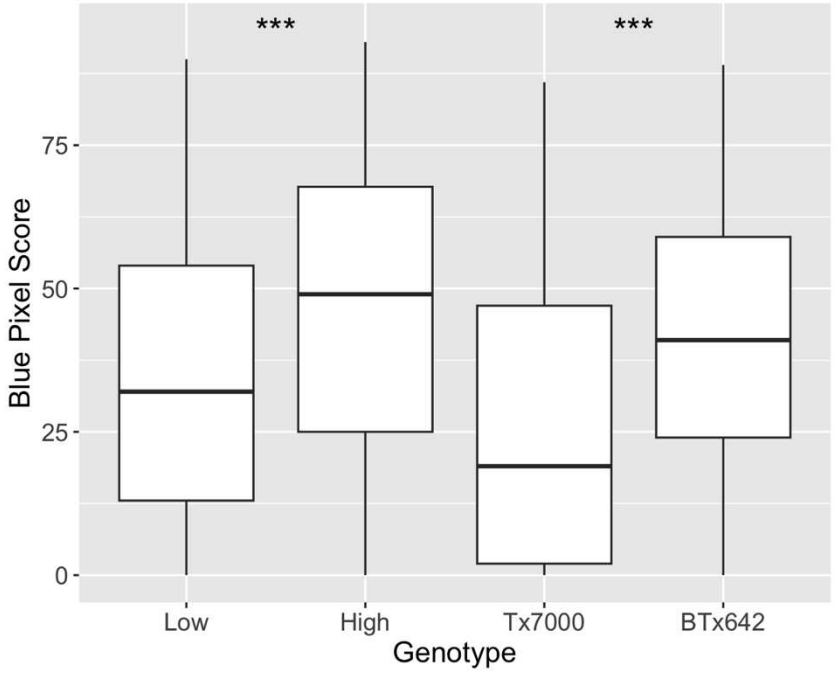


**Figure 2.6: HCNp semi-quantitative measurements for each classified low and high and low dhurrin genotypes A)** A pairwise *t*-test (***= $p<0.005$) was performed to confirm the HCNp assay detects known high and low dhurrin genotypes in the StgRIL population. The bold horizontal line represents the median (.5). The means for each group was 34, 47, 26, and 41, respectively.

***HCNp variation of StgRIL population in greenhouse and field is highly heritable***

To test the hypothesis that HCNp variation found in the population is due to genotypic differences, broad-sense heritability ($H^2$) was calculated for each sampling time. To do this, a

MLM was performed in R using the lme4 package (R Core Team, 2022). Genotypic and residual variance components per sampling time was included in the $H^2$ calculation. For each stage $H^2$ was estimated as 0.48, 0.64, 0.83, and 0.91, respectively (Figure 2.10).

$H^2$ around flowering time in the controlled environments is relatively high, therefore, we conclude it is best for breeders to sample at a later growth stage due to the genetic factors significantly impacting HCNp phenotypic variability. The $H^2$ increased due to biosynthetic gene cluster and catabolic gene cluster expression differences over plant growth. To validate the HCNp assay in the field, $H^2$ was estimated as 0.64. This heritability value is acceptable enough to detect HCNp variability found in a breeding population. In addition, the StgRIL population included additional genotypes with different known dhurrin values.

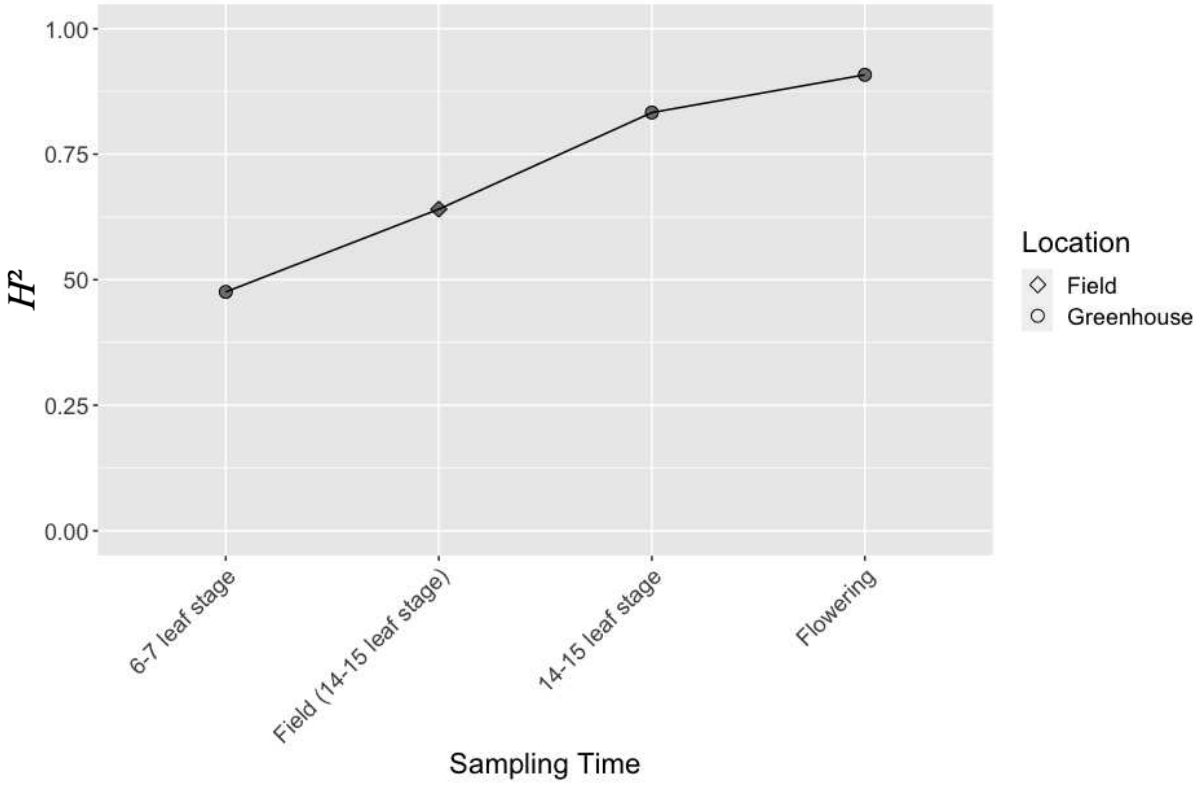


**Figure 2.7: Broad sense heritability ($H^2$) estimations for each sampling time for the StgRIL population and field experiment**. Each value is 0.48, 0.64, 0.83, and 0.91, respectively where

sampling time is indicated on the x-axis. The sampling locations of the field and greenhouse are represented by different point shapes.

***Phenotyping of SA resistant and susceptible parents reveal no difference in HCNp***

To test the hypothesis that SA is resistant to HCN, both the susceptible (RTx430) and resistant (IRAT204) parents were phenotyped for HCNp. We phenotyped the parents first because if there was a significant difference between the parents, we would predict to see the same in the NIL+ and NIL- genotypes in support of a previous observation that *RMES1* confers aphid resistance (Muleta et al., 2021; VanGessel et al., in prep). There was no significant difference found between the HCNp visual scores of RTx430 and IRAT204 so we can conclude HCNp does not confer SA resistance. Although the semi-quantitative measure of blue pixel score was not used in this study, this could contribute to decreased accuracy.

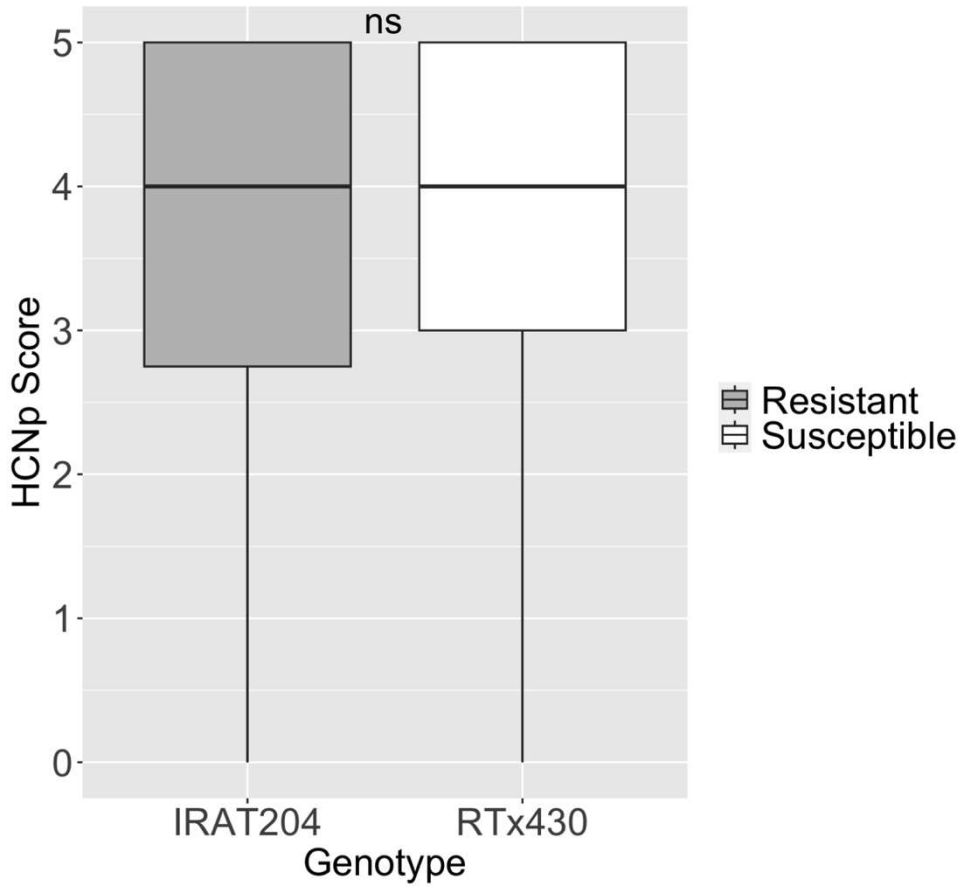


**Figure 2.8: No association of HCNp scores with sorghum aphid resistant (IRAT204) and sorghum aphid susceptible (RTx430) genotypes**. A Wilcoxon rank-sum test was performed. A *p*-value of 0.66 was calculated (n = 48) concluding a non-significant (ns) difference between each group.

## Discussion

### *The HCNp phenotyping method utilization for breeders*

The release of HCN, otherwise known as cyanogenesis, is commonly found in many plant species. Cyanogenesis in sorghum is regulated by the combination of dhurrin and dhurrinase, where each component is separated in different plant cell types. Upon plant tissue disruption, the dhurrin and subsequent catabolic enzymes combine producing the release of HCN. Factors such as environment, genetics, plant maturity, and tissue type plays a role in

dhurrin content, contributing to HCN variability found in diverse sorghum germplasm (Jones et al., 1999; Burke et al., 2013; Gruss et al., 2023; Hayes et al., 2015). HCNp is known to play a role in pest defense but affects forage quality (Gruss et al., 2022; Krothapalli et al., 2013; Wheeler et al., 1990). Tradeoffs exist, therefore, a phenotyping method to semi-quantitatively determine HCNp can inform future plant breeding decisions.

In sorghum, multiple HCN phenotyping methods have been used previously (De Nicola et al., 2011; Feigl & Anger, 1966; Nakasagga et al., 2022; Reddy et al., 2016; Vinutha et al., 2021). The method developed was adapted from the FA test, but utilized commercial test strip paper without the need of paper preparation. The FA test has been utilized to detect for HCN presence, yet the preparation of the paper involves chemicals that may not be widely accessible to smallholder plant breeding operations. Additionally, the extra step of preparing the paper could introduce variability that can be avoided by utilizing commercial test strips. Other semi-quantitative methods, such as Near Infrared Reflectance (NIR), have been developed but they require additional time and wet-lab equipment that this assay does not require (Feigl & Anger, 1966; Nakasagga et al., 2022; Reddy et al., 2016; Vinutha et al., 2021).

The Cyantesmo test strips chosen have been used in previous studies but only to qualitatively determine HCN presence in food, animal, and plant science studies (Ahmad et al., 2022; Grueter et al., 2016; Rella et al., 2004; Strano et al., 2017; Yamashita et al., 2010). Cyantesmo paper has been used in sorghum leaf tissue, specifically to test for forage quality (Motis, 2016; Smith et al., 2018). To make the assay high-throughput, a 96 well plate was used but only 48 samples could be analyzed to allow space for the strips. Each sample included two hole punches per well because some samples did not appear to have enough HCN for the paper to change color. Sometimes bleeding over of the samples did occur, but to prevent HCN gas

from leaking well-to-well, a tofu press was used to apply pressure on both sides of the plate. To help this issue, a 3-D printed jig was developed but needed additional fixes after the pilot jig was tested (Rife et al., in prep). Visual scores are used frequently in high-throughput phenotyping techniques, yet subjective values can vary person-to-person (Burke et al., 2013; Emendack et al., 2017; Reddy et al., 2016). As a result, semi-quantitative measurements (blue pixel score) using CIE Color lab space can increase the accuracy between users, as well as speed up the phenotyping process. NIR and other colorimetric methods have been used to semi-quantitatively determine cyanide content in a given sample (Fox et al., 2012; Nakasagga et al., 2022; Sen et al., 2008). CIE Color lab space is similar to other colorimetric methods, where the equation uses different color wavelengths to distinguish color differences using a handheld colorimetric device (Nakasagga et al., 2022). The goal of this assay is to develop a cell phone application which could be used in both the lab and field, allowing for a more high-throughput phenotyping method compared to other semi-quantitative methods.

***StgRIL population validates HCNp assay***

To test the hypothesis that the HCNp assay can be used as a good proxy for HCN content, post-flowering drought tolerant StgRILs containing high and low dhurrin concentrations were used to represent a plant breeding population varying in HCN content. Under drought-stress conditions, sorghum plants that possess the *Stg* trait can maintain their photosynthetic capabilities due to more green leaf material during the post-flowering growth stage (Borrell et al., 2000; Harris et al., 2006; Xu et al., 2000). Burke et al. (2013) found *Stg* lines to contain higher dhurrin than pre-flowering drought tolerant lines.

The F13 RILs developed contained variants that modulate leaf dhurrin level where the dhurrin biosynthesis genes had the largest effect on total dhurrin leaf content (Burke et al., 2013;

Hayes et al., 2016). Ten previously known low dhurrin RILs and ten high dhurrin RILs as well as both parents (BTx642 & Tx7000) were phenotyped for HCNp and grouped into known high and low dhurrin concentrations to confirm the HCNp assay quantified HCNp appropriately for each group and each parent (Fig. 2.6).

To determine if the HCNp assay captures the phenotypic variation attributable to genetic differences in the StgRIL population, heritability was calculated for each sampling time and location (Fig. 2.7). The HCNp assay was heritable at all sampling time points and locations but highly heritable at later growth stages. Burke et al. (2013) reported that leaf dhurrin content distinction between low and high dhurrin lines was most noticeable from fully grown leaves of mature plants. As sorghum matures, relative dhurrin concentrations decrease due to an increase in turnover (Busk & Møller, 2002; Cowan et al., 2021). Dhurrin variation encoded by the biosynthesis and catabolism genes strongly influence HCNp (Gleadow & Møller, 2014; Hayes et al., 2015). The higher $H^2$ values in later growth stages could be due to lower expression of the dhurrin biosynthesis genes and higher expression of the catabolic gene cluster (Gleadow et al., 2021), which could contribute to more HCNp variation. Although the flowering growth stage had the highest $H^2$ estimate, other sampling growth stages may be important for breeders to test a hypothesis such as pest resistance and forage quality. If a breeder wants to determine pest feeding preference, they would want to sample at an earlier growth stage where dhurrin content is highest (Burke et al., 2013; Gruss et al., 2022). In addition, if a breeder wants to determine forage quality, it would be best for them to sample when the fresh sorghum forage will be consumed. In U.S. agriculture systems, this would be at a younger growth stage (Cassida, 2012) and in international agriculture systems, it would be best to sample when the sorghum grain is mature, when HCN levels are low in the leaf (Busk & Møller, 2002; Vinutha et al., 2021)

To test the hypothesis that the assay works in the field, the same StgRIL population, with other genotypes differing in dhurrin content, was phenotyped for HCNp and $H^2$ was estimated. The StgRIL population in the field had a relatively high heritability estimate (Fig. 2.7), although additional field sites and sampling time points could affect this estimate (Nakasagga et al., 2022). Currently, the HCNp assay is being utilized in a field setting during different time points and environments so supplemental studies will be performed for further assay improvement. In addition, it is important that breeders take into account limitations that could affect the end-use of the assay. Growth stage, in this case leaf number, is most important for breeders to consider and has been found to affect HCNp variation in other sorghum HCNp phenotyping assays (Nakasagga et al., 2022; Zahid et al., 2012). Therefore breeders should be aware of the maturity stage of the plants, especially when introducing other variables such as environment in a field experiment.

***HCNp could still confer SA resistance***

SA has defestated sorghum populations worldwide, yet *RMES1*, a SA resistant allele, has served as a source of resistance to SA worldwide (Muleta et al., 2021). HCN release from dhurrin serves as a source of chewing insect defense (Gruss et al., 2022; Krothapalli et al., 2013), yet stylet, phloem feeding species such as SA and the effects of HCNp in sorghum have not been studied. Other studies have found that HCN deterred two different species of aphids from dhurrin containing *A. thaliana* (Pentzold et al., 2015).

Observations by a sorghum breeder in Haiti found SA to accumulate on the older leaves on the same plant and decrease on younger leaf tissue. These observations led to testing the hypothesis that HCNp confers SA resistance using the HCNp assay. The results do not support our hypothesis, where no significant HCNp difference was found between the resistant

(IRAT204) and susceptible (RTx430) genotypes, although only subjective visual scores were used in this assay (Figure 2.8). Subjectivity could add some additional inaccuracies, where the semi-quantitative measure is able to assign a larger range of values and decrease user variability. Further metabolomics analyses in tandem with the use of an electronic penetration graph (EPG) could uncover the feeding mechanisms of SA as well as quantitatively determine the dhurrin content in the leaf (Nalam et al., 2018, 2019). The HCNp assay is being utilized in Haiti since this was not performed in an environment where SA pressure is present. Performing resistance studies in the target environment could influence plant-insect interactions that are important for pest resistance. The HCNp assay in tandem with targeted metabolomics will conclusively determine if HCNp plays a role in SA resistance.

**Conclusion**

The development of phenotyping tools in large-scale breeding populations requires efficient methods to screen for a trait of interest. Biochemical traits, such as cyanogenic glucosides, are important to quantify due to the release of HCN, yet methods to detect these traits, such as HPLC/LC-MS, are expensive. The goal of this study was to create a high-throughput, affordable, semi-quantitative HCN phenotyping assay that could be used primarily in smallholder breeding programs. To do this, select sorghum genotypes with different known dhurrin content were chosen to confirm the methodology developed. We used commercial test strip paper to reduce variability in the assay and provide increased accessibility for breeders to test HCNp in their own breeding populations.

We performed routine analyses using the commercial test strips to obtain semi-quantitative measurements using CIE LAB color space. The accuracy of the assay was confirmed by the addition of a StgRIL population. Broad-sense heritability values confirmed flowering to

be an important growth stage to capture the phenotypic variability in the population. SA resistance due to HCNp was evaluated using the HCNp assay. It is important that breeders consider limitations that could affect the end-use of the assay. No differences were found between the resistant (IRAT204) and susceptible (RTx430) parent, concluding HCNp may not play a role in SA resistance although further studies utilizing the semi-quantitative measure or a quantitative measure such as HPLC could parse out dhurrin/HCNp differences. Altogether this assay can be used to screen a sorghum breeding population in both a greenhouse and field setting, allowing for an effective phenotyping method for smallholder breeding programs looking to advance their breeding generations more efficiently.

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# CHAPTER III: GENETICS OF DHURRIN AND CYANOGENIC POTENTIAL IN GLOBAL SORGHUM DIVERSITY

## Introduction

The effects of climate change will greatly impact plant production worldwide due to the increasing effects of drought. There is evidence that plant populations are able to adapt to spatial climatic gradients and precipitation gradients can be utilized to make conclusions about evolutionary drought adaptation traits. Developing crops to withstand this environmental variability is necessary to ensure a food secure future, particularly in smallholder breeding programs (Mundia et al., 2019). Understanding the mechanisms that underlie environmental stressors such as drought can advance the overall fitness of grain crops. Sorghum [*Sorghum bicolor* (L.) Moench] is a staple grain crop grown worldwide in semi-arid/arid environments, originating from Africa. Local landraces possess diverse adaptive traits that make them suitable for evolutionary studies (Blum, 2011). The known drought tolerance of sorghum provides an opportunity to dissect drought tolerant traits to further understand sorghum landraces where it is not fully understood (Mullet et al., 2014). The diverse precipitation gradients present in Africa offer a range of environmental conditions suitable for conducting a trait-by-environment association study.

Secondary plant metabolites (PSMs) are compounds that assist in plant defense, and enable adaptation to biotic and abiotic stressors (Ahmad et al., 2022; Yang et al., 2018). Cyanogenic glucosides are known PSM's that produce hydrogen cyanide (HCN) upon hydrolysis where they serve as a defense mechanism against herbivores and pathogens. Cyanogenic glycosides are a class of glycosides containing 2-hydroxynitrile, which can be broken down into cyanohydrin and subsequently release HCN through the action of β-glucosidase (Gleadow &

Møller, 2014), where they are constitutively expressed. This HCN release process is known as cyanogenesis.

The cyanogenic glucoside found in sorghum is known as dhurrin. Dhurrin is synthesized by the amino acid, L-tyrosine, and is converted into dhurrin by the biosynthesis gene cluster (BGC) consisting of two cytochrome P450 enzymes (*CYP79A1, CYP79E1*) and a UDP-dependent glycosyltransferase (*UGT85B1*) located on chromosome 1 (Cicek & Esen, 1998; Conn, 1979; Kojima et al., 1979). The biosynthetic pathway of sorghum is also shared in other cyanogenic glucoside-producing species, therefore meaning the pathway is evolutionary conserved (Bak et al., 2006; Zagrobelny et al., 2004). The catabolic gene cluster (CGC) located on chromosome 8 is able to convert dhurrin to HCN when the tissue is disrupted. *Dhurrinase-1* (*dhr1*) and *dhurrinase-2* (*dhr2*) cleaves glucose off dhurrin, allowing the release of HCN (Kojima et al., 1979). *Dhurrinase-like*3 (*dhr-like3*) and *dhurrinase-like*4 (*dhr-like4*) have also been identified to aid in this process. Each enzyme is expressed in different plant tissues (Gleadow et al., 2021), yet the relationship between the CGC cluster and its influence on HCN concentrations is unknown. Not all dhurrin is catabolized to HCN. Several other pathways are involved in detoxification and recycling of dhurrin, with no release of HCN (Bjarnholt et al., 2018; Jenrich et al., 2007; Pičmanová et al., 2015). In the recycling process, a glutathione transferase (*GST1/GST1B*) and *NIT4A/NIT4B* convert dhurrin to *p*-hydroxyphenylacetic acid and ammonium ($NH_3$). The recycling process supports the claim that dhurrin aids in other developmental processes, dependent on plant growth stage and environment (Bjarnholt et al., 2018; Gleadow & Møller, 2014).

Dhurrin variability in a sorghum population is dependent on genotype, environment, growth stage, and plant organ (Burke et al., 2013; Busk & Møller, 2002; Emendack et al., 2018;

Gleadow et al., n.d., 2021; Hayes et al., 2015). Dhurrin is also hypothesized to recycle nitrogen, a process that could aid in the ability for sorghum to survive in drought environments (Møller, 2010; L. J. Nielsen et al., 2016), as well as function as an osmoprotectant (Burke et al., 2013; Busk & Møller, 2002; Hayes et al., 2015) Furthermore, dhurrin was found to be higher in post-flowering drought tolerant sorghum lines, also known as Stay-Green, (*Stg*) compared to pre-flowering senescent lines (Burke et al., 2013). As a result, dhurrin may contribute to drought tolerance (Burke et al., 2013; Busk & Møller, 2002; Hayes et al., 2016; Laursen et al., 2016; K. A. Nielsen et al., 2008). Although dhurrin has favorable characteristics, forage quality is negatively impacted due to the potential for HCN poisoning in foraging animals (Vough & Cassel, 2006).

Tradeoffs for dhurrin and HCNp exist therefore it is important for breeders to phenotype for both depending on their traits of interest. Dhurrin is hypothesized to serve as an osmoprotectant, yet dhurrin affects HCN concentrations, thus influencing pest resistance and forage quality (Busk & Møller, 2002; Gruss et al., 2022; Krothapalli et al., 2013; L. J. Nielsen et al., 2016). The simple, high-throughput HCNp assay aims to phenotype for both dhurrin and HCNp, where they serve as direct proxies, yet the mechanisms underlying these differences are not understood (M. Cowan et al., 2022; Gleadow et al., 2021; Rosati et al., 2019). The goal of this study was to understand the variation of dhurrin BGC, CGC, and other important candidate genes, as well as investigate the relationship between drought and dhurrin using diverse sorghum landraces. To test this hypothesis, landraces were phenotyped for HCNp and quantified for dhurrin content using ultra-high performance liquid chromatography (UPLC-MS), to establish an environment-trait association between dhurrin and drought. In addition, a genome-wide

association study (GWAS) was performed to understand how the genetic variability underlying dhurrin biosynthesis, catabolism, and recycling contributes to HCNp variability.

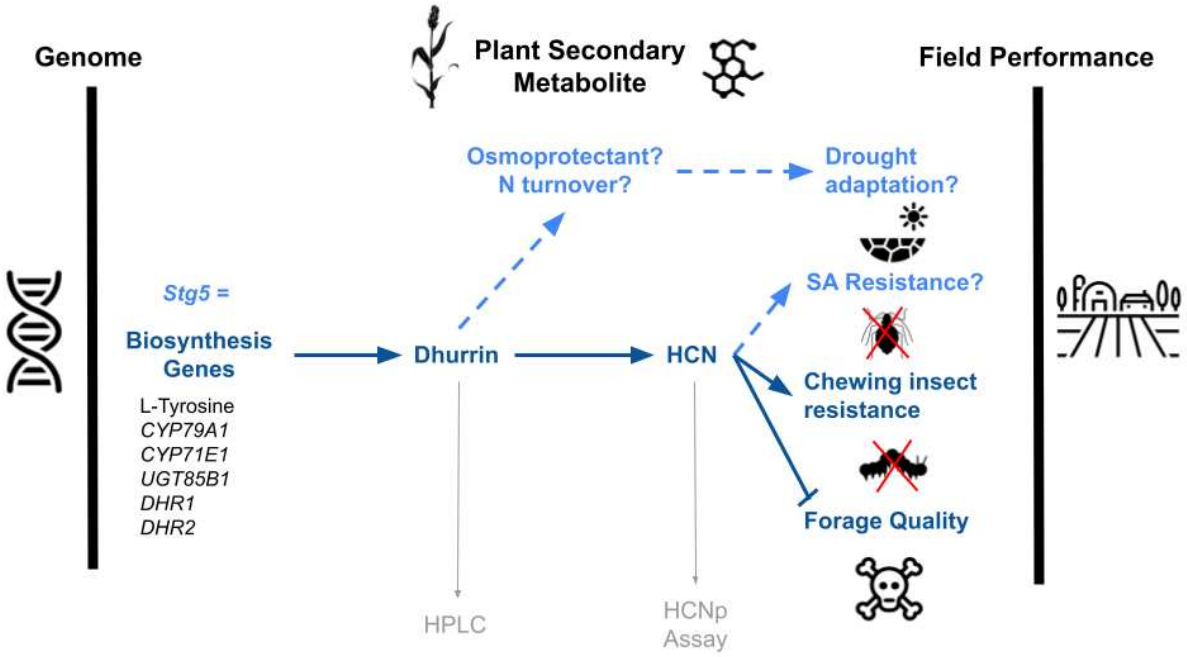


**Figure 3.1**: **The influence of dhurrin and HCN in mitigating abiotic and biotic stressors. A** genotype to phenotype (G2P) map of dhurrin and HCN with its effect on field performance.

## Materials & Methods

### *Plant materials*

The germplasm for this study were globally diverse sorghum landraces (n=373) originating from a variety of different geographic locations in Africa (Faye et al., 2022). The landrace accessions were obtained from the Germplasm Resources Information Network (GRIN) and evaluated in the Plant Growth Facilities (PGF) at Colorado State University. The daytime temperatures were set to heat at 23℃ and cool at 21℃. Nighttime temperatures were set to 16℃ and cooled at 18℃. Relative humidity was ambient and fluctuated as humidity was not controlled. Lighting was set for a 16-hour photoperiod. In addition, Tx7000, a pre-flowering

drought tolerant line and BTx642, a post-flowering drought tolerant line, were used as controls. The latitude, longitudes, country of origin, and population were identified previously (Lasky et al., 2015).

The landrace accessions were planted in two replicates of a complete randomized block design (two blocks) where four different accessions were sowed in a 3-gallon pot filled with Pro-Mix HP and a tablespoon of Osmocote. Two separate populations of the same genotypes were planted. The first population had higher germination (n=330) compared to the second population planted (n=222). Corn leaf aphid pressure was present so biological insecticides were applied to both populations.

The accessions in the PGF contained corresponding environmental parameters obtained from WorldClim (2.5 minute spatial resolution) where the known latitudes and longitudes from each landrace accession were paired with the long-term historical precipitation averages in millimeters (Fick & Hijmans, 2017). To merge the WorldClim data frame and genotype data frame, the latitudes and longitudes were transformed to contain one decimal point (Figure 3.2).

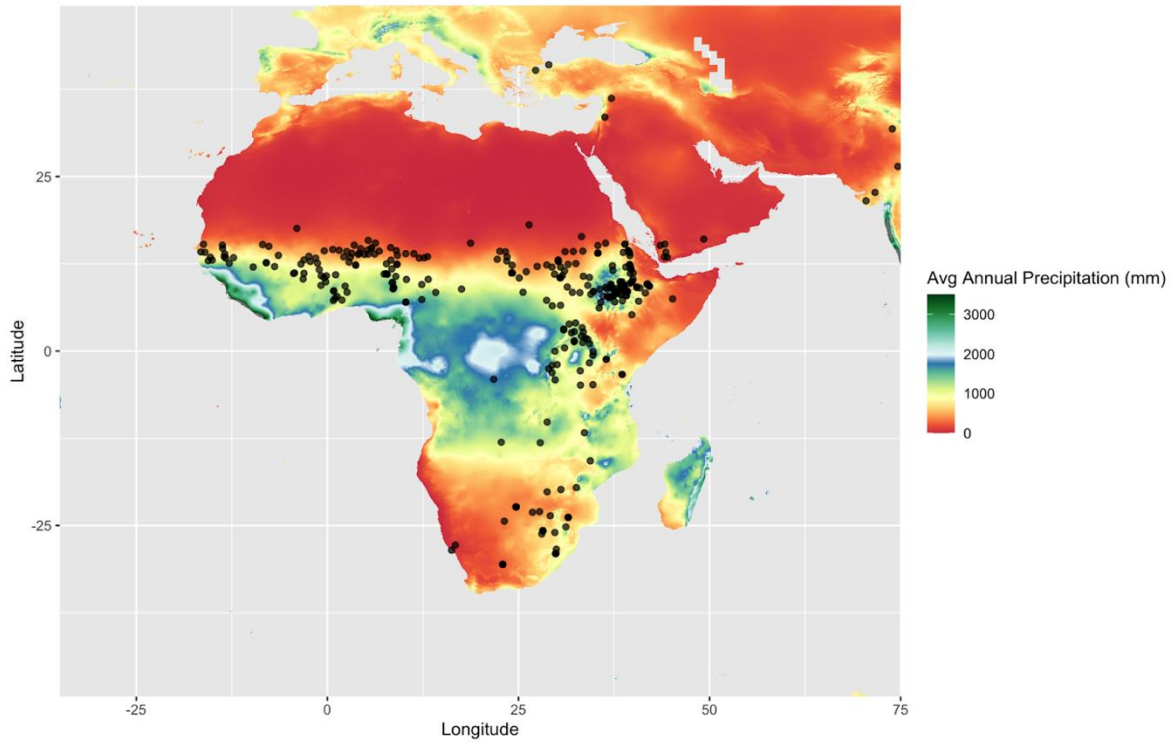


**Figure 3.2: The average annual precipitation gradient and location of globally diverse sorghum accessions** The geographic distribution of the landrace accessions corresponding with the precipitation gradient. The color scale represents average annual precipitation in millimeters with dark green representing higher precipitation and dark red representing low precipitation.

All landraces were phenotyped at two growth stages using the HCNp assay. Each genotype was hole punched twice on both sides of the leaf, excluding the midrib. The Cyantesmo test strip paper was used to detect cyanide content in each genotype, where cyanide content is equivalent to blue dots on the strip. Blue intensity of each identifiable blue dot was analyzed using CIE LAB Color Space which quantifies blue intensity into semi-quantitative values using three values of L* for lightness, a* and b* for the color opponents green-red and blue-yellow. These values correspond to the perception of color by the human eye and the detection of blue color differences represent the HCNp in each hole punch of sorghum leaf tissue. The detailed equations used for this analysis are in data chapter 1.

***Ultrahigh-performance LC-MS***

Targeted metabolomics using ultrahigh-performance liquid chromatography (LC) mass spectrometry (MS) (UPLC-MS) was performed to quantitatively determine dhurrin content found in the landrace accessions using dried leaf tissue. The modified extraction protocol was based on a method developed by (Vinutha et al., 2021). The youngest leaf tissue was harvested at around the 8-10 leaf growth stage then oven-dried for 7 days at 60 ℃. The dried leaf tissue for each genotype was grinded using an Omni Bead Ruptor Elite then extracted for metabolomic quantification.

After tissue grounding, 100 mg was weighed, transferred to a 2 mL Eppendorf tube, then 750 µL of prepared 50% methanol was added. The tubes were placed in a 75 ℃ water bath for 15 minutes. Afterwards, the tubes cooled off for about 10 minutes and another 750 µL of 50% methanol was added and immediately placed in a microcentrifuge at 11,000 rpm for 5 minutes. 30 µL of the supernatant and 270 µL of LC-MS grade water was transferred to a 2 mL HPLC glass vial. The final dilution factor was 0.15 mL/mg, with a final concentration of 5% of MeOH. Each glass vial was stored in a -80 ℃ until HPLC analysis to determine dhurrin content in each sample.

One microliters extract was injected onto an LX50 UHPLC system equipped with a LX-50 solvent delivery pump (20-µL sample loop, partial loop injection mode) (PerkinElmer, Shelton, CT). An ACQUITY UPLC HSS T3 column (1 x 50 mm, 1.8 µM; Waters Corporation, Milford, MA, USA) was used for chromatographic separation. The column was maintained at 45°C, Mobile phase A consisted of LC-MS grade water with 0.1% formic acid and mobile phase B was 100% acetonitrile. Elution gradient was initially at 1% B for 0.5 min, which was increased to 99% B over a span 4.5 min (5 min), then decreased to 1% B at 5.2 min. The column was re-equilibrated for 2.8

min for a total run time of 8 min. The flow rate was set to 400 µL/min. Detection was performed on a PerkinElmer QSight 420 triple quadrupole mass spectrometer (MS) with an electrospray ionization source operated in selected reaction monitoring (SRM) using positive mode ionization. SRM transitions for dhurrin were optimized through analysis of an authentic standard. The MS had a drying gas temperature at 120°C, an Hot-surface induced desolvation (HSID) temperature of 200°C, electrospray voltage was kept at 5000 eV, and a nebulizer gas flow at 350. The MS acquisition was scheduled by retention time (1.14 min) with 1 min windows. The dwell time for each compound was set to 100 msec.

The Simplicity 3Q™ software (Version 3.0, PerkinElmer) was utilized for instrument control, data acquisition, and data processing. Quantification (ng/mL) of dhurrin was determined by applying the corresponding linear regression equation obtained from each curve. Only one replicate was analyzed for each accession. The quantification was further adjusted for the weight of leaf tissue for each sample (µg/g) and a dilution factor based on volumes used in sample extraction. Accessions with the lowest analyte concentration (LOD) were excluded from analyses.

***Genotypic data***

The germplasm was previously genotyped using whole-genome resequencing (Faye et al., 2019). Missing genotypes were imputed with Beagle 5.4 (Pook et al., 2019). Kinship (***K***) between accessions was estimated using single nucleotide polymorphisms (SNPs). Prior to estimation, SNPs in linkage disequilibrium of 0.9 or greater within a 10 kb window with other SNPs were filtered. Kinship was estimated as

$$\boldsymbol{K}=\mathbf{XX'p} \quad \textbf{(Eq. 1)},$$

where $\mathbf{X}$ is the incidence matrix of markers with dimension of number of accessions (n) by number of markers ($\mathbf{p}$). $\mathbf{X'}$ is the transpose of $\mathbf{X}$.

***Narrow-sense heritability and genome-wide association study***

A mixed linear model (MLM) was performed in R with the lme4 package (Bates et al., 2015; R Core Team, 2022). The best linear unbiased predictors (BLUPs) for each genotype was obtained using Eq 2. Blue pixel score, genotype, and block were all variables in the model. Correlation coefficients (r) for each sampling time were calculated using the estimated BLUPs from the model to represent HCN content in each genotype. $\sigma_G^2, \sigma_\epsilon^2$ represent variance components of *G* and ∈, respectively, and $n_r$ is the number of replicates.

*lmer(formula=BPS~1 + (1|Genotype) + (1|Block))* **(Eq. 2)**

Narrow-sense heritability ($h^2$) was calculated as

$$h^2 = \frac{\sigma_G}{\sigma_G + \sigma_\epsilon} \quad \textbf{(Eq. 3),}$$

where $\sigma_G$ is the additive genetic variance and $\sigma_\epsilon$ is the residual error variance estimated using average REML from the following linear model

$$Y = G + \epsilon \text{ , with } G \sim N(0, \boldsymbol{K}\sigma_G^2) \text{ and } \epsilon = N(0, I\sigma_\epsilon^2) \quad \textbf{(Eq. 4),}$$

*Y* is the vector of BLUPs obtained from **Eq. 2**, *G* is individual genotypes with an underlying covariance matrix equivalent to the relationship matrix (***K***; **Eq.5**) and ∈ is the residual error.

Genome-wide association scans (GWAS) were conducted using all available markers that had a minor allele frequency greater than 0.05. A generalized linear model was applied to each marker independently and Wald's test statistic was computed. Linkage pruning, kinship, $h^2$, and GWAS were conducted using the software package LDAK (Speed et al., 2012).

**Results**

***HCNp phenotype and dhurrin concentrations are not strongly correlated with precipitation variables***

To test the hypothesis that HCNp and dhurrin are drought adaptive, yearly precipitation values of each landrace were correlated against the blue pixel score from the HCNp assay. To confirm this hypothesis, we would expect to see a strong negative correlation ($r$>-0.7) comparing average historical precipitation values to the blue pixel scores. The best linear unbiased predictors (BLUPs) for each genotype were estimated using a MLM (Eq.1). Each sampling time for each replicate planted was combined. The Pearson correlation coefficients for each time point were low ($r$= 0.21, $r$= 0.08). Overall, we can conclude that HCNp and precipitation clines are not associated with each other, and maybe other environmental factors could drive dhurrin variation.

To test the hypothesis that dhurrin confers drought tolerance, UPLC-MS was performed to determine dhurrin concentrations in each accession. The UPLC-MS includes only one replicate per accession. The landrace accessions' known average annual precipitation (mm) values were plotted against the quantitative dhurrin values (μg/g dry leaf material) obtained from running UPLC-MS. Under this hypothesis we would predict to see a strong negative Pearson correlation coefficient value ($r$ > -0.7) between historical average precipitation and dhurrin. Alternatively, we conclude there is no strong relationship between dhurrin and annual precipitation represented with an $r$-value of -0.14 (Figure 3.3). Thus, dhurrin and drought may not confer drought adaptation, but could confer other adaptive evolutionary roles.

To test the hypothesis that the phenotyping methods for both dhurrin and HCNp are adequate, HCNp and the dhurrin levels corresponding to each accession were plotted to obtain the Pearson correlation coefficient. Dhurrin and HCNp have been reported to serve as proxies so

we would expect to see a strong positive correlation between the two phenotypes (Rosati et al., 2019; Nielsen et al., 2015; Gleadow et al., 2021; Cowan et al., 2022). We conclude that the Pearson's correlation coefficient for both phenotyping methods are relatively low (Figure 3.4), therefore the lack of correlation between dhurrin and HCNp could be due to biological

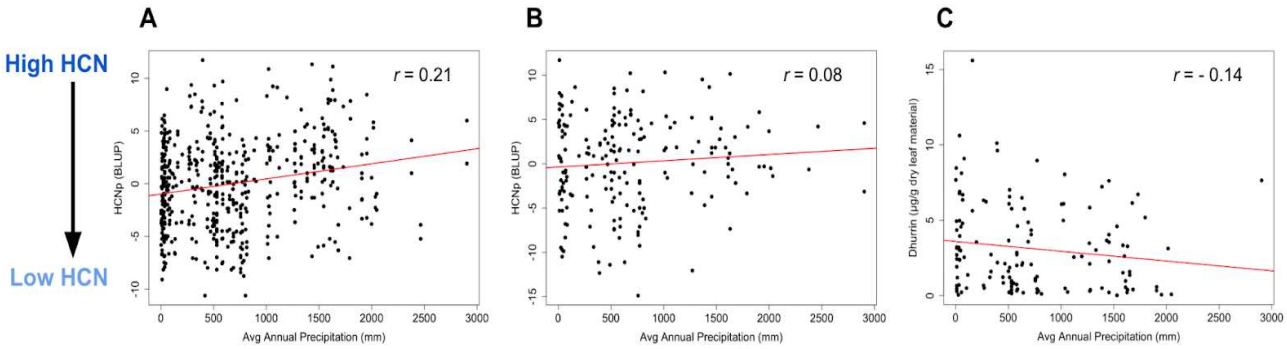


**Figure 3.3: A weak correlation between HCNp and precipitation.** A) Correlation plots indicating the Pearson correlation coefficient (r) for each replicate (n=513, n=393). The x-axis represents average yearly precipitation (mm) obtained from WorldClim and the y-axis represents the estimated genotypic BLUPs derived from the MLM. C) A weak negative correlation between dhurrin (µg/g dry leaf material) and avg annual precipitation (mm). A Pearson's correlation coefficient plot (n=138) of known average annual precipitation values (mm) of each landrace accession and their corresponding dhurrin value (µg/g dry leaf material).

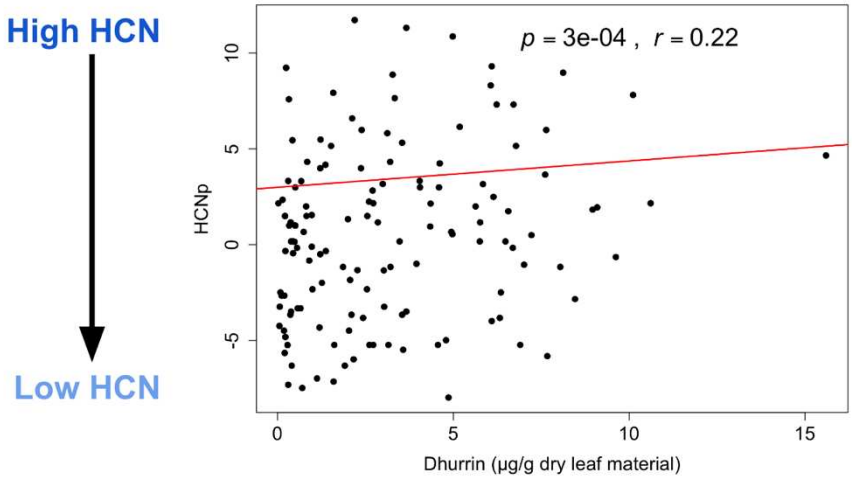

**Figure 3.4: Dhurrin and HCNp values are not strongly correlated.** A) Dhurrin concentrations and HCNp are slightly correlated. Pearson's correlation coefficient (*r)* was calculated ($r$=0.22) to determine the relationship between dhurrin (μg/g dry leaf material) and HCNp using the HCNp assay (n=179). A *t*-test was performed to calculate the *p*-value ($p$<0.05).

***Narrow-sense heritability estimates suggest variation for the HCNp and dhurrin phenotype***

To test the hypothesis that the lack of correlation between HCNp and dhurrin is due to biology, we would expect to see moderate narrow-sense heritability ($h^2$) estimates for both phenotyping methods. $h^2$ for each HCNp phenotyping replicates were 0.52, 0.64, 0.21, 0.28, respectively. The $h^2$ estimate for dhurrin was 0.28 (Table 3.1). The $h^2$ estimates are moderate for both HCNp and dhurrin phenotypes, but enough to consider that the lack of correlation between the phenotypes is due to biology, not noise in the data. To investigate the biology behind this finding, a genome-wide association study (GWAS) was conducted.

**Table 3.1: Dhurrin and HCNp narrow sense heritability ($h^2$) estimates**

| Trait | Sampling | $H^2$ |
|---|---|---|
| HCNp | S1T1 | 0.52 |
| HCNp | S1T2 | 0.64 |
| HCNp | S2T1 | 0.21 |
| HCNp | S2T2 | 0.28 |
| Dhurrin | S1T2 | 0.28 |

Narrow-sense heritability ($h^2$) was estimated for each HCNp phenotyping time point. Sample size is 257, 257, 198, 208, and 179, respectively. S (sampling stage) and T (time) were taken into account. Sampling means the same maturity leaf stage where S1 represents the first population and S2 represents the second population. T1 represents sampling time 1 (6-8 leaf stage) and T2 represents sampling time 2 (9-11 leaf stage).

***GWAS reveals dhurrin gene variation underlies HCNp variation***

To test the hypothesis that the variation in dhurrin biosynthesis, catabolic, and recycling genes can explain the variation of HCNp, a genome wide association study (GWAS) was performed using georeferenced landrace accessions. Four different Manhattan plots were generated. The gray horizontal lines and individual red dots on each plot correspond to the a-priori dhurrin candidate genes (Table 3.2). Each Manhattan plot represents different population replicates and sampling time points to understand if any genetic variation exists between different population replicates and growth stages (Fig. 3.6). The results reveal significant a-priori genes underlying HCNp variation. The first sampling time had less significant single nucleotide polymorphisms (SNPs) corresponding to the known candidate genes represented by a gray horizontal line labeled with each gene name. Many of the significant single nucleotide polymorphisms (SNPs) were associated with all the known dhurrin candidate genes.

Under the hypothesis that HCNp and dhurrin are good proxies for each other, we expected to see the BGC and CGC significantly associated with HCNp for each replicate (Hayes et al., 2015). For all the GWAS for HCNp, we found significant associations on *GST1B* (Sobic.001G065800), *CGTR1* (Sobic.001G133900), *GSTL1* (Sobic.002G421200), *NIT4B1* (Sobic.004G225000), *HNL* (Sobic.004G335500), *CAS* (Sobic.006G016900), CGC (Sobic.008G079800; Sobic.008G080100; Sobic.008G080400; Sobic.008G080600), and *GSTL2* (Sobic.009G033200). Interestingly, the BGC was not significant for HCNp (Fig. 3.6). Only the first sampling population contained significant associations, particularly the 9-11 leaf stage (Fig. 3.6B). Other *a priori* candidate genes were identified other than the CGC we saw. A majority of the dhurrin recycling pathway genes were significantly associated with HCNp (*GST1B, GSTL1, NIT4B1, GSTL2*). The dhurrin recycling pathway is able to metabolize dhurrin over the course of plant development (Busk & Møller, 2002; L. J. Nielsen et al., 2016), resulting in no HCN

release. This has been hypothesized to contribute to N-remobilization in later stages of growth (Bjarnholt et al., 2018; Pičmanová et al., 2015).

In addition, a transporter gene (*SbCGTR1*) and detoxification gene (*CAS*) identified was significant. The nitrate/peptide family (NPF) *SbCGTR1* gene is a sorghum homolog of *MeCGTR1*, a transporter for linamarin, a cyanogenic glucoside found in cassava (Jørgensen et al., 2017). The transporters may be able to transfer dhurrin to the vacuole or other enclosed cell types (Darbani et al., 2016). The other replicate did not identify any SNPs associated with the dhurrin pathway (Fig. 6C; Fig. 6D).

To investigate the genetic variation underlying dhurrin concentration, we conducted a second GWAS (Fig. 3.6) with the quantified dried leaf samples from the UPLC-MS analysis (n= 179). We tested the same hypothesis that HCNp and dhurrin are good proxies for each other. So we expected to see the BGC and CGC significantly associated with dhurrin (Hayes et al., 2015). We found the BGC (Sobic.001G012100; Sobic.001G012200; Sobic.001G012300; Sobic.001G012400; Sobic.001G012500; Sobic.001G012600; Sobic.001G012700) and *NIT4B1* (Sobic.004G225000) were significantly associated for dhurrin (Fig. 3.7). This is different from the HCNp GWAS, where the BGC was not significantly associated. In addition, the CGC was present for HCNp but not for dhurrin. This is different from what we should see if dhurrin concentrations and HCNp are a proxy for each other (Hayes et al., 2015; O'Donnell et al., 2013). *NIT4B1* was also significant for both dhurrin and HCNp. This could mean that the recycling pathway contributes to both HCNp and dhurrin variation. Many other uncharacterized loci were significant, but further analyses would need to confirm their potential role in HCNp and dhurrin variation. In addition, a larger sample size for dhurrin concentrations could confirm adequate statistical power (Klein, 2007; Spencer et al., 2009).

**Table 3.2: The *a priori* candidate genes for dhurrin**

| Gene name | Gene ID | Chromosome | Category |
|---|---|---|---|
| Dhurrin biosynthesis | Sobic.001G012100 | Chr 1 | BGC |
| *CYP71E1* | Sobic.001G012200 | Chr 1 | BGC |
| *CYP79A1* | Sobic.001G012300 | Chr 1 | BGC |
| *UGT85B1* | Sobic.001G012400 | Chr 1 | BGC |
| *GST1* | Sobic.001G012500 | Chr 1 | BGC |
| *MATE2* | Sobic.001G012600 | Chr 1 | BGC |
| Dhurrin biosynthesis | Sobic.001G012700 | Chr 1 | BGC |
| *GST1B* | Sobic.001G065800 | Chr 1 | Recycling |
| *NPF transporter, SbCGTR1* | Sobic.001G133900 | Chr 1 | Transport |
| *GSTL1* | Sobic.002G421200 | Chr 2 | Recycling |
| *GST3* | Sobic.003G416300 | Chr 3 | Recycling |
| *NIT4B1* | Sobic.004G225000 | Chr 4 | Recycling |
| *NIT4B2* | Sobic.004G225100 | Chr 4 | Recycling |
| *NIT4A* | Sobic.004G225200 | Chr 4 | Recycling |
| *HNL* | Sobic.004G335500 | Chr 4 | Catabolic |
| *POR* | Sobic.007G088000 | Chr 7 | Biosynthesis |
| *CAS* | Sobic.006G016900 | Chr 6 | Detoxification |
| *Dhr-1* | Sobic.008G079800 | Chr 8 | CGC |
| *Dhr-like3* | Sobic.008G080100 | Chr 8 | CGC |
| *Dhr-2* | Sobic.008G080400 | Chr 8 | CGC |
| *Dhr-like4* | Sobic.008G080600 | Chr 8 | CGC |
| *GSTL2* | Sobic.009G033200 | Chr 9 | Recycling |

The Gene ID, chromosomal location, and corresponding function of each candidate gene. The categories include the biosynthetic gene cluster (BGC), catabolic gene cluster (CGC), dhurrin recycling, and detoxification genes identified as candidate genes (Darbani et al., 2016; Gleadow et al., 2021; Hayes et al., 2015; Nielsen et al., 2016).

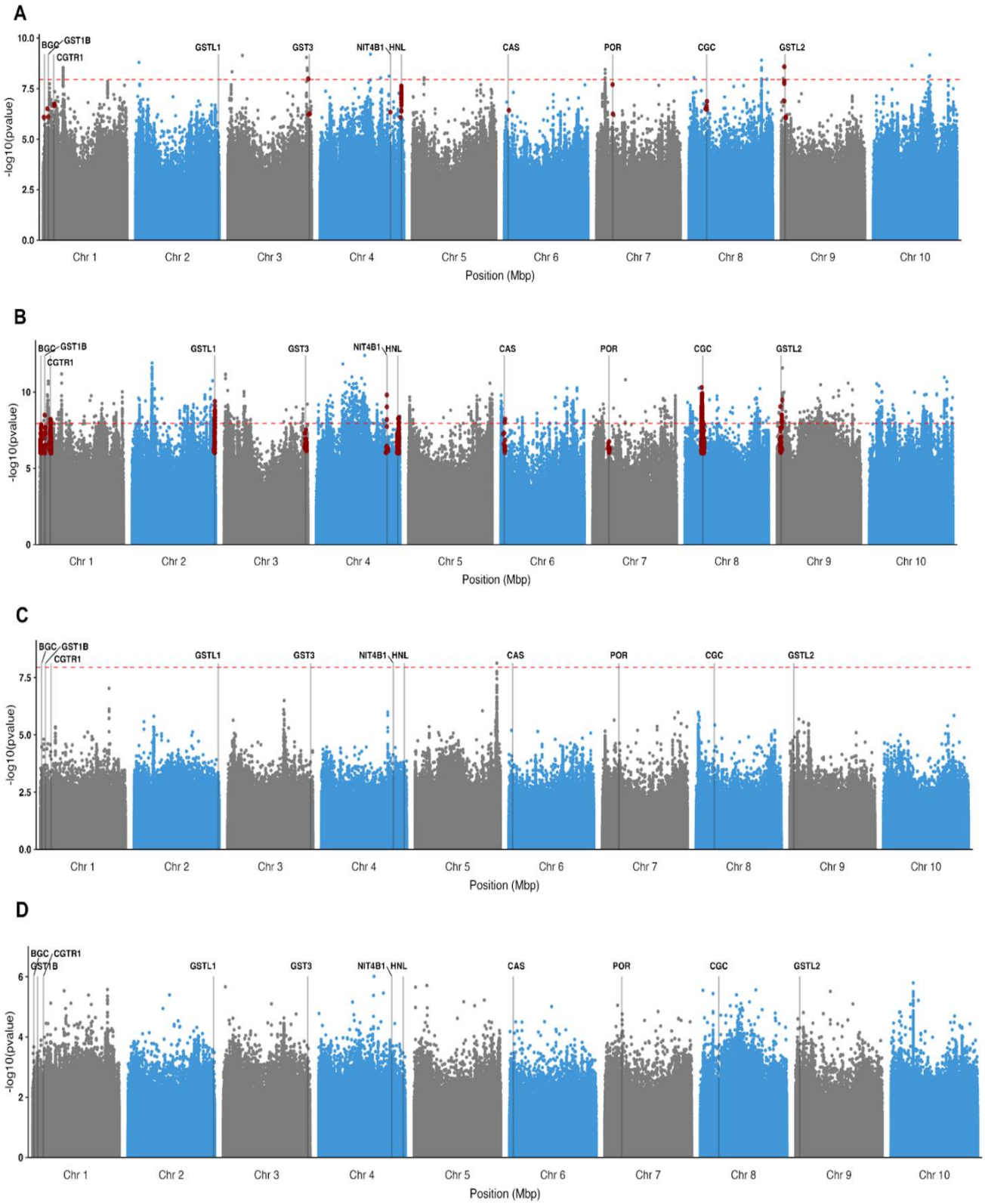


**Figure 3.5: Genome-wide association study for HCNp reveals a significant association with the CGC. In addition, population replicates and sampling time contributes to HCNp variation.** The -$\log_{10}$ *p*-value (*y*-axis) is plotted against the position (Mb) of each chromosome (*x*-axis). Chromosome (Chr) number is noted on the bottom. Figure A and B are one population replicate (n= 257, 257) C and D are another population replicate all consisting of the

georeferenced accessions (n=198, 208). Each horizontal line represents the location of a-priori candidate genes for dhurrin and individual red dots and markers within 1 Mbp and a -log10(*p*-value) > 6. Red dashed line is the Bonferroni cutoff of 0.1.

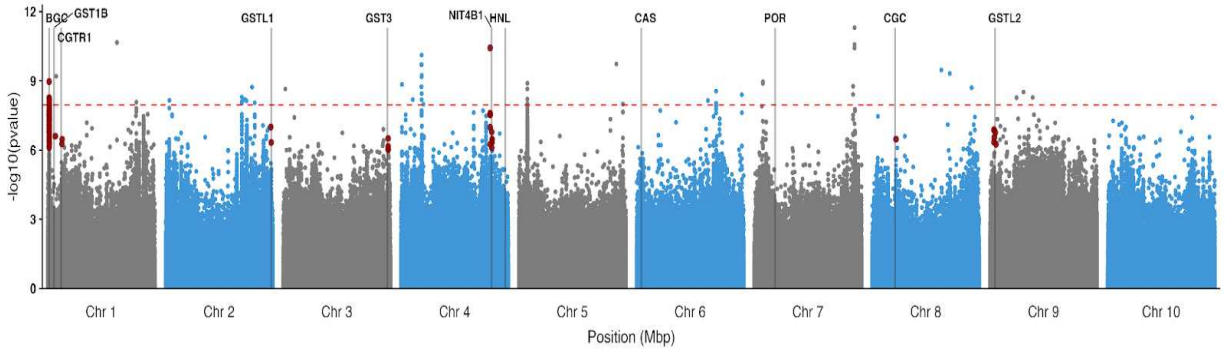


**Figure 3.6: Genome-wide association study for dhurrin concentrations in dried sorghum leaves indicate a significant association with the BCG.** A Manhattan plot of dhurrin concentrations for the first replicate. The -$\log_{10}$ *p*-value (*y*-axis) is plotted against the position (Mbp) of each chromosome (*x*-axis). Chromosome (Chr) number is noted on the bottom. The horizontal line represents the location of a-priori candidate genes for dhurrin and individual red markers within 1Mb and a -log10 (*p*-value) > 6.

## Discussion

### *The diversity of global sorghum germplasm can capture adaptive traits*

The success of crop improvement relies on the utilization of the available genetic diversity found in crop plants. Utilizing diverse sorghum landraces has led to the understanding of adaptive traits that increases crop performance (Devnarain et al., 2016; Faye et al., 2019; Lasky et al., 2015). The goal of this study was to investigate the role of dhurrin and HCNp in African landraces to infer adaptation strategies utilizing the corresponding precipitation values. In addition, identify loci contributing to HCNp and dhurrin variability.

The 373 different African landraces captured the environmental variability necessary for performing these analyses. The 22 different countries and six major climatic zones were represented, with many landraces originating from the semi-arid regions of Africa (Fig. 3.2). We obtained average annual precipitation data from WorldClim, and found many of the landraces

sharing similar precipitation values due to close geographic proximity. Most if not all of the landraces share similar growing seasons and photoperiod sensitivity. Plant growth with photoperiod sensitive germplasm was stunted due to reduced photoperiod, especially for the second replicate. Low germination rates were also common which could have been due to cooler greenhouse temperatures and shorter day lengths in the winter. This reduced the sample size for both HCNp phenotyping and dhurrin. Even though there may be noise in the data, the heritability estimates and GWAS results can confirm the hypotheses we aimed to test.

***Low correlation between HCNp/dhurrin and drought indicates other pressures could drive variation***

The use of 373 different African landraces captured the global genetic diversity of sorghum in tandem with the diverse climatic conditions to make strong inferences for a trait-by-environment study. HCNp and dhurrin concentrations were phenotyped then correlated with the average annual precipitation data corresponding to the georeferenced germplasm (Fig. 3.2). Although these inferences were weak, other factors such as pest pressures and population size could influence dhurrin/HCNp levels (M. Cowan et al., 2022; Dritschilo et al., 1979; Santangelo et al., 2022).

Cyanogenic glucosides are evolutionarily conserved where the biosynthesis, catabolic, and compartmentalization abilities are shared among different plant species, indicating roles in pest and environmental adaptation (Bak et al., 2006; Jensen et al., 2011). Sorghum was found to have a higher frequency of cyanogenic glycosides in crop plants compared to wild species (M. F. Cowan et al., 2021; Gleadow & Møller, 2014; Jones, 1998). One hypothesis suggests that these plants were indirectly selected due to their pest resistance and the ability to outcompete neighboring plants (Cowan et al., 2021; Gioria & Osborne, 2014; ). Durra and durra-caudatum

sorghum botanical types are found predominantly in the arid parts of Africa while durra-bicolor is prevalent in humid environments (Harlan & Wet, 1972). Additional analyses such as comparing dhurrin concentrations based on botanical types could uncover a different relationship.

Environmental factors can make a significant impact on the interactions between plants and insects. Studies on white clover have found cyanogenic clines based on temperature and herbivore pressure (Dritschilo et al., 1979; Wright et al., 2018) where more cyanogenic white clover populations are found in warmer environments rather than cooler. Population size has also been found to affect cyanogenesis in clover. Overall, they found that rural areas with high herbivore pressure exhibit increased HCN production in response to herbivory, whereas in areas with less vegetation, drought becomes the primary selective factor for HCN production (Santangelo et al., 2022). Cassava produces a cyanogenic glucoside known as linamarin where environmental conditions have shaped HCN concentrations. A negative correlation was found between cyanide content and monthly rain amounts (mm) (S.L. Tan & Mak, 1995). Further research could uncover the relationship between dhurrin and the pest pressures that influenced HCNp variation over time.

***Dhurrin loci underlying variation in HCNp and dhurrin production in global sorghum germplasm***

A GWAS is able to capture the genetic variability that could explain genotype-to-phenotype (G2P) mechanisms underlying phenotypic variability. We used known candidate genes for dhurrin to understand the genotypic variation associated with HCNp and dhurrin variation. The *a priori* candidate genes used for this analysis were previously reported. Before performing the GWAS, we performed a correlation analysis to determine if dhurrin is the only

variable underlying HCNp (Fig.3. 4A). Narrow-sense heritability was calculated to validate each phenotyping method (Fig. 3.4B) and each value was acceptable enough to conclude that biological variation is underlying the dhurrin to HCNp phenotype. Sample size differences due to one replicate for the dhurrin quantitative measurement could affect these values, so additional analyses can validate these results.

The BGC and CGC have been identified to contribute to dhurrin variation (Hayes et al., 2015). The two-component defense system of dhurrin is driven by both gene clusters, so we would expect both clusters to be associated with the dhurrin and HCNp phenotypes (Gleadow et al., 2021; Hayes et al., 2015). To biologically understand dhurrin and HCNp variation, we utilized characterized candidate genes coding for dhurrin biosynthesis, catabolism, and recycling pathways associated with both HCNp and dhurrin. The major finding for the GWAS found the BGC as significant for dhurrin concentration, but not for HCNp (Fig. 3.5; Fig. 3.6). The opposite was found for variation in HCNp, where the CGC cluster was significant, but not for dhurrin concentrations. This indicates that dhurrin may not serve as a direct proxy for HCNp concentrations so other underlying genetic mechanisms influencing this difference. A previous expression study found higher BGC activity during early plant development stages. Alternatively, the CGC is more active in the leaf blade during later development stages (Gleadow et al., 2021). Another study found that the use of dry sorghum leaves for UPLC-MS analysis inactivated the catabolic enzyme, dhurrinase-2 (Dhr2) (Gruss et al., 2023). This finding could support why the CGC was not associated with the dhurrin phenotype because our protocol also used dried sorghum leaves for dhurrin quantification. The decoupling of the BGC and CGC suggests that the CGC and recycling pathway may be responsible for modulating HCN

concentrations, but not dhurrin concentrations. Further studies utilizing germplasm with known dhurrin variation could confirm these differences.

The main differences were found between phenotyping traits (HCNp & dhurrin) and population replicates for HCNp. The first population replicate (Fig. 3.6A; Fig. 3.6B) had higher sample sizes compared to the second population replicate (Fig. 3.6C; Fig. 3.6D). Sample sizes can affect the results of a GWAS. A GWAS requires a large sample size to have enough statistical power to detect significant associations, yet some of the phenotyping data sets were lacking in sample sizes. This could have led to false negatives or underpowered analyses (Spencer et al., 2009). Other significant loci were uncharacterized in this study, but future research could identify the contribution they may have on HCNp and dhurrin variation. Another consideration is the influence of environmental factors. In another study, N application influenced associations for the BGC and CGC for dhurrin concentrations (Hayes et al., 2015). Therefore, conducting in-field phenotyping could be a crucial future measure, particularly when the target audience are breeders.

***The utilization of G2P data sets to improve future breeding prospects***

The use of G2P data sets has improved plant breeding by providing an understanding of the genetic mechanisms underlying a trait of interest (Houle et al., 2010; Marjoram et al., 2014). The advancement of high-throughput phenotyping methods and increased availability of genotyping data allows for these analyses to be made. This type of research can be useful for breeders to successfully advance a breeding population with their trait of interest, even when the trait could be a complex metabolite such as dhurrin (Hall et al., 2022).

HCNp is regularly used as a proxy for dhurrin content in phenotyping studies (Fukuda et al., 2010; Gleadow et al., 2012; Rosati et al., 2019). Under this observation, breeders using the

semi-quantitative measure such as the HCNp assay would expect a positive correlation for HCNp and dhurrin concentrations. However, the findings from this study indicate that variation for dhurrin and its visual phenotype, HCNp, are not direct proxies for each other. The variation in the catabolic gene cluster (CGC) could affect HCN levels, thus affecting the phenotype. For example, dhurrin has been hypothesized to act as a compatible solute, therefore influencing drought tolerance (Heraud et al., 2018; Møller & Laursen, 2021). But based on the variation and activity of the CGC, HCNp can be greatly affected. High levels of HCN can deter chewing insects. With these two separate beneficial traits, it is important to understand the variation that underlies the biological differences between dhurrin and HCNp. To confirm our results, in-field phenotyping could account for data variation and add to the current phenotyping data set we have now. Overall, the use of G2P data sets allows for breeders to appropriately phenotype for traits that can improve field performance.

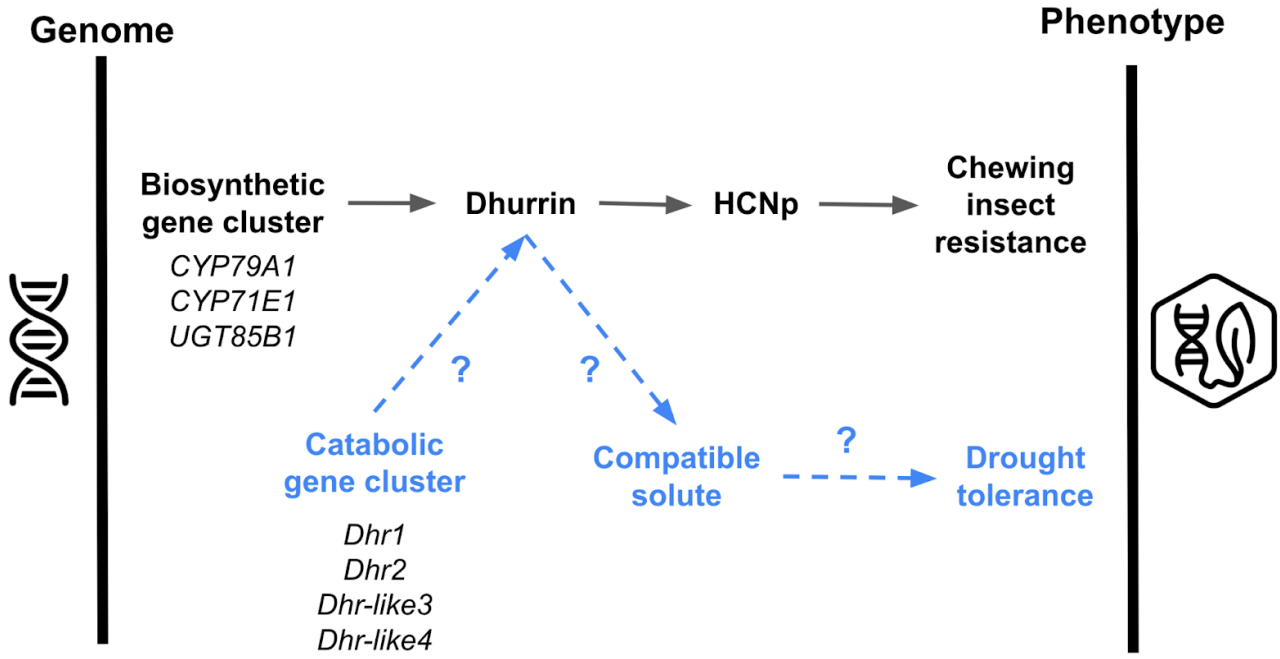

**Figure 3.8: The decoupling of dhurrin and HCNp based on the variation in the CGC.** A G2P map visualizing the roles dhurrin and HCNp have on biotic and abiotic factors based on the observed variation in the dhurrin gene cluster.

## Conclusion

Climate change can impact the evolutionary and ecological phenomena found in local populations where plants are able to exhibit phenotypic plasticity and evolve to changing environments. Utilizing diverse global sorghum germplasm corresponding with historical precipitation gradients can uncover adaptive traits. Dhurrin, a PSM in sorghum, has been observed to potentially adapt to drought, functioning as a compatible solute (Burke et al., 2013; Heraud et al., 2018; Laursen et al., 2016). A trait-environment association study was conducted to determine if there is an association between dhurrin and drought adaptation. The findings conclude that there is no direct relationship between dhurrin and drought, yet other variables may contribute to this relationship. To further understand the role dhurrin plays in drought adaptation, the variation of known dhurrin biosynthetic, catabolic, and recycling genes underlying HCNp variation could provide additional insight on these differences. The GWAS uncovered variation for both traits, suggesting dhurrin may not be a direct proxy for HCN content. Other analyses such as dhurrin metabolon formation can uncover the molecular mechanisms which drive evolutionary adaptation of dhurrin in sorghum.

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