GENETIC ANALYSIS OF INTERVEINAL CHLOROSIS AND REDUCED SEEDLING VIGOR AS RELATED TO AGRONOMIC PERFORMANCE IN SORGHUM RESISTANT TO ALS INHIBITOR HERBICIDES

by

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AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of Agriculture

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Abstract

The lack of effective post-emergence weed control options is often highlighted as one of the major factors behind dwindling acreage under sorghum (*Sorghum bicolor* (L.) Moench) in the United States. The discovery of herbicide resistance sources in wild sorghum population and subsequent efforts to incorporate them into cultivated sorghum was received with much optimism to change weed management practices in sorghum. As the development of the technology advances, especially of the Acetolactate synthase (ALS) resistance, concerns over the temporary interveinal chlorosis and reduced seedling vigor in some of the resistant families became heightened. This thesis research is designed to shed light on the genetic basis of seedling chlorosis and assess its impacts on yield potential.

The study has three parts; the first part is focused on identifying the genetic causes and plant mechanisms associated with the chlorotic phenotype. ALS herbicide resistant sister-lines expressing normal and chlorotic phenotypes were analyzed via RNA sequencing at four time points during seedling growth. The study identified several variants of genes coding chloroplast precursors and those that cause epigenetic modifications. Once confirmed, genetic markers can be developed to track these gene variants in the breeding population and eliminate segregates genetically prone to chlorosis/yellowing.

The second part of the study focuses on assessing the effect of ALS resistance associated chlorosis on agronomic and nutritional parameters of sorghum inbred lines. A set of ALS resistant lines expressing different levels of the chlorotic phenotype were evaluated in replicated field trials and laboratory methods. Results showed that interveinal chlorosis delays flowering but does not have negative effect on yield and nutritional parameters with and without herbicide treatment. The last part addresses whether there is any yield drag that may be associated with herbicide resistance

traits and foliar interveinal chlorosis. For this, we synthesized a large set (182) of hybrids from ALS resistant, ACCase resistant and regular (susceptible) seed and pollinator parents. The hybrids were then evaluated in three sets at multiple locations during the 2014 and 2015 crop seasons along with commercial checks. The results revealed that resistance to both herbicides do not cause any drag to grain yield. The traits also do not have any negative impact on grain and nutritional quality of resistant hybrids.

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Abbreviations

Abbreviation	Explanation
ALS	Acetoacetate synthase
ACCase	Acetyl Co-enzyme-A carboxylase
KN	Kernel number per panicle
TKW	Thousand kernel weight

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Dedication

With respect and gratitude to his profound teachings, I dedicate this dissertation to my spiritual teacher Venerable Ajahn Brahmavamso.

Though oceans apart, staying close to my heart,

you stood by my side both through deepest pains and ecstasies in life,

sprinkled me with your unconditional love, compassion and forgiveness,

and accepted me for who I am.

Moved by your audacity, simplicity and delight,

I feel blessed to have known you and, to trail your footsteps,

for you showed me

how not to get drowned and dive deep in life's ocean of uncertainty…

General Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) ranks fifth among the major cereal crops grown in the world. Because of its unique adaptation to low input marginal growing conditions, sorghum remains the primary source of energy, protein, vitamins and minerals for millions of impoverished people in the world (Unger and Baumhardt, 1999; Khalil et al., 2015). It also remains the second most important feed source and biofuel feedstock in the developed world. Sorghum's distinct biology that allows the crop to conserve water while still producing high grain and biomass yields on marginal lands have made it an ideal crop for the dry and arid regions in the world (Lux et al., 2002). Thus, as compared to other major crops such as maize (*Zea mays*), wheat and rice (*Oryza sativa*), sorghum stands out as a very promising cereal crop that could survive a range of stressful environmental conditions. These unique characteristics of the crop make sorghum one of the most viable food grains that is capable of reliable production where other crops fail (Dendy, 1995). Thus, sorghum plays a significant role in meeting ever-increasing demand for food and feed for millions of the most vulnerable and food insecure people worldwide.

Enhancing the competiveness of sorghum and realization of its potential as the 21st century food and feed grain will require bridging key gaps that kept the productivity and utilization of the crop at bare minimum for the past half century. Among the many such gaps is the lack of effective post-emergence weed control options for the crop. Commercial sorghum production in the United States met several setbacks in the past decades both from the dwindling acreages and the difficulty in crop management primarily attributable to poor post-emergence grass weed control. While recent advancements in sorghum has proven potential for deployment of the resistance based weed control technologies (Tesso et al., 2011, Kershner et al., 2012) two important concerns that draw attention of sorghum farmers as well as the industries are the possible consequences of the yellow

seedling phenotype that is observed in many of the Acetolactate synthase (ALS) inhibitor herbicide resistant lines and the potential yield penalty due to linked deleterious genes dragged from the wild herbicide resistance gene donor. This study is aimed at addressing these prominent concerns of the sorghum growers and research community. The study encompasses various disciplines including plant breeding and genetics, molecular biology, bioinformatics, and weed science to determine the cause and biological impacts of seedling chlorosis on sorghum and the possible yield compromise that may be caused as a result of deployment of the herbicide resistance trait.

This thesis is organized in to three parts. The first part which follows a thorough review of the state of sorghum production in the U.S. and the world and, the major gaps undermining sorghum productivity is focused on determining the genetic causes and mechanisms behind the interveinal chlorosis and associated reduced seedling vigor that is observed in many ALS herbicide resistant sorghums. Here various techniques and analytical approaches including RNA-sequencing, gene ontology and metabolic pathway analysis and gene variant discovery approaches were used to identify the associated genes. The results of this experiment is discussed with an emphasis of significantly altered genes, plant metabolic pathways and mechanisms in relation to observed unusual seedling phenotype. The second part describes results of experiments performed to assess the physiological and agronomic characteristics of ALS herbicide resistant lines varying for seedling interveinal chlorosis with and without and herbicide treatment. The possible effects on nutritional attributes of the resistant genotypes was also investigated. The last part describes the results of the study aimed at addressing the real concerns of the growers, the possible yield penalty that may be caused by genes dragging along with the herbicide resistance gene. This part reports on data obtained from evaluation of series of hybrids with homozygous or heterozygous

resistance to ALS, ACCase or both ALS and ACCase as well as normal susceptible hybrids grown at multiple locations over two years.

These studies together provide important information both to growers and producers alike in choosing strategies for herbicide resistance breeding and also to provide experimental evidence to assist farmers make sound production decisions.

Chapter 1 - Literature Review

Sorghum production in the United States

Originated in Africa thousands of years ago, sorghum is grown as one of the world's major cereal grains. While it is used as food and beverages throughout Africa and Asia, sorghum is primarily used as animal feed in the United States and other parts of the world. The emergence of sorghum as a widely grown cereal crop in the United States is the culmination of three distinct events over the last century, the introduction of the crop during the latter part of the nineteenth century followed by cultivar improvement through selection and selective hybridization and the discovery of the commercial hybrid technology (Smith and Frederiksen, 2000). Sorghum introduction in the United States has been reported to have coincided with western rail road developments where the crop was grown on the marginal ranch lands on which any other crop could not be grown due to extreme hot and dry conditions. Successful performance on land areas with such poor conditions led the shift of ranch lands to intensive farming systems directed towards expanded feed grain production. The first improved sorghum cultivar in the U.S. was released in 1916 by H. Willets from Kansas (Quinby and Martin, 1954) which was followed by the development of relatively short cultivars with stable but low yields. The sorghum industry in the U.S. expanded during the 1950's with the development of mechanized agriculture and eventually gained popularity as an important cereal crop which could be grown in rotation with wheat. However, sorghum's inherent self-pollinated nature remained a bottleneck to the development of the hybrid system until the revolutionary discovery of cytoplasmic male sterility (CMS) system during the latter part of the twentieth century (Stephens and Holland, 1954). CMS offered enormous opportunity to plant breeders to exploit heterosis, which led to yield increases as much as three times as that of an open pollinated cultivar. Thus sorghum acquired a considerable

commercial interest during the decades that followed the discovery of CMS leading to increased private investment on research, seed production and distribution. Increased crop yields achieved following the deployment of the hybrid system and other improved management practices led to increase in acreages and thus to surplus production that opened up export opportunities for the sorghum growers. By 1960's 95% of the total sorghum production in the U.S. was from hybrids (Smith et al., 1999). The rapid acceptance of the hybrids by the growing community and further improvement in crop resistance/tolerance to various stress conditions were the key ingredients for increased production and the unprecedented increase in export market. However, with development of technologies in other disciplines, it became easier to grow other crops in areas where sorghum was once the only viable crop. This disproportionate investment and undue preference for alternative crops reduced sorghum acreage eventually leading to the dwindling of acreage grown to the crop. In the last three decades, sorghum acreage has reduced by about 70%. The U.S. is the largest producer and exporter of sorghum in the global grain market. At present, the United States accounts for just about 9% of the world's sorghum acreage but contributes about 25% to the global grain sorghum output with over half of this coming from the state of Kansas (Hamman et al., 2001). The crop is grown in 14 states in the U.S. on a total of 8.46 million acres with the total production is valued at $2.08 billion in 2015 (NASS, 2015).

Utilization and nutritional attributes of sorghum

Sorghum is used in various feed formulations and food applications throughout the world. It is also the major ingredient for production of various beverages in the developing world. Because of its versatility in utilization, adaptation to marginal conditions and its inherent high yield potential, sorghum is poised as a key source of food and energy in the 21st century. In the developed world, sorghum grains are commonly fed to cattle, poultry and swine, while sorghum stalks and

leaves remain important source of feed in developing countries providing a vital feed alternative during dry season. The importance of the crop is growing beyond these traditional uses that it has become the second major feedstock for ethanol production in the United States and elsewhere. The crop also has gained wide recognition as gluten free and non-GM food alternative for millions of people living in various parts of the world. In terms of nutrient content, sorghum is generally comparable to many other cereals such as maize and wheat (FAO, 1972) while it is believed to be richer in several essential nutrients including iron and zinc, vitamin B1, B2 and niacin (Parthasarathy Rao and Basavaraj, 2015). There are tremendous opportunities for improving the nutrient content of sorghum both for animal and human food including its protein content while the availability of sorghum proteins continue to present significant challenge that needs more research emphasis (Singh and Axtell, 1973). Moreover, sorghum is also praised for enormous health benefits it offers primarily due to the unique phytochemicals that it carries in its bran layer including tannins, phenolic acids, anthocyanins, phytosterols and policosanols. These chemicals have been shown to reduce the risk of certain cancers and promote cardiovascular health (Awika and Rooney, 2004).

Weed infestation as a key constraint to sorghum production

Like many other crops, sorghum suffers from various production constraints in different parts of the world. Among the many factors affecting sorghum production, especially in mechanized agriculture, is infestation by grass weeds. No effective weed control options are available for controlling post emergence grass weeds in sorghum. While 2,4-D has been used to manage post emergence broad leaved weeds, grass weeds remained difficult to control in sorghum fields. While Concep III ^{TM} treatment of sorghum seeds allowed the use of pre-emergence

herbicides which is critical for controlling early emerging weeds, the slow growing habit of sorghum seedlings have made it a weak competitor to post emergence weeds, thus, even a mild weed infestation during early growth stage can have marked impacts on yields (Peng, 2012). Therefore, early-season weed management is the most critical management step for successful sorghum production. Experimental evidences show that a single pigweed plant (*Amaranthus* spp.) per three feet of row if left uncontrolled until sorghum reaches the three-leaf stage can reduce yields by 10% (Smith and Scott, 2010). Though not specifically recommended for sorghum, broadleaf weeds in sorghum fields could be managed by several different chemicals used for other crops. The biggest challenge for sorghum, however, is on management and control of post emergence grass weeds. The abundance of wild and weedy relatives of sorghum such as Johnsongrass (*Sorghum halepense* (L.) Pers.) and Shattercane (*Sorghum bicolor*) which both morphologically and physiologically mimic sorghum, further complicate efforts to manage grass weeds. The presence of these weeds obviously have imposed restrictions to identification and utilization of new over the top herbicides for sorghum. Heavy infestation of grass weeds during early weeks of germination have reported to account for up to 20% yield reduction (Smith and Scott, 2010). Though late emerging weeds have less effect on yield, they impact harvesting efficiency, reduce harvestable yields and may further increase the weed seed bank in the soil.

While the problem with weed infestation is not unique to sorghum, the development and deployment of glyphosate resistance has made this problem a history in crops that benefited from the technology. Today the most problematic weeds in glyphosate resistant crops are glyphosate resistant volunteers from previous season crop or glyphosate resistant weeds that seem to be on the rise in several states (Chahal and Jhala, 2015). While this technology obviously resolved weed management issues for crops such as maize, soybean, sunflower, cotton, etc., it had negative

effects on sorghum production and seriously undermined its competitiveness. Sorghum acreages continuously dropped over the last two decades with majority of lost acres picked up by glyphosate resistant maize. The better weed control option that glyphosate resistant maize offers and the existing farm policy that favors maize over sorghum seem to be the major incentive for farmers to switch from sorghum to maize even when this does not necessarily translate to increased profit, especially under dryland production. Though, the same technology could be deployed for sorghum, the agricultural community seems reluctant to grow glyphosate resistant sorghum due to the well-founded fear of resistance gene escape to wild and weedy relatives. While the concern may be valid especially in the absence of effective stewardship mechanisms, the move has greatly undermined traditional sorghum growers and unfairly affected the sorghum industry not only by enhancing the productivity of the competing crops but also by slashing acreages from sorghum. The apparently little or no investment to develop alternative technology for enhancing sorghum may eventually lead to further reduction in acreage under the crop despite the enormous benefit it can offer in the face of dwindling irrigation water resources and increasing drought and high temperature stress that could be detrimental to the future of global agriculture. In order to remain a viable alternative as food and feed source, sorghum needs to benefit from modern production technologies. Among others, effective and low cost grass weed control technology is needed to curb losses incurred due to grass weed infestation and change the current seemingly unsustainable trend of declining acreage under sorghum.

The role of herbicides in modern agriculture

Weeds primarily interfere with the quality and quantity of agricultural produce. The presence of weeds in crop fields has been a serious issue since around 10,000 BC (Hay, 1974) and

represents one of the major restraining factors in agriculture (Avery, 1997). The United States is not exceptional to this problem; almost 70% of the world's invasive weeds are reported to have found in the country (Zimdahl, 2013). To date, there are around 30,000 weed species identified worldwide which accounts for about 10% of all plant species (Kostov and Pacanoski, 2007). Among these, around 1,800 are known to cause severe economic losses and of those about 300 species interfere with cultivated crops throughout the world (Ware and Whitacre, 2004). The difficulty of practicing mechanical weed control methods in large scale production systems is the major driving force that led to the discovery of chemical weed killers or herbicides.

The first used chemical herbicide was copper sulphate to control charlock (*Sinapis arvensis*) in oats (Cobb and Reade, 2010). Historical accounts of chemical herbicides to abet agricultural production dates back to the end of the 19th century where weed control using organic chemicals which are substances containing carbon and its derivatives, commenced in 1932 with the use of 4,6-dinitro-o-cresol (DNOC) as a weed-controlling agent. This was followed by phenoxyacetic acids such as 2, 4-D and MCPA that was introduced in 1940s (Hay, 1974). However, the chemical weed control in crop production was not widely put into use until the availability of ureas (1951), triazines (1955) and bipyridiniums (1960). From there onwards, agricultural production took a huge step forward in terms of production, profitability and minimized labor use for weed control (Van Rensen, 1989). At present, in countries where intensive and highly mechanized agriculture is practiced chemical weed killers have largely substituted mechanical means of weed control. It is projected that the reported growth in herbicide market worldwide between 2002 and 2011 has been 39% while the projected growth by 2016 is expected to be further 11% (McDougall, 2013).

Herbicides contribute to crop production in a number of ways. Primarily, they help increase the crop yield by reducing competition by weeds, improve crop management operations during harvesting, reduce the risk of pest and disease outbreaks and reduce soil erosion due to reduced tillage, this ultimately leads to reduced fuel consumption and thus reduced emission of greenhouse gases. For example, a row-crop cultivator and a moldboard plow requires four times and 17 times more diesel fuel per unit area, respectively, than a herbicide sprayer per trip across a field (Gianessi, 2013). On the other hand, increased use of herbicides in turn promotes fertilizer use, which eventually leads to yield increases (Manda, 2011). Hence, the use of herbicides has become a crucial factor for worldwide increase in agricultural production. Herbicide use in the U.S. has contributed 20% increase in maize yields and 62% increase in soybean yields from 1964 to 1979 (Manda, 2011; Schroder et al., 1981; Schroder et al., 1984). On the other hand, the increased use of herbicides has created considerable concern for human health and environment (Pacanoski, 2007). Further research to develop new herbicide products needs to heighten emphasis on addressing concerns about increased use of the chemical on humans and environment and devise ways of mitigating the risks.

Since the commercialization of herbicides in mid-1940s, extensive studies conducted on herbicide research led to discovery of a variety of modes of action for selective as well as non-selective herbicides. Numerous herbicides representing each mode of action have been developed and commercialized. However, resistance development of weeds with the continuous use of herbicides was unavoidable, thus more advanced weed control options are needed. With the availability of the novel biotechnological tools in early 1980s, the major breakthrough technology came into picture and deployed under a variety of nomenclature such as genetically modified (GM), transgenic or biotech crops. Scientists in both public and private sectors embraced this

technology as an advanced tool which would create opportunities towards increased profits. So far, there is no other technique considered equivalent to this modern biotechnological approach of GM crops which brought about a breakthrough to weed control strategies.

The deployment of GM crops which was initially focused on virus resistance in tobacco quickly expanded to insect resistance (BT crops) and glyphosate resistance to rapidly revolutionize weed control in several crops. Of all GM based technologies, glyphosate resistance (roundup ready) technology received the greatest market and was widely deployed (Nap et al., 2003). The technology offered enormous advantage to the farmer by offering simple but very effective weed control during the entire growing season (Stein and Rodríguez-Cerezo, 2009). This was achieved through the exogenous gene constructs introduced into the crop that either enabled fast degradation of the active ingredient in the herbicide or made the target site insensitive to the herbicide, thus, rendering it harmless to crops carrying this construct. Soybean was among the first commercialized herbicide tolerant crop in the USA (1996), which was followed by cotton (*Gossypium* spp.) (1997), maize (*Zea mays*) (1998) and oilseed rape (*Brassica napus*) (1998). After few years of its introduction, farmers worldwide picked up this technology basically owing to the numerous benefits it carries. As compared to traditional weed control strategies, this technology offers excellent broad spectrum weed control for a wide range of grasses including, annuals to perennial grasses, broad leaved weeds as well as invasive species. It offers a long-term control thus a single application may be sufficient to control weeds for entire season making it far more cost-effective than any other approach. Nevertheless, the popularity and rapid adoption of this technology is partly due to the characteristics of the active ingredient of the "Roundup" (glyphosate) herbicide itself which offers a broad spectrum control of weed in a field grown with Roundup-ready crops, the minimal toxicity to humans, high absorbance with no or little mobility

in the soil, low persistence in the soil and little or no movement from target area by factors such as run-off as compared to other herbicides in the market (Henderson et al., 2010). Thus, ground and surface water pollution through glyphosate is limited as it is readily degradable by soil microbes into a non-toxic aminomethylphosphonic acid (AMPA) and carbon dioxide (Peltier et al., 1985). GM crop technology also offers easy plant establishment and improves harvesting efficiencies which in turn save labor and reduce fuel cost.

To date, glyphosate resistance remains the most widely adopted GM crop technology with 90% of the all GM crops grown in the world carrying glyphosate resistant trait (Duke and Powles, 2008). In the 16 years since its introduction, the technology is in 29 countries worldwide (James, 2010). Reports indicate that globally the total area cultivated to GM crops during 2013 accounted for about 175.2 million hectares with a recorded annual growth rate of 3%. The acreage allocated to GM crops in 2013 was higher by 5 million hectares than the previous year 2012 and the great majority of these are located in developed countries in four crops, soybean, maize, cotton and canola (Brief, 2013). Of the total area planted to these four crops, around 16% fall under the two dominating GM traits that are insect resistance and herbicide tolerance (Sateesh, 2010). In addition, according to the USDA's National Agricultural Statistics Service, the reported land area grown with GM crops in the USA included 94% of soybeans, 96% of cotton and 93% of maize (NASS, 2013) while no GM crop releases have been reported for sorghum yet. Conversely, biotech crops have not been well received in several other parts of the world including North and South America, owing to the consumer perception about genetic modification. On the other hand, there has been a major boost in adaption of this technology by developing countries and approximately 18 million farmers worldwide contribute to GM crop production (Brief, 2013).

Encouraged by widespread adoption of the technology, seed companies have expanded their research horizon and are seeking to develop crop varieties into which genes conferring resistance to multiple non-selective herbicides are incorporated. This initiative if successfully deployed will bring even more flexibility to farmers in choosing herbicides and will reduce dependence on glyphosate which is the single most important herbicide widely used by farmers growing Roundup-ready crops (Duke and Powles, 2008). Moreover, the herbicide rotation that will be possible through this approach will help reduce selection pressure and will markedly reduce probability of resistance development in the weeds. However, as any other technology, this may also carry its own risks and with time, a need may arise for more reliable means for preventing multiple resistance development in weeds.

Can sorghum benefit from resistance based weed control technology?

Exploiting herbicide resistance in sorghum

Considered an orphan crop (NRC, 1996), sorghum has always been either left out or never received full package of technological breakthroughs that benefited other crops. Soil fertility management, irrigation water supply, seed treatment packages, weed control practices, etc., were used only at half rate of that of maize or soybean or were never developed for sorghum at all. As a result of these and the persistent neglect on its utilization, the interest to grow sorghum has always been low except in areas where other crops do not fit. Thus sorghum acreage in the U.S. has steadily declined over the past few decades with much of the lost acreage picked up by maize (Smith and Frederiksen, 2000).

One of the areas where sorghum trails behind other crops is weed control. Sorghum suffers from weed infestation as bad as any other crop. Yet not enough efforts were made by both public and private institutions to develop post emergence weed control tools for sorghum. The tools currently used are primarily adapted from related crops such as maize and none of them are effective against grass weeds. The two major problematic grasses that haunt sorghum fields are johnsongrass (*Sorghum halepense* (L.) Pers.) and shattercane (*Sorghum bicolor* spp.) (Hoffman and Buhler, 2009). Both weeds cause damage to other crops as well but the peculiar morphological similarity of the weeds with sorghum makes them particularly important in sorghum fields. Johnsongrass is increasingly becoming the major weed in the southern United States (McWhorter, 1989) which is also known in more than 58 countries throughout the world (Holm et al., 1979). The major morphological characteristics that make these weeds difficult to control are the underground vegetative propagules (rhizomes) of johnsongrass and the dormant seeds of shattercane (Mallory-Smith and Sanchez Olguin, 2010). It has been reported that, the major driver for the switch from sorghum to maize is due to a better grass weed control option that the Roundup technology accorded to the latter (Wishart, 2004). While sorghum has never enjoyed any post emergence weed control practices targeted to benefit the crop, its way towards Roundup technology is also discouraged by the industries that are concerned about the potential risk of roundup ready volunteer sorghums becoming a weed in other roundup ready crop fields. Development of new herbicides for sorghum is mainly challenged by the presence of wild weedy relatives that closely resemble sorghum both morphologically and physiologically. As a result, there is not a single herbicide that can be used to control grass weeds in sorghum fields without harming the sorghum itself. Unlike for other crops, the development of transgenic herbicide resistant sorghum did not attract much enthusiasm due to the possible risk of gene flow from the

transgenic plants to the wild and weedy relatives. There is not a genetic barrier between cultivated sorghum and its wild relatives, thus pollen mediated transgene escape from cultivated sorghum is possible if the field is close enough to wild or weedy relatives. The fact that sorghum is recalcitrant to tissue culture and plant regeneration makes transformation more difficult (Zhu et al., 1998). Though selection of the potent tissues and cultivars with better potential has eased this problem, the general public concern about transgenic sorghum left out the crop from capturing the benefits that modern science offers. With transgenic herbicide resistance being not an option, efforts over the last several years focused on identification of natural sources of resistance within sorghum and its wild relatives to develop a non-GM but resistance based weed control option for sorghum. Efforts to that end enabled identifying two sources with strong resistance to completely different herbicide chemistries. One of the sources that confer resistance to acetolactate synthase (ALS) inhibitor herbicides was discovered among a shattercane population in a maize field in Kansas that was treated with ALS herbicides (Tuinstra and Al-Khatib, 2007). The other source that provides resistance to Acetyl Co-enzyme-A carboxylase (ACCase) inhibitor herbicides was discovered in Bolivia in a sudangrass population. Both resistance traits have been effectively incorporated into cultivated sorghum genome, and several elite seed and pollinator parental lines possessing resistance to these herbicides have been developed and tested.

ALS inhibitor herbicides

Acetolactate synthase (EC 2.2. 1.6) which is also referred to as acetohydroxyacid synthase (AHAS), is the first common enzyme in the branched-chain amino acid biosynthetic pathway. It is a thiamin diphosphate dependent protein that acts by catalyzing reactions whose initial step is decarboxylation of pyruvate and condensation of 2-ketoacid molecules with pyruvate leading to

the formation of acetolactate and acetohydroxybutyrate, respectively (Chipman et al., 1998; McCourt and Duggleby, 2006). Thus, it leads to the production of three main amino acids, valine, leucine, and isoleucine. Encoded by nuclear genes, the ALS enzyme contains both catalytic and regulatory subunits (Yu et al., 2010). The enzyme once synthesized in the cytosol moves to the chloroplast where it involves in the biosynthesis of these three key amino acids. In order to be functional the transit peptide is cleaved once it enters the chloroplast (Smith et al., 1999). Molecular investigations have looked at interactions between ALS enzyme of *Arabidopsis thaliana*, enzyme cofactors and various ALS herbicides and elucidated that herbicide binding site of ALS enzyme lies deep within a channel and therefore the herbicide binds across the binding domain that spans the channel entry blocking substrate access to the catalytic site (McCourt and Duggleby, 2006).

ALS acts as the common target site for five different herbicide chemistries which involves sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl-thiobenzoates, and sulfonyl-aminocarbonyl-triazolinones (Powles and Yu, 2010; Yu et al., 2010). Thus, there is also evidence of overlapping between the binding sites for sulfonylurea and imidazolinone (McCourt et al., 2005). The first commercialized sulfonylurea herbicide, chlorosulfuron came in to the market in early 1980s followed by the introduction of the first imidazolinone, imazaquin which was recommended for soybeans. The capability of the ALS inhibitor herbicides to offer a broad spectrum control of weed species that commonly interfere with crops at very low use rates (Kershner, 2010) coupled with its very low mammalian toxicity (Brown, 1990; Newhouse et al., 1991) have triggered intensive use of ALS herbicide chemistries in many different crops over huge land areas. Additionally, ALS herbicides demonstrate excellent crop safety over a wide range of crop growth stages while they do not pose a considerable risk to human health.

Resistance to ALS inhibitor herbicides

For nearly three decades, ALS inhibiting herbicides have been in widespread commercial use in global agriculture. There are several reports on resistance development in weeds due to the persistent use of ALS inhibitor herbicides (Tardif et al., 2006; Tranel et al., 2004; Warwick et al., 2008). In circumstances where weeds evolved resistance, target site resistance is the most predominant type which occurs due to one or more specific point mutations in the ALS gene. The resistance conferring amino acid substitutions give rise to structural changes in the ALS protein leading to effective prevention of herbicides binding to the protein (Duggleby et al., 2008; McCourt and Duggleby, 2006). To date, a total of 22 mutations conserved at seven amino acid residues have been identified to confer resistance to ALS-inhibitor herbicides in a number of weed biotypes (Powles and Yu, 2010; Tranel and Wright, 2002; Tranel et al., 2004; Yu et al., 2010). These mutations occurred at alanine 122 (Ala122), proline 197 (Pro197), alanine 205 (Ala205), aspartate 376 (Asp376), tryptophan 574 (Trp574), serine 653 (Ser653), and glycine 654 (Gly654) (Ashigh and Tardif, 2009; Délye et al., 2009; Imaizumi et al., 2008; Kolkman et al., 2004; Laplante et al., 2009; Patzoldt et al., 2001; Patzoldt and Tranel, 2009; Powles and Yu, 2010; Sales et al., 2008; Tranel and Wright, 2002; Warwick et al., 2010). Among these, Trp574 substitutions grant strong resistance to both sulfonylurea and imidazolinone herbicides (Duggleby et al., 2008; Tranel and Wright, 2002). Table 1.1 presents summary of the ALS herbicide resistance mutations found in different weed species under natural conditions. Based on the results of the international survey conducted during 1995/1996, 33 ALS inhibitor herbicide resistant biotypes have been reported in 11 different countries (Heap, 2016).

Resistance mutations are common among cultivated crops as well. Spontaneous mutations followed by selection for ALS resistance have resulted in identification of ALS resistant variants

in maize (*Zea mays* L), wheat (*Triticum aestivum* L), rice (*Oryza sativa* L), oilseed rape (*Brassica napus* L) and sunflower (*Helianthus annuus* L) eventually resulting in development and commercialization of cultivars of these crops that are resistant to ALS inhibitor herbicides (Al-Khatib and Miller, 2000; Bernasconi et al., 1995; Gealy et al., 2003; Newhouse et al., 1991; Shaner et al., 1996; Swanson et al., 1989; Tan et al., 2005). Furthermore, few other crops that have been identified as prospective for the development of ALS resistant trait include sugarbeet (*Beta vulgaris* L), cotton (*Gossypium hirsutum* L), soybean (*Glycine max* (L) Merr), lettuce (*Lactuca sativa* L), tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana tabacum* L) (Tan et al., 2005). While non-target-site resistance to ALS inhibitor herbicides is also found, the resistance is mainly endowed via enhanced rates of herbicide metabolism often involving P450 (Yu and Powles, 2014).

ACCase inhibitor herbicides

The other major herbicide for which resistant sorghums are being developed is the Acetyl-Coenzyme-A Carboxylase (ACCase) inhibitor herbicides. ACCase is an enzyme involved in the first step de novo lipid biosynthesis that occurs in the chloroplast stroma (Page et al., 1994). It is a high molecular weight, multifunctional protein that carries three distinct functional regions. ACCase inhibitors mainly act by inhibiting the chloroplastic ACCase and preventing the synthesis of fatty acids (Délye, 2005). This limits cell growth and disrupts the cell membrane integrity allowing metabolite leakage followed by rapid plant death. ACCase inhibitors were introduced to the market in the late 1970s (Heap, 1997). Two important families of herbicides belonging to ACCase inhibitors are aryloxyphenoxypropionates (APPs) and cyclohexanediones (CHDs). Both these families exhibit effective grass weed control with concurrent safety to broadleaf crops and

thus have been used extensively for controlling many monocotyledoneous species since their introduction in mid 1970s. During 2006, another chemical family which is phenylpyrazoline (PPZ) was introduced to this group with the herbicide pinoxaden (Hofer et al., 2006). Though all these herbicides are structurally different (Délye, 2005) their biochemical activity with regards to inhibiting the ACCase enzyme is similar (Hamdani, 2013). They all act through inhibiting chloroplastic ACCase thus averting the synthesis of fatty acids (Délye, 2005) which may lead to restricted cell growth and concession of cell membrane integrity and eventually plant death.

Resistance to ACCase inhibitor herbicides

Like the ALS, several resistance developments to ACCase inhibiting herbicides have been reported in several species. The first report of resistance to ACCase came just five years after the release of APP and CHD herbicides (Heap, 2016). It appears that several mutations at different locations of the ACCase gene are capable of conferring resistance. Mutations conferring resistance in wild oats were shown to have resulted from five amino acid substitutions; Ile-1,781 to Leu, Trp-1,999 to Cys, Trp-2,027 to Cys, Ile-2,041 to Asn, and Asp-2,078 to Gly. One of these substitutions, Ile-1,781 to Leu was known to grant resistance to both group of ACCase inhibitor herbicides (APPs and CHDs) in wheat (Liu et al., 2007). At the same time, though mechanistic basis was not characterized, there are several reports on non-target-site resistance to ACCase inhibitor herbicides which may be due to enhanced capacity to metabolize herbicides (Délye et al., 2007). According to the 1995/1996 international survey on herbicides resistant weeds, about 13 ACCase inhibitor herbicide resistant biotypes have been reported in 11 different countries (Heap, 2016).

The ALS and ACCase inhibitor herbicide resistance mutations in sorghum

Genetic segregation studies for resistance to ALS inhibitor herbicides have shown that resistance is controlled by one major locus and two modifier loci (Tuinstra and Al-Khatib, 2007). Efforts to identify the DNA sequence of the sorghum ALS gene had been carried out via aligning the DNA sequence reads of the ALS resistant sorghums to the amino acid residues corresponding to the *Arabidopsis thaliana* AHAS gene (GenBank accession X51514). The only sequence close to the *A. thaliana* AHAS gene reported in the sorghum genome is the Sb04g020680 which is reported to have two exons separated by an intron (Kershner, 2010). Based on the DNA sequencing results, the resistant ALS gene has been identified to carry two point mutations at Val-560 and Trp-574 which converted these residues into isoleucine and leucine, respectively. Val-560 is a non-factor mutation where residue 560 is not conserved and is of unknown importance, however, Trp-574 is a conserved residue where its mutated form (Leu-574) is known to provide strong cross resistance to both sulfonylurea and imidazolinone herbicides (Yu and Powles, 2014).

As reported in other species, the ACCase herbicide resistance was reported in wild relatives of sorghum as well. The most stable resistance source reported to date is the one discovered in Sudangrass population in Bolivia. A further greenhouse evaluation and dose response studies have indicated that this source is highly stable at a very high application rates which was later confirmed under multiple environments in the field. Genetic segregation studies on resistance to APP herbicides from this source provided strong evidence for a single major gene providing ACCase herbicide resistance. Although APP and/or CHD herbicide resistance is known to be associated with multiple mutation sites in the carboxyl transferase domain of the ACCase gene (Délye, 2005), sequence analysis of the resistance gene in wild sorghum identified a single causal mutation that rendered the substitution of amino acid tryptophan to cysteine (Trp-2027-Cys) in the ACCase

gene. This mutation is previously known to provide resistance to APPs but not to CHDs (Kershner, 2010). This mutation located in the sorghum gene Sb06g003090 closely corresponded with the amino acid residue 2027 of ACCase gene in *A. myosuroides* (GenBank accession AJ310767).

Table 1.1. Major point mutations conferring resistance to ALS inhibitor herbicides in crop plants.

Amino Acid Position	Resistance Substitution	Reported Resistance	Source
Alanine-122	Threonine/Tyrosine	IM	(Powles and Yu, 2010)
Proline-197	Methionine/Lysin/ Typtophan	SU	(Délye et al., 2009; Kolkman et al., 2004; Warwick et al., 2008)
Alanine-205	Val	IM	(Ashigh and Tardif, 2009; Kolkman et al., 2004; Powles and Yu, 2010)
Aspartate-376	Glu	SU and IM	(Imaizumi et al., 2008)
Tryptophan-574	Leu	SU and IM	(Patzoldt et al., 2001; Patzoldt and Tranel, 2009; Warwick et al., 2010)
Serine-653	Threonie/Asparagine/ Isoleucine	IM	(Laplante et al., 2009)
Glycine-654	Glutamine/Asparagine	IM	(Laplante et al., 2009; Sales et al., 2008)

Potential risks associated with deployment of herbicide resistance in crop plants

The use of any technology or product has risks associated with it. The risk level should be determined and weighed against the benefits before such products are made accessible for general public use. Deployment of herbicide resistant genes in every crop has certain level of risk and so is with sorghum. Since there is no herbicide resistant commercial sorghum on the market yet, risks associated with the technology is not known. However, based on the biology of the crop and experience from other crops, there are certain level of risks anticipated with the deployment of herbicide resistant traits in the crop. Some of the potential risk factors commonly raised by sorghum stakeholders include environmental risk from expanded use of the chemicals, yield drag associated with the resistance mutation, resistant development in weeds, and escape of the resistance gene (gene flow) to wild and weedy relatives. These risk factors should not be considered minor and appropriate stewardship mechanisms need to be in place to mitigate the risks or prevent them from happening.

Environmental risk

Even though a substantial benefit can be gained through the use of herbicides to control unwanted vegetation in crop fields, herbicide application on crops pose certain risks to the environment (Fletcher et al., 1993; Madsen and Streibig, 2003). Important risks may arise due to the direct toxic effect of certain herbicides on humans during chemical application process or may affect both humans and wildlife alike through indirect exposure such as through drift and water contaminations. Generally there are two ways by which herbicide use due to the introduction of herbicide resistant crops may increase environmental risks. The first is that herbicide resistant

crops may enable farmers to use herbicides on their crops by adding more chemicals to environment. The second reason is the issue of development of resistant weeds which may compel farmers to use increased doses of herbicides on the crop fields in order to suppress the resistant weeds (Madsen and Streibig, 2003). On the other hand, there can also be several indirect effects that include inadvertent damages occurring on the sprayed site as well as offsite. The change in vegetation caused due to spraying of the herbicide may alter the habitat of animals such as birds and mammals which leads to disruption of biodiversity, particularly in the areas near natural vegetation (Taylor et al., 2006). Herbicides sprayed using a tractor or an aircraft may frequently deposit the chemicals on sites beyond the intended spray zone mainly due to drift (Marrs et al., 1989). This may also bring unintended damages to the vegetation. Therefore, a lot of controversy does exist with considerable amount of negative opinions about the broadcast spraying of herbicides.

In the past few decades, the amount of chemical applied to obtain weed control has been significantly reduced and this may be cited as one of the major successes of the GM industry in the recent decades. Some of this reduction amounts from kilograms to grams of active ingredients per hectare. But it is not clear how much contribution this may have in terms of reducing potential risks. It appears that reduction in the amount should be coupled with other properties of the chemical such as interaction with soils and low persistence in order to reduce risks.

Development of resistance in weeds

Modern crop production is heavily reliant on herbicide use. This has tremendously increased in recent years perhaps due to the no till production option that the use of herbicides offer which in turn was acclaimed for its perceived positive role in protecting soil erosion and

minimizing energy use (Papendick and Parr, 1997). Although herbicides offer great flexibility in crop weed management acting as an integral part of the agricultural systems for more than 30 years, it also poses the potential risk of selection for herbicide resistant weeds (Heap, 2016). In crop production system based on chemical weed control, the susceptible weeds are killed due to the herbicide effect while weed plants resilient to herbicides may survive and produce seed (Prather et al., 2000). Repeated occurrence of this process would lead to shift of weed population resulting in the buildup of hard-to-control weeds. This phenomenon will eventually result in a situation where the weeds will no longer respond to herbicide application. The International Survey of Herbicide Resistant Weeds recorded 388 unique cases of herbicide-resistant weeds in 210 different species (Heap, 2016). However, due to the reliability that herbicides offer as a tool for weed management, herbicides are likely to remain as the most effective weed management option. But if not managed with proper attention and vigilance, herbicides can worsen the weed problem by increasing the population of resistant weeds. Herbicide resistance occurs either due to genetic mutations that are induced by the herbicide effect itself or shifts that occur in weed biotypes (Prather et al., 2000) both of which can occur as a result of improper use of a rather effective herbicide.

The continuous buildup of resistant weed populations against a particular herbicide renders the herbicide ineffective and will eventually lead to termination of its use. In order to overcome this, researchers have come up with alternative weed control strategies that would prolong the field life of herbicides. Many of such strategies advocate for integrated weed management which involved combined or sequential deployment of several control options including biological control agents, use of allelopathy, mulching, cover crops, manipulation of the soil fertility, crop rotation and rotation of herbicides of different mode of action (Buhler, 2009; Swanton et al., 2009).

Combination of these techniques with chemical herbicides would provide effective and sustainable control of weeds, delay or prevent development of resistant weeds thereby ensuring long term use of the herbicides.

Gene flow

Increasing popularity of herbicide resistant crops has created widespread concern about the resistant gene escaping from cultivated fields to the wild relatives. Gene flow through pollen has been documented in both traditional and transgenic herbicide resistant crops (Ellstrand, 2003; Rieger et al., 2002). Under a typical situation where pollen mediated gene flow is likely, the wild relative receives a resistant gene which was naturally absent in the wild population, resulting in rapid replacement of the wild type allele in the weeds by resistant allele eventually creating herbicide resistant weeds (Haygood et al., 2003). A field investigation in Canada has reported the presence of crop seed residue of oilseed rape with multiple herbicide-resistance in an area where oilseed rape with resistance to different herbicides had been grown on neighboring fields (Hall et al., 2009).

Sorghum is not immune to such conflict between technology and nature. In fact when the relative abundance of wild and weedy relatives that do not seem to have genetic barriers with cultivated sorghum is high, the risk of pollen mediated gene flow from sorghum crop to wild relatives is likely. Several reports indicated evidence for hybridization between cultivated sorghum and different species of wild relatives under experimental conditions (Arriola and Ellstrand, 1996; Paterson et al., 1995). Therefore, the deployment of herbicide resistant sorghum should weigh in to such risks and put forward effective educational programs and stewardship mechanisms to prevent unwanted spread of the resistance gene.

In addition to the risk of pollen mediated resistance gene escape, the increased use of herbicide resistant crops raised significant concern about herbicide-resistant crops themselves becoming resistant weeds in other crop fields. It is very common to see Roundup-ready maize growing as volunteers (weeds) in soybean fields throughout the mid-west.

Next generation sequencing (NGS) platforms and gene expression analysis

Genetic variability observed at the DNA sequence level is the causal factor for changes in gene expression which leads to phenotypic variability in individuals. The very first investigation on rapid determination of DNA sequence was published in 1970's by Fred Sanger and Alan Coulson (Sanger and Coulson, 1975; Sanger et al., 1977). This technique ascribed as Sanger sequencing method, involves DNA sequencing based on the selective incorporation of di-deoxynucleotides by DNA polymerase during in vitro DNA replication that causes a chain-termination reaction (Sanger et al., 1977). Sanger sequencing remained the only method used for DNA sequencing for almost 30 years following its invention. However, owing to the unprecedented developments in the past decade primarily driven by the interest to understand and manipulate human genome, numerous high throughput sequencing technologies have been developed. Such developments essentially included laboratory automation and parallelization of processes and significant cost reduction leading to the establishment of sequencing facilities in several public institutions to accommodate the growing interest in human, animal and plant functional genomic studies.

The power of NGS along with novel molecular tools have also enabled gene expression profiling. Just like DNA sequencing technology, gene expression profiling also evolved overtime growing both in accuracy and depth in terms of number of genes that can be investigated at a time.

The very first gene expression profiling method, microarray technology, relied on DNA hybridization. Hybridization-based techniques involve incubation of fluorescently labeled cDNA with custom-made or commercial high-density oligo microarrays. These hybridization-based approaches are high throughput and fairly low cost, however, these techniques offered limited capacity to obtain the complete set of transcripts representing expressed RNA molecules at a given condition due to its heavy reliance upon the existing knowledge about the organism's genome sequence and possible cross-hybridization (Okoniewski and Miller, 2006; Royce et al., 2007). On the other hand, depending on the nature of the experiment, the expression level comparisons between experiments can require complicated normalization methods. The most recent sequence-based approaches provide a better alternative to the hybridization based gene expression analysis. The first sequencing-based high-throughput method for gene expression analysis is called Serial Analysis of Gene Expression (SAGE). This was followed by Massively Parallel Signature Sequencing (MPSS) (Chu and Corey, 2012; Morin et al., 2008) which employs a series of considerably different biochemical and sequencing steps. These techniques which were, however, less popular as compared to microarrays were followed by NGS technologies that revolutionized sequence oriented molecular research. Owing to their exceptional level of sensitivity and high-throughput nature, NGS technologies have become the method of choice for gene expression analysis (Ozsolak and Milos, 2011; Wang et al., 2009). To date, the power of NGS technologies together with novel molecular biological and computational tools have allowed researchers to conduct gene expression profiling at an unprecedented pace and scale. Thus, NGS technologies have been gaining popularity in the scientific research arena through enabling researchers to answer several biological questions relating to the transcriptional complexity of organisms' genomes that were never possible before. However, there is limited research conducted on plant

transcriptomes as compared to human and animal research but this too have shown a significant boost in the recent past.

RNA sequencing (RNA-seq)

RNA-seq is a widely used high-throughput deep sequencing technology that allows deep sampling of the transcriptome of an organism at a specific time point (Chu and Corey, 2012). To date, this technology has gained enormous popularity in the scientific community that deal with functional genomics. The transcriptome is the complete set of expressed transcripts in a cell of an organism. Therefore, a complete interpretation and understanding of the functional elements of the genome that is associated with a certain phenotype requires detailed analysis and understanding of the transcriptome. RNA-seq combined with appropriate bioinformatics tools can provide a better approach to study gene expression profiles of organisms under different biological conditions. Several published studies based on RNA-seq technique attest to the power of the technique for studying gene expression dynamics over the microarray technique (Garg et al., 2011; Morozova et al., 2009; Weber et al., 2007). Nevertheless, this technique is still under active development and has capacity to improve.

Typically, the workflow involved in an RNA-seq experiment requires isolation of mRNA from the extracted total RNA which is then converted to libraries of complementary DNA (cDNA) fragments with attached adaptors. Each cDNA library is then sequenced on a high-throughput NGS platform to obtain millions of short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The length of these sequence reads could generally range from 30–400 bp depending on the sequencing platform used. For the sequencing step in a regular RNA-seq experiment, any high-throughput sequencing technology such as Illumina IG and Life Ion Torrent

can be used. Figure 1 provides a simplistic summary of how gene expression profiling experiment is conducted.

RNA-seq in sorghum

The recently completed whole genome sequence and comprehensive annotation of the sorghum genome in 2009 (Paterson et al., 2009) along with the developments in functional genomics resources have played a key role in deployment of RNA-seq technology for sorghum. Introduced in 2009, RNA-seq technology (Wang et al., 2009) was rapidly taken up by the scientific community. However, animal research that applied RNA-seq technology took precedence over plant studies and was largely used in medical research (Feng et al., 2013; Martens-Uzunova et al., 2014; Miyamoto et al., 2015; Oshlack et al., 2010; Raghavachari et al., 2012; Ren et al., 2012). The very first study on transcriptome profiling in sorghum appeared in 2011 which focused on revealing the transcriptional changes associated with adaptations and plant responses under abiotic and biotic stresses (Dugas et al., 2011). This investigation which involved RNA-seq of plants subjected to abscisic acid (ABA) or polyethylene glycol treatments at different developmental stages was able to discover more than 50 gene orthologs that associate with plant drought response and are in conjunction with published transcriptome analyses for rice, maize, and Arabidopsis. Another study on comparison of transcriptomes between nitrogen stress tolerant and sensitive sorghum genotypes revealed several common differentially expressed genes that showed higher expression levels in tolerant genotypes (Gelli et al., 2014). Sorghum RNA-seq study linked to a sorghum disease condition included the transcriptomic analysis of sorghum infected by the fungus *Bipolaris sorghicola* which elucidated high resolution expression information on plant responses to pathogens (Yazawa et al., 2013) while a different study on genes responsible for the gradual

variation of colors in sorghum leaves infected with *B. sorghicola* revealed a flavonoid 3′-hydroxylase gene on chromosome 4 to have likely caused the observed variability (Mizuno et al., 2014). However, many of these studies show the need of further proteomics studies in sorghum due to the considerable number of the differentially expressed genes being currently annotated as proteins that are either predicted, similar to expressed or putative uncharacterized (Dugas et al., 2011). Thus, sorghum transcriptome analysis carries much potential as a powerful impetus towards mining the genetic causes underlying numerous abnormal conditions that occur within the sorghum genome.

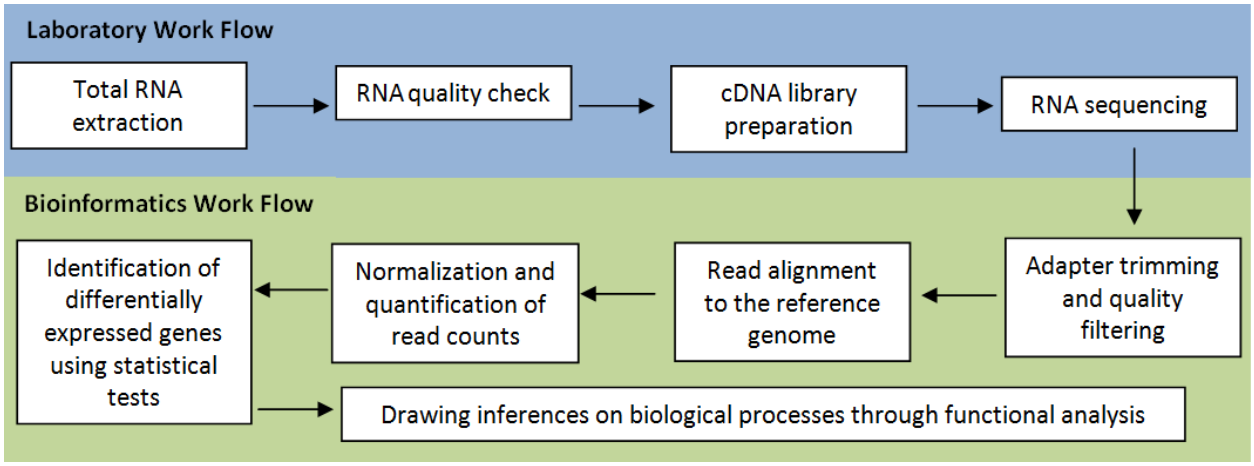

Figure 1.1. A simplistic summary of a gene expression analysis experiment using RNA-seq.

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Chapter 2 - RNA-seq analysis of ALS resistant sorghums (*Sorghum bicolor*) with contrasting seedling chlorotic phenotypes

Abstract

Acetolactate synthase (ALS) inhibitor herbicides are among widely marketed herbicide chemistries that act both against grass and broad-leaved weeds. ALS resistant sorghums (*Sorghum bicolor* (L.) Moench) were developed as a viable post-emergence weed control option in sorghum. However, many of the lines resistant to ALS herbicides show marked interveinal chlorosis during early stages of growth causing reduced seedling vigor. Though affected genotypes green-up at advanced seedling stage, the persistence of this unusual phenotype may undermine adoption of ALS resistant sorghums. The objective of this study was to identify genes, metabolic pathways and mechanisms associated with the reduced vigor and yellow seedling phenotype. Two ALS resistant genotypes expressing yellow and normal (green) phenotypes were grown and tissues were harvested at four time points with the fourth sampling conducted after the genotypes have fully re-greened. RNA was extracted from the tissues and subjected to RNA-seq analysis. Differential gene expression analysis was performed using DESeq2 software package. Gene Ontology enrichment and SorghumCyc pathway analysis revealed significant regulatory activity in several genes related to chloroplast and plant defense responses in chlorotic genotypes. Variant analysis on chloroplast genes resulted in one high impact variant and several other variants that showed moderate effects on gene expression. The high impact variant and majority of moderate impact variants represented genes linked to chlorophyll metabolism and chloroplast precursors while few others represented genes with a role in epigenetic modifications. The color transformation in affected genotypes appears to be due to altered regulation in chloroplast linked genes. The stress condition created

due to low leaf chlorophyll content provoked defense response mechanisms that are often reflected under abiotic stress. Once confirmed, the identified SNP variants would serve as valuable markers for early elimination of affected backgrounds from the breeding population.

Key words: *Sorghum bicolor*, RNA-seq, gene variants, ALS herbicide resistance, interveinal chlorosis.

Introduction

The United States accounts for about 9% of the world's sorghum (*Sorghum bicolor* (L.) Moench) acreage but contributes 25% to the global grain sorghum production with over half of this produced in Kansas (Hamman et al., 2001). Despite the tremendous progress in genetic improvement of the crop over the last several decades, numerous bottlenecks still remain and present challenges to global sorghum production. One of the outstanding bottlenecks in mechanized sorghum production system is the lack of effective post emergence grass weed control options.

In the United States, post emergence weeds, particularly grasses, cause a significant management problem for sorghum. Farmers consistently ranked weed management as priority research area (Tuinstra and Al-Khatib, 2007). Apart from causing considerable economic loss, the lack of effective post-emergence weed control options has forced farmers to switch production to riskier crops such as maize because it offers better weed control tools. As a result sorghum acreage has been halved over the last two decades. Though glyphosate resistance technology has been available for major crops including maize, sorghum has not benefitted from such technology due

to the concern about the possible escape of resistance genes to the wild and weed relatives (Arriola and Ellstrand, 1996).

The discovery of the Acetolactate synthase (ALS) resistance brought some optimism for addressing weed control issues in sorghum. Hybrids with resistance to ALS inhibitor herbicides are poised to provide viable post-emergence weed control option (Tesso et al., 2011) and efforts are underway to make the technology available to growers. ALS inhibitor herbicides are among widely marketed herbicide chemistries that act both on grass and broad-leaved weeds. They act by inhibiting the activity of ALS enzyme, the first enzyme in the branched-chain amino acid biosynthetic pathway which leads to the production of amino acids, valine, leucine, and isoleucine. In 2007, a strong source of resistance to ALS inhibitor herbicides which carries double mutations in the gene coding for the ALS enzyme was discovered among a shattercane (*Sorghum bicolor*) population in Kansas. The point mutations on amino acid residues Val-560 and Trp-574 resulted in the change of these amino acids to Ile and Leu, respectively, however, only the latter substitution prevents the binding of ALS inhibitor herbicides. Thus resistant plants continue to function and produce branched chain amino acids while susceptible plants with wild type ALS protein suffer from loss of function when treated with ALS inhibitor herbicides. Of these mutations, only Trp-574 is a conserved residue thus Leu-574 was found to be associated with strong resistance to ALS inhibitor herbicides (Kershner, 2010; Tuinstra and Al-Khatib, 2007). Over the past few years, breeders have been able to successfully incorporate the resistant gene into adapted sorghum backgrounds. Hence, a large number of sorghum germplasm and parent lines with strong resistance to all classes of ALS inhibitor herbicides have been developed. These resistant sorghums can tolerate herbicide concentrations that are 6 to 10x the normal use rate (Kershner, 2010).

However, many ALS resistant lines tend to show variable degree of leaf yellowing and reduced seedling vigor at early stages of growth. Even though, the plants eventually turn green and effectively grow out of these symptoms after few weeks, such yellow and stunted seedlings may be unpleasant to human eyes and may undermine adoption of the ALS resistance technology. The expression of such phenotype is variable with some genotypes displaying intense yellowing and stunting while others show moderate chlorosis. Few of the resistant genotypes seem to be not affected at all indicating that expression of the phenotype may be dependent on background. Field scoring of chlorosis conducted on families derived from different backgrounds confirmed this variability (Weerasooriya et al., 2012). While traditional selection against yellow phenotypes was successful in eliminating the extreme undesirable phenotypes, the genetic causes for expression of these phenotypes remain unclear. Knowledge of the underlying cause for the phenotype will help eliminate parental genotypes with extreme yellowing tendency from breeding programs.

The availability of the whole genome sequence of sorghum and the advent of new molecular techniques such as RNA-seq have facilitated development of better tools to address complex problems similar to this one. The recent release of sorghum draft genome sequence which revealed ~34, 500 sorghum genes including ~27,640 bona-fide protein coding genes (Paterson et al., 2009) is a remarkable improvement in sorghum genetic studies using next generation sequencing platforms (Dugas et al., 2011; Johnson et al., 2014; Mizuno et al., 2012; Olson et al., 2014). The objective of this study was to identify genes, metabolic pathways and mechanisms associated with the reduced vigor and yellow seedling phenotype using the RNA-seq technique.

Materials and Methods

This study will explore the potential of RNA-seq technique to track gene expression profiles of genetically related yellow and green ALS resistant genotypes to elicit gene expression differences in seedlings. The gene expression result will be combined with the Gene Ontology (GO) and metabolic pathway analysis to characterize the sorghum transcriptome and locate genes functionally related to the expression of yellow seedling phenotypes.

Genetic materials

ALS resistant sister lines of sorghum derived from several F_4 families representing the two extremes for seedling phenotype (green and yellow) were grown in 2012 main crop season for further phenotyping. A sister line pair derived from a pedigree family 'Berhan × (Macia//Macia/Tw) – 3 that express the most extreme seedling color was selected for this study. Berhan is a tropically adapted *Striga* resistant variety developed and released (release No. PSL5061) by Purdue University. Macia (PI 565121) is another tropical variety with broad adaptation to sub-Saharan Africa preferred for its white bold seeds. Tailwind (Tw) is an ALS resistant wild sorghum (shattercane) discovered in 2003 in an ALS treated maize field in Kansas. Seeds from the sister lines representing green and yellow phenotypes were harvested from the 2012 crop. Following standard seed treatment (Maxim, Apron XL, Concep III) the seeds were planted side by side in Puerto Rico the following winter to increase the seeds and also confirm the phenotypes under a different environment. The sister lines maintained clear differences with all plants from the yellow plot (PR12/13-764) expressing yellow phenotype and the sister line grown on the nearby plot (PR12/13-763) expressing normal green phenotype. The use of such genetically

related genotypes was found to be useful in reducing background noise during gene expression studies that may occur due to variation in genomic regions other than the target loci.

Experimental design, tissue sampling and RNA extraction

Seeds representing the yellow and green sister lines from the selected pedigree family were grown in the field during 2013 main season at Ashland Bottoms KSU agronomy research farm, Manhattan. Field grow out was preferred to greenhouse as it represents the actual production condition and also offers better expression of the phenotypes. Plants from each sister line showed uniform seedling phenotype in terms of the degree of observed leaf yellowing (Figure 2.1a). For RNA-Seq analysis, replicated tissue samples were collected at four stages in weekly intervals starting on day 14 after planting (S0) and subsequent samplings made on 21, 28 and 35 days after planting representing S1, S2 and S3 stages, respectively (Figure 2.1b). Since the greatest dynamism in seedling vigor and leaf chlorosis occurs until four weeks after emergence, sampling during this time was assumed to capture most of the genes that are differentially expressed between green and yellow sister lines. The first (S0) sampling was done prior to herbicide application to mimic actual production conditions while this would allow comparison of gene expression profiles between yellow and green backgrounds before and after herbicide application. The subsequent three samplings were done after herbicide treatment. A control (non ALS resistant) genotype was included as a check in the first sampling but not for subsequent samplings since the genotype died by ALS herbicide treatment. After the first sampling (on day 15 after planting) the plants were sprayed with ALS inhibitor herbicide Accent® (Dupont Pioneer, USA) at the rate of 105.08 g a.i. ha^{-1} 15 days after planting. Accent contains nicosulfuron which is a sulfonylurea that works mainly by systemic action where susceptible plants die within two weeks after application. At each

sampling stage, three plants were randomly tagged and approximately 100 mg of leaf tissue samples were collected from each tagged plant. The tissues were immediately frozen in liquid nitrogen to prevent mRNA degradation and stored at -80˚C until use. Total RNA extraction was performed using RNeasy Plant Mini isolation Kit (Qiagen Inc., Valencia, CA, USA) and extracted total RNA was treated with Amplification Grade DNAse I (Invitrogen Corporation, Carlsbad, CA, USA) before further analysis. RNA samples were diluted with RNase free water to obtain samples with required concentration (100-200ng/ul). RNA integrity and quantity were checked using Agilent 2100 Bioanalyzer (Agilent Technologies Genomics, Palo Alto, CA, USA).

In addition, several other ALS resistant genotypes expressing variable levels of leaf chlorosis were grown alongside the two sister lines used for RNA-seq analysis. Leaf chlorophyll content was measured using SPAD 502 chlorophyll meter (Spectrum Technologies, Aurora, IL) at all sampling stages in all genotypes to monitor changes in leaf greenness as additional evidence.

cDNA library construction and sequencing

cDNA libraries were constructed using the Illumina TruSeq^{TM} RNA sample preparation kit according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). RNA from each genotype was subjected to two rounds of enrichment for poly-A mRNAs using "oligodT" attached magnetic beads. Purified mRNA was chemically fragmented and converted to single-stranded cDNA according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). cDNA samples from each genotype was separately barcoded with adapter indexes and pooled. Sequencing was performed on a HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA) at Genome Sequencing Facility of Kansas University Medical Center using 100bp single-end sequencing runs and 15x multiplex.

Differential gene expression analysis and gene clustering

Single-end sequencing reads obtained from HiSeq 2000 runs were subjected to adapter trimming and quality filtering with "Cutadapt" which is a stand-alone adapter trimmer (Martin, 2011). The *Sorghum bicolor* reference genome (Sbicolor_v1.4) (Paterson et al., 2009) was used to perform read alignment using Genomic Short-read Nucleotide Alignment Program (GSNAP) (Wu and Watanabe, 2005). Read counting per gene in each sample was conducted using an in-house script. Differential gene expression among yellow and normal genotypes was analyzed using 'DESeq2' which employs a method based on the negative binomial distribution, with variance and mean linked by local regression. A q-value (Benjamini and Hochberg, 1995) was determined for each gene to account for multiple tests. To control false discovery rate (FDR) at 5%, the differentially expressed genes were required to have q-values smaller than 0.05. Additionally, we only included genes shown at least two fold-change in the list of significantly differentially expressed genes. The RPKM value per gene in each sample represents read counts per kilobase of transcribed region per million reads (Mortazavi et al., 2008). The analysis was further extended to test the null hypothesis, no interaction between genotypes and sampling stages, in order to identify patterns of changes in differential gene expression among sampling stages between the two genotypes using DESeq2 software package. The 5% FDR was used as a threshold to obtain a set of genes with significant interaction between genotypes and sampling stages. The Log2 expression ratios between green and yellow genotypes of this set of genes were used as input for cluster analysis with the R package "mclust" (Fraley et al., 2012) using the model "VVV". The differentially expressed genes were functionally annotated using current sorghum gene annotations in Phytozome (Goodstein et al., 2012).

Gene Ontology (GO) enrichment

The Gene Ontology (GO) enrichment analysis was performed to identify over-represented GO terms in the differentially expressed gene lists using an R software package, goseq. GO functional annotations for sorghum gene products were downloaded from Agrigo (http://bioinfo.cau.edu.cn/agriGO/). GO categories were considered significantly enriched based on the p-value cutoff of 0.05. Based on the results for the GO analysis, the genes related to significant GO terms were extracted and the expression pattern of related genes in log2 fold change at each stage were visualized using a heatmap generated via R package Heatplus (Figure 2.4).

SorghumCyc pathway analysis followed by visualization via Mapman

Metabolic pathway enrichment using SorghumCyc genome database was performed for each differentially expressed gene using the Z-score method suggested by (Dugas et al., 2011) in order to derive functional annotations to infer metabolic pathways of sorghum (Youens-Clark et al., 2011). Pathways were considered significantly enriched if the following criteria were met; Z-score ≥ 2 and the expected number of genes for a family >1. Mapman has the capability of lightening the redundancy that occurs in other commonly used ontologies. Hence, Mapman was used to collect and classify the calculated fold change values in to a set of hierarchical functional categories called 'bin's which then were organized and displayed according in a desired format. Herein, using Mapman alone was not preferred as it may not provide a holistic view of the significant pathways in order of significance as would a Z-core method. However, Mapman provides a better graphical output of the expression under a certain cell component/pathway of choice. Thus, a combination of two methods was used for visualization of results.

Discovery of gene variants

Based on the gene expression results, variant analysis was performed for 31 chloroplast genes that had elevated expression levels during S0, S1 and S2. For the selected set of genes, variants between sequence reads and reference genome were identified using Genome Analysis Toolkit (GATK) (McKenna et al., 2010) and SnpEff variant annotation and effect prediction tool (Cingolani et al., 2012). This analysis displayed the effect of each SNP variants on all related genes. All of the SNP calls were filtered based on the quality of base calls and SNP effect on the gene models were determined using SnpEff (Cingolani et al., 2012). The SNP variants were further filtered to remove non-homologous variants throughout sampling stages that only true SNP variants were used for interpretations. Variant annotation was used to remove the variants with synonymous effect.

Results

Physiological measurements

Phenotypic differences between yellow and normal genotypes monitored using chlorophyll meter (SPAD-502) parallel to RNA-seq analysis showed significant difference between yellow and green genotypes during first three stages of sampling while no difference was observed at stage 3 (S3) when most of the affected genotypes have recovered from the phenotypic disorder (Table 2.1, Figure 2.1a and 2.1c). The difference in seedling phenotype was highest and most significant at S0 stage and progressively reduced but resulted significant at S1 and S2 stages with the difference virtually disappearing at S3 stage.

Mapping of transcriptome to the sorghum genome

Out of the 34,496 gene models reported earlier (Paterson et al., 2009), this study revealed activity for 27,608 unique sorghum gene models. Approximately 512.6 million reads were generated across all three biological replicates for yellow and green genotypes. Of those, 497.2 million (~95-98%) passed quality filtering standards and about 461.8 million (~88-91%) of those uniquely mapped to the sorghum reference genome. The read mapping summary for all yellow and normal samples used in the study can be found in appendix Table 1. Pearson's correlation analysis for quality assessment of quantile normalized reads showed significant positive correlations with an average of 0.98 between biological replicates belonging to a specific genotype at each sampling stage (Figure 2.2a). This, analysis provided a clear picture of high correlations between samples from S0 and S1 vs. S2 and S3. P-value histograms for the normalized read counts for each comparison showed acceptable read count distribution (Figure 2.2b). The differential expression analyses for the comparison between yellow and green genotypes performed separately for each growth stage from S0 through S3 stages resulted in 7510, 6787, 5709 and 3575 differentially expressed genes, respectively.

Clustering pattern of differentially expressed (DE) genes and resulted GO terms and pathways linked to significant DE genes

Out of the total of 27,608 gene models resolved in this study, 5321 were identified to possess significant interactions between genotype and sampling stages. Clustering performed based on the Log2 expression ratio between green and yellow genotypes identified 11 major gene clusters (groups) (Figure 2.6). The clustering patterns varied from stable expression ratios to irregular patterns. However, major consideration was drawn towards gene clusters that showed

change in expression ratios from high to low or low to high along the sampling stages. Thus, clusters 9, 10 and 11 that contained 327, 46 and 28 genes which comprised stable, increasing and decreasing gene expression ratios were excluded from further consideration based on the assumption that they may either comprise genes that contribute to plant developmental processes or genes that do not relate seedling color dynamics that is the focus of this study. The rest of the clusters were divided into two major groups based on their patterns. Thus, clusters 1, 5 and 7 that showed initial decrease followed by an increase in gene expression ratio (from S0 through S3) were considered as cluster set1, while clusters 2, 3, 4, 6 and 8 that showed more or less opposite pattern of variability to the first set of clusters were considered as cluster set 2. The variability seen in the genes captured in cluster set1 reflected up-regulated activity in yellow genotypes during early sampling stages and down regulated at the later sampling stages. Common functions of the genes captured in cluster set 1, in general, were related to photosystem I and II reaction centers, chlorophyll binding proteins, chloroplast precursors, signal transduction involving calmodulin, plant hormones such as auxin, cytokinin and ethylene, oxidative stress response genes involving glutamate cycle genes, heatshock proteins, cytochrome P450, oxidoreductases and specifically chlorophyll catabolic genes and drought induced proteins. In contrast, cluster set 2 which showed increased initial expression ratios that decreased at later stages generally involved; expansins, anthocyanins, aquaporins and UDP-glucosyltransferases. Few other genes that were commonly found in both sets of clusters included heatshock proteins, cytochrome P450, oxidoreductases, peroxidase precursors. At the same time, a large number of genes could not be classified under a specific cluster due to their intermediary involvement in protein synthesis, transcription, cellular transport, signal transduction and other cellular processes.

The GO term enrichment based on differentially expressed genes identified 136 – 174 GO categories throughout the sampling stages based on involvement of each differentially expressed genes to specific molecular functions, cellular components and biological processes. The significance of each GO category was declared by considering significant activity of both up and down regulated genes grouped under each category. These enriched GO categories primarily included chloroplast and its structural components, response to abiotic stresses, substrate metabolism and numerous pathways related to toxic catabolite detoxification. Pathway enrichment using SorghumCyc annotations throughout the sampling stages revealed significant regulatory activity in 34 - 49 metabolic pathways based on the Z-score analysis. This analysis facilitated filtering and identification of pathways exhibiting high confidence differentially expressed genes. Pathways with significant regulatory activity in yellow backgrounds accounted for phenotypic changes in leaf tissues, defense responses, hormonal networks and other processes perhaps due to the significantly low chlorophyll content.

GO term analysis in combination with pathway analysis was useful to identify pathways with higher regulatory activity. Thus it was evident that a considerable number of genes linked to chlorophyll degradation pathway showed altered regulation. At the same time, a large number of differentially expressed genes captured under the GO term, chloroplast, were not categorized under a specific pathway due to deficiencies in pathway annotation. Therefore, despite the considerably increased regulatory activity observed for genes coding chloroplast precursors, photosynthesis, chlorophyll binding proteins, they were not assigned to a specific pathway via SorghumCyc.

Apart from GO terms related to chloroplast, each stage comprised a large number of abiotic stress related GO terms such as oxidative stress due to reactive oxygen species (ROS), singlet oxygen, response to oxidative stress, response to hydrogen peroxide, protein kinases and toxin

catabolic processes involving glutathione S-transferase (GST) activity. Pathway analysis further supported this through significant activity in chlorophyll degradation, betanidin degradation, glutathione (GSH) mediated detoxification, gamma glutamyl cycle (which involves GSTs), nicotine degradation, phospholipases which were relatively common to all sampling stages. Thus, most prominent among pathways that linked with detoxification processes were processes mediating antioxidant molecules. Simultaneously, GO terms linked to stress response hormonal networks were observed throughout the sampling stages. Thus, a lot of variability in transcript abundance was observed in hormonal networks related to defense responses (Figure 2.4). This was reflected through altered regulation of genes coding abscisic acid (ABA), gibberellin (GA), brassinosteroid (BRs), jasmonate (JA), ethylene (ET) and cytokinin (CK) biosynthesis. Overall, differentially expressed transcripts between yellow and green genotypes at stage S0 and stage S1 based on the GO categories and pathways showed a large overlap. These overlapped categories commonly comprised chloroplast, thylakoid membrane, thylakoid lumen, electron carrier activity, and several terms related to toxin catabolic processes. Phospholipid biosynthesis, phospholipases and triacylglycerol degradation, starch degradation and sucrose biosynthetic process were also among the important GO terms observed under first two samplings. On the other hand, gene expression profiles between S2 and S3 stages showed more or less similar patterns while a considerable number of genes linked to chloroplast were both up and down regulated throughout the sampling stages as also revealed via GO enrichment (Figure 2.3a).

The procedure utilized for data analysis helped create a bigger picture that could be dissected to different areas based on the significance. However, many pathways that comprised less number of annotated genes than what is required to declare the significance in the Z-score limited our capacity to draw conclusions primarily based on the pathway analysis. Thus, GO term

analysis in combination with pathways could provide a better view of the results. At the same time, a considerable number of clustered genes annotated as "expressed proteins" limited their usefulness in drawing inferences.

While SNP variants are known to carry important functional roles via changing the genetic code through point mutations at certain genomic regions, SNP variant analysis deployed in this study primarily allowed identifying single base polymorphisms in different genomic loci related to expression of chloroplast related genes in tested genotypes. Overall, SNP variant analysis for chloroplast related genes revealed SNP variant calls affecting expression of 21 genes (Table 2.2). Though not all genes tested contained SNP variants, some of the resulted variants showed impacts on the gene containing the SNP as well as on adjacent genes. In general, the SNP variants contained one high impact variant and, few moderate and low impact variants while rest of the variants that included the majority had only modifier effects.

Chloroplast related genes with modified expression levels

A total of 308 active genes related to chloroplast were resolved out of which 274 were associated with chlorophyll metabolism and showed differential expression in at least one of the sampling stages. The differentially expressed genes list was higher for S0 (39) followed by S1 (19), S2 (19) and S3 (14). Stages S0 and S2 shared the highest (39) number of differentially expressed genes while the least (7) differentially expressed genes were shared between S0 and S3 stages. There were several genes that were consistently expressed at two or more of the sampling stages including S3 which are likely not related to the phenotype of interest. Some 31 genes were over-expressed during S0, S1 and S2 stages while few genes were activated in all four stages. S0 and S3 stages contained the highest (187) and lowest (95) differentially expressed gene counts,

respectively (Figure 2.3a). The lower differentially expressed counts at S3 agree with the phenotypic measurement on leaf chlorophyll content which was the lowest at S3 stage (Figure 2.1b). Overall, variability in expression profiles of the chloroplast related genes seemed to have reduced from stage S0 to S3. Genes that were up and down regulated between S0 and S3 during the photosynthesis light reaction (Tables 2.3b and 2.3c indicating the marked difference in gene expression levels between the two stages.

Furthermore, a closer inspection of 31 chloroplasts related genes that showed significantly altered expression during first three sampling stages using variant discovery revealed several important variants including a high impact variant which acts both as a splice donor and an intron variant located on chromosome 3 position 11194286-11202586 bp (Table 2.2). Gene coding geranylgeranly reductase (Sb03g010330), which contained this variant is involved in chlorophyll biosynthesis pathway (Wang et al., 2014) and carried two other missense variants with moderate effects and another low impact variant in both a splice region and an intron region.

Other chlorophyll biosynthesis genes which carried SNP variants included coproporphyrinogen III oxidase (Sb06g028140) that contained a moderate effect gene variant, two other genes coding for FAD binding domain containing proteins (Sb03g010340 and Sb04g028050), a cysteine proteinase inhibitor precursor protein (Sb09g024230), and a magnesium-protoporphyrin O-methyltransferase (Sb10g002100) that carried several variants with modifier effects. Among the genes involved in chlorophyll degradation, pheophorbide a oxygenase (Sb01g047120) and red chlorophyll catabolite reductase gene (Sb01g029900) contained several SNPs. No variants affecting chlorophyllase gene involved in chlorophyll catabolism were obtained while variants for Mg-dechelatase gene could not be tested as the structure and sequence of this gene still remains elusive to researchers.

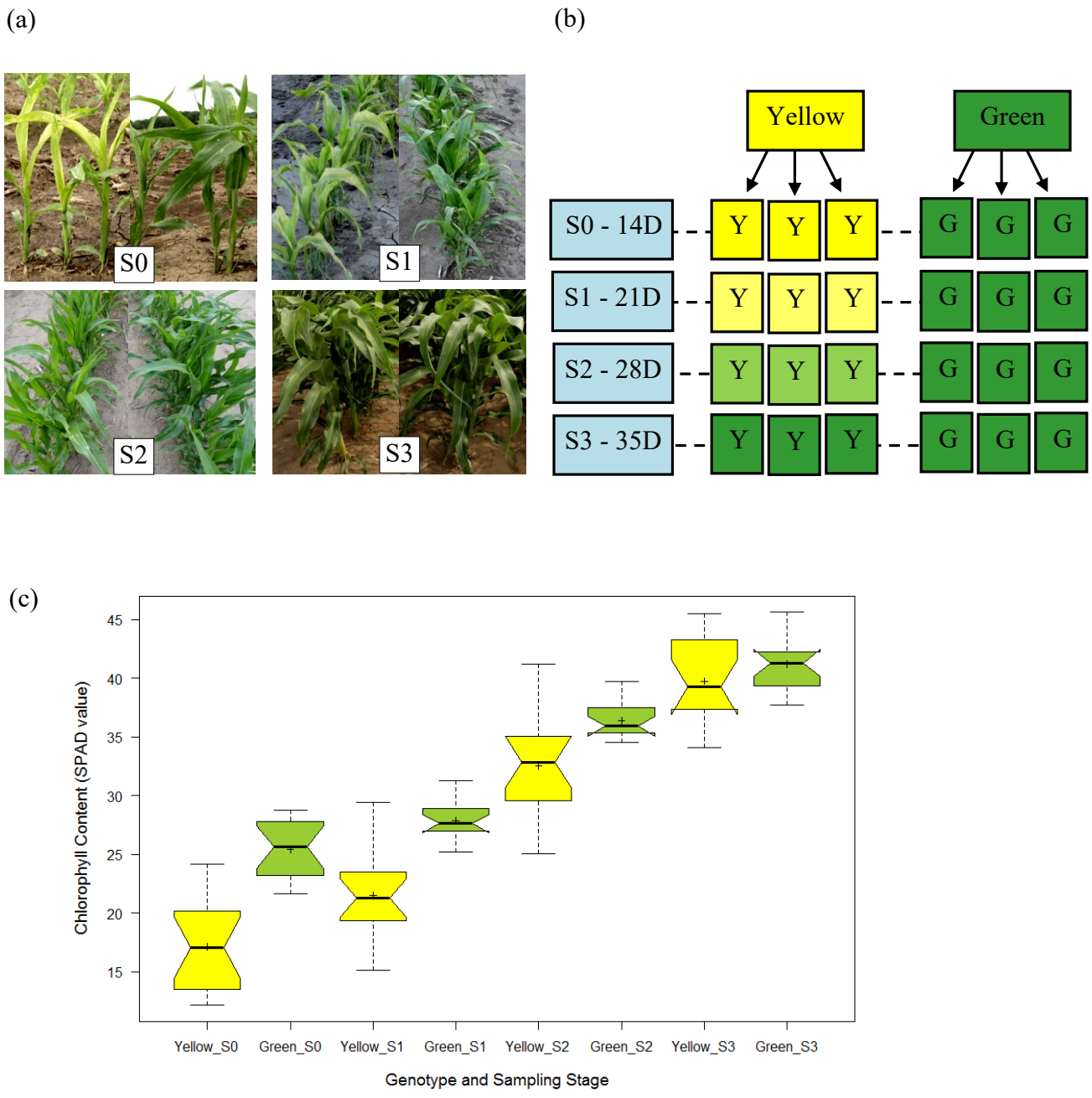
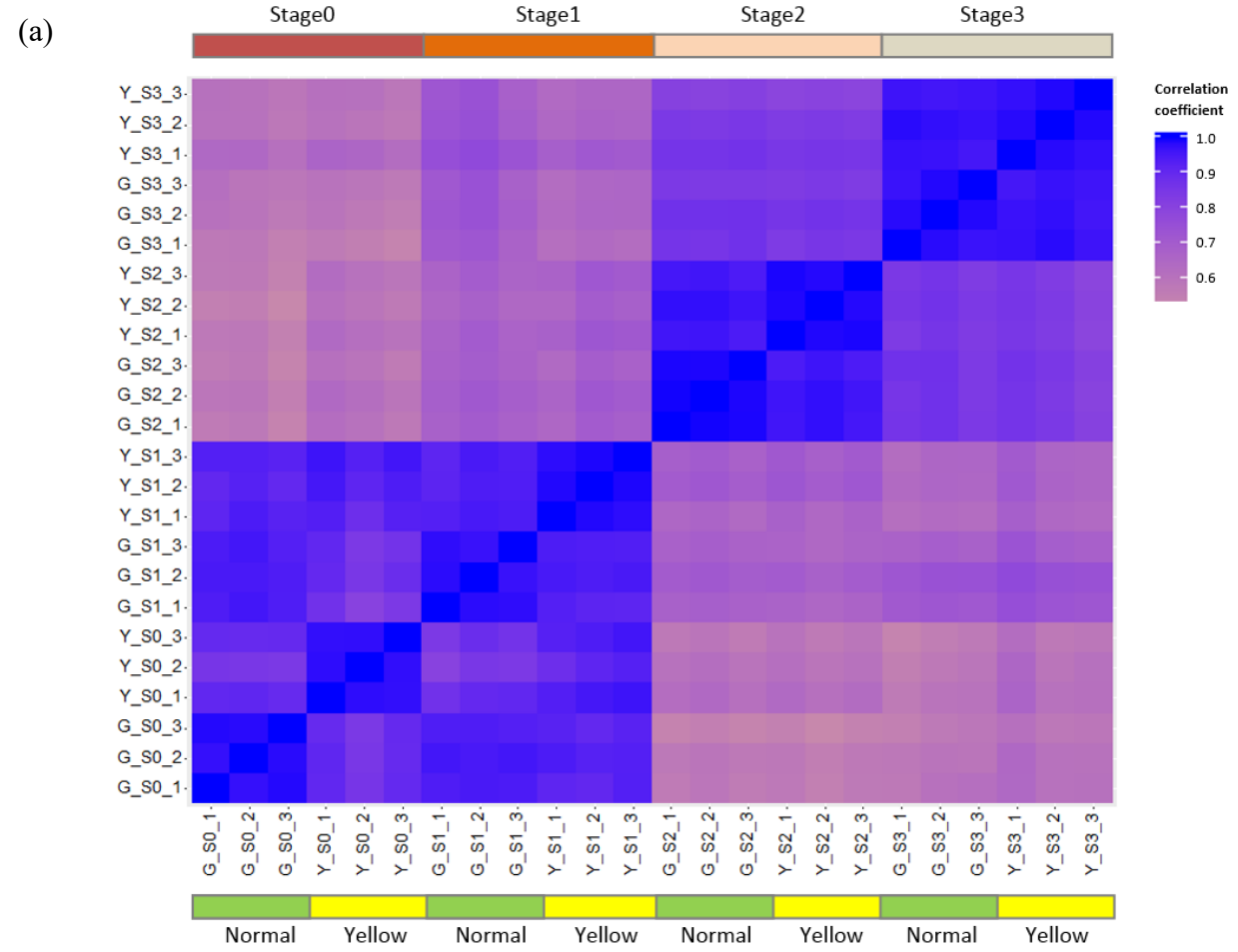


Figure 2.1. (a) Variability in leaf color between yellow (left) and green (right) genotypes during four sampling stages (S0-S3); (b) RNA-seq experimental design with replicated tissue samples collected at S1 through S3 stages (color charts represent change of leaf phenotypes at different growth stages); (c) Variation in leaf chlorophyll content between yellow and green genotypes at four sampling stages.

(a)



(b).

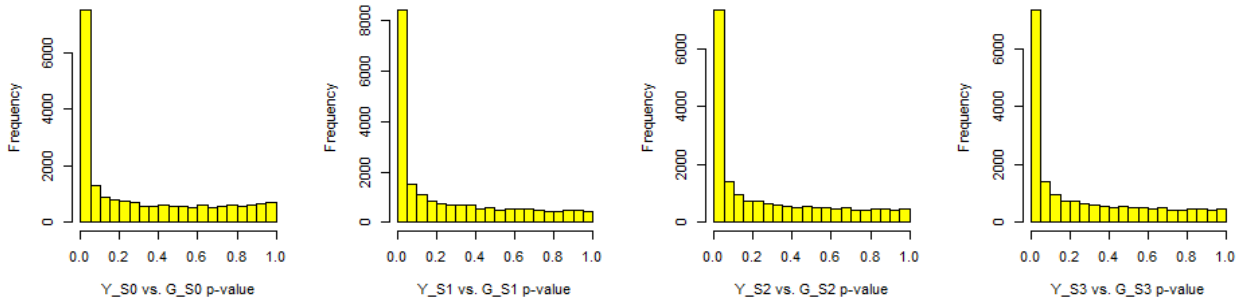
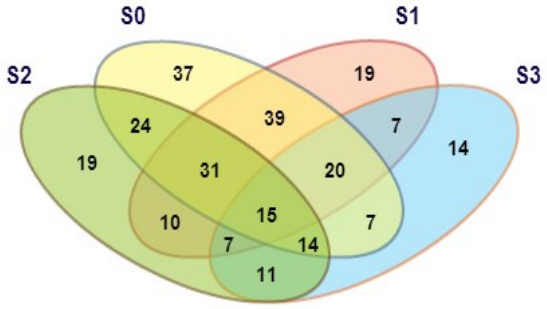
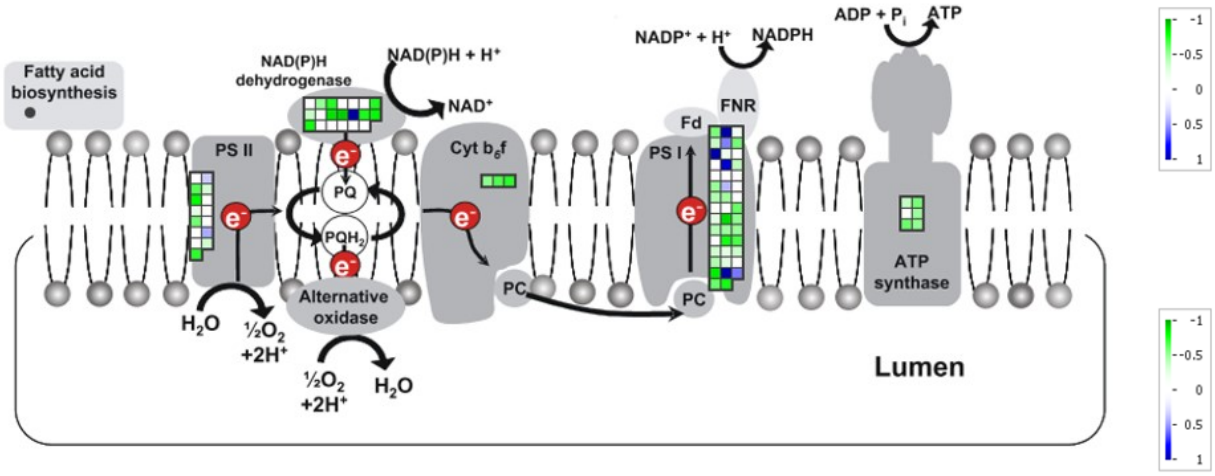


Figure 2.2. (a) Pearson's correlation matrix of the whole dataset. Pair-wise Pearson correlation coefficients were calculated from the gene expression values of the whole transcriptome (27,608 genes) in all 24 samples. The color scale indicates the degree of correlation. Sample names are in the sequence of genotype yellow (Y) or normal (G), stage (S0 through S3) and sample number (1 through 3); (b) P-value histograms of the read counts after normalization for yellow and green genotypes for comparisons at all stages.

(a).



(b).



(c).

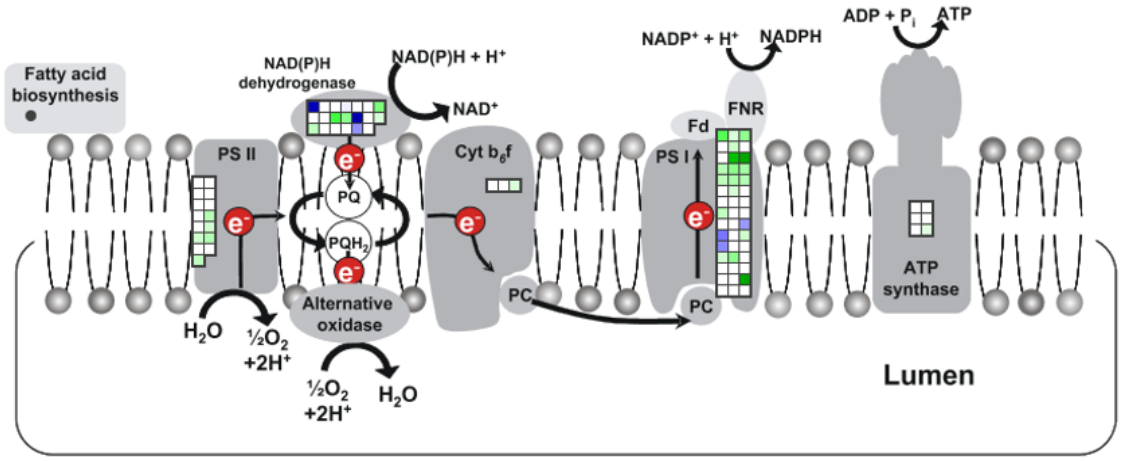


Figure 2.3. (a) Venn diagram showing DE genes involved in chlorophyll metabolism between yellow and green genotypes at each sampling stage. Up and down-regulated gene bins involved in photosynthesis light reaction occurring within chloroplast (b) at S0 and, (c) at S3 stages. The altered regulation of chloroplast genes at S0 are reflected by higher number of down regulated gene bins as compared to S3 showing majority of not differentially expressed gene bins lead

towards recovery of chlorosis symptom (Blue = up-regulated, Green = down-regulated, white = not DE).

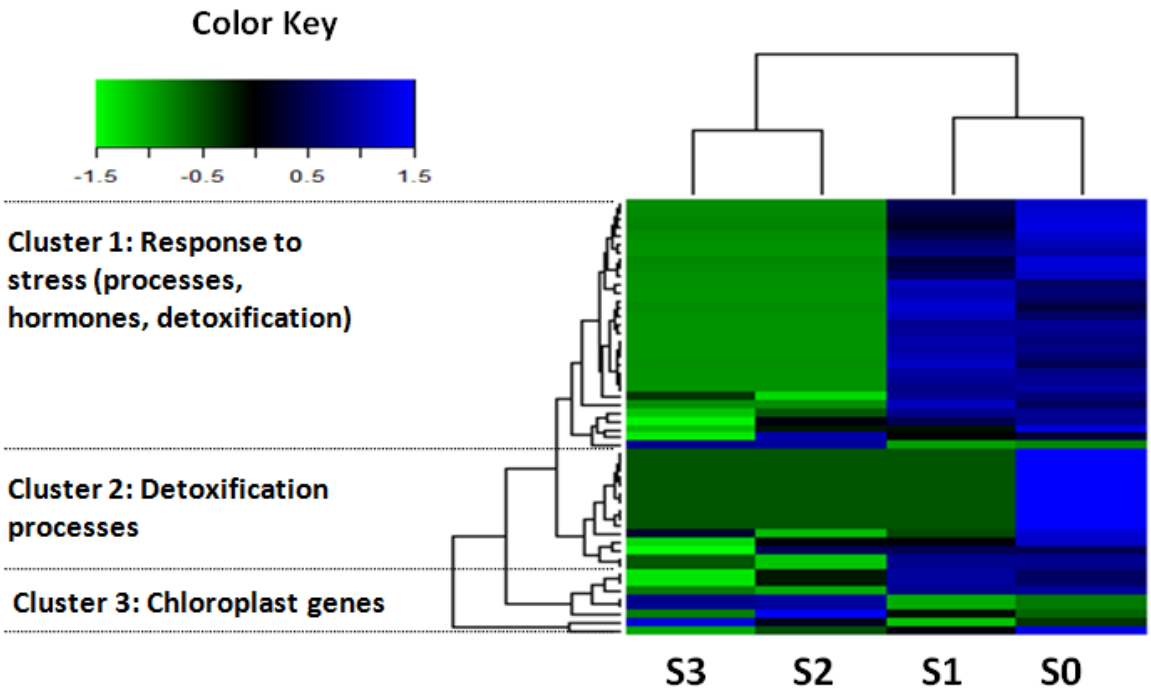


Figure 2.4. Heatmap showing clustering pattern of the genes related to chloroplast and stress response mechanisms (Green= down-regulated, Blue = up-regulated, Black= not DE) at four sampling stages from S0 through S3.

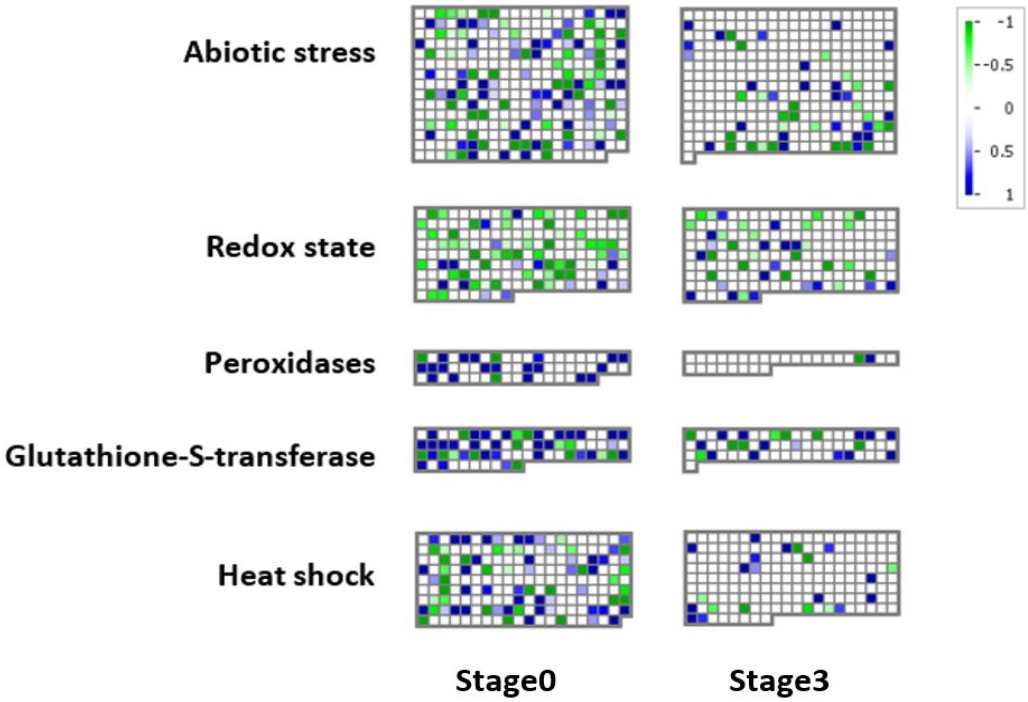


Figure 2.5. Selected abiotic stress response mechanisms that showed altered gene regulation between yellow and green genotypes during S0 and S3 stages. Defense response gene bins that were significantly up or down-regulated during S0 stage including glutathione-S-transferase, peroxidases, heat shock proteins and some defense related hormonal pathways have shown recovery of altered gene regulation by S3 stage (Green= down-regulated, Blue = up-regulated, white= not DE).

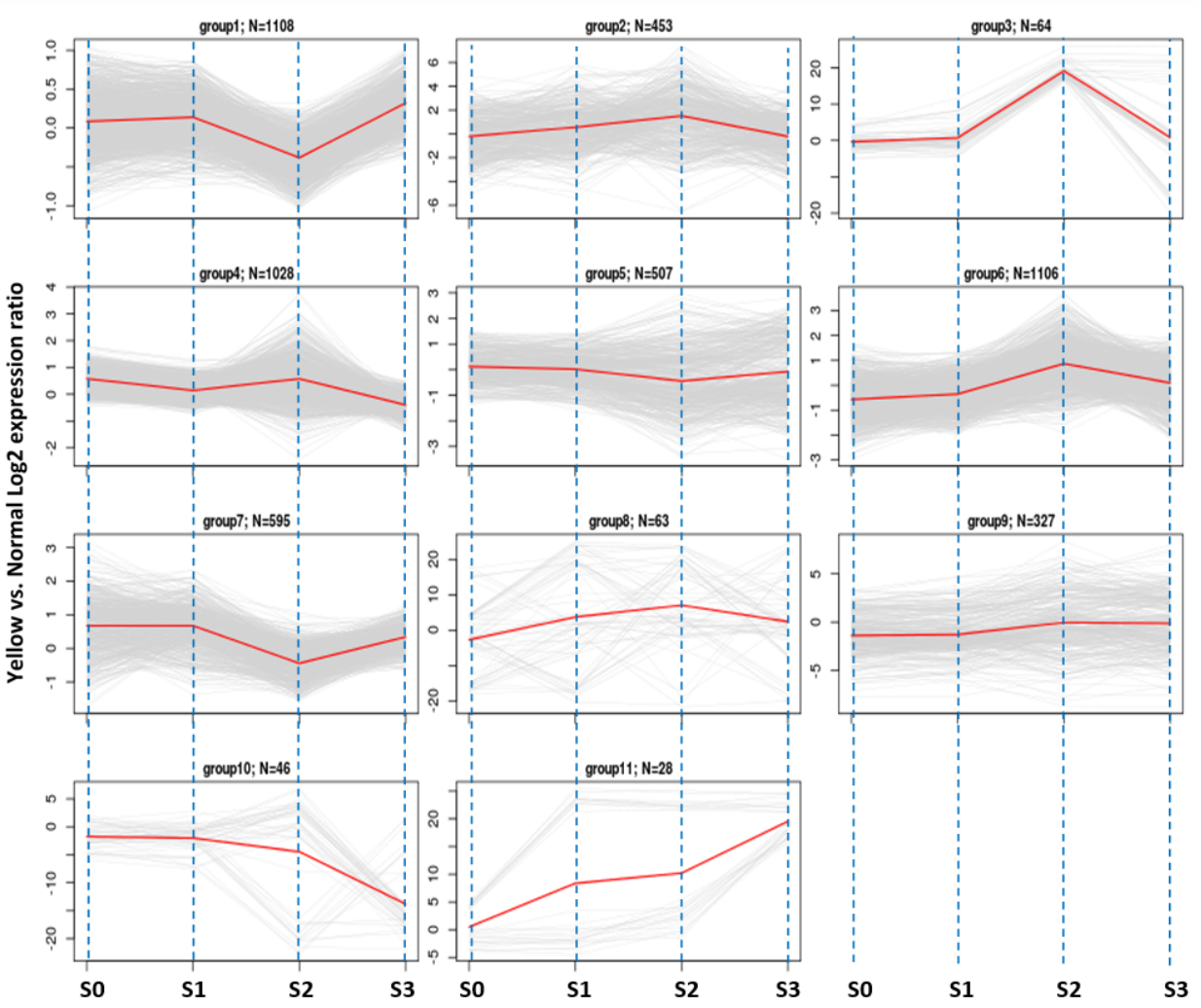


Figure 2.6. Clustering pattern of significant DE genes based on Log2 fold expression ratio between yellow and green genotypes. The total number of significant genes that had significant interactions with the sampling stage were grouped into 11 basic clusters. Clusters 9, 10 and 11 that contained 327, 46 and 28 genes which comprised stable, increasing and decreasing gene expression ratios were excluded from further considerations.

Table 2.1. Summary of pairwise t-test comparisons for leaf chlorophyll content between yellow and normal genotypes at each stage of sampling from S0 through S3.

Stage	Mean of yellow genotype	Mean of green genotype	Estimated difference between G vs. Y	P value
S0 (14D)	17.14	25.44	8.30	4.35E-08
S1 (21D)	21.40	27.88	6.48	1.93E-06
S2 (28D)	32.50	36.40	3.90	4.00E-03
S3 (35D)	39.75	41.19	1.44	0.18

Table 2.2. Summary of protein coding gene variants of chloroplast related genes differentially expressed from S0 through S3.

Gene containing or adjacent to SNP	SNP physical position (bp)	Effect and number of variants	Impact Type	†Gene Annotation	Related metabolic pathway, cellular component or biological process
Sb01g047120	Chr1: 70232083-70240223	3' UTR variant (1)	Modifier	PAO	Chlorophyll degradation
		upstream gene variant (1)	Modifier		
Sb01g029900	Chr1: 52020259-52030063	5' UTR variant (2)	Modifier	RCCR	Chlorophyll degradation
		intron variant (2)	Modifier		
		downstream gene variant (14)	Modifier		
Sb01g029910	Chr1: 52028751-52030063	missense variant (4)	Moderate	PPR	RNA editing
		upstream gene variant (1)	Modifier		
Sb03g010330	Chr3: 11194286-11202586	splice donor variant & intron variant (1)	High	GGR	Chlorophyll biosynthesis
		missense variant (2)	Moderate		
		splice region variant & intron variant (1)	Low		
		3' UTR variant (5)	Modifier		
		downstream gene variant (3)	Modifier		
Sb03g010340	Chr3: 11194286-11203488	upstream gene variant (29)	Modifier	FAD	Chlorophyll biosynthesis
		downstream gene variant (2)	Modifier		
Sb03g046660	Chr3: 73748631-73755943	missense variant (1)	Moderate	CAO	Chloroplast precursor
		5' UTR variant (4)	Modifier		
		intron variant (1)	Modifier		
		downstream gene variant (8)	Modifier		
Sb04g028050	Chr4: 57992915-57994773	downstream gene variant (1)	Modifier	FAD	Chlorophyll biosynthesis
Sb06g028140	Chr6: 56988353-56992081	missense variant (1)	Moderate	CPOX	Chlorophyll biosynthesis
		upstream gene variant (1)	Modifier		
Sb06g033030	Chr6: 61063154-61067556	downstream gene variant (1)	Modifier	POR_A	Chloroplast precursor
Sb08g018560	Chr8: 48758696-48777519	missense variant (2)	Moderate	DAPE	Chloroplast precursor
		upstream gene variant (23)	Modifier		
		intron variant (1)	Modifier		
		downstream gene variant (1)	Modifier		
Sb09g023130	Chr9: 52782099-52789596	missense variant (1)	Moderate	IF-2	Chloroplast precursor
		upstream gene variant (3)	Modifier		

Gene containing or adjacent to SNP	SNP physical position (bp)	Effect and number of variants	Impact Type	†Gene Annotation	Related metabolic pathway, cellular component or biological process
Sb09g023140	Chr9: 52786439- 52789596	missense variant (1)	Moderate	MT	Epigenetic modifications
		downstream gene variant (2)	Modifier		
Sb09g024220	Chr9: 53767326- 53771188	missense variant (2)	Moderate	HIL-TF	Regulate Gene expression
		splice region variant (1)	Low		
		3' UTR variant (1)	Modifier		
		downstream gene variant (4)	Modifier		
Sb09g024230	Chr9: 53767326-53771188	upstream gene variant (3)	Modifier	CPI	Chlorophyll biosynthesis
		downstream gene variant (13)	Modifier		
Sb09g029170	Chr9: 57931700-57934815	upstream gene variant (1)	Modifier	KARI	Chloroplast precursor
Sb10g000400	Chr10: 163724-175981	upstream gene variant (1)	Modifier	GARS	Chloroplast/mitochondrial precursor
		downstream gene variant (2)	Modifier		
Sb10g001390	Chr10: 1139316-1150458	upstream gene variant (2)	Modifier	DAG	Chloroplast precursor
		downstream gene variant (22)	Modifier		
Sb10g001410	Chr10: 1148096-1148761	missense variant & splice region variant (1)	Moderate	DnaJ	Heatshock chaperone
		3' UTR variant (2)	Modifier		
		intron variant (1)	Modifier		
		downstream gene variant (16)	Modifier		
Sb10g002100	Chr10: 1772503-1774438	downstream gene variant (7)	Modifier	ChlM	Chlorophyllide a biosynthesis
Sb10g003480	Chr10: 3052061-3067638	missense variant (1)	Moderate	CSase	Chloroplast/chromoplast precursor
		intron variant (8)	Modifier		
		upstream gene variant (3)	Modifier		
Sb10g029300	Chr10: 59141483-59145440	splice region variant (1)	Low	TL-16.5	Chloroplast precursor
		upstream gene variant (1)	Modifier		
		downstream gene variant (1)	Modifier		

†CAO= chlorophyllide a oxygenase, ChlM =magnesium-protoporphyrin O-methyltransferase, CPI= cysteine proteinase inhibitor precursor protein, CPOX= coproporphyrinogen III oxidase, CSase = cysteine synthase, DAG=diacylglycerol protein, DAPE= diaminopimelate epimerase, DnaJ = chaperone protein DnaJ, FAD= FAD binding domain containing protein, GARS= glycyl-tRNA synthetase 2, GGR = geranylgeranlyl reductase, HIL-TF= histone-like transcription factor, IF-2= translation initiation factor IF-2, PAO= pheophorbide a oxygenase, KARI= ketol-acid reductoisomerase, MT=methyltransferase, POR_A= protochlorophyllide reductase A, PPR= Pentatricopeptide repeat domain containing protein, RCCR= red chlorophyll catabolite reductase, TL-16.5= thylakoid lumenal 16.5 kDa protein.

Discussion

The total number of gene models revealed in our study was relatively lower compared to the number revealed through the sorghum draft genome sequence reported in 2009 (Paterson et al., 2009). Apart from lack of strand-specific information in cDNA alignments, this could perhaps be due to low transcriptional activity of some of the genes during seedling stages. Many of the undetected genes could be expressed late thus are activated at adult plant stage or even further later around physiological maturity. However, percentage of uniquely mapped reads observed in the present study was higher as compared to some earlier RNA-seq studies that reported 67.1% (Dugas et al., 2011) and 83.1% (Lu et al., 2010). The p-value distributions for read count comparisons at each stage (Figure 2.2b) and markedly high Pearson correlations for gene expression results between biological replicates (Figure 2.2a) well agreed with previous RNA-seq studies (Dugas et al., 2011; Guo et al., 2013; Lu et al., 2010). This result attested the high reproducibility of the data and further validated the quality of the data set.

On the other hand, interaction of gene expression between genotypes and sampling stages was evident. Gene cluster set 1 that contained genes related to chloroplast and plant defense responses showed increased early expression ratios that decreased towards last sampling, the opposite behavior was observed in cluster set 2. Cluster set 2 genes included expansins and aquaporins, which are expressed under drought stress towards proper water channeling (Jones and McQueen-Mason, 2004; Maurel et al., 2002), UDP-glucosyltransferases, which that help in sucrose synthesis (Singh et al., 1978), anthocyanins, which reduce photo-oxidative damage due to degenerating chloroplasts under stress and promote nitrogen recovery from senescing leaves (Hoch et al., 2001) provided initial clues on an activated stress condition within yellow genotype. The occurrence of some genes with similar functions in both sets of clusters could be explained in two

ways. First, this kind of observation may be possible when certain pathways show both up and down regulated genes throughout the sampling stages. Secondly, genes in a particular pathway may comprise variability in expression profiles, thus some genes could be highly expressed while others are low expressed to assist the recovery process form the stress condition. For instance, considering a particular stress response pathway, while majority of the genes are up regulated, few genes could still be down regulated (Figure 2.5). These genes that are down regulated may probably possess a repressor activity on one or more up regulated genes.

Plant response to different types of stresses has been shown to be associated with the generation of ROS. ROS which is a common signal of plant stress responses (Xia et al., 2009) can damage cellular components through disturbing cellular redox homeostasis (Cruz de Carvalho, Maria Helena, 2008). Over-reduction of various molecules is frequently accompanied with rapid increases in superoxide, hydrogen peroxide and hydroxyl radicals that belong to ROS (Cruz de Carvalho, 2008). In the present study, enriched GO terms related to oxidative stress, phospholipid and starch degradation, and observed over-reduced redox state (Figure 2.5) suggested generation of ROS due to early chlorophyll breakdown. While glutathione-s-transferase (GST) is considered as a ROS scavenging system that carries high antioxidant properties (Alscher, 1989; Grant et al., 1996) owing to its redox-active thiol group that conjugates with potentially dangerous xenobiotics (Marrs, 1996), significant up regulation in GST in current study seemed to have helped alleviate oxidative damage caused by generated ROS (Cruz de Carvalho, Maria Helena, 2008). Nevertheless, our interpretation was further supported by the observed increased activity of several other strong antioxidants such as betanidin (Wybraniec and Michałowski, 2011), Cytochromoe 450 (Sahoo et al., 2013; Saijo et al., 2000), ascorbate peroxidases (Triantaphylides et al., 2008), and glycein betain (Lv et al., 2007; Quan et al., 2004) that are well known to play an important

role in acquired tolerance to abiotic stresses such as drought and salinity. The effectiveness of these antioxidants is well reflected in Figure 2.5 that shows reduced redox states during S0 and S3 stages. Heatshock proteins (HSPs) has an important role in assisting proper protein folding (Borges and Ramos, 2005; Walter and Buchner, 2002) and expression of HSPs is one of the most common immediate responses to plant stress. Thus, simultaneous expression of HSPs in current study implied possible misfolding of proteins in yellow genotype. The resultant heatshock gene variant in Sb10g001410 coding for DnaJ chaperone in the current study implied possible variant effect towards increased heatshock protein expression upon created stress condition.

Because hormones play vital role in abiotic stress responses in plants (Wilkinson and Davies, 2002) promoting survival or escape mechanisms through modifying signal transduction (Franklin, 2008), changes to hormonal networks was investigated. The result showed wide variability in transcript abundance tied to hormonal networks in current study evidenced a series of defense responses activated due to yellow seedling phenotype. Rapid increases in endogenous ABA levels is characteristic to abiotic stresses (Goda et al., 2008; Kilian et al., 2007; Zeller et al., 2009) and up to 10% of protein-encoding genes are transcriptionally regulated by ABA (Nemhauser et al., 2006). ET on the other hand, has shown to play a significant role in response to heat and osmotic stress on Arabidopsis (Suzuki et al., 2005) and salinity stress in soybean (Ma et al., 2012). Accordingly, increased ET and ABA signaling in yellow genotype in current study assented induced stress tolerance mediated by these hormones. Several previous studies have reported altered CK levels and mutants lacking functional CK receptors expressing resistance to abiotic stresses (Jeon et al., 2010; Kang et al., 2012; O'Brien and Benková, 2013; Tran et al., 2007). This matched with significant number of down regulated genes CK biosynthetic genes in our study. SA is required for inducing stress resistance proteins such as antioxidants and HSPs and

therefore SA-deficient plants cannot create an effective abiotic stress defense system (Clarke et al., 2004; Nawrath et al., 2002). Though not listed among pathways with significant activity, probably due to reduced representation of the genes involved, up-regulated HSPs confirmed the involvement of SA pathway in stress response (Figure 2.5). GA signaling has shown to be quite variable depending on the type of stress abiotic condition on plants (Colebrook et al., 2014) where, in species such as rice (*Oryza sativa*) under submergence stress (Bailey-Serres and Voesenek, 2010), arabidopsis (*Arabidopsis thaliana*) and maize (*Zea maize*) under salinity, have shown to trigger reduced GA signaling towards stress escape (Achard et al., 2006; Magome et al., 2008; Wang et al., 2008) while there is emerging evidence that GA may integrate multiple hormone pathways in response to stress (Achard et al., 2006). Thus, altered regulation in GA biosynthesis pathway genes observed during early stages suggested involvement of GA towards stress escape. BRs carry a prospective role towards induced stress tolerance under elevated hydrogen peroxide levels (Xia et al., 2009), drought, high or low temperature, salinity and heavy metals (Bajguz and Hayat, 2009; Hayat and Ahmad, 2010), BRs have also been described as a booster of net photosynthetic rate (Hasan et al., 2011). Observed increased BR levels in our study further confirmed its role towards stress tolerance. The overall hormonal coordination observed in present study corroborated several studies on plant hormonal cross-talk (Jaillais and Chory, 2010; Santner and Estelle, 2009; Xiong and Yang, 2003) suggesting their imperative role directed towards stress escape through modifications in gene regulation.

The gene expression profile between genotypes of contrasting leaf phenotype reveals some gene expression mechanism that either support increased activity of chlorophyll degradation or poor chlorophyll biosynthesis in yellow genotype during early samplings. Generally, ALS herbicide injury symptoms are slow thus upon herbicide application, takes up to two weeks to

develop (Gunsolus and Curran, 2007). The large overlap in DE transcripts between yellow and green genotypes observed at S0 and S1 stages (Figure 2.2a and 2.4) implied no interaction between seedling leaf color and herbicide treatment thus plant mechanisms activated towards leaf yellowing seems to be similar to the responses to herbicide treatment. Thus, it's obvious that yellow genotype has undergone a stress condition that provoked more or less similar transcriptional activity as in a plant sprayed with the ALS herbicide. Conversely, similar expression profiles resulted for S2 and S3 stages (Figure 2.2a and 2.4) implied recovery processes to have initiated during S2 stage. Thus, it is apparent that observed plant responses are consequences of a "domino effect" created due to altered regulation in genes specifically linked to chloroplast (Figure 2.3a). Among the resultant SNP variant calls, variants on geranylgeranyl reductase (Sb03g010330) that carry high, moderate, low and modifier effects on protein function seems to play a central role in reduced chlorophyll production. Geranylgeranyl reductase (GGR) involves in a key step in chlorophyll a biosynthesis and provides phytol for chlorophyll (Chl) synthesis (Wang et al., 2014). Two other genes coding FAD binding domain containing proteins (Sb03g010340 and Sb04g028050), that resulted with several variants also catalyzes GGR in two major steps in chlorophyll biosynthesis (Keller and Bouvier, 1998) thus carry a high likelihood of contributing to leaf yellowing symptom. Moderate and modifier variants resulted for Coproporphyrinogen III oxidase (CPOX) gene (Sb06g028140) which is a major enzyme in chlorophyll *a* biosynthesis pathway I and II and, chlorophyll cycle suggests an important role of this gene towards chlorotic symptom development. Cysteine proteinase inhibitor precursors (CPI) in chlorophyll biosynthesis involved in the reaction which gives rise to coproporphyrinogen III from uroporphyrinogen III while magnesium-protoporphyrin O-methyltransferase (ChlM) gene is involved in chlorophyllide *a* biosynthesis. Thus variants for CPI (Sb09g024230) and ChlM (Sb10g002100) may also pose a negative effect on initial steps of

chlorophyll biosynthesis. The reaction involving pheophorbide *a* oxygnase (PAO) and red chlorophyll catabolite reducates (RCCR) complex is responsible for loss of green color in leaves thus is considered a key step in chlorophyll catabolic pathway. These two enzymes are also known to physically interact with each other during chlorophyll catabolism (Pruzinska et al., 2007; Rodoni et al., 1997). Typically PAO and RCCR are highly up-regulated when plants enter senescence while their increased activity is restricted to senescence (Hörtensteiner et. al., 1995). Conversely, stay-green genotypes of certain plant species are known to carry defective PAO activity which delays chlorophyll catabolism (Roca and Mínguez-Mosquera, 2006; Thomas and Howarth, 2000; Vicentini et al., 1995). Modifier effect variants of PAO (Sb01g047120) and RCCR (Sb01g029900) genes (Table 2.2) resulted in current study suggests that chlorotic phenotype may partly be due to altered PAO-RCCR regulation.

Thylakoid luminal 16.5 kDa protein coding gene (Sb10g029300) which carried a low impact splice variant, the gene coding chloroplast/chromoplast precursor (Sb10g003480) that carried one moderate and few missense variants, and other chloroplast precursor genes Sb03g046660, Sb06g033030, Sb08g018560, Sb09g023130, Sb09g029170, Sb10g001390, Sb10g000400 with several variants perhaps could be associated with chloroplast structural transformations throughout the sampling stages. For instance, when over-expressed, chlorophyllide *a* oxygenase (CAO) is known to enlarge the antenna size of photosystem II in Arabidopsis (PSII) (Tanaka et al., 2001). The missense variant and few other variants resulted for CAO gene (Sb03g046660) in present study suggests a possibility for altered PSII antenna size leading to decreased capacity for chlorophyll production in yellow genotype. Proteins containing PPR motifs are important for expression of organelle genomes and organelle biogenesis (Delannoy et al., 2007). PPR motifs, histone-like transcription factors, Methyltransferases independently help

regulate gene expression through creating epigenetic modifications (Burley et al., 1997; Manna, 2015). Thus, moderate effect variants on PPR repeat domain containing protein (Sb01g029910), Sb09g023140 coding for methyltransferase, and gene coding histone-like transcription factors (Sb09g024220) perhaps have initiated or aggravated the regulation of chloroplast related genes via gene editing events. Though, not on grounds similar to conditions observed in the present study, temporary yellowing followed by re-greening has been observed in various plant species due to transient differentiation stages of the chloroplasts (Egea et al., 2010; Mayfield and Huff, 1986; Prebeg et al., 2008; Zavaleta-Mancera et al., 1999). Thus, gradual re-greening of chlorotic leaf tissues mirrored through changes in leaf chlorophyll contents in yellow genotype (Figure 2.1a and 2.1b) suggests developmental shifts in chloroplasts directed towards re-greening process.

Conclusion

Grower satisfaction and cultivar stability are important considerations in the development and deployment of new hybrids. The current study addressed concerns (seedling chlorosis and stunting) that may arise following the deployment of herbicide resistant sorghum hybrids. The result provided some clue on the pattern of gene expression in sorghums suffering from leaf chlorosis that the bizarre phenotype was implicated to be the result of mutations in major chloroplast associated genes that resulted in their altered expression levels. Corroborating results from GO term and pathway analysis, some of the SNP variants were directly associated with genes responsible for chlorophyll metabolism and chloroplast structural components resulting in modified gene expression in yellow genotypes that is similar to plant defense responses under abiotic stress. Other SNP variants appeared to occur in genes responsible for regulating gene expression. The study laid groundwork on understanding the genetic basis behind unusual

chlorotic phenotype observed in ALS herbicide resistant sorghums. Once confirmed, the SNP loci associated with unique expression pattern in affected genotypes may be targeted for marker assisted elimination from breeding populations.

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Chapter 3 - Agronomic adaptability, yield components and nutritional attributes of ALS herbicide resistant sorghum genotypes

Abstract

Resistance to Acetolactate synthase (ALS) inhibitor herbicides is anticipated to offer an effective post-emergence weed control option in sorghum (*Sorghum bicolor* (L.) Moench). Introgression of the resistance trait from a wild relative into cultivated sorghum resulted in the development of numerous ALS resistant parental lines. Depending on the genetic background, many resistant lines show various levels of interveinal chlorosis at seedling stages. The objective of this study was to examine the effects of leaf yellowing and herbicide treatment on plant performance. Thirty-three ALS resistant lines expressing varying degree of chlorosis were evaluated for agronomic, yield and nutritional attributes along with three checks from 2013 through 2015. The study consisted of two experiments separately focused on evaluating the physiological and yield performance of the tested breeding lines both with and without the herbicide treatment. Data were collected on seedling height, leaf chlorophyll content and biomass 14 days after planting. At later stages, data were recorded on days to anthesis, adult plant chlorophyll content, height and biomass, panicle length, panicle width, panicle weight, panicle yield, 1000 kernel weight and grain yield. Additional analysis on grain nutritional attributes included grain protein, starch, fat and ash. Based on the results, interveinal chlorosis on ALS resistant inbreds appears to delay flowering and perhaps maturity. But, both chlorotic phenotype and herbicide treatment did not seem to have negative effects on final yield and other crop parameters. While a few of the resistant parents have higher protein content, the majority have nutritional attributes comparable to that of regular lines.

Key words: *Sorghum bicolor*, ALS herbicide resistance, interveinal chlorosis, chlorophyll content, yield, nutritional traits.

Introduction

Ranked fifth among the major world cereals, sorghum (*Sorghum bicolor* (L.) Moench) is considered an important food, feed and bio-energy crop of global importance. Sorghum feeds millions of people in more than 30 countries in Africa and Asia while it is used as the second largest source of animal feed and bio-fuel feedstock in the United States. As the number one sorghum producer and exporter in the world, the U.S. contributes to the global grain sorghum production of about 25% (Hamman et al., 2001). However, the area planted with sorghum in the United States accounts only for about 9% of the total land area cultivated to sorghum worldwide. While this seems to indicate a very positive achievement in improving sorghum productivity, there has been a sharp decline in sorghum acreage in the U.S. over the past few decades. Evaluation of crop production trends in one of the largest sorghum producing states, Kansas, indicated that much of the lost sorghum acres were picked up by maize. According to local farmers, the major driver for the switch from sorghum to maize was due to a better weed control option that the latter offers (pers. comm.). Thus the key issue in sorghum production in the United States that hasn't long been answered is the lack of effective post-emergence weed control options.

Continued research in this area led to identification of sources of resistance to "Acetolactate Synthase (ALS) inhibitor herbicides" among wild sorghum population which was expected to provide resistance based post-emergence weed control option for sorghum (Tesso et al., 2011). ALS herbicides are amongst the broadly marketed herbicide chemistries that act both on grass and broad-leaved weeds. Resistance gene from the wild sorghum was successfully introgressed into

cultivated sorghum with the derivates expressing stable resistance as strong as in the original resistance gene donor. Over the last few years, large number of sorghum germplasm and parent lines (inbreds) with strong resistance to all classes of ALS inhibitor herbicides have been developed by sorghum breeding program at Kansas State University. These resistant sorghums have shown to tolerate herbicide doses that are 6 to 10x the normal use rate. Despite their relatively long persistence in the soil, residual activity, ALS inhibitor herbicides have made them widely popular in the farming communities due to their potency against both grass and broad-leaved weeds, low cost and very low use rates (Kershner, 2010). As the technology awaits commercialization, one key concern among both the industry and producers is the interveinal chlorosis commonly observed in ALS resistant lines.

Many of the ALS resistant lines tend to show reduced seedling vigor and variable degree of leaf yellowing at seedling stages. But the expression of such phenotype seems to be dependent on genetic backgrounds and is heritable. This was confirmed from evaluation of families derived from backgrounds expressing different levels of the phenotype (Weerasooriya et al., 2012). Though, the chlorotic plants turn green and effectively grow out of these symptoms after few weeks of emergence, this abnormal seedling phenotype may become disturbing to growers and undermine adoption of the ALS resistance technology.

Previous studies have shown that chlorophyll loss under biotic and abiotic stresses can lead to major yield and biomass reductions in crops (Hayatu and Mukhtar, 2010) perhaps due to compromised photosynthesis (Rharrabti et al., 2001). Although the yellowing phenotype observed in ALS resistant sorghums is not the result of biotic or abiotic stresses, the significant reduction in leaf chlorophyll content may be reflected in overall biomass accumulation or yield formation. While such phenotype is expressed even without herbicide treatment, the yellowing intensity

seems to increases when herbicides are applied. Therefore, the objectives of this study were, 1) to evaluate the extent of leaf chlorophyll loss among ALS resistant lines derived from diverse genetic backgrounds and, 2) to investigate the effect the of seedling chlorosis and herbicide spray treatment on plant physiological activities, yield components and nutritional properties of sorghum grains.

Materials and Methods

Two experiments were conducted in this study with both experiments consisting of the same set of genetic materials. Experiment I was conducted in single row plots during 2013 and 2014 season and was aimed at evaluating the impacts of ALS gene induced early season leaf chlorosis on seedling dry matter accumulation and plant growth characteristics with and without herbicide treatment. Whereas Experiment II was carried out in two row plots during 2014 and 2015 seasons in order to evaluate the agronomic and yield parameters with and without herbicide application.

Genetic materials

A total of 36 sorghum inbred lines resistant to ALS inhibitor herbicides were included in the study. The test genotypes comprised 27 ALS resistant B-lines (female parents), 6 ALS resistant R-lines (male parents) and another 3 ALS resistant lines from the 2007 releases. Selection of the entries was primarily based on the variation in the degree of leaf chlorosis that the materials suffer and, not on their fertility reaction. Thus the entries selected for the study captured the spectrum of the interveinal seedling chlorosis observed in the larger nursery. Figure 3.1a depicts the phenotypic appearance of the typical yellow and green genotypes grown side by side. As a negative control, ALS herbicide susceptible standard pollinator parent Tx430 was also included. The list of test

entries is provided in Table 3.1 and the same test genotypes were used in both experiments I and II.

Experimental design and field management

Prior to planting, seeds were cleaned and surface-sterilized using standard sorghum seed treatment (a mixture of Maxim 4FS ™, Apron XL ™, Concep III ™, and colorant). In preparation for planting, three grams of seeds enough to plant 5 m long single row were packeted for Experiment I. A plot was represented by a single 5 m long row in Experiment I and by a double row in Experiment II. The design in both experiments was split-plot with randomized complete block replicated two and three times in Experiment I and II, respectively. The herbicide treatment was assigned to the main plot whereas the 36 genotypes were assigned to the sub-plot unit.

At planting, the seeds were drilled into 5-meter-long rows spaced 0.75 m apart using a cone planter. Fertilizer nitrogen (urea) and phosphorous (di-ammonium phosphate, DAP) were applied at the rate of 90 kg ha-1 and 40 kg ha^{-1}, respectively. Pre-emergence weeds were controlled with 0.55 kgha-1 Atrazine ™, 0.76 kg ha^{-1} Dual II Mg ™, and, 0.16 kg ha^{-1} Callisto ™ while post-emergence weeds were removed manually. Three weeks after planting, whole plot units designated for herbicide treatment in Experiment I and II were treated with 2x rate (105.08 g a.i. ha^{-1}) of herbicide accent.

Data collection

Data were collected on a number of agronomical, physiological and yield parameters in both experiments. In Experiment I, leaf chlorophyll content, plant height, days to flowering and above ground biomass were measured at seedling and adult plant stage (at 4 and 10 weeks after

planting, respectively). Visual ratings of leaf coloration was recorded using a "1-4" scale with 1=extreme yellow, 2= greenish yellow, 3= yellowish green, 4= normal green. Chlorophyll measurements were taken using SPAD-502 chlorophyll meter (Spectrum Technologies Inc.) using three plants per plot. At seedling stage, chlorophyll reading was made on the second fully expanded leaf from top whereas the pre-flag leaf was used later at grain filling. Seedling height was recorded as the length of the plant measured from the soil level to the tip of the most top leaf and from the base to the tip of the panicle in adult plants. Total above ground biomass was measured using destructive sampling of three plants per plot at both early and adult plant stages. Fresh plants were harvested at the base, and weighed on a tabletop balance and oven dried at 70°C for three days. The biomass was then adjusted to 14% moisture content for statistical analysis. Days to flowering was recorded on plot basis as the number of days from planting to when half of the plants in a plot reached half-bloom stage.

The main parameters collected under Experiment II include adult plant chlorophyll content, days to flowering, adult plant height and, grain yield and yield components, panicle weight, yield per panicle, kernel number per panicle, thousand kernel weight, panicle length, and panicle width. The following outlines the procedures for determination of yield components under Experiment II. Panicle samples (three from each plot) were harvested after maturity and oven dried at 65°F for three days. Panicle weight on plot basis was determined as the average weight of three individual panicles harvested. The dry panicles were then threshed using a belt thresher (Model SVPT, Almaco). Kernel weight per panicle was measured as the weight of kernels threshed per individual panicle. Number of kernels per panicle was determined by counting the kernels threshed from each panicle using a seed counter (Model 850-3, International Marketing and Design Corp.). Thousand kernel weight was estimated by dividing the panicle yield by the number of kernels per panicle

multiplied by 1000. The mean yield component data from three panicles was used to represent a plot for statistical analysis. Additional samples were collected from the herbicide treated whole plot unit for determining the nutritional profile of herbicide treated ALS resistant genotypes. Seeds from three panicles harvested in each plot were bulked to pool large enough sample (40g or more). The nutritional quality analysis was performed using near infrared spectroscopy (NIR) system (Perten Instruments Inc.) pre-calibrated for use on sorghum grain. The seed samples were carefully cleaned to remove chaffs and broken seeds and the intact seed samples were scanned to obtain protein, fat, starch, ash, and moisture content from the intact seed samples. The results were adjusted to 12.5% moisture before statistical analysis.

Statistical analysis

For both experiments, statistical analysis was performed using SAS software version 9.4 (SAS Institute, 2008). Analysis of variance (ANOVA) was performed using a mixed model (PROC GLIMMIX) procedure with environments and replicates treated as random effects. Significant means between herbicide treatments and genotypes were separated using Fischer's protected LSD in SAS. The data in both experiments was re-arranged to test the effects of seedling color on both seedling growth parameters and adult plant performance including yield and yield components. Pearson correlation coefficient was run using PROC CORR procedure to determine the degree of correlation between measured parameters.

Results

Figure 3.1a shows the visually observable variability in seedling phenotype between the yellow and green genotypes while Figure 3.1b shows the appearance of the same genotypes after the leaves were re-greened. A close-up of a seedling leaf bearing interveinal yellowing symptom is shown in Figure 3.1c. Figure 3.2a presents variability for leaf chlorophyll content observed between herbicide treated genotypes expressing chlorotic and normal phenotypes under each environment. Based on the results of the t-tests, during seedling stage, the difference between SPAD values under both environments were highly significant. However, at the adult plant stage SPAD value difference between yellow and normal genotypes was not significantly different as inferred from the p-values. On the other hand, above ground biomass showed significant differences between chlorotic and normal genotypes during seedling stage under environment 1 but was not significant under environment 2 (Figure 3.2b). Differences between two groups of genotypes for adult plant biomass and chlorophyll content were non-significant under both environments.

The effect of herbicide treatment and interveinal chlorosis on plant growth characteristics

Tables 3.2 and 3.3 present the analysis of variance for the effect of herbicide treatment and seedling chlorosis across the range of genotypes on crop growth and phenology evaluated under Experiment I. Herbicide treatment had significant effect on seedling height, seedling and adult plant biomass, while the effect on other parameters was not significant (Table 3.2). On the other hand, the effect of genotypes was significant for all parameters collected while the interaction between herbicide treatment × genotype, genotype × environment and the three way interaction

(herbicide treatment × genotype × environment) were also significant for all parameters except for seedling and adult plant height (Table 3.2). The effect of environment was significant for all parameters except adult plant chlorophyll content and seedling height. When ANOVA was run after the data was rearranged by seedling color, the analysis revealed that herbicide treatment again has significant effect on all parameters except on adult plant chlorophyll content, adult plant height and days to flowering (Table 3.3). The effect of seedling color on these parameters, on the other hand, was highly significant except for adult plant height and adult plant biomass. The effect of environment was again significant for all parameters except for seedling height. The interaction between seedling color and herbicide treatment was not significant except for seedling chlorophyll content and seedling biomass while seedling color × environment interaction effect was significant for seedling biomass and days to flowering. The three way interaction between these factors (seedling color × herbicide treatment × environment) was significant only for seedling biomass. The herbicide treatment, genotype and seedling color effects for individual environment analysis was fairly consistent with the combined data for most of the parameters (Tables 3.2 and 3.3).

Mean seedling chlorophyll content between herbicide treated and untreated plots was 28.3 and 29.1 SPAD units, respectively, and was virtually the same (55.4 and 54.4) in adult plant showing that ALS herbicides do not have any effect on chlorophyll biosynthesis in ALS resistant genotypes (Table 3.4). Herbicide treatment also did not have an effect on days to flowering and adult plant height. However, seedling height, seedling and adult plant biomass were significantly lower in herbicide treated plots (Table 3.4). On the other hand, genotypes with conspicuous seedling chlorosis tended to have reduced seedling growth characteristics. Accordingly chlorophyll content, height and biomass in genotypes with yellow seedling phenotype were significantly lower than those not affected by yellowing. Mean seedling chlorophyll content in

yellow genotypes was 25.4 units compared 35.2 in normal green genotypes. Also seedling height and biomass in affected genotypes was 16.8cm and 32.4g as compared to 18.2cm and 43.6 g, respectively, in genotypes not affected by yellowing. However, all of these characteristics were not affected in adult plant except days to flowering which took an average of 73 d in yellow genotypes vs. 71 d in normal green genotypes.

The mean performance of the 36 genotypes for various parameters with and without herbicide treatment is presented in Table 3.5. Seedling chlorophyll content was markedly different between genotypes ranging from the lowest of 13.4 units in herbicide treated yellow background (PR12/13-764-6) to 40.4 units in MN13-7450 also under herbicide treatment. The non-ALS resistant check Tx430 had mean seedling chlorophyll 38.3 SPAD units in untreated control which was comparable with 39.1 units in MN13-7450, 35.9 units in MN13-7458 and few other resistant genotypes. No comparison of chlorophyll content was made between the herbicide treated and untreated control of the susceptible check as it died after being treated with the herbicide (Figure 3.1d). Chlorophyll content was generally low in yellow backgrounds than the normal and near normal green genotypes. While the majority of the entries had lower chlorophyll contents, the chlorophyll content of a large portion of the herbicide treated entries were slightly higher than the untreated control after treated with the herbicide and this is especially true in the green backgrounds while chlorophyll content in many of the yellow genotypes was either reduced or remained the same after herbicide treatment resulting in significant seedling color × herbicide treatment interaction for the trait.

Days to flowering among the entries ranged from as early as 59 d in MN13-7838 to 84 d in PR11/12-851 in untreated plots. The check entry Tx430 took 68 d to bloom. Herbicide treatment appear to have caused a little delay in flowering which was consistent across genotypes except a

handful of entries with yellow backgrounds that seem to have been not affected by herbicide treatment or even had accelerated flowering under herbicide treatment. Seedling height and biomass were visibly affected by herbicide treatment with treated plots having reduced height and lower biomass and this was consistent across all genotypes (Table 3.5).

The centerpiece of this study was to determine if the yellow seedling phenotype has an impact on growth and adult plant performance. Hence correlation analysis was run between seedling chlorophyll content and various seedling and adult plant characteristics. The result shows that seedling chlorophyll content was positively and significantly correlated with seedling height and seedling biomass under herbicide treated and untreated conditions in both test seasons (Table 3.6). Whereas, it was negatively correlated with days to flowering and adult plant height in environment 1 and the correlation was not significant in environment 2. Other adult plant characteristics including biomass and adult plant chlorophyll content were not correlated with seedling chlorophyll content indicating that the effect of this phenotype was limited to seedling growth only (Table 3.6)

Effect of herbicide treatment and interveinal chlorosis on adult plant performance

The analysis of variance on the effects of herbicide treatment and seedling chlorosis on phenology, yield and yield components tested under Experiment II are presented in Tables 3.7 and 3.8. The analysis shows that both herbicide treatment and seedling chlorosis had significant effect on days to flowering and panicle length while the effect on all other parameters was not significant. On the other hand, the genotype, environment and genotype × environment interaction effects were significant for all parameters studied except the genotype effect on grain yield and environment on chlorophyll content were not significant (Table 3.7). Genotype × herbicide treatment effect was

significant for days to flowering and panicle length while genotype × herbicide treatment × environment interaction effect was significant only for days to flowering. The effect of these factors under individual environment analysis was generally consistent with the combined analysis. Further analysis was conducted after sorting the genotypes by seedling color and the result shows that herbicide treatment effect was again significant for days to flowering and panicle length whereas seedling color effect was significant only for days to flowering, panicle width (diameter) and TKW and not significant for all other traits. All interaction effects were not significant for all traits except seedling color × environment effect for grain yield (Table 3.8). Again the individual environment ANOVA was similar to the combined analysis except few traits showed significant for seedling color effect.

The combined mean for the various traits of genotypes subjected to herbicide treatment and untreated control is presented in Table 3.9. As indicated in the ANOVA in Table 3.8, only days to flowering and panicle length were significantly affected by herbicide treatment. That is herbicide treatment delayed flowering by an average of 3 days while panicle length was 1.2cm longer in the treated plots. Individual location analysis shows that the difference for these traits between treated and untreated plots was significant only under environment 1 and not under environment 2 (Tables B.5 and B.6). All other traits were not affected by herbicide treatment with grain yield, though not significant, was slightly higher under herbicide treatment (3.3 vs. 3.5 tons/ha). The effect of seedling color on adult plant performance was not significant for most of the traits except days to flowering that was delayed in the chlorotic genotypes by an average of 2 days and panicle width that was slightly larger in the normal green genotypes (Table 3.9).

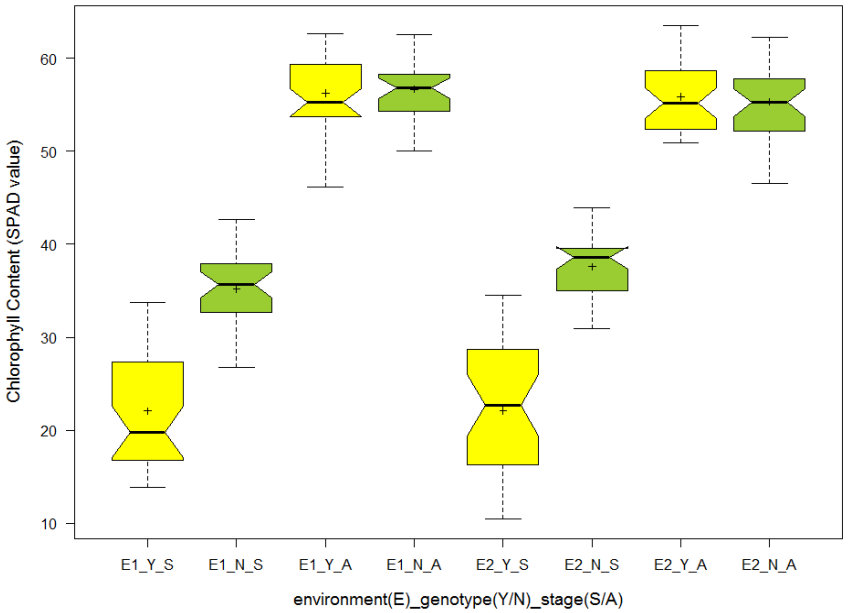
The effect of genotypes on all traits measured was significant under both herbicide treated and untreated conditions except grain yield. Mean plant height of ALS resistant genotypes ranged from

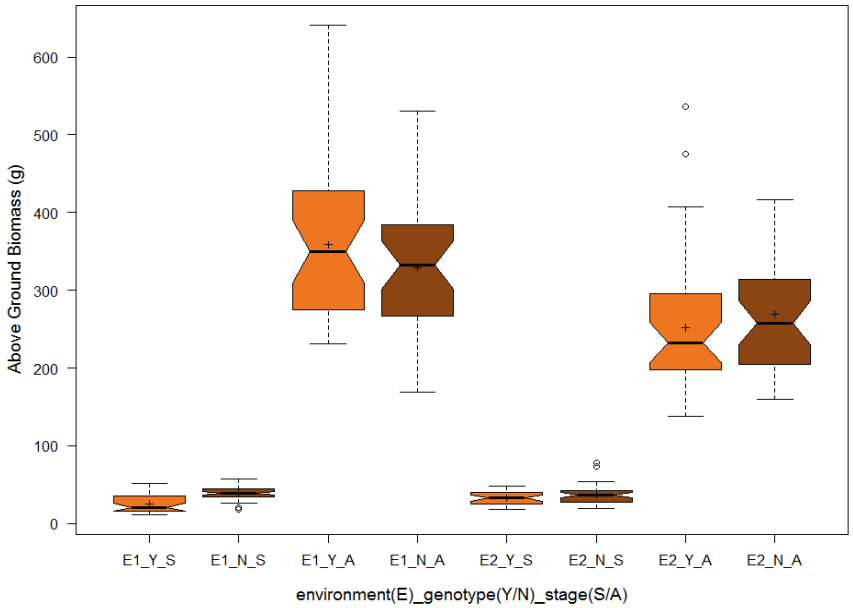
89 to 138cm in the control and 90 to 131 in herbicide treated plots of genotypes MN07-2165 and MN13-7500, respectively (Table 3.10). The check genotype Tx430 was about 116cm tall without herbicide treatment. Similar variability was observed for all other traits. The longest maturing genotype flowered 14 days after the normal check genotype Tx430. However, 13 genotypes out 36 were earlier than this check. For the yield components, out of the total of 36 ALS resistant genotypes included, 16, 18, 20 and 33 of them had mean panicle width, panicle length, panicle weight and KN larger or equivalent to the check genotype Tx430 (Table 3.10). However, only three and six genotypes had mean panicle length and grain yield higher than the check while none were superior to the check for TKW. Comparison with the check was not possible under herbicide treatment, but except for days to flowering, herbicide did not affect any other trait measured. The result was fairly consistent across locations as well though few genotypes apparently showing differential response for some of the traits. Correlation between the different yield components is presented in Table 3.11. All measured yield components as well as chlorophyll content and pant height showed positive and significant correlations with the grain yield across environments except for days to flowering that showed significant inverse correlations with all measured parameters except with panicle length. No correlations were observed for chlorophyll content with plant height or kernel number. At the same time, kernel number per panicle did not show any correlations with thousand kernel weight. All correlations between other parameter pairs were significant and positive across environments.

Because the resistance trait is introduced from wild background, it is possible that undesirable alleles can drag with the resistance genes that may compromise productivity or utilization of the crop. Hence, in addition to the agronomic parameters, tests were conducted to evaluate nutritional profile of the ALS resistant materials. The results are presented in Tables 3.12-

3.14). The combined analysis of variance showed significant genotype effect for all nutritional parameters including protein, starch, fat and ash content (Table 3.12). Protein content among genotypes ranged from 12.4 to 17.0 while starch ranged from 71.6 to 76.8%. As expected the genotype with the highest protein content had the lowest starch. The fat content ranged from 4.8 to 5.2 % while ash was between 1.33 and 1.7% (Table 3.14). There was no significant difference between yellow and green genotypes for any of these traits both in the combined as well as individual location analysis (Table 3.13).

Figure 3.1. ALS resistant genotypes expressing yellow (left) and green (right) seedling phenotypes (a) at seedling stage (three weeks old) and (b). after recovery of the yellowing symptom (six weeks old). (c). A close-up of interveinal yellowing symptom on leaves. (d). Appearance of an ALS herbicide resistant genotype (left) and herbicide susceptible check (Tx430) one week after the herbicide treatment.





Pair tested	t value	P-value	†Y mean	‡N mean
E1_Y_S vs. E1_N_S	-11.89	<0.0001	22.06	35.22
E1_Y_A vs. E1_ N_A	-0.50	0.62	56.24	56.66
E2_Y_S vs. E2_ N_S	-12.16	<0.0001	22.12	37.58
E2_Y_A vs. E2_ N_A	0.58	0.56	55.85	55.33

Pair tested	t value	P-value	†Y mean	‡N mean
E1_Y_S vs. E1_ N_S	-5.41	<0.0001	25.59	39.02
E1_Y_A vs. E1_ N_A	1.34	0.18	358.39	329.09
E2_Y_S vs. E2_ N_S	-1.83	0.07	32.51	37.34
E2_Y_A vs. E2_ N_A	-0.91	0.37	251.95	269.59

Figure 3.2. A modified version of multiple boxplots displaying variability between genotypes showing seeding yellow and normal phenotypes for (a) seedling and adult plant chlorophyll content and (b) above ground biomass measured in herbicide treated conditions under two environments. The '+' sign denotes the sample mean and the notch represents 95% confidence interval for the median. The table shows results of each two sample t-test performed between yellow and normal genotypes for each stage and environment. X axis labels read as, E1 and E2=Environment 1 and 2, Y and N = genotypes showing yellow and normal seedling phenotypes, S and A = seedling and adult stage of growth. †Y mean=mean of yellow genotypes, ‡N mean=mean of normal genotypes

Table 3.1. List and some of the characteristics of the ALS resistant breeding lines included in the study.

Genetic Material	Fertility Reaction with A1 cytoplasm	Seedling Color	Resistance to ALS Herbicides
PR12/13-764-4	Maintainer (B)	Yellow	Resistant
MN13-7450	Maintainer (B)	Green	Resistant
MN07-2118	Maintainer (B)	Yellow	Resistant
PR12/13-763-5	Maintainer (B)	Yellow	Resistant
PR12/13-762-2	Maintainer (B)	Yellow	Resistant
MN13-7455	Maintainer (B)	Yellow	Resistant
PR12/13-763-3	Maintainer (B)	Yellow	Resistant
PR11/12-873	Restorer (R)	Yellow	Resistant
MN13-7458	Maintainer (B)	Green	Resistant
PR12/13-764-6	Maintainer (B)	Yellow	Resistant
PR12/13-763-1	Maintainer (B)	Green	Resistant
PR12/13-761	Maintainer (B)	Yellow	Resistant
MN07-1916	Restorer (R)	Green	Resistant
MN13-7840	Restorer (R)	Yellow	Resistant
MN13-7498	Maintainer (B)	Green	Resistant
MN13-7462	Maintainer (B)	Green	Resistant
PR9/10-4720-1	Maintainer (B)	Yellow	Resistant
PR11/12-984	Maintainer (B)	Yellow	Resistant
PR12/13-764-1	Maintainer (B)	Yellow	Resistant
MN11-10362	Restorer (R)	Yellow	Resistant
PR12/13-762-1	Maintainer (B)	Yellow	Resistant
PR11/12-850	Restorer (R)	Yellow	Resistant
PR11/12-1026	Maintainer (B)	Green	Resistant
MN13-7499	Maintainer (B)	Yellow	Resistant
PR12/13-763-4	Maintainer (B)	Yellow	Resistant
MN13-7463	Maintainer (B)	Yellow	Resistant
PR11/12-851	Restorer (R)	Green	Resistant
PR12/13-764-2	Maintainer (B)	Yellow	Resistant
PR12/13-764-3	Maintainer (B)	Yellow	Resistant
MN13-7838	Restorer (R)	Yellow	Resistant
MN13-7500	Maintainer (B)	Yellow	Resistant
MN07-2165	Maintainer (B)	Yellow	Resistant
PR12/13-763-2	Maintainer (B)	Green	Resistant
MN13-7439	Maintainer (B)	Green	Resistant
PR11/12-852	Restorer (R)	Yellow	Resistant
MN13-7923	Restorer (R)	Green	Resistant
Tx430	Restorer (R)	Green	Susceptible

Table 3.2. Analysis of variance for physiological and agronomic characteristics of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment.

Source of variation	df	Chlorophyll Content (SPAD)		Plant height		Biomass		Days to flowering
		Seedling	Adult plant	Seedling	Adult plant	Seedling	Adult plant	
Combined analysis								
Environment (E)	1	1360.6**	98.0	3.1	5191.5*	3476.53*	188616**	606.6*
Block/E	2	7.7	22.8	1.3	5.9	42.3	1656.4	18.7
Herbicide treatment (T)	1	54.0	69.5	430.3*	33	1790.9*	41744*	203.3*
T x E	1	115.3	82.1	18.3	99.1	54.3	7753	11.6
Error a	2	22.2	9.1	5.4	42.1	86.3	97.7	10.8
Genotype (G)	35	401.2**	108.5**	23.7**	152**	811.6**	35774**	337.5**
G x T	35	25.1**	41.7**	5.3	101*	180.6**	7774.6**	14.6**
G x E	35	22.2**	42.7**	7.3	53.2	358.7**	5792.5**	65.3**
G x T x E	35	25.4**	34.3**	4.4	9.9	137.5**	4392.3**	21.2**
Error b	140	9.3	6.3	4.5	5.3	54.8	55.2	4.0
Environment 1								
Block	1	11.3	5.84	1.9	0.6	6.2	2756.2	5.4
T	1	746.7**	153.4	318.8**	265.5**	6836.8**	2777.2	58.7
Error a	1	0.92	4.0	3.6	0.1	188.2	2916	1.4
G	35	226.0**	117.8**	21.5**	158.9**	879.3**	21875**	129.2**
G x T	35	12.8**	64.2**	8.47	8.2	153.7*	7063**	8.2**
G x Block	35	3.4	2.6	6.9	0.9	68.5	2314.7	1.6
Error b	35	3.0	2.4	7.6	0.7	102.1	2462	0.7
Environment 2								
Block	1	3.9	54.2	0.55	11.1	57.7	556.5	32.1
T	1	1422.5*	0.3	134.4*	66.7	757.1*	116719*	156.2
Error a	1	43.2	7.1	7.3	64.0	46.2	2753.6	20.2
G	35	197.4**	32.5**	8.8**	46.3**	281.0**	19692**	273.6**
G x T	35	37.6**	12.2	1.3	11.8	161.0*	5104.1**	27.6**
G x Block	35	13.6	14.0	1.5	9.8	28.8	743.1	10.5
Error b	35	17.5	6.5	1.9	9.9	22.4	1183.8	7.3

* and ** statistically significant at $P \leq 0.05$ and 0.01, respectively.

Table 3.3. Analysis of variance for physiological and agronomic characteristics of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes of contrasting seedling color (yellow vs. green) evaluated with and without herbicide treatment.

Sources of variation	df	Chlorophyll content		Days to flowering	Plant height		Biomass	
		Seedling	Adult plant		Seedling	Adult plant	Seedling	Adult plant
Combined analysis								
Environment (E)	1	1130.0**	149.2*	838.5**	9.2	4157.5**	5508.7**	140997**
Block/E	2	7.6	21.6	18.7	1.3	5.8	35.5	38883
Herbicide treatment (T)	1	163.2*	80.0	181.1	399.8**	49.9	2633.6*	1656.4*
T x E	1	13.0	81.3	30.7	10.0	23.5	325.8	546
Error a	2	22.0	10.0	10.8	5.2	42.0	91.0	2834.8
Color(C)	1	6087.9**	23.7	192.5*	122.6**	109.1	8043.2**	16071
C x T	1	305.9**	14.2	0.001	0.7	24.4	1176.0**	187.1
C x E	1	12.1	67.3	296.1*	15.8	107.3	2685.2**	10383
C x T x E	1	20.5	0.8	48.4	5.5	25.2	741.9*	80.9
Error b	4	41.5	31.5	56.2	6.8	30.3	170.8	7567.3
Environment 1								
Block	1	12.7	5.7	2.1	0.6	0.7	138.9	2462.2
T	1	514.9**	62.6	31.3	270.2**	313.9**	9338.8**	1966.1
Error a	1	0.9	4.0	1.3	3.6	0.09	135.9	2916.0
C	1	2778.2**	5.6	483.0**	114.2**	216.4*	9938.3*	309.3
C x T	1	83.9	11.0	23.9	1.1	49.7	1886.3	257.0
C x Block	1	1.3	0.1	4.7	2.6	0.1	662.7	0.1
Error b	1	41.7	47.6	31.9	10.5	41.1	219.5	8609.1
Environment 2								
Block	1	3.8	42.3	32.0	0.5	5.8	48.0	520.0
T	1	1661.2*	23.5	180.5	140.4*	59.5	589.3*	104463**
Error a	1	43.2	4.9	20.2	6.9	84.0	46.2	2753.6
C	1	3321.8**	84.2	5.5	24.9**	0.003	719.4*	26144*
C x T	1	242.5	4.2	24.5	5.0	0.003	24.9	10.9
C x Block	1	0.08	15.8	0.8	0.03	4.7	0.5	2.8
Error b	1	42.0	15.8	81.2	3.2	19.8	120.6	6636.0

* and ** statistically significant at $P \leq 0.05$, and 0.01, respectively.

Table 3.4. The combined mean of the effect of herbicide treatment and seedling phenotype on physiological and agronomic characteristics of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment.

Treatment effect	Chlorophyll content (SPAD units)		Days to Flowering	Plant height (cm)		Biomass(g)	
	Seedling	Adult Plant		Seedling	Adult plant	Seedling	Adult plant
Herbicide							
Untreated	28.3 (±0.32)	55.4 (±0.46)	71.6 (±0.32)	18.5 (±0.22)	116.0 (±1.45)	38.7 (±1.5)	326.8 (±7.6)
Treated	29.1 (±0.32)	54.4 (±0.46)	73.2 (±0.32)	16.0 (±0.22)	114.3 (±1.45)	33.7 (±1.5)	302.7 (±7.6)
Mean	28.7	54.9	72.4	17.3	115.2	36.2	314.8
†LSD	ns	ns	ns	0.62	ns	3.65	21.2
Seedling color							
Yellow	25.4 (±0.52)	54.7 (±0.4)	73.0 (±0.55)	16.8 (±0.20)	116.3 (±1.25)	32.4 (±1.3)	309.5 (±6.6)
Normal	35.2 (±0.74)	55.3 (±0.6)	71.1 (±0.78)	18.2 (±0.29)	113.0 (±1.77)	43.6 (±1.8)	325.4 (±9.3)
Mean	30.3	55.0	72.1	17.5	114.7	38.0	317.5
†LSD	1.80	ns	1.88	0.71	ns	3.69	ns

†LSD = Least significant difference; ns = not significant.

Table 3.5. Combined data for phenology and growth characteristics of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment during the 2013 and 2014 seasons.

Genotype	Chlorophyll content (SPAD units)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
MN13-7450	39.1	40.4	56.3	57.2	69.0	69.0	19.9	17.3	105.8	99.0	22.7	19.1	477.2	345.7	G
MN07-1916	34.4	38.8	63.0	60.9	65.3	66.5	19.0	17.6	111.0	103.5	44.5	41.4	248.0	253.7	G
PR11/12-1026	34.6	38.6	55.0	58.7	68.3	68.5	19.8	16.9	119.3	108.8	47.0	58.8	256.0	302.2	G
PR12/13-763-4	34.4	33.4	48.2	41.9	77.5	76.3	17.5	16.6	99.5	112.3	37.1	35.0	404.0	256.9	Y
MN13-7462	34.5	38.3	57.6	58.7	66.0	65.8	19.3	16.8	110.8	104.0	29.4	15.8	322.7	215.2	G
MN13-7458	35.9	37.9	50.3	54.7	68.3	66.8	20.9	18.3	111.0	112.5	42.6	38.1	328.2	209.0	G
MN11-10362	31.4	33.2	56.5	56.1	74.8	79.5	19.0	17.8	131.3	132.0	40.6	32.2	417.5	461.2	Y
PR12/13-763-1	31.1	37.9	52.6	45.1	78.5	75.0	17.8	15.0	107.8	108.0	41.7	38.3	460.0	374.3	G
PR12/13-763-2	31.8	37.1	51.6	45.0	78.5	83.8	17.0	14.4	112.0	114.5	58.8	54.9	246.4	248.5	G
PR12/13-763-5	33.1	32.9	55.3	38.1	73.8	80.3	17.9	16.2	111.0	113.0	23.2	22.4	332.3	285.8	Y
MN13-7439	29.2	36.8	56.1	55.9	67.5	66.8	21.4	17.4	128.8	123.3	46.4	29.8	356.7	297.9	G
PR12/13-763-3	28.4	31.5	52.7	55.0	77.5	81.0	17.6	16.2	108.0	112.8	20.8	20.6	388.8	249.3	Y
MN13-7923	34.3	35.3	57.3	49.0	63.3	71.0	19.9	16.8	105.8	103.8	26.6	34.2	325.7	293.1	G
MN13-7498	32.8	34.6	55.3	54.1	63.0	65.0	20.9	19.2	116.3	107.0	49.6	31.8	293.7	344.8	G
MN07-2118	29.7	33.1	55.0	53.6	65.0	65.5	18.0	16.1	114.8	108.5	62.1	41.3	245.1	214.0	Y
PR11/12-851	31.2	32.2	62.6	59.0	84.0	89.0	19.5	16.2	120.8	118.5	56.0	38.7	331.1	401.1	G
MN13-7463	34.8	31.7	54.3	57.7	67.0	67.0	19.7	18.0	115.5	111.0	22.2	30.6	321.5	273.4	Y
PR11/12-852	30.4	31.4	54.9	55.4	83.3	88.5	18.5	16.2	125.5	126.3	41.4	26.6	315.2	367.2	Y
MN13-7838	29.6	31.4	57.4	59.3	59.5	63.0	18.6	16.5	107.3	99.0	22.7	28.7	215.1	209.9	Y
MN13-7840	30.7	30.3	58.2	57.2	61.5	63.5	20.7	18.0	106.3	103.3	50.1	33.3	279.3	220.6	Y
PR11/12-873	28.2	29.0	56.1	58.8	75.3	79.0	24.5	16.0	113.3	113.0	24.1	26.8	438.8	408.2	Y
PR11/12-850	28.3	29.5	58.5	58.7	76.8	84.3	18.6	16.1	125.0	134.5	38.0	37.1	390.2	355.0	Y
PR11/12-984	28.6	28.7	61.4	51.1	67.8	69.0	17.1	15.3	110.0	111.8	61.9	41.9	275.5	300.4	Y
MN13-7455	26.8	26.4	56.0	60.1	67.3	66.8	20.4	17.6	106.3	107.0	32.1	44.1	329.3	272.9	Y
MN13-7499	29.1	26.0	56.5	56.9	62.8	64.0	19.7	17.9	106.3	106.0	35.8	32.1	292.8	273.2	Y
MN07-2165	22.3	21.8	56.6	61.1	74.8	73.5	18.3	14.2	92.3	96.0	48.5	38.7	295.5	271.4	Y
PR12/13-762-2	23.3	21.3	56.1	55.2	74.3	78.3	15.4	14.3	109.3	110.3	43.7	22.3	339.0	330.1	Y
PR12/13-762-1	22.6	20.6	56.2	56.4	73.0	73.3	16.5	14.5	114.5	118.3	20.0	18.1	293.8	273.5	Y
PR12/13-764-2	18.9	18.9	52.5	53.2	72.5	73.0	16.8	13.8	134.5	129.5	25.7	20.8	295.7	276.8	Y
MN13-7500	19.9	17.9	52.3	55.3	74.3	76.0	18.7	15.8	138.5	131.3	39.6	44.5	496.1	471.9	Y
PR9/10-4720-1	19.4	17.8	56.0	57.6	76.5	78.8	15.6	15.1	127.5	124.5	33.0	34.6	321.2	426.6	Y
PR12/13-764-3	22.0	17.7	49.1	52.4	73.0	71.8	18.2	14.6	128.5	134.5	34.5	27.6	295.4	251.5	Y

Genotype	Chlorophyll content (SPAD units)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
PR12/13-761	19.4	17.2	53.8	53.5	79.0	79.3	13.8	12.6	106.0	104.0	31.4	38.2	295.2	265.6	Y
PR12/13-764-4	18.2	15.9	53.6	53.4	75.3	75.5	16.7	15.1	142.8	128.8	50.9	43.7	250.9	239.7	Y
PR12/13-764-1	19.4	14.3	58.3	52.7	74.0	74.5	15.7	14.9	133.8	128.5	31.5	30.7	269.4	321.7	Y
PR12/13-764-6	20.7	13.4	53.2	51.9	73.0	72.8	16.8	13.8	124.5	121.8	56.3	40.3	325.0	238.7	Y
Tx430	38.3	-	57.3	-	67.8	-	17.8	-	108.3	-	37.6	-	352.6	-	G
Mean	28.3	29.1	55.4	54.4	71.6	73.3	18.5	16	116	114.2	38.7	33.7	327.5	300.0	-
‡LSD	4.8	4.2	4.5	5.1	5.7	5.8	4.03	3.8	20.2	18.4	12.5	11.3	24.0	26.4	-

†Seedling color Y= yellow and G = Green.

‡ LSD = Least significant difference.

Table 3.6. The correlation between the seedling chlorophyll contents and physiological parameters measured at seedling and adult plant stage.

Parameter tested against Seedling chlorophyll content	Environment 1		Environment 2	
	Untreated	Treated	Untreated	Treated
Seedling height	0.41**	0.71**	0.34**	0.27*
Seedling biomass	0.69**	0.72**	0.29*	0.25*
Days to flowering	-0.52**	-0.50**	-0.11	-0.15
Adult plant height	-0.58**	-0.50**	-0.05	-0.04
Adult plant biomass	-0.13	-0.25	-0.12	-0.15
Adult plant chlorophyll content	0.13	0.16	0.29	0.30

* and ** statistically significant at $P \leq 0.05$ and 0.01, respectively.

Table 3.7. Mean squares for agronomic characteristics and yield components of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment under Experiment II.

Sources of variation	df	Chlorophyll content	Plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
Combined analysis											
Environment (E)	1	127.1	6348.0**	569.4*	952.6*	44.2**	220237**	66035**	54373**	896.1**	952711*
Block/E	4	51.0	33.2	67.5	6.4	0.30	453.0	374.4	1567	11.7	11522
Herbicide Treatment (T)	1	47.8	3.0	675.0**	142.9*	0.02	436.4	0.2	205.2	6.0	42986
T x E	1	4.0	149.3	37.0	2.4	0.7	324.3	0.8	5690	3.9	40401
Error a	4	16.7	39.3	16.4	10.7	1.0	295.0	157.6	3174	1.7	29270
Genotype (G)	35	38.0**	150.5**	433.2**	41.8**	4.1**	3476.2**	1849.7**	30130**	75.1**	36202
G x T	35	7.8	8.2	44.7*	6.3*	0.4	493.9	298.2	3545	11.6**	54478**
G x E	35	18.3*	38.8	107.3**	9.8*	1.1**	1679.1**	826.2**	12310**	12.2**	54372**
G x T x E	35	8.3	8.0	48.2**	7.2	0.38	447.0	267.5	3556	8.6**	54372
Error b	136	8.7	6.6	20.1	3.8	0.47	381.1	212.5	2963	4.7	30373
Environment 1											
Block	2	59.9	27.3	98.2	13.2	0.5	623.2	296.2	119056	9.8	26105
T	1	15.9	97.3	856.0**	222.1*	2.6	4.6	4.3	216230	54.5**	55972
Error a	2	4.6	59.0	0.7	19.4	0.8	224.5	146.7	211682	2.7	24642
G	35	36.9**	73.5**	410.4**	30.7**	2.0**	1926.8**	1088.9**	1410415**	42.7**	151073*
G x T	35	11.3	11.3*	64.6	9.6	0.4	544.8*	288.3	367180	16.8**	48387
G x Block	70	9.8	9.0	18.7	4.9	0.4	378.3	230.3	374295	7.0	32262
Error b	70	12.1	6.2	18.6	3.6	0.5	436.1	223.5	347011	7.3	45544
Environment 2											
Block	2	43.6	39.1	36.9	0.02	0.1	268.5	445.5	180260	11.9	106426
T	1	40.1	55.0	56.0*	22.7*	3.2	764.8	0.08	277924	17.7	2119.0
Error a	2	30.6	19.6	32.2	1.0	1.1	392.6	169.3	444663	1.3	466089
G	35	18.5**	115.7**	130.1**	21.0**	3.2**	3217.6**	1582.0**	2813183**	44.1**	3616335*
G x T	35	5.1	74.9*	48.3	12.8	0.3	314.7	237.0	291438	3.2	441975
G x Block	70	6.6	4.9	22.0	3.1	0.4	316.8	183.5	215840	2.4	195551
Error b	70	6.6	6.4	21.1	3.5	0.5	395.7	213.4	251142	2.1	270025

†KN = Kernel number per panicle; ‡TKW = thousand kernel weight; * and ** statistically significant at $P \leq 0.05$ and 0.01, respectively.

Table 3.8. Mean squares for agronomic characteristics and yield components of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes with contrasting seedling color evaluated with and without herbicide treatment under Experiment II.

Sources of variation	df	Chlorophyll content	Plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
Combined analysis											
Environment	1	106.8**	5964.0**	663.2*	825.4**	36.0**	185031**	55613**	4289522**	840.8**	992194*
Block/E	4	51.2	33.2	67.5	7.4	0.3	504.1	392.3	160946	11.6	123020
Herbicide Treatment (T)	1	32.1	4.4	585.0**	108.1**	0.02	329.9	3.0	1308.2	4.2	23377
T x E	1	1.7	111.2	33.3	81.1	4.7	167.8	6.2	746830	60.8	69935
Error a	4	16.9	39.3	16.4	12.1	1.0	335.0	193.8	367695	1.9	31382
Color(C)	1	57.1	23.3	321.4*	0.2	4.9*	2774.5	667.1	157753	159.0**	3842
C x T	1	8.3	2.0	0.8	3.1	0.05	11.2	1.2	719.0	1.1	13583
C x E	1	0.2	40.0	95.3	4.6	0.9	2228.7	726.9	2361440*	6.1	24384
C x T x E	1	15.8	8.5	5.1	0.4	0.2	408.8	226.1	400246	0.2	55944
Error b	8	11.7	21.6	65.8	8.0	0.8	756.8	411.4	612431	11.9	35789
Environment 1											
Block	2	42.3	10.6	99.2	21.3	1.2	1116.1	488.9	209496	11.9	2753638
T	1	3.2	80.0	778.7**	185.0*	2.1	8.5	0.2	299127	38.1	7713986
Error a	2	3.3	59.0	0.7	23.3	0.9	282.5	237.4	313044	2.4	1781969
C	1	53.7	1.1	33.3	3.2	5.0*	429.4	149.4	1727806	50.3*	89186
C x T	1	23.3	1.1	0.9	0.7	0.2	247.5	98.6	169965	1.2	490706
C x Block	2	3.9	30.0	9.7	17.6	1.5	1819.3	279.4	1146043	0.8	5738478
Error b	2	15.3	19.3	93.1	9.8	0.7	667.8	378.5	542189	15.1	4322051
Environment 2											
Block	2	44.2	27.6	43.5	1.0	0.2	392.7	549.7	239630	16.7	1187869
T	1	38.8	35.5	39.7	0.9	2.7	487.6	9.1	410515	16.7	650567
Error a	2	30.6	19.6	32.2	1.0	1.1	392.6	169.3	444663	1.3	4660897
C	1	43.7	62.2	383.4**	1.3	0.7	15.0	97.3	657306	115.2**	2482073
C x T	1	0.6	9.4	5.1	3.0	0.01	143.2	97.9	185796	0.1	6502150
C x Block	2	2.7	7.5	11.3	6.9	0.2	203.9	119.6	96173	7.8	611853
Error b	2	8.2	23.9	39.6	6.2	0.9	839.5	441.8	681141	8.9	2897860

†KN = Kernel number per panicle; ‡TKW = thousand kernel weight; * and ** statistically significant at $P \leq 0.05$ and 0.01, respectively.

Table 3.9. The combined mean of the effect of herbicide treatment and seedling phenotype on agronomic parameters and yield components of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment.

Treatment Effects	Chlorophyll content	Adult plant Height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†TKW	‡KN	Grain yield
Herbicide										
Untreated	55.9(±0.39)	115.3(±1.0)	72(±0.45)	27.2(±0.16)	5.8(±0.04)	98.8(±1.34)	65.2(±1.15)	25.5(±0.20)	2563(±36.62)	3334.7(±136.60)
Treated	56.6(±0.39)	114.0(±1.0)	75(±0.45)	28.4(±0.14)	5.9(±0.05)	100.8(±1.44)	65.1(±1.21)	25.3(±0.19)	2567(±37.94)	3509.7(±137.49)
Mean	56.3	114.7	73.5	27.8	5.8	99.8	65.1	25.4	2565	3422.2
§LSD	ns	ns	1.55	0.61	ns	ns	ns	ns	ns	ns
Seedling color										
Yellow	55.9(±0.20)	114.3(±0.82)	74(±0.48)	27.7(±0.19)	5.8(±0.06)	98.0(±1.61)	63.8(±1.40)	24.95(±0.20)	2548(±50.76)	3457.1(±118.36)
Normal	56.9(±0.29)	114.1(±1.01)	72(±0.68)	27.8(±0.27)	6.0(±0.08)	103.4(±2.29)	68.0(±1.99)	26.26(±0.28)	2588(±72.04)	3397.4(±168.29)
Mean	56.4	114.2	73	27.7	5.9	100.7	65.9	25.6	2568	3427.2
§LSD	ns	ns	1.65	ns	0.19	ns	ns	0.75	ns	ns

†TKW = Thousand kernel weight; ‡KN = Kernel number per panicle;
§LSD = Least significant difference; ns = not significant.

Table 3.10. Combined mean for agronomic characteristics and yield components of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment.

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle yield		Panicle weight		‡KN		†TKW		Grain yield		§SC
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
MN07-2165	88.8	90.5	75.7	72.5	24.0	26.7	5.1	5.5	43.4	52.1	68.4	79.2	2234	2160	20.5	24.0	2076.1	4502.9	Y
PR11/12-984	113.8	112.5	67.8	69.7	24.6	27.8	5.7	6.3	55.1	63.6	84.1	95.9	2230	2603	24.7	24.3	3481.5	5092.5	Y
MN13-7450	107.0	105.0	76.5	74.3	26.7	28.7	6.8	6.9	72.5	72.3	113.7	119.8	2772	2738	26.2	25.7	3914.0	3414.4	G
PR11/12-851	108.8	118.3	74.7	85.8	28.2	29.1	5.2	4.4	57.0	35.3	85.6	61.4	2024	1327	28.3	26.2	3109.0	3296.7	G
PR11/12-873	110.0	114.5	75.7	81.2	30.4	31.6	5.3	5.4	67.2	75.1	97.7	109.0	2314	2545	29.2	29.0	3558.7	4278.3	Y
PR11/12-1026	122.0	114.5	71.2	70.7	29.1	28.4	6.9	7.0	85.5	80.8	125.0	115.0	2895	3029	29.5	26.6	3081.0	4924.5	G
MN07-1916	110.5	109.5	59.8	67.7	24.0	24.9	5.3	5.2	40.0	38.0	66.1	67.2	1632	1516	24.5	25.3	3691.2	3619.1	G
MN13-7455	113.0	109.3	70.2	68.7	32.4	33.2	6.4	7.0	75.6	79.5	117.2	124.8	3227	3353	23.4	23.6	4143.2	3722.4	Y
MN11-10362	126.3	127.5	75.5	81.2	29.6	30.9	5.3	5.4	70.9	64.8	102.9	106.0	2504	2817	24.9	22.5	3501.9	3312.2	Y
MN13-7439	124.5	123.8	70.2	73.7	29.9	29.1	6.6	6.2	89.2	74.7	129.1	107.4	3446	2945	25.8	25.3	3027.9	4205.0	G
PR9/10-4720-1	120.0	119.5	75.3	80.3	27.9	28.1	5.9	5.9	78.8	65.7	112.4	106.8	3321	2647	23.5	24.2	2769.8	3384.2	Y
PR11/12-850	125.5	128.3	78.5	86.7	26.1	30.1	5.3	4.7	63.3	53.6	81.0	80.1	2160	1927	28.6	27.0	2522.6	5154.5	Y
MN13-7923	112.5	110.5	69.0	69.5	29.0	27.7	5.3	5.0	54.2	52.2	84.6	80.6	2254	2116	24.1	24.4	2859.0	2970.5	G
MN13-7463	120.5	116.8	64.5	69.8	32.6	31.5	6.3	6.0	80.5	78.2	120.4	120.2	3476	3339	23.1	23.3	3348.0	2950.7	Y
MN13-7840	106.3	101.8	67.2	71.0	24.6	25.7	5.7	5.6	53.4	54.0	82.0	81.5	2154	2272	24.5	23.9	2993.0	5889.9	Y
PR12/13-763-3	107.0	107.0	78.0	83.8	26.5	26.8	5.8	5.2	68.2	60.7	102.7	97.2	2945	2584	22.6	22.7	2679.1	4074.1	Y
PR11/12-852	114.3	121.3	85.8	89.8	25.5	29.2	4.4	4.4	38.8	27.5	48.1	59.8	1126	1334	24.9	22.5	2882.3	4506.3	Y
MN13-7838	111.8	103.8	58.8	66.7	24.0	25.9	5.2	5.1	50.8	52.8	78.0	80.3	1894	1834	26.5	28.5	3640.5	4102.6	Y
MN13-7462	116.8	115.8	68.5	68.7	28.3	28.1	7.0	6.5	86.5	75.3	131.8	120.8	3698	3074	23.2	24.6	3697.8	2757.4	G
PR12/13-762-2	102.0	103.3	74.3	78.2	25.6	27.1	5.9	5.7	60.6	58.7	94.7	95.0	2442	2455	24.8	23.4	4264.5	2349.4	Y
MN13-7499	118.3	118.0	64.0	65.3	28.2	28.8	6.2	5.6	71.8	76.2	105.6	107.1	2534	2630	28.5	28.8	2740.1	2526.8	Y
PR12/13-764-1	125.0	122.0	71.8	72.5	27.4	27.6	6.3	5.9	73.7	73.4	113.9	110.5	3024	2956	24.4	24.7	4257.0	3544.6	Y
MN13-7500	138.3	131.3	72.0	73.3	29.3	30.7	5.7	5.9	77.1	73.6	112.3	103.7	2242	2534	34.4	29.0	2724.7	2383.5	Y
PR12/13-763-2	113.3	120.8	74.5	84.7	27.7	28.3	5.7	6.2	70.2	78.0	104.9	119.4	2806	3252	24.6	23.1	3493.5	3007.7	G
PR12/13-762-1	109.3	113.0	73.3	73.7	27.8	28.4	5.9	6.2	67.8	62.2	105.2	103.2	2724	2593	24.7	24.0	4891.1	2613.1	Y
PR12/13-761	96.3	100.8	76.8	80.2	26.1	29.8	5.7	6.4	57.8	72.0	90.9	118.3	2429	3014	23.0	23.2	3417.6	2621.1	Y
MN07-2118	110.0	114.5	69.3	69.5	26.7	27.4	5.6	5.9	53.1	55.6	88.6	89.7	2042	2103	26.1	26.3	2999.5	3457.1	Y
PR12/13-763-4	113.3	115.5	78.0	81.5	27.6	27.6	5.7	5.6	69.8	58.4	106.0	92.2	2771	2520	25.7	22.7	4028.0	2459.9	Y
PR12/13-763-1	104.5	109.5	79.8	76.3	25.6	30.4	5.3	6.2	56.6	87.0	89.9	129.6	2631	3424	21.1	25.4	3533.1	3949.6	G
MN13-7458	115.5	119.5	69.8	68.7	26.1	28.7	6.3	7.1	61.2	83.3	90.2	120.1	2141	2550	28.4	32.4	3122.9	2814.1	G
PR12/13-764-4	126.8	127.0	73.5	78.8	27.4	28.3	6.1	5.7	68.4	67.8	105.2	100.5	2764	2740	24.6	24.0	3720.2	2903.9	Y
PR12/13-764-6	113.8	112.5	72.8	73.0	27.2	26.1	6.0	6.0	73.4	59.9	110.0	96.7	3005	2390	24.3	24.6	4080.7	3865.4	Y

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle yield		Panicle weight		‡KN		†TKW		Grain yield		§SC
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
PR12/13-764-2	126.3	124.5	73.2	70.7	28.1	28.4	6.4	6.5	74.5	76.5	119.5	118.5	3113	3103	23.5	24.4	2445.3	3208.0	Y
PR12/13-764-3	118.8	123.0	73.3	71.3	25.6	27.7	6.3	6.2	61.6	71.2	100.4	111.0	2526	2921	24.2	24.1	2960.2	2683.2	Y
PR12/13-763-5	110.0	112.0	81.7	85.0	25.6	27.0	5.4	5.5	57.8	57.0	87.0	90.1	2543	2483	22.4	22.5	2774.6	2482.1	Y
MN13-7498	118.0	115.5	65.5	62.3	24.3	25.8	5.9	6.2	70.6	78.8	102.3	113.4	2223	2480	31.8	31.8	3610.1	3321.0	G
Tx430	115.8	-	72.3	-	30.0	-	5.9	-	68.1	-	99.4	-	1974	-	35.0	-	3956.0	-	G
Mean	115.3	114.0	72.4	74.9	27.2	28.4	5.8	5.9	65.2	65.1	98.8	100.8	2562	2563	25.4	25.2	3334.7	3509.7	-
¶LSD	16.5	13.8	5.8	5.3	3.58	3.75	0.8	1.0	23.8	25.2	36.9	39.9	192	184	3.09	3.68	504.9	510.2	-

†KN = kernel number per panicle; ‡TKW = thousand kernel weight;

§ SC= Seedling color Y = Yellow G = Green;

¶ LSD = Least significant difference.

Untrt.= without herbicide treatment, Trt. = Herbicide treated.

Table 3.11. Correlation coefficients between all tested parameters across environments under Experiment II.

Parameter	Chlorophyll content	Plant height	Days to Flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW
Plant height	0.08								
Days to Flowering	-0.13*	-0.12*							
Panicle length	0.18**	0.51**	-0.04						
Panicle width	0.11*	0.27**	-0.31**	0.37**					
Panicle weight	0.12*	0.55**	-0.27**	0.64**	0.79**				
Panicle yield	0.12*	0.51 **	-0.29**	0.59**	0.77**	0.96**			
†KN	0.08	0.39**	-0.21**	0.56**	0.76**	0.90**	0.92**		
‡TKW	0.12*	0.38**	-0.32**	0.25**	0.23**	0.36**	0.43**	0.04	
Grain yield	0.14*	0.39**	-0.21**	0.33**	0.26**	0.48**	0.45**	0.42**	0.19**

†KN = kernel number per panicle; ‡TKW = thousand kernel weight;
* and ** statistically significant at P ≤ 0.05, and 0.01, respectively.

Table 3.12. Analysis of variance for nutritional parameters among ALS resistant genotypes subjected to herbicide treatment.

Sources of Variation	df	Protein%	Fat%	Starch%	Ash%
Combined analysis					
Environment (E)	1	421.3*	1.39	171.47**	0.39*
Block/E	4	0.5	0.10	0.16	0.02
Genotype (G)	35	5.10**	0.05**	9.27**	0.04**
G x E	35	25.93**	0.03*	1.28**	0.01**
Error	130	0.36	0.01	0.463837	0.01
Environment 1					
Block	2	0.42	0.03	0.003	0.01
G	35	3.26**	0.04*	5.46**	0.02**
Error	70	0.39	0.02	0.46	0.002
Environment 2					
Block	2	0.65	0.003	0.32	0.004
G	35	2.58**	0.04***	5.09**	0.02**
Error	70	0.33	0.02	0.45	0.005

* and ** statistically significant at $P \leq 0.05$, and 0.01, respectively.

Table 3.13. Analysis of variance for nutritional parameters among ALS resistant sorghum expressing normal and yellowish seedling phenotypes following herbicide treatment.

Sources of Variation	df	Protein%	Fat%	Starch%	Ash%
Combined analysis					
Environment (E)	1	349.6**	1.4*	153.8**	0.4*
Block/E	4	0.5	0.1	0.2	0.02
Seedling Color(C)	1	7.8	0.03	0.03	0.01
C x E	1	0.3	0.05	0.7	0.02
Error	4	1.6	0.03	2.9	0.01
Environment 1					
Block	2	0.4	0.2	0.02	0.01
C	1	2.6	0.08	0.2	0.03
Error	2	1.8	0.03	3.0	0.01
Environment 2					
Block	2	0.6	0.003	0.3	0.04
C	1	4.6	0.002	0.5	0.01
Error	2	1.3	0.02	2.8	0.02

* and ** statistically significant at $P \leq 0.05$, and 0.01, respectively.

Table 3.14. Mean protein content, starch, fat and ash among ALS resistant sorghum genotypes subjected to herbicide treatment.

Breeding line	Protein%	Fat%	Starch%	Ash%	Seedling color
PR11/12-851	17.0	5.0	71.6	1.70	Green
MN07-2118	16.7	5.1	73.1	1.51	Yellow
MN13-7838	16.1	4.8	70.4	1.69	Yellow
PR11/12-852	15.7	5.1	71.7	1.61	Yellow
MN07-1916	15.6	4.8	72.5	1.66	Green
PR12/13-762-2	14.8	5.0	74.1	1.67	Yellow
PR11/12-1026	14.8	4.9	75.1	1.53	Green
MN13-7458	14.7	5.3	76.8	1.38	Green
MN11-10362	14.7	5.0	74.2	1.44	Yellow
MN13-7923	14.6	5.0	73.9	1.62	Green
PR12/13-764-2	14.5	5.1	74.2	1.52	Yellow
PR11/12-850	14.3	5.1	72.8	1.53	Yellow
PR12/13-764-4	14.2	5.1	74.	1.54	Yellow
MN13-7439	14.2	5.1	73.9	1.45	Green
PR11/12-984	14.1	4.8	73.8	1.62	Yellow
MN13-7499	14.1	5.0	74.5	1.50	Yellow
PR11/12-873	14.0	5.0	73.2	1.41	Yellow
PR12/13-762-1	13.8	5.1	74.8	1.58	Yellow
MN13-7462	13.8	5.0	74.1	1.53	Green
MN13-7498	13.7	5.1	76.5	1.38	Green
PR12/13-763-1	13.6	4.9	71.6	1.47	Green
MN13-7500	13.6	5.2	76.1	1.33	Yellow
MN13-7840	13.6	4.9	73.8	1.63	Yellow
PR12/13-763-3	13.4	4.9	71.6	1.48	Yellow
MN13-7450	13.4	4.9	73.7	1.47	Green
PR12/13-764-1	13.3	5.1	74.7	1.46	Yellow
PR12/13-764-6	13.3	5.1	74.6	1.43	Yellow
PR12/13-764-3	13.2	5.0	74.6	1.47	Yellow
PR12/13-763-5	13.0	4.9	71.7	1.51	Yellow
MN13-7455	12.9	5.0	74.0	1.35	Yellow
PR12/13-763-4	12.9	4.9	71.8	1.47	Yellow
PR12/13-761	12.9	5.1	75.1	1.47	Yellow
MN07-2165	12.8	5.2	76.0	1.35	Yellow
PR12/13-763-2	12.8	4.9	72.5	1.46	Green
MN13-7463	12.5	4.9	74.7	1.41	Yellow
PR9/10-4720-1	12.4	5.1	74.9	1.41	Yellow
Tx430	13.9	4.9	72.9	1.58	Green
Mean	14.0	5.0	73.8	1.5	-
†LSD	2.9	0.3	2.1	0.14	-

†LSD = Least significant difference.

Discussion

Crop establishment is an important prerequisite for successful crop production. This includes optimum germination and vigorous seedling growth (Brar and Stewart, 1994; Maulana and Tesso, 2013; Yu and Tuinstra, 2001). In sorghum, some of the yield components are determined as early as 30 d after germination (Maman et al., 2004; Wrather, 2009) hence in addition to fixing the crop stand and reducing weed competition, timely germination and seedling vigor have important agronomic implications. While germination is not an issue in herbicide resistant sorghums, reduced seedling vigor and low photosynthetic efficiency of chlorotic plants observed in some of the ALS resistant genotypes have been a source of concern (Weerasooriya et al., 2012). These characters are visible before herbicides are applied hence the concern about yield drag associated with ALS resistance seems real.

In this study we investigated the effects of both herbicide application and reduced seedling growth associated with interveinal chlorosis in some of the ALS resistant genotypes. In general, herbicide treatment markedly reduced seedling growth which appears to have interfered with biomass accumulation in adult plants (Table 3.4). Though our speculation was that the effect of herbicide on adult plant biomass may also be reflected on grain production, the second experiment showed not significant effect of herbicide treatment on grain yield. Nevertheless, not all genotypes are equally sensitive to herbicide treatment that some 30% of the treated entries had higher or comparable adult plant biomass with the untreated plots while 33% of the entries have higher or similar seedling biomass (Table 3.5). The significant herbicide treatment × genotype interaction observed in this study (Table 3.2) perhaps arises from differential response of genotypes. This result agrees with previous observations with other chemicals (atrazine and mesotrione) where sorghum genotypes respond differently to herbicide treatments (Abit et al., 2009; Ahrens et al., 1981). In addition, the arbitrary chemical dose used in this study may also be partly responsible for the current result. Since ALS

herbicides are not labeled for use on sorghum, the current rates, which is twice the recommended use rate for maize, may be too high that crop recovery from herbicide injury was not fast enough. ALS herbicides can kill susceptible sorghums at 35.03 g a.i. ha^{-1} (Anonymous., 1993) whereas the rate used in this experiment was 105.08 g a.i. ha^{-1}. In addition, this sampling is conducted on individual plant basis which introduces some sampling bias, and the result needs to be confirmed on larger plot experiments on non-inbred genotypes.

On the other hand, seedling chlorosis had a significant effect on seedling growth and biomass (Table 3.4). Reduction in leaf chlorophyll content (chlorosis) is often considered as an indicator of plant stress (Tjoelker et al., 1993). Thus, plants subjected to various types of abiotic stresses express different degree of chlorophyll breakdown (Jnandabhiram and Sailen Prasad, 2012; Sanchez et al., 1983). However, the chlorotic phenotypes being studied in this experiment are not caused by any of these stresses. As compared to the normal (green) genotypes, the chlorotic genotypes were 28, 8 and 26% less in seedling chlorophyll content, seedling height and biomass, respectively (Table 3.4). Perhaps due to the effect on seedling growth, flowering in chlorotic plants is delayed by two days. All these parameters were not affected in adult plant indicating that the yellowish seedling phenotype is a temporary growth phenomena that may not have any effect on adult plant performance.

The effect of herbicide treatment and seedling color on yield components and other adult plant characteristics was markedly different from that of the seedlings. Despite its significant effect on adult plant biomass as tested under Experiment I (Table 3.4), all agronomic and yield parameters were not affected except days to flowering for both seedling color and herbicide treatment, panicle length for herbicide treatment and, panicle width and thousand kernel weight for seedling color, grain yield and yield components were not affected by herbicide treatment under Experiment II (Tables 3.7-3.9). The data on Table 3.4 where adult plant biomass was affected by herbicide treatment was collected on individual plant as opposed to Table 3.9 where grain yield was recorded on whole plot basis. The

yellowish phenotype in ALS resistant lines express in different ways. Most genotypes emerge yellow and the phenotypes persist for a substantial period of seedling growth before it disappears which again is different in different backgrounds, whereas others emerge fairly green but suffer from yellowing at later stage. This phenomenon is further complicated by herbicide treatment. Thus it is possible that some genotypes may continue to express the yellow phenotype until later seedling growth stage that there was a concern this phenomenon might affect some of the yield components. However, the current result removes some of these worries. Grain yield in 50% of the tested genotypes was higher in treated plots than in the untreated and over 60% of these are genotypes from yellow background (Table 3.10) showing that herbicide treatment does not compromise yield and yield components even in genotypes that are suffering from yellowing. Therefore, despite the fact that seeing sorghum field turning yellow is not pleasant, the phenomena has no impact on yield potential thus though other genes dragging from the wild resistance gene donor may compromise yield, the yellow phenotype in itself do not seem to cause a yield penalty. Moreover, not all genotypes are sensitive to seedling chlorosis thus selection for backgrounds expressing little or no yellow phenotype can eliminate the undesirable characteristics. Moreover, neither herbicide treatment nor seedling color has impact on nutritional composition of ALS resistant sorghums. Although, there is significant difference among the genotypes for all nutritional attributes measured, the values are within published range of normal sorghum not subjected to herbicide treatment (Deosthale et al., 1970; Deyoe and Shellenberger, 1965; Edwards, 1943; Hubbard et al., 1950; Jambunathan and Subramanian, 1988; Mohan, 1968; Singh and Axtell, 1973b; Wall and Blessin, 1970). Moreover, genotypes of yellow seedling background as well as the susceptible normal sorghums were not different from the green background types for grain nutritional composition.

Conclusion

Loss of greenness upon stress conditions is a general tendency observed in crop plants. Despite the unattractive appearance and delayed flowering associated with growth retardation observed during seedling stages, ALS resistant genotypes carrying leaf chlorosis symptom did not seem to cause any substantial effect on plant performance in terms of both yield and yield components, agronomic and nutritional attributes. Though, the tested genotypes showed differential response to the herbicide treatment, majority of the genotypes that showed improved yield performance upon herbicide treatment implied potential for practicing selection among the backgrounds with ALS herbicide resistance. While, further research is needed for correcting the observed abnormal seedling phenotype towards timely delivery of the ALS resistant technology to the industries, current results revealed considerable capacity of ALS resistant genotypes towards improved agronomic and yield traits accompanied by reduced season-long weed competition.

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Chapter 4 - Agronomic performance of sorghum hybrids resistant to ALS and ACCase inhibitor herbicides

Abstract

Though still placed second among the major feed crops grown in the U.S., Sorghum (*Sorghum bicolor* (L.) Moench) acreage took a sharp decline over the past few decades. Among the many factors responsible for this decline include better weed control options that competing crops have offered. Sorghum farmers craved for years for an effective post-emergence weed control option. The discovery of the ALS and ACCase resistance traits in feral relatives of sorghum opened the way for breeding herbicide resistance in to cultivated sorghum. These developments have been received with much optimism for improving management of grass weeds in sorghum. The ALS trait offers resistance to a wide range of ALS herbicide chemistries, but many resistant plants tend to show interveinal chlorosis and reduced seedling vigor during seedling stages. While this phenotype persist for only few weeks, it is obviously not desirable. Moreover, the phenotype may possibly harm yield potential and grain quality. Moreover, because the resistance gene donors for both ALS and ACCase inhibitors come from wild relatives, there is a growing concern that some wild characteristics may drag in to the cultivated types to undermine yield potential. The industry is already speculating on how much yield penalty farmers are willing to accept while at the same time paying more for the resistance technology. It will be of significant interest to both growers and industries to clear these to facilitate the deployment of herbicide resistant hybrids in production fields. The objective of this study was to evaluate the agronomic adaptability, yield potential and

grain and nutritional quality attributes of ALS and ACCase resistant hybrids as compared to regular grain sorghum hybrids.

Key words: *Sorghum bicolor*, ALS herbicide resistance, ACCase herbicide resistance, yield drag, nutritional attributes.

Introduction

In the commercial agriculture of the western world, use of herbicides has become the major production input. Almost all major crops have benefited from the revolution that discoveries in herbicide chemistry have brought to weed management in farmers' fields. Unfortunately, despite the second most important feed grain in the world, sorghum was almost left out of any major breakthroughs that transformed the production of other crops. Hence, regardless of its inherent high yield potential, sorghum trails other crops in productivity. One of the technologies where sorghum was left behind is the technology that facilitate weed control.

Sorghum is naturally resilient to marginal growing conditions but due to its slow growth at crop establishment phase sorghum is relatively a poor competitor against early season weed flushes. Grass weed infestation, in particular is the greatest constraint to sorghum production in the mechanized world. While the adoption of modified rate of pre-plant weed control options from other crops have helped to control pre-emergence weeds in sorghum (Phillips and Ross, 1964; Wiese and Rea, 1962), the lack of post emergence weed management options have severely undermined the productivity of the crop.

Grass weeds such as crab grass, rye grass, shattercane and johnsongrass are among the most problematic weeds in sorghum fields. The morphological similarity of these weeds to the

sorghum crop make their management even more complicated as herbicides effective against these weeds can equally harm the crop. The development of glyphosate resistance has made these problems a history in many other major crops. The technology allowed over the top use of the "Roundup" to burn any vegetation in crop fields without harming the resistant crops. In 2015, 89% of maize, 94% of soybeans, and 89% of cotton produced in the United States were glyphosate resistant (roundup-ready) (USDA-ERS, 2015). Sorghum failed to benefit from this technology both due to its recalcitrance to genetic transformation (Zhu et al., 1998) and simple reluctance to deploy the technology in sorghum. The technology did not only leave out sorghum from benefiting but also put it at a disadvantage by increasing the fitness of the competitive crops and as result led to its displacement by others causing unfair harm to growers whose livelihoods are largely dependent on sorghum.

A limited grower funded research support at Kansas State University provided to study weed management options in sorghum led to the discovery of resistance sources to Acetolactate synthase (ALS) inhibitor herbicides among shattercane population. A parallel effort elsewhere also identified sources of resistance to acetyl co-enzyme-A carboxylase (ACCase) inhibitor herbicides. Both sources expressed stable resistance to all families of the ALS chemistry and the FOP family of the ACCase chemistry by tolerating the chemicals up 10 times the recommended use rates. This discovery attracted so much enthusiasm because of the possibility to develop and deploy herbicide resistant sorghum on to which over the top grass and broad-leaf killer herbicides can be applied. If successful this will be the first non-GM herbicide resistant sorghum ever developed. The resistance traits were quickly intorgressed into cultivated sorghum and by 2007 partially introgressed families of both B- and R-line backgrounds were transferred to the industry.

The ALS is the first enzyme in the biochemical pathway that leads to the synthesis of branched chain amino acids, valine, leucine, and isoleucine (McCourt and Duggleby, 2006; Shaner et al., 1984; Yu et al., 2010). It is the target of five ALS-inhibiting herbicide chemistries, namely sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyl-thiobenzoates, and sulphonyl-aminocarbonyltriazolinone (Oard et al., 2006; Yu et al., 2010). Resistance to these herbicides is resulted from a mutation that caused a tryptophan residue to be replaced by leucine (Trp-574-Leu) in the ALS protein. ACCase on the other hand, catalyzes the first step in lipid biosynthesis in plants by adding a carboxyl group onto the common metabolite Acetyl-coenzyme-A to form Malonyl-coenzyme-A (Délye, 2005; Ohlrogge and Browse, 1995). There are three distinct chemical families that are known to inhibit ACCase, namely aryloxyphenoxypropionate (APP), cyclohexanedione (CHD), and phenylpyrazolin (PPZ) (Hofer et al., 2006; Yu et al., 2007). Resistance in the new sources was conferred by a mutation at 2027 residue of the of ACCase protein resulting in replacement of tryptophan by cysteine (Trp-2027-Cys) (Petit et al., 2010; Raghav et al., 2016; Yu et al., 2007). Resistance to both herbicide families was due to altered chemical property of the ALS and ACCase proteins caused by the mutations which prevented the chemicals from binding effectively countering their inhibitory activity.

While the deployment of ACCase resistance trait is awaiting clearance from the country where the source originated, efforts to deploy the ALS resistance trait is in progress. Due to the fact that both resistance traits were introduced from a wild species of no agronomic desirability, there is concern that unwanted wild traits may have dragged along that can compromise yield potential. Though target site-based genetic mutations endowing herbicide resistance like these ones have not been shown to negatively affect plant fitness and growth (Délye, 2005; Yu et al., 2010). The low seedling vigor and interveinal chlorosis observed in ALS resistant sorghums have

elevated this concern that this phenomenon may translate into lower yields. Seed producers are speculating how much yield penalty farmers are willing to accept in addition to the premium price they are expected to pay for the trait. Thus this study was initiated to address these concerns by evaluating the agronomic adaptability, yield potential and grain and nutritional quality attributes of hybrids carrying the ALS, ACCase and both ALS and ACCase resistance traits as compared to regular grain sorghum hybrids.

Materials and Methods

The study involved evaluation of different sets of hybrids (ALS resistant, ACCase resistant, ALS and ACCase resistant as well as ALS and ACCase susceptible hybrids) at three locations, in three replications during the 2014 and 2015 crop seasons. In 2014 the tests were conducted at the Ashland Bottoms KSU agronomy research farm located approximately 10 miles south of the city of Manhattan. Tests in the 2015 season were conducted at four locations: the Ashland Bottoms KSU agronomy research farm, the north campus agronomy research farm, the KSU Agricultural Research Center Hays and the East Central Kansas Experiment Field at Ottawa, Kansas. The soils at Ashland bottoms are Chase silty clay loam while the north campus agronomy research farm is a Wymore silty clay loam. Soils at Hays is a Harney-Carlson silt loams while Ottawa is Woodson silt loam (Web Soil Survey, 2016). Soils at all locations have moderately fine texture and high cation exchange capacity (CEC).

Genetic materials and hybrid synthesis

Test entries included in these studies were derived among elite breeding lines from Kansas State University sorghum breeding program. The entries comprised of hybrids that are

homozygous or heterozygous resistant to ALS and ACCase herbicides and those that are susceptible to these chemicals including two commercial checks. A set of advanced ALS resistant, ACCase resistant and susceptible regular R- and A-lines were randomly selected among the advanced nursery of the KSU sorghum breeding program. Crosses were made in all possible combinations during the 2013/14 wintery nursery season in Puerto Rico generating large number of hybrids of the following categories: ALS × ALS, ALS × ACCase, ALS × Regular, ACCase × ACCase, ACCase × ALS, ACCase × Regular, Regular × Regular, Regular × ALS, and Regular × ACCase. Depending on success with seed set, the number of hybrids in each category was different. Similar crosses were made during the 2014/15 winter nursery to synthesize additional hybrids of similar category for testing during 2015 season. In both seasons the crosses were manually harvested and threshed using head thresher and the seeds shipped to Kansas State University. Up on arrival, the seeds were placed in a dryer to remove excess moisture and carefully cleaned and treated using standard experimental seed treatment protocol (a mixture of Maxim 4FS ™, Apron XL ™, Concep III ™, and colorant). Three grams (approximately 100 seeds) of the treated seeds were then packeted in to a seed envelop in preparation for planting. The list of hybrids grouped by herbicide resistance categories is presented in Tables 1 and 2.

Experimental design and management

The entries were grouped into eight categories based on the kind and dose of herbicide resistance alleles they carry. These categories include: ALS × ALS, ALS × Regular, ACCase × ACCase, ACCase × Regular, Regular × Regular, Regular × ALS, Regular × ACCase and commercial checks. The study consisted of three sets of experiments with each set consisting different categories. Set I and II consisted of 68 and 62 entries, respectively, that included all eight

categories whereas set III consisted of 56 entries that included seven of the eight categories. Set I experiment was conducted at one location during 2014 season and two locations during 2015 season whereas sets II and III were conducted at two locations during 2015 season. Altogether a total of 189 hybrids including two check entries were evaluated in three sets over two seasons.

All experiments were laid out in randomized complete block design with three replications. Seeds were sown into 5 m long double row plots spaced 0.75 m apart. Fields at Manhattan and Hays were prepared following the standard tillage practices while Ottawa was strip tilled after burning the weeds with glyphosate. Fertilizer nitrogen in the form of urea and phosphorous in the form of DAP were applied at the rate of 100 kg ha^{-1} N and 45 kg ha^{-1} P_2O_5 at Manhattan; and 78 kg ha^{-1} N and 33.5 kg ha^{-1} P_2O_5 at Hays. At Ottawa, fertilizer was applied at the rate of 42.5 kg ha^{-1} N and 13.5 kg ha^{-1} P_2O_5. Pre-emergence weeds at Manhattan were controlled with 1.34 kg ha^{-1} Atrazine™, 1.94 L ha^{-1} Dual II Mg™ and, 0.42 L ha^{-1} Callisto™ and 4.67 L ha^{-1} Atrazine™ and 1.17 L ha^{-1} Metolachlor™ at Hays. At Ottawa, weeds were burned down using Glyphosate™ at the rate of 1.75 L ha-1 and 2,4-Dichlorophenoxyacetic acid (2,4-D LV6™) at the rate of 0.39 L ha^{-1} followed by Atrazine™, Liberty™ and Callisto™ at the rates of 3 L ha^{-1}, 1.6 L $ha^{-1}$and, 0.22 L ha^{-1}, respectively prior to planting. Post-emergence weeds at all locations were removed manually as necessary.

Data collection

Data were collected on a range of physiological, phenological and yield parameters at all locations and testing seasons. Because the entries in experiment set I included homozygous ALS resistant hybrids, leaf chlorophyll content was measured on the seedlings and adult plants using SPAD-502 chlorophyll meter (Spectrum Technologies Inc.) on ALS resistant hybrids and the

commercial checks at all locations and test seasons. The chlorophyll readings were taken on day 14 after planting on the second fully expanded leaf from the top and recorded as the mean of readings from three plants in a plot. As quantitative indicators of seedling vigor, height and biomass of seedlings were also measured from the same three plants on day 14 after planting. To avoid impact on grain yield destructive sampling of seedling dry matter was not taken.

In order to determine whether the effect of seedling yellowing at early growth stage had persisted to have impact on photosynthesis and grain development, chlorophyll fluorescence was measured at grain fill stage on pre-flag leaves in all test entries in set I. The measurements were made on three plants per plot using OS30p+ hand held chlorophyll fluorometer (Opti-Sciences, Inc.).

A number of agronomic and yield parameters were measured on the hybrids in all experiments including days to flowering, plant height, panicle length, panicle width, panicle weight, kernel weight per panicle (panicle yield), kernel number per panicle, thousand kernel weight and grain yield per plot basis. Seedling height was measured from the soil level to the leaf whorl and adult plant height was recorded as the length of the plant from the base to the tip of the panicle on plot basis.

Days to flowering was measured as the number of days from planting to when half of the plants reached half-bloom stage. Yield components were determined based on measurements taken on three panicles sampled from each plot at physiological maturity. After harvest, the panicles were dried at 65°F for three days and their weight, length and width were taken before threshing. After threshing, the weight of the kernels was taken for individual panicles and kernel numbers per panicle was determined using the seed counter (Seed Counter Model 850-3, International

Marketing and Design Corp.). Thousand kernel weight was determined by dividing panicle yield by the number of kernels per panicle and multiplying by 1000.

About 40g seeds of the bulked samples from the three panicles in each plot were saved for grain and nutritional quality analysis. The grain hardness and diameter were determined using single kernel characterization procedure (Perten SKCS 4100 (Perten Instruments Inc, Chatham, IL). The nutritional quality parameters were determined using NIR methods (Perten Instruments Inc, Chatham, IL) after adjusting the grain moisture content to 12.5% (Miller et al., 1964). Major nutritional parameters determined include: protein, starch, fat and ash contents from whole grains. Grain mineral contents including Nitrogen (N), Phosphorous (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Zinc (Zn), Iron (Fe), Copper (Cu) and Manganese (Mn) were also determined. Nitrogen content was determined from flour samples using an indophenol blue colorimetric procedure (Lindner, 1944) using the Rapid Flow Analyzer (Model RFA-300, Alpkem Corporation, Clackamas, OR). For determining mineral composition, perchloric digestion method described by (Gieseking et al., 1935) was performed using an inductively coupled plasma (ICP) optical emission spectrometer (Model 720-ES ICP, Varian Austrailia Pty Ltd, Victoria, Australia). Samples for the physical grain quality and mineral profiles were determined only for ALS resistant hybrids and the commercial checks while other nutritional parameters were determined for all entries.

Statistical analysis

Statistical analysis for all experiments was performed using SAS software version 9.4 (Institute, 2008). The analysis of variance for hybrid entries was performed across environments for each experimental set and also for individual environment. The data were then rearranged in to hybrid groups and the analysis was re-run to obtain the comparison between hybrid groups for

all traits. In all cases PROC GLM was used with hybrid entries and hybrid groups treated as fixed effects and environment and block as random effects. Test of significance was performed using appropriate error terms for each effect which was specified using the random statement in GLM. Significant means were separated using the LSD test.

In additional multivariate analysis performed for nutrient composition among the entries, Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering procedure were conducted using XLSTAT software version 2014. XLSTAT analysis was used for clustering the hybrids as well as for visualization of the clusters in the principal component space.

Results

The analysis of the variance on the physiological and yield parameters for hybrids and hybrid groups evaluated under Experiment I is presented in Table 4.2. The hybrid effect and hybrid × environment interaction effect were significant for all measured traits except for seedling and adult plant height, adult plant chlorophyll content and panicle width for hybrid × environment effect (Table 4.3). Similarly, the hybrid group and hybrid group × environment interaction effects in Experiment set I was significant for all parameters except seedling height and panicle length (Table 4.2). The environment effect was highly significant both in entire hybrid and hybrid group analysis. Individual environment analysis revealed similar result with hybrid group effect being significant for all parameters under all environments except for seedling height and adult plant chlorophyll content (Table C.1).

Analysis of variance for Experiment set II and set III are also presented in tables 4.3 and 4.4. In Experiment set II, the hybrid effect was significant for most of the parameters measured except panicle width, panicle yield and TKW. Whereas, hybrid × environment interaction effect

was significant for grain yield, panicle length, plant height and days to flowering (Table 4.3). The effect of hybrid group, on the other hand, was significant for grain yield, panicle length, panicle weight and plant height while the interaction between hybrid group and environment was significant only for days to flowering and panicle weight (Table 4.3).

The results from Experiment set III were fairly similar to that of Experiment sets I and II that the hybrid effect was again significant for all parameters while hybrid × environment interaction effect was significant only for days to flowering and panicle length (Table 4.4). This was similar for hybrids groups as well except the effect of panicle length and panicle width was not significant. The interaction between hybrid groups and environment was significant only for plant height, days to flowering and panicle length (Table 4.4).

Summary of the mean performance of the hybrids tested under Experiment set I is presented in Table 4.5. As expected, the lowest seedling chlorophyll content of 35.3 SPAD units was recorded in ALS resistant hybrid while the highest (43.4 SPAD unit) was recorded in the herbicide susceptible commercial check. But some of the ALS × ALS hybrids had mean chlorophyll contents that were comparable with that of the heterozygous hybrids indicating that the traits can be improved through selection. Seedling height was not affected by the seedling chlorosis. Nevertheless, the seedling chlorosis does not seem to have effect on photosynthetic efficiency of adult plants and other parameters. Although there is significant difference among hybrids for several other parameters, none of them seem to be associated with the ALS resistant trait. The homozygous ALS resistant entries were among the highest yielding hybrids with seven out of the eight ALS × ALS entries out yielding the top commercial check (Table 4.5). Similar result was obtained for yield components as well. The trend was the same for other yield components including TKW, panicle weight, panicle yield and number of kernels per panicle

where few entries among the ALS × ALS hybrids were among the top among the entire entries. Among the hybrid groups, the ALS × ALS hybrids apparently had the lowest seedling chlorophyll content of 37.9 vs. 42.6 in the susceptible commercial check (Table 4.6). The ALS × Regular and Regular × ALS hybrids had 40.9 and 41.1 SPAD units which was not significantly different from 42.6 in the susceptible check showing that unlike the herbicide resistance which was controlled by a partially dominant gene, the seedling chlorosis appear to be a recessive trait that it only displays itself under a homozygous condition. Furthermore, the absence of apparent difference between the ALS × Regular and Regular × ALS hybrids indicate that maternal effect has little or no role in determining seedling chlorosis. Most of the parameters were significantly different between the different hybrid groups but none of them appear to be associated with herbicide resistance. Grain yield was highest (5133 kg ha^{-1}) among the Regular × ACCase group followed by the ALS × ALS group (4999 kg ha^{-1}) while the lowest was in the Regular × Regular and Regular × ALS group. The commercial checks had mean grain yield of 4482 kg ha^{-1}. On tests conducted on inbred parents as well as from common field observations ALS resistant materials tend to have delayed flowering. Such phenomena was not observed in the current study where all ALS resistant hybrids were found to be earlier than the commercial checks by an average of 3-4 days.

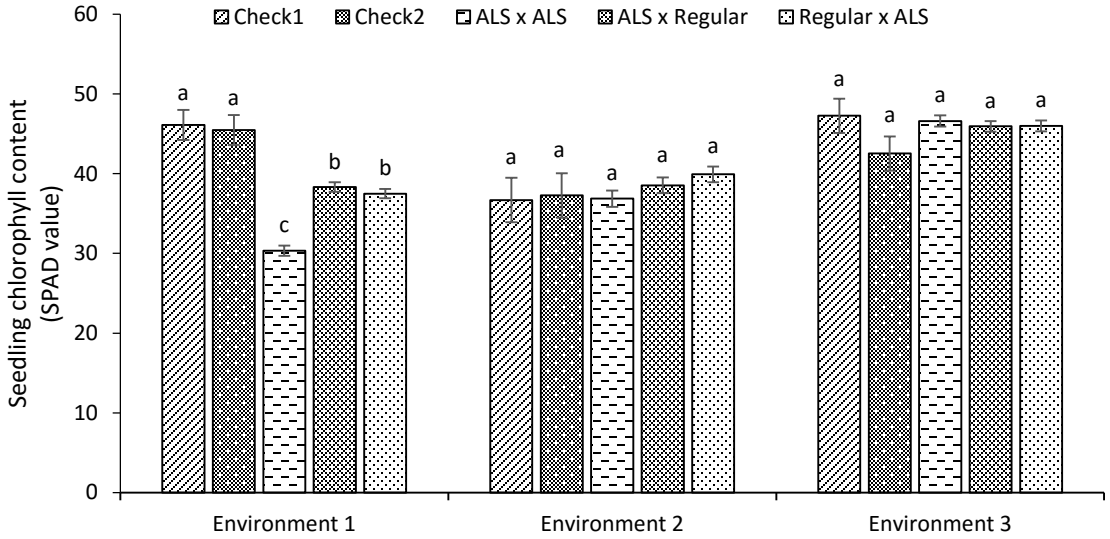
Results from Experiment set II are similar to that of set I (Tables 4.7 and 4.8). Four entries out yielded the commercial checks while 11 of them were statistically the same as the checks. All of the hybrids that topped the highest check are from the ALS × Regular group while many from the other groups were in a statistical tie with the checks (Table 4.7). Comparison of the commercial checks with the different hybrid groups showed that the checks significantly out yield the other groups (Table 4.8). However, a scrutiny to the different yield components does not show any trait that was particularly high in the commercial checks. This may be due to a poor stand caused by

low stand establishment in 2015 season. Likewise, in the set III experiment, about sixteen hybrids were found to have out yielded either of the commercial checks and they represent all of the categories indicating that yield potential is not specific to a certain category (Table 4.9). The highest yield (5748 kg ha^{-1}) among the hybrid groups under Experiment set III was obtained in the ACCase × Regular group followed by the commercial checks (5378 kg ha^{-1}) which was not significantly different from that of ALS × Regular, ACCase × ACCase and Regular × ACCase groups (Table 4.10). Again the specific yield component that contributed to higher yield in one group vs. the other is not clear. It may have to do with other parameters that were not accounted for in this experiment. The Pearson correlation analysis between many of the measured traits showed some significant association between the traits. Chlorophyll florescence of the leaves was significantly correlated with chlorophyll content (Table 4.11). It is also significantly correlated with yield components including panicle weight, kernel number and grain yield. Correlation among yield components is similar to what is generally expected.

Analysis of the nutritional composition of the herbicide resistant hybrids revealed that the new hybrids have similar nutritional composition with that of the check hybrids. The major nutrients (protein, starch and fat) were similar among all hybrids groups and the checks. But there was significant location effect with the 2014 tests having higher content of these nutrients than those recorded from the 2015 samples. Measurement of mineral profile and physical grain quality parameters was conducted only in set I Experiment from the 2014 samples. Phosphorus, calcium and magnesium tend to be higher among the herbicide resistant groups compared to the commercial check. Similarly micronutrients such as zinc copper and manganese are higher in herbicide resistant groups compared to the checks whereas iron content was similar among all hybrid groups. Among physical grain quality characteristics measured, grain hardness appear to

be uniquely high in the ALS resistant group while measured grain size and grain diameter was higher in the commercial check group. Correlation analysis among these traits showed fat content as negatively correlated with all other nutritional parameters while all other parameters have either near zero or positive correlation with each other except that of potassium with copper, iron, protein and nitrogen. Starch was also negatively correlated with potassium, calcium and ash (Figure 4.1). Principal component analysis based on the nutritional parameters sorted the hybrids according to their nutritional profile. Hybrids with higher fat content were clearly separated in the principal component space while those with similar micronutrient profile converged (Figure 4.2). This agrees with summary results in Table 4.12.

(a).



(b).

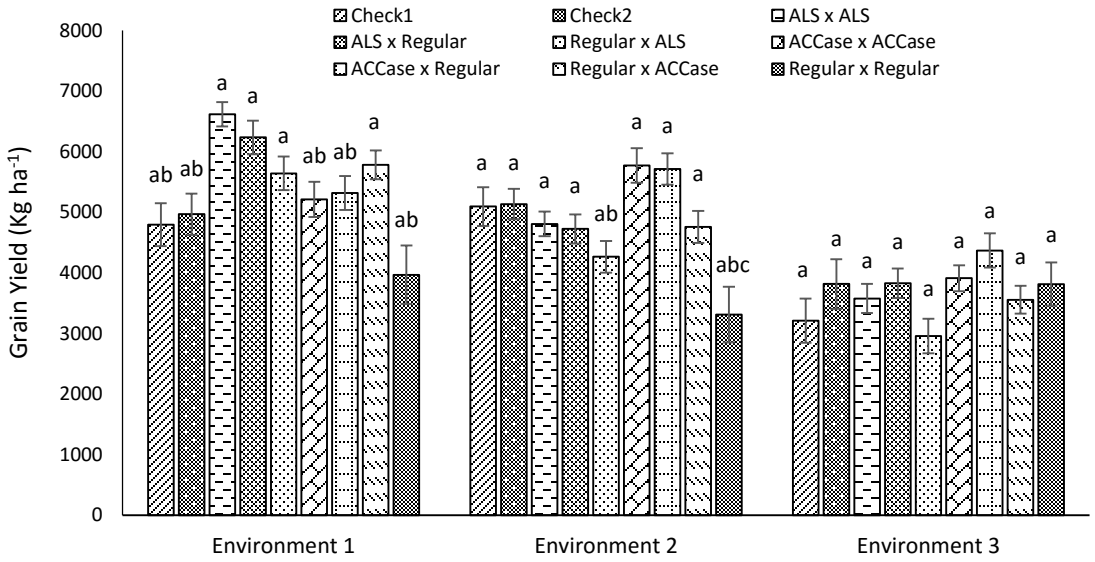


Figure 4.1. Grain yield is not affected by seedling chlorophyll content: a) variability for seedling chlorophyll content among hybrid categories; b) Variation for grain yield among the hybrid categories. Check1= P84-G62, Check 2 = Dekalb54-00.

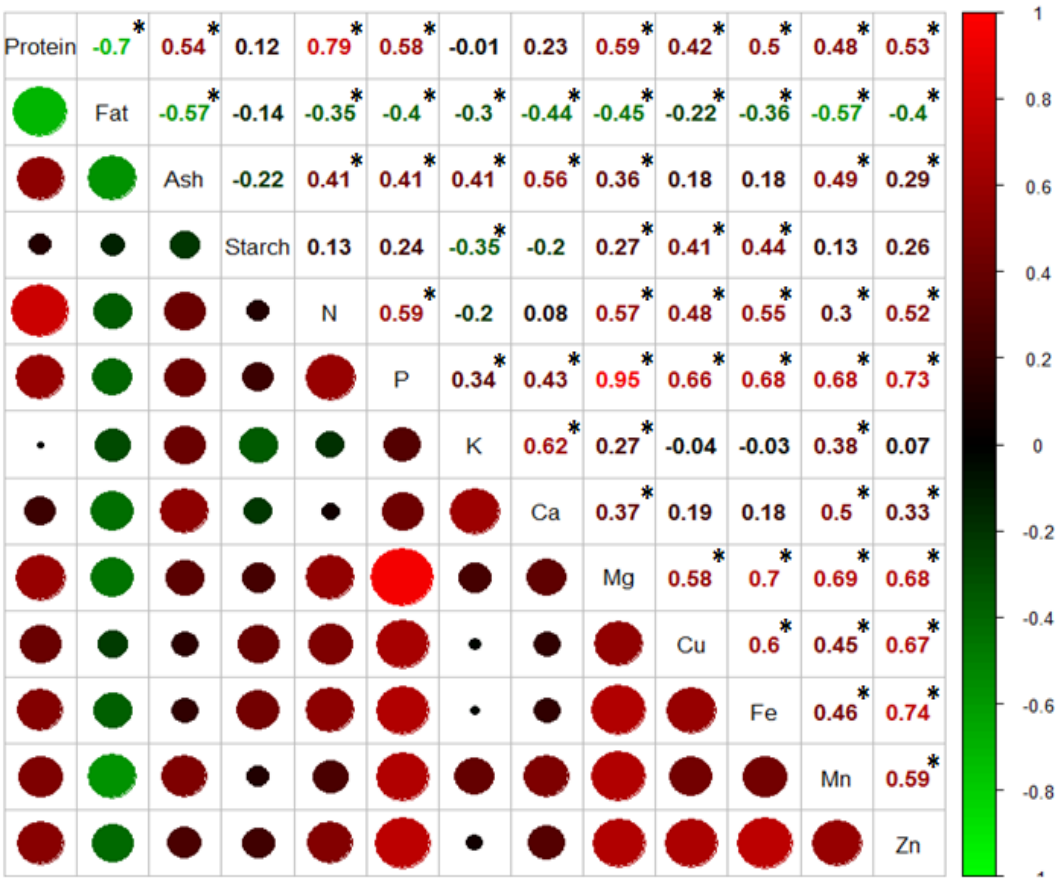


Figure 4.2. Correlations observed between seed nutrient content of ALS herbicide resistant hybrid groups tested under Experiment Set I. Size of the circle represents the magnitude of correlation and color of the circles (red and green) represents positive and negative relationships, respectively. * Statistically significant at $P \leq 0.05$.

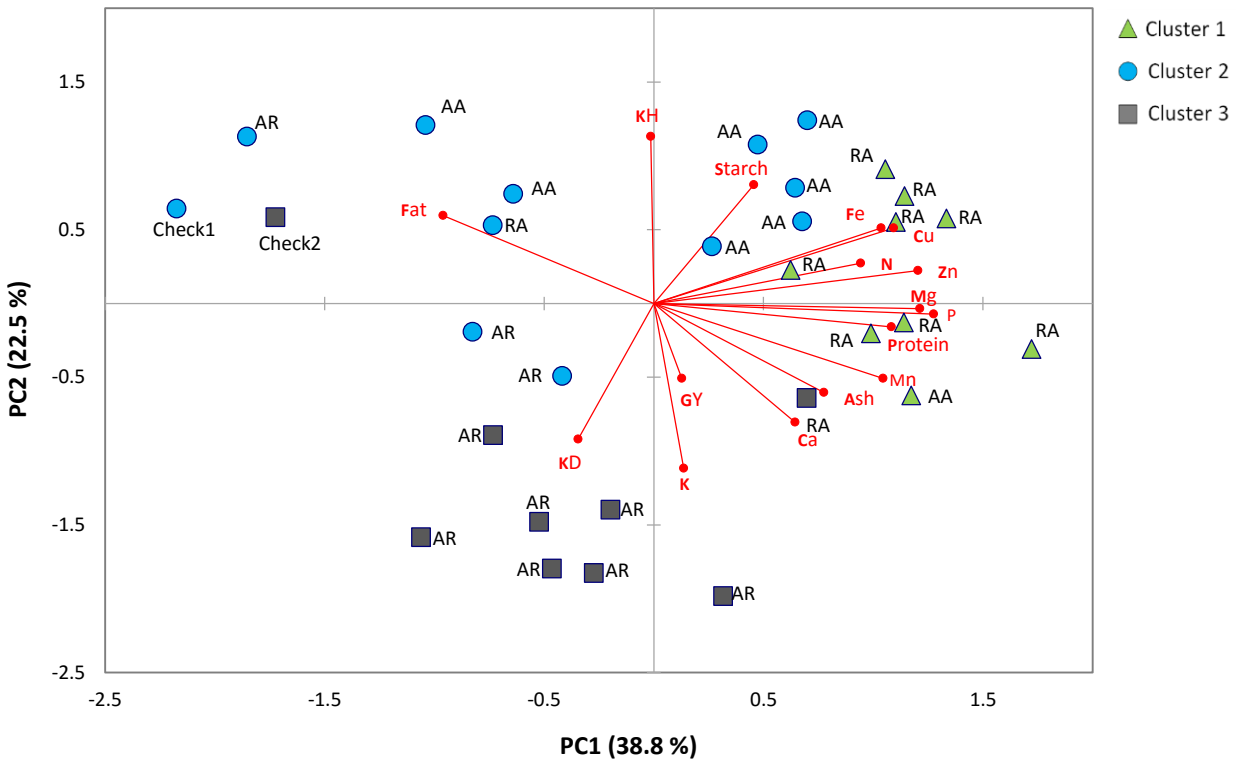


Figure 4.3. The distribution based on nutritional profile of the ALS herbicide resistant hybrids and commercial checks in the principal component space. KD = kernel diameter, KH = kernel hardness, Check1= P84-G62, Check 2 = Dekalb54-00, AA = ALS × ALS resistance, AR = ALS × regular , RA = Regular × ALS.

Table 4.1. Different groups of hybrids developed and tested under each experimental set.

†Hybrid Groups	Category based on herbicide resistance	Number of hybrids			Seed parent	Pollinator parent
		Set I	Set II	Set III		
Homozygous ALS	ALS × ALS	08	-	-	ALS resistant	ALS resistant
Heterozygous ALS	ALS × REG	10	21	04	ALS resistant	Herbicide susceptible
Heterozygous ALS and ACCase	ALS × ACCase	00	21	27	ALS resistant	ACCase resistant
Heterozygous ALS	REG × ALS	10	-	03	Herbicide susceptible	ALS resistant
Homozygous ACCase	ACCase × ACCase	08	03	02	ACCase resistant	ACCase resistant
Heterozygous ACCase	ACCase × REG	10	08	07	ACCase resistant	Herbicide susceptible
Heterozygous ALS and ACCase	ACCase × ALS	00	02	05	ACCase resistant	ALS resistant
Heterozygous ACCase	REG × ACCase	10	05	06	Herbicide susceptible	ACCase resistant
Regular hybrids	REG × REG	10	-	-	Herbicide susceptible	Herbicide susceptible
Commercial checks	Checks	02	02	02	Herbicide susceptible	Herbicide susceptible
Total		68	62	56		

†Homozygous ALS=Homozygous for ALS resistance trait, Heterozygous ALS= Heterozygous for ALS resistance trait, Homozygous ACCase= Homozygous for ACCase resistance trait, Heterozygous ACCase= Heterozygous for ACCase resistance trait.

Table 4.2. Combined ANOVA for entire hybrids (a) and hybrid groups (b) for physiological, agronomic traits and yield components of sorghum (*Sorghum bicolor* (L.) Moench) hybrids carrying different combinations of herbicide resistance genes as evaluated at three environments under Experiment set I.

Source of variation	df	Seedling		Adult plant		Days to flowering	†Chl. Fluo. (Fv/Fm)	Panicle weight	Panicle length	Panicle width	Panicle yield	‡KN	§TKW	Grain yield
		Chlorophyll content	Height	Chlorophyll content	Height									
a) Entire hybrids														
Environment (E)	2	2509.1**	881.4**	4383.7**	4674.8**	1329.1**	0.07**	8934.1**	1152.3**	32.4**	4823.0**	2228155**	126.1**	123159514**
Block/E	6	19.1	11.3	29.6	45.0	67.1	0.0005	782.0	10.1	1.1	1678.1	1632440	15.1	2631970
Hybrid	67	41.2**	3.8	16.7	64.4*	87.3**	0.002**	958.7**	17.5**	1.3**	618.3**	757070**	20.6**	3535708**
Hybrid x E	134	34.2**	4.5	18.6	27.6	21.6**	0.001	845.9**	8.0**	1.1	485.7**	522834**	6.7**	3906045**
Error	402	14.7	3.3	13.1	14.8	9.2	0.0004	607.0	6.2	0.7	313.4	346717	4.7	2299406
b) Hybrid groups														
Environment (E)	2	1208.1**	541.6**	3463.0**	3332.8*	957.1**	0.05**	6396.3	923.4**	27.0*	3581.0	1486358	122.5**	80700689
Block/E	6	18.2	11.1	29.8	43.7	67.8	0.001	2791.9	10.1	1.0	1626.2	1561081	14.6	26017649
Hybrid group (HG)	7	210.7**	6.0	24.0	171.7**	335.7**	0.02**	2628.6**	54.9**	2.7**	1848.7**	1770781**	73.0**	13147599**
HG x E	14	193.3**	6.8	48.5**	55.4**	61.3**	0.007**	2339.0**	8.7	4.7**	1524.0**	1398640**	19.5**	16168829**
Error	42	15.7	3.6	13.8	20.8	16.1	0.004	638.4	7.3	0.7	340.7	393713	5.8	2356403

†Chl. Fluo.= chlorophyll fluorescence; ‡KN = kernel number per panicle, §TKW = thousand kernel weight;
* and ** statistically significant at P ≤ 0.05 and 0.01, respectively.

Table 4.3. Combined ANOVA for entire hybrids (a) and hybrid groups (b) for agronomic and yield components of sorghum (*Sorghum bicolor* (L.) Moench) hybrids with varying combinations of herbicide resistant traits as evaluated under Experiment set II.

Source of variation	df	Adult plant height	Days to flowering	Panicle weight	Panicle length	Panicle width	Panicle yield	†KN	‡TKW	Grain yield
a) Entire hybrids										
Environment (E)	2	4704.2**	15490**	222457**	286.0**	28.2	160090**	10576319**	1816.8*	69807524**
Block/E	6	31.5	10.1	5649.7	7.9	15.1	2039.4	1872180	197.1	7780558
Hybrid	61	36.5**	23.4**	2893.7	29.1**	7.2	1167.7	1556281	163.6	3887164**
Hybrid × E	122	17.3**	19.5**	2314.7	9.6**	6.4	1240.1	777839	165.3	3371029**
Error	366	5.1	8.6	2305.2	6.4	6.9	1018.2	658515	154.7	2443732
b) Hybrid groups										
Environment (E)	2	2373.2**	5710.0**	38142.0**	112.6**	7.7	52991.0**	31946837**	696.1*	19690556**
Block/E	6	31.6	39.9	5738.9	8.2	15.1	2054.0	1915791	196.4	7648441
Hybrid group (HG)	6	50.8**	23.1	16844.0**	60.0**	2.2	437.6	1611949	228.1	6911120*
HG × E	12	34.3	47.8**	14934.0**	10.7	3.1	928.0	392875	174.6	2592525
Error	36	10.2	11.6	1847.8	8.9	7.0	1097.2	784962	156.7	2762342

†KN = kernel number per panicle, ‡TKW = thousand kernel weight;
* and ** statistically significant at P ≤ 0.05 and 0.01, respectively.

Table 4.4. Combined ANOVA for entire hybrids (a) and hybrid groups (b) for agronomic and yield components of sorghum (*Sorghum bicolor* (L.) Moench) hybrids with different combinations of herbicide resistant traits as evaluated under Experiment set III.

Source of variation	df	Plant height	Days to flowering	Panicle weight	Panicle length	panicle width	panicle yield	†KN	‡TKW	Grain yield
a) Entire hybrids										
Environment (E)	1	10859**	1491.0*	102883*	34.4	28.3**	72500**	36378217*	981.3**	207139489*
Block/E	4	5.6	17.1	1124.1	7.6	0.2	611.5	450229	5.5	4853941
Hybrid	55	162.7**	34.8**	1257.5**	13.3*	0.8*	655.9**	806319**	15.5**	1782251**
Hybrid × E	55	84.5	29.9*	594.7	8.9*	0.7	361.9	360817	5.6	1383500
Error	220	30.1	3.9	458.5	5.4	0.5	242.2	281473	2.1	1051068
b) Hybrid groups										
Block	2	5.6	16.0	1.0	0.4	0.07	8.2	10042	0.03	1837292
Environment (E)	1	427.5**	322.2**	46738**	19.8	6.9**	32168**	5079586**	535.7**	36388292**
Block/E	4	4.6	13.5	504.7	6.9	0.2	594.5	206299	4.4	3622057
Hybrid group (HG)	7	191.6*	145.1**	3113.2**	10.2	0.6	1864.1**	2303551**	42.5**	5517284**
HG × E	7	213.3**	21.4*	987.8	17.2*	0.9	583.2	643454	1.7	1796385
Error	28	68.7	8.3	608.8	7.7	0.6	316.6	361836	5.4	1137322

†KN = kernel number per panicle, ‡TKW = thousand kernel weight;
* and **statistically significant at P ≤ 0.05 and 0.01, respectively.

Table 4.5. Mean performance of each of tested sorghum (*Sorghum bicolor* (L.) Moench) hybrid across the environments for all physiological and yield characteristics evaluated under Experiment set I.

Entry Code	Seedling		Adult plant		Days to flowering	†Chl. Fluo. (Fv/Fm)	Panicle length	Panicle width	panicle weight	panicle yield	‡KN	§TKW	Grain yield	Hybrid group
	chlorophyll content	height	chlorophyll content	height										
132	35.3	15.3	58.3	124.7	64.7	0.76	34.5	6.3	124.1	77.3	2842	27.3	4798.8	ALS x ALS
131	35.5	14.0	55.0	123.0	66.0	0.74	33.0	5.9	110.4	72.7	2677	26.9	4991.1	ALS x ALS
135	36.1	15.6	56.1	120.0	66.2	0.75	33.1	6.0	123.4	79.6	2775	28.4	5536.3	ALS x ALS
130	37.5	14.9	57.6	118.0	70.2	0.75	32.0	6.7	133.0	84.8	3215	26.0	5636.1	ALS x ALS
136	38.2	15.2	58.1	113.5	69.2	0.74	30.2	6.8	137.7	91.0	3331	27.3	4442.8	ALS x ALS
133	38.0	14.6	54.7	123.5	67.4	0.77	31.6	5.9	112.6	72.1	2692	26.6	4690.6	ALS x ALS
129	39.1	16.4	57.9	125.7	65.2	0.74	33.8	6.5	120.7	78.0	2904	26.4	4959.2	ALS x ALS
137	39.6	15.2	55.1	126.0	68.6	0.76	31.6	6.0	125.5	90.0	3123	28.6	4920.5	ALS x ALS
147	39.5	14.2	56.3	122.0	66.2	0.73	32.3	6.6	134.7	88.9	3268	26.9	4481.5	ALS x Regular
151	39.9	15.3	57.3	128.5	64.0	0.73	31.2	6.7	122.9	78.7	3062	25.6	5067.2	ALS x Regular
149	39.0	14.4	57.4	133.2	64.3	0.72	32.0	6.4	109.6	67.5	2544	26.3	5613.0	ALS x Regular
152	40.4	15.1	57.1	126.0	66.6	0.74	32.2	6.3	114.0	68.4	2448	27.7	5747.8	ALS x Regular
143	40.5	15.7	58.2	126.0	62.0	0.74	31.8	6.2	117.2	75.3	2952	25.6	4367.6	ALS x Regular
145	41.7	15.6	56.5	127.7	64.0	0.72	33.1	6.4	110.8	68.7	2680	25.3	4628.4	ALS x Regular
146	41.0	15.8	56.7	125.7	65.2	0.73	33.9	6.7	128.5	82.8	3019	27.4	5697.3	ALS x Regular
144	42.1	15.9	57.6	126.7	63.7	0.75	32.5	6.3	126.2	83.7	2995	27.9	4107.3	ALS x Regular
150	42.4	15.9	59.8	130.5	63.3	0.73	33.7	6.3	132.1	85.6	2917	29.2	5158.7	ALS x Regular
148	42.7	15.6	59.0	125.7	65.6	0.73	33.7	6.8	127.4	91.6	3287	27.5	4407.7	ALS x Regular
164	38.6	16.1	55.8	124.2	64.3	0.75	29.8	6.2	106.2	67.0	2461	26.6	4483.9	Regular x ALS
163	39.2	15.7	57.4	126.5	65.8	0.75	31.3	6.6	108.8	68.9	2937	23.6	4444.4	Regular x ALS
167	39.5	14.8	55.5	123.5	67.3	0.76	33.8	6.1	95.6	56.6	2453	22.6	4784.7	Regular x ALS
160	41.4	16.3	59.7	125.2	60.9	0.76	30.6	5.9	112.5	71.5	2652	26.5	4608.3	Regular x ALS
165	41.5	15.6	58.7	127.7	66.6	0.76	30.5	7.1	135.6	88.7	3496	25.3	4512.3	Regular x ALS
162	41.8	15.3	61.2	128.5	61.4	0.75	30.2	6.3	107.9	68.7	2553	26.8	4431.3	Regular x ALS
161	42.1	15.9	55.8	131.7	66.3	0.75	30.5	6.2	106.7	68.9	2728	25.7	4365.7	Regular x ALS
158	42.2	15.6	56.3	131.5	61.2	0.75	32.6	6.1	114.2	72.1	2883	25.1	3645.2	Regular x ALS
166	42.0	15.5	57.3	126.5	62.8	0.76	32.7	6.7	110.0	66.5	2867	23.2	3622.8	Regular x ALS
159	43.2	15.3	56.8	127.0	65.1	0.75	31.3	6.4	118.0	79.0	3234	24.3	4134.2	Regular x ALS
114	-	-	59.8	116.2	68.3	0.76	31.6	6.3	118.8	73.8	2927	25.0	4290.0	Regular x ACCase
107	-	-	59.3	129.7	62.6	0.77	32.1	7.0	127.2	79.6	2903	27.3	5402.8	Regular x ACCase
112	-	-	58.9	120.2	63.0	0.76	27.3	7.2	111.0	63.9	2723	23.2	4770.1	Regular x ACCase
111	-	-	58.4	127.2	60.7	0.77	30.6	6.6	106.6	65.1	2504	25.8	3819.0	Regular x ACCase
116	-	-	58.4	120.0	67.0	0.76	32.2	6bc	103.6	67.1	2703	24.4	4696.4	Regular x ACCase
115	-	-	58.3	130.0	61.8	0.77	31.8	6.3	112.0	72.1	2771	26.0	5451.4	Regular x ACCase
110	-	-	58.2	123.0	66.3	0.77	31.3	7.7	127.2	76.5	3253	23.5	4592.4	Regular x ACCase
108	-	-	57.9	125.2	69.4	0.76	31.7	7.4	124.4	79.1	3004	25.7	5064.0	Regular x ACCase

Entry Code	Seedling		Adult plant		Days to flowering	†Chl. Fluo. (Fv/Fm)	Panicle length	Panicle width	panicle weight	panicle yield	‡KN	§TKW	Grain yield	Hybrid group
	chlorophyll content	height	chlorophyll content	height										
113	-	-	57.5	131.0	61.9	0.77	33.2	6.7	127.6	79.4	3087	25.7	4488.6	Regular x ACCase
109	-	-	56.7	125.0	60.9	0.78	31.9	7.3	122.7	75.2	2950	25.4	4283.3	Regular x ACCase
080	-	-	58.7	136.2	64.5	0.78	33.0	6.6	127.9	84.6	3426	24.8	5211.3	ACCase x ACCase
073	-	-	58.5	126.0	60.4	0.78	29.0	5.9	93.3	55.4	2269	24.4	5514.9	ACCase x ACCase
074	-	-	58.0	130.0	61.8	0.78	30.6	6.1	117.5	68.2	2568	26.4	4173.0	ACCase x ACCase
075	-	-	57.1	135.2	68.0	0.78	30.9	6.8	114.1	72.3	2718	26.2	4485.5	ACCase x ACCase
079	-	-	56.2	126.7	68.1	0.78	30.3	6.3	122.6	78.5	3171	24.7	5187.9	ACCase x ACCase
078	-	-	56.0	133.0	65.4	0.79	30.2	6.5	119.5	76.4	2923	26.1	5450.2	ACCase x ACCase
077	-	-	55.5	123.0	64.2	0.78	29.0	6.1	107.4	69.7	2646	26.3	5602.0	ACCase x ACCase
082	-	-	54.2	123.2	63.9	0.78	30.2	5.9	103.6	63.6	2540	24.6	5288.2	ACCase x ACCase
098	-	-	59.4	128.2	64.1	0.76	31.8	6.5	109.1	65.9	2712	24.3	4818.5	ACCase x Regular
099	-	-	58.2	124.7	64.1	0.77	31.9	6.7	124.4	79.2	3288	24.1	4884.4	ACCase x Regular
102	-	-	58.2	119.5	69.2	0.76	31.6	6.6	119.7	75.3	2883	26.0	6369.2	ACCase x Regular
094	-	-	57.9	128.0	62.2	0.77	31.2	6.0	98.8	59.9	2556	23.5	5122.9	ACCase x Regular
100	-	-	57.1	138.2	65.7	0.77	30.5	6.5	111.4	71.2	2835	25.0	4191.0	ACCase x Regular
095	-	-	57.1	124.2	65.3	0.76	28.8	6.3	115.0	70.2	2934	23.9	5872.9	ACCase x Regular
096	-	-	56.9	125.0	63.8	0.77	32.2	6.8	126.6	80.5	3256	24.7	5559.4	ACCase x Regular
101	-	-	56.8	117.5	65.9	0.77	33.2	6.9	129.5	80.4	3030	26.3	4434.6	ACCase x Regular
093	-	-	56.3	132.5	64.9	0.77	31.2	6.3	103.0	63.3	2640	23.6	5559.9	ACCase x Regular
097	-	-	56.3	114.2	66.3	0.76	29.4	6.1	107.2	64.4	2635	24.5	4709.2	ACCase x Regular
051	-	-	59.5	117.2	73.6	0.77	29.9	6.6	116.1	73.5	2789	26.6	3574.4	Regular x Regular
053	-	-	58.5	126.5	67.7	0.76	32.2	6.6	115.2	72.4	2519	28.4	4047.8	Regular x Regular
052	-	-	58.2	130.0	62.3	0.76	31.7	6.1	98.0	62.5	2209	28.5	3326.4	Regular x Regular
046	-	-	57.6	113.2	67.1	0.77	30.3	6.6	110.0	69.7	2827	24.7	4271.2	Regular x Regular
047	-	-	56.9	107.5	73.0	0.76	31.1	5.6	99.4	62.8	2768	22.6	3885.6	Regular x Regular
055	-	-	56.8	120.7	70.7	0.76	30.8	6.1	102.2	63.8	2394	26.4	4335.5	Regular x Regular
050	-	-	56.5	124.5	67.2	0.75	31.3	6.5	104.4	65.0	2569	25.3	4269.5	Regular x Regular
048	-	-	56.2	111.7	66.1	0.76	29.8	6.8	109.8	65.6	2627	24.7	4596.5	Regular x Regular
049	-	-	55.7	110.2	73.7	0.76	29.7	6.6	102.1	61.4	2361	25.6	3275.9	Regular x Regular
054	-	-	55.4	132.0	66.7	0.76	31.6	6.5	112.5	71.9	2548	27.9	4239.9	Regular x Regular
C1	43.4	15.4	57.1	109.7	70.9	0.76	30.2	6.2	105.5	65.6	2347	27.6	4261.5	Check1
C2	41.8	14.9	55.3	119.7	72.4	0.76	29.8	6.9	117.6	67.8	2488	27.0	4639.3	Check2
Mean	40.2	15.3	57.3	124.7	65.7	0.8	31.4	6.4	115.3	72.8	2808	25.8	4700.9	-
¶LSD	5.9	ns	ns	14.25	4.1	0.03	3.1	0.9	25.07	18.4	226	2.2	435.3	-

†Chl. Fluo.= chlorophyll fluorescence; ‡KN = kernel number per panicle, §TKW = thousand kernel weight;
¶LSD = Least Significant difference; ns = not significant.

Table 4.6. Mean performance of each sorghum (*Sorghum bicolor* (L.) Moench) hybrid group across environments for all physiological and yield characteristics evaluated under Experiment set I.

Parameter	Hybrid Group								Mean	¶LSD
	ALS × ALS	ALS × Regular	Regular × ALS	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Regular × Regular	Checks		
Seedling chlorophyll content	37.9(±0.5)	40.9(±0.4)	41.1(±0.4)	-	-	-	-	42.6(±0.9)	40.6	2.8
Seedling height	14.9(±0.3)	15.3(±0.2)	15.6(±0.2)	-	-	-	-	15.2(±0.4)	15.3	ns
Adult plant chlorophyll content	56.7(±0.5)	57.6(±0.4)	57.5(±0.4)	56.9(±0.5)	57.4(±0.4)	58.3(±0.4)	57.1(±0.4)	56.2(±0.8)	57.2	ns
Adult plant height	122.0(±1.5)	127.3(±1.2)	127.2(±1.2)	128.8(±1.5)	126.3(±1.2)	125.0(±1.2)	119.3(±1.2)	114.8(±2.5)	123.7	4.8
Days to flowering	67.2(±0.6)	64.5(±0.5)	64.2(±0.5)	64.5(±0.6)	65.2(±0.5)	64.1(±0.5)	68.8(±0.5)	71.7(±0.9)	66.2	1.5
†Chl. Fluo. (Fv/Fm)	0.8(±0.002)	0.7(±0.002)	0.8(±0.002)	0.8(±0.002)	0.8(±0.002)	0.8(±0.002)	0.8(±0.002)	0.8(±0.005)	0.7	0.1
Panicle length	32.5(±0.3)	32.6(±0.2)	31.3(±0.2)	30.2(±0.3)	31.2(±0.2)	31.4(±0.2)	30.8(±0.2)	30(±0.6)	31.2	1.2
Panicle width	6.3(±0.3)	6.5(±0.2)	6.4(±0.2)	6.2(±0.3)	6.5(±0.2)	6.8(±0.2)	6.4(±0.2)	6.5(±0.9)	6.4	0.4
Panicle weight	123.3(±3.4)	122.1(±3.2)	111.4(±3.2)	111.5(±3.4)	114.4(±3.2)	118.2(±3.2)	106.9(±3.2)	111.5(±6.4)	114.9	9.2
Panicle yield	80.6(±2.5)	78.9(±2.4)	70.7(±2.4)	69.5(±2.5)	71.0(±2.4)	73.6(±2.4)	66.9(±2.4)	66.7(±2.6)	72.2	6.5
‡KN	2946(±85.4)	2914(±79.6)	2821(±79.6)	2703(±85.4)	2877(±79.6)	2893(±79.6)	2561(±79.6)	2418(±154.2)	2766	207.8
§TKW	27.2(±0.3)	26.9(±0.2)	25(±0.2)	25.6(±0.3)	24.6(±0.2)	25.3(±0.2)	26.1(±0.2)	27.3(±0.5)	26	0.8
Grain yield	4999(±271.5)	4927(±256.2)	4286(±256.2)	4964(±271.5)	5133(±256.2)	4696(±256.2)	3997(±256.2)	4482(±422.3)	4686	633

†Chl. Fluo. = chlorophyll fluorescence; ‡KN = kernel number per panicle, §TKW = thousand kernel weight;
¶LSD = Least Significant difference; ns = not significant.

Table 4.7. Mean performance of each of tested sorghum (*Sorghum bicolor* (L.) Moench) hybrid across the environments for agronomic and yield characteristics evaluated under Experiment set II.

Entry code	Plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield	Hybrid group
04	138.1	58.1	31.2	6.9	138.7	87.7	3243.0	26.6	5207.0	ACCase × ACCase
40	153.1	63.4	34.3	5.8	124.7	77.0	3081.4	24.7	5951.5	ACCase × ACCase
23	150.6	62.2	34.9	6.8	140.9	89.9	3383.7	26.2	5833.9	ACCase × ALS
41	152.8	63.6	34.4	6.2	135.0	85.9	3209.6	26.8	4643.2	ACCase × ALS
11	145.3	60.4	35.1	6.6	116.6	70.7	2890.7	24.0	5828.4	ACCase × Regular
16	147.5	63.0	35.0	6.5	136.1	81.1	3131.8	25.4	6316.0	ACCase × Regular
17	144.7	61.6	32.3	6.5	123.4	76.4	2695.9	28.3	6677.6	ACCase × Regular
24	142.5	59.3	34.7	7.3	141.6	89.6	3027.4	28.8	5648.2	ACCase × Regular
27	142.8	60.9	34.7	7.0	149.1	98.3	3497.1	27.7	5880.6	ACCase × Regular
39	143.6	60.6	31.7	6.9	124.3	78.0	2680.3	28.7	6189.6	ACCase × Regular
42	147.2	59.6	34.3	6.2	123.9	105.5	2742.6	57.9	5849.0	ACCase × Regular
43	151.4	62.9	35.6	6.9	134.8	84.2	2984.4	28.1	5768.5	ACCase × Regular
13	148.1	62.0	34.2	6.5	139.8	84.9	3178.4	26.2	6887.4	ACCase × ACCase
02	151.1	63.8	32.1	7.4	148.6	96.1	3318.1	27.9	4256.6	ALS × ACCase
03	143.1	59.6	29.1	6.6	120.1	73.6	2794.4	26.2	5819.1	ALS × ACCase
12	142.2	61.4	32.4	6.8	129.6	81.9	3152.6	25.8	5493.5	ALS × ACCase
18	146.9	61.7	34.8	6.6	134.9	89.9	3188.1	28.1	5187.8	ALS × ACCase
25	156.7	63.3	34.3	6.1	133.9	87.1	3272.1	26.3	5132.8	ALS × ACCase
26	143.6	61.0	31.5	6.6	131.6	84.4	2971	27.9	6680.1	ALS × ACCase
28	145.8	62.9	31.1	6.9	150.6	98.6	3729.1	26.1	6195.7	ALS × ACCase
30	151.7	60.1	34.8	6.4	140.9	95.1	3422.7	28.0	6151.3	ALS × ACCase
31	142.8	60.1	33.4	6.3	120.4	79.3	2893.0	26.9	6097.4	ALS × ACCase
35	143.1	59.4	34.9	6.4	121.3	73.7	3203.0	22.7	5930.1	ALS × ACCase
36	144.4	60.0	34.2	6.2	135.2	90.7	3370.9	26.7	5397.4	ALS × ACCase
47	156.4	64.2	37.9	6.1	137.6	87.5	3547.9	24.7	5706.6	ALS × ACCase
49	156.9	65.3	33.3	6.8	123.7	78.6	2717.1	28.1	6083.6	ALS × ACCase
50	147.5	59.8	35.6	7.5	134	89.9	5423.7	22.1	5942.7	ALS × ACCase
52	156.1	63.6	33.3	6.5	147.8	91.4	3616.1	24.7	5153.8	ALS × ACCase
54	143.1	62.1	33.4	6.7	136.4	86.5	3338.1	25.8	5009.9	ALS × ACCase
55	158.1	63.0	35.6	6.9	149.2	99.5	3735.6	26.4	5103.7	ALS × ACCase
56	143.2	59.6	33.0	6.0	109.1	67.5	2702.3	24.7	5737.0	ALS × ACCase
57	144.2	60.6	33.3	6.1	112.8	69.5	2761.9	24.9	5602.6	ALS × ACCase
59	144.4	63.3	30.1	7.2	140.5	86.8	3235.9	26.3	4567.7	ALS × ACCase

Entry code	Plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield	Hybrid group
60	150.8	58.9	36	6.5	137.4	90.7	3552.6	25.2	5668.6	ALS × ACCase
01	148.1	62.0	31.8	7.1	143.8	91.2	3526.1	25.6	5916.5	ALS × Regular
05	141.9	59.8	33.7	6.0	123.2	81.3	3030.6	26.6	6823.1	ALS × Regular
07	143.9	59.3	33.2	6.8	142.4	89.6	3365.6	26.3	5761.2	ALS × Regular
08	149.2	61.2	33.6	6.5	112.7	67.6	2452.2	27.4	5353.1	ALS × Regular
09	147.2	63.3	35.7	6.7	153.3	96.4	3562.7	26.5	5041.3	ALS × Regular
14	152.8	62.9	36.2	6.8	143.3	90.4	3642.8	24.6	5135.4	ALS × Regular
15	147.8	61.9	33.5	6.7	127.4	81.9	3108.7	26.0	6036.1	ALS × Regular
20	150.3	60.3	36.5	6.1	117.1	73.6	3025.3	24.3	5712.9	ALS × Regular
21	148.1	62.8	36.8	6.5	148.0	94.8	3518.4	26.3	5239.9	ALS × Regular
22	144.2	60.9	35.2	7.1	137.0	83.8	3271.2	25.3	7091.4	ALS × Regular
29	142.2	62.2	32.8	6.3	125.1	82.6	3191.9	25.8	5714.3	ALS × Regular
32	147.5	61.1	34.9	7.0	156.1	100.3	3906.7	25.4	5479.4	ALS × Regular
34	146.7	59.7	33.9	6.3	134.2	89.2	3053.3	28.7	7027.5	ALS × Regular
37	143.3	61.1	36.5	7.3	147.8	97.7	3570.6	27.1	5711.1	ALS × Regular
44	145.6	60.1	33.4	6.4	129.9	79.7	3136.4	25.3	5695.8	ALS × Regular
45	156.1	62.7	33.8	6.6	142.5	86.7	3163.7	27.5	5831.7	ALS × Regular
46	143.9	60.6	33.7	6.4	125.0	77.7	3014.9	25.8	5666.1	ALS × Regular
48	147.8	59.4	35.4	6.6	140.5	92.0	3233.1	27.9	6993.2	ALS × Regular
51	154.4	61.6	34.4	6.5	136.4	89.1	3022.1	28.9	6281.4	ALS × Regular
53	152.8	61.6	34.1	6.8	139.9	87.2	3306.6	25.7	5721.3	ALS × Regular
58	158.3	64.1	34.4	6.7	138.4	86.9	3111.4	27.1	4178.6	ALS × Regular
06	149.7	61.4	34.1	6.6	128.1	83.2	3047.1	27.0	6052.2	Regular × ACCase
10	155.0	61.6	35.0	7.4	137.2	91.2	3464.1	25.8	6657.2	Regular × ACCase
19	148.3	62.1	33.4	6.6	139.3	89.7	3369.8	26.2	5477.2	Regular × ACCase
38	156.1	63.7	35.1	6.4	143.0	89.2	3377.4	25.6	5133.9	Regular × ACCase
33	147.5	63.7	37.2	7.3	149.3	96.9	3789.9	25.1	6100.1	Regular × ACCase
C1	137.5	60.8	31.1	6.5	123.1	78.9	2756.6	28.0	6990.2	Check
C2	147.8	64.0	29.3	7.3	147.9	91.4	3129.6	28.6	6846.2	Check
Mean	148	61.6	34	6.7	134.8	87	3239.4	26.9	5778.5	-
§LSD	12.2	6.4	2.7	ns	ns	ns	ns	ns	732.4	-

†KN = kernel number per panicle, ‡TKW = thousand kernel weight;
§LSD = Least Significant difference; ns = not significant.

Table 4.8. Mean performance of each sorghum (*Sorghum bicolor* (L.) Moench) hybrid group across environments for all agronomic and yield characteristics evaluated under Experiment set II.

Parameter	Hybrid Group							Mean	[§]LSD
	ALS × ACCase	ACCase × ALS	ALS × Regular	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Checks		
Adult plant height	148.2 (±0.3)	151.6(±0.8)	148.2(±0.3)	146.3(±0.7)	145.6(±0.4)	151.3(±0.5)	142.6(±0.7)	147.7	6.2
Days to flowering	61.6(±0.4)	62.8(±0.8)	61.3(±0.4)	61.1(±0.7)	61.0(±0.5)	62.4(±0.6)	62.3(±0.8)	61.7	ns
Panicle length	33.5(±0.2)	34.6(±0.7)	34.4(±0.2)	33.2(±0.5)	34.1(±0.3)	34.9(±0.4)	30.1(±0.7)	33.5	1.4
Panicle width	6.6(±0.2)	6.5(±0.6)	6.8(±0.2)	6.4(±0.5)	6.7(±0.3)	6.8(±0.4)	6.8(±0.6)	6.6	ns
Panicle weight	133.2(±4.2)	137.9(±11.2)	136.3(±4.2)	134.3(±9.3)	131.2(±6.0)	139.3(±7.4)	135.5(±11.2)	135.3	5.9
Panicle yield	85.8(±2.7)	87.9(±7.9)	86.6(±2.7)	83.2(±6.4)	91.1(±4.1)	90.0(±5.0)	85.1(±7.9)	87.1	ns
[†]KN	3336(±78)	3296(±213)	3248(±78)	3167(±176)	2956(±113)	3409(±139)	2943(±213)	3193.6	ns
[‡]TKW	25.9(±1.0)	26.5(±3.0)	26.3(±0.9)	25.8(±2.4)	31.1(±1.5)	25.9(±1.9)	28.3(±3.0)	27.1	ns
Grain yield	5514(±153)	5238(±402)	5829(±152)	6015(±332)	6019(±216)	5884(±264)	6918(±402)	5917	624

[†]KN = kernel number per panicle, [‡]TKW = thousand kernel weight;
[§]LSD = Least Significant difference; ns = not significant.

Table 4.9. Mean performance of each of tested sorghum (*Sorghum bicolor* (L.) Moench) hybrid across environments for all agronomic and yield characteristics evaluated under Experiment set III.

Entry Code	Adult plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield	Hybrid Group
077	141.3	62.5	35.5	6.6	145.7	92.3	3475.8	26.4	5273.6	ACCase × ACCase
082	130.0	61.8	35.0	7.4	161.2	104.7	3672.8	28.5	4530.2	ACCase × ACCase
074	125.6	64.0	33.9	6.6	144.8	90.3	3657.0	24.6	4516.5	ACCase × ALS
093	128.8	61.3	35.8	7.6	159.8	105.0	3774.0	27.9	4123.0	ACCase × ALS
095	121.3	66.5	30.1	6.8	130.0	88.8	3256.8	27.1	5207.8	ACCase × ALS
096	140.6	66.5	33.6	5.9	132.2	85.7	3330.0	25.5	4486.5	ACCase × ALS
109	138.1	64.0	34.5	5.5	124.7	81.6	2977.0	27.1	4572.7	ACCase × ALS
070	140.6	61.0	33.5	6.3	131.8	83.1	3099.5	26.6	5791.0	ACCase × Regular
075	131.3	64.5	31.5	6.6	139.3	88.1	3013.8	28.4	5641.2	ACCase × Regular
081	131.3	62.8	31.8	6.1	133.2	85.2	3045.0	27.8	5620.9	ACCase × Regular
083	139.4	60.8	33.8	6.8	161.8	103.4	3217.3	32.0	5724.6	ACCase × Regular
092	141.3	61.5	35.8	6.6	132.3	82.5	3047.8	27.0	6076.9	ACCase × Regular
097	145.6	66.5	37.0	6.1	141.8	89.1	3249.8	27.0	5700.9	ACCase × Regular
112	114.4	63.0	29.3	6.2	108.2	68.3	2597.0	26.3	5684.7	ACCase × Regular
061	135.6	65.0	32.9	6.8	140.4	87.5	3601.0	24.2	4234.4	ALS × ACCase
062	131.9	60.5	35.4	6.8	151.8	93.8	3418.8	27.3	4084.1	ALS × ACCase
063	142.5	60.0	36.0	6.2	148.9	97.1	3789.3	25.5	4269.0	ALS × ACCase
065	137.5	64.0	35.5	7.4	154.1	99.4	3855.5	25.6	3120.1	ALS × ACCase
066	126.3	63.3	33.5	6.1	134.9	81.5	3171.8	25.5	4626.0	ALS × ACCase
068	138.1	59.0	34.0	6.4	131.6	84.5	3580.0	23.5	4285.0	ALS × ACCase
069	130.0	62.5	36.0	6.4	139.6	89.3	3347.8	26.6	5657.6	ALS × ACCase
072	135.6	63.8	31.5	6.6	150.5	98.9	3575.3	27.2	4529.9	ALS × ACCase
073	140.6	63.5	32.1	6.2	124.9	80.4	3309.5	24.3	3930.5	ALS × ACCase
076	143.1	67.0	32.1	7.8	191.6	129.8	4517.3	28.1	6178.1	ALS × ACCase
078	133.1	63.0	34.8	6.6	146.0	100.3	3794.3	26.2	4541.6	ALS × ACCase
079	131.9	62.0	36.1	6.4	140.3	90.8	3353.8	26.9	4324.0	ALS × ACCase
080	135.0	61.0	31.5	6.2	119.6	78.0	2903.0	26.3	4861.5	ALS × ACCase
085	140.0	60.5	35.8	6.5	153.4	103.2	4066.3	25.0	3775.5	ALS × ACCase

Entry Code	Adult plant height	Days to flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield	Hybrid Group
086	136.3	65.0	30.0	7.3	150.2	97.8	3567.3	27.1	4979.1	ALS × ACCase
087	125.6	63.5	30.9	6.7	133.8	87.3	3063.0	28.5	5454.8	ALS × ACCase
088	136.9	64.5	34.6	6.9	172.1	111.9	4228.0	26.4	4225.4	ALS × ACCase
089	136.9	63.5	34.0	5.8	136.4	90.3	3317.8	26.9	4576.3	ALS × ACCase
091	141.9	64.0	35.0	6.6	164.6	108.2	4021.5	26.6	5007.5	ALS × ACCase
099	124.4	63.5	30.1	7.4	151.6	104.2	3571.3	28.4	4406.8	ALS × ACCase
100	132.5	64.5	32.8	6.4	148.0	94.5	3761.8	25.1	5025.8	ALS × ACCase
103	132.5	59.8	31.8	6.2	127.8	85.0	3443.0	24.2	4407.1	ALS × ACCase
104	141.3	64.8	34.4	7.3	146.4	92.8	3614.3	25.3	4804.8	ALS × ACCase
105	135.0	60.0	34.5	7.0	157.4	105.8	4244.5	24.9	4505.4	ALS × ACCase
108	128.1	61.5	35.5	6.4	139.7	94.6	3424.5	27.3	5253.4	ALS × ACCase
111	138.8	60.5	33.6	6.5	137.5	95.5	3323.8	28.1	4793.7	ALS × ACCase
113	133.8	62.5	32.4	5.9	114.8	73.5	2777.5	26.0	4271.6	ALS × ACCase
094	138.1	61.3	33.9	6.6	161.6	104.9	3556.0	28.7	5216.4	ALS × Regular
101	143.8	67.5	34.8	6.6	170.2	110.2	4091.8	26.7	4391.0	ALS × Regular
106	134.6	64.1	32.5	6.3	128.2	89.3	2607.8	34.5	5904.3	ALS × Regular
107	140.0	65.8	33.1	6.1	137.0	92.0	2923.8	31.0	5601.5	ALS × Regular
064	132.5	64.5	33.9	6.4	150.2	98.0	3731.8	26.3	4419.8	Regular × ACCase
067	136.3	72.5	33.1	6.5	166.2	114.3	4120.8	27.6	5610.2	Regular × ACCase
071	137.5	68.5	33.1	6.9	158.4	108.2	4204.5	25.2	4194.6	Regular × ACCase
098	139.4	68.3	35.8	6.7	175.7	119.8	3905.3	30.5	4388.7	Regular × ACCase
102	132.5	70.0	30.9	7.1	190.6	129.7	4130.8	30.8	4710.0	Regular × ACCase
110	125.6	66.5	32.0	7.0	187.3	122.7	4587.8	26.5	4833.2	Regular × ACCase
084	138.8	70.8	33.1	7.3	151.4	99.9	3651.0	26.8	4087.3	Regular × ALS
090	135.6	69.5	32.6	6.2	130.3	88.0	3113.5	27.6	4815.9	Regular × ALS
114	131.9	66.0	32.3	6.5	128.3	85.4	3373.5	25.0	3580.9	Regular × ALS
C1	121.3	67.0	31.8	6.6	139.8	94.3	3077.3	30.4	5058.9	Check
C2	129.4	65.5	31.4	6.7	153.0	100.7	3774.3	26.8	5698.9	Check
Mean	134.5	64	33.4	6.6	146.1	95.7	3516.1	27	4814.1	-
§LSD	14.7	5.4	3.6	1.2	46.8	37.4	324	4.2	783	-

†KN = kernel number per panicle, ‡TKW = thousand kernel weight; §LSD = Least Significant difference.

Table 4.10. Mean performance of each sorghum (*Sorghum bicolor* (L.) Moench) hybrid group across environments for all agronomic and yield characteristics evaluated under Experiment set III.

Parameter	Hybrid Group								Mean	§LSD
	ALS × ACCase	ACCase × ALS	ALS × Regular	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Regular × ALS	Checks		
Plant height	135.0(±0.8)	130.9(±1.8)	139.2(±2.1)	135.6(±2.9)	134.8(±1.6)	134.0(±1.7)	135.4(±2.4)	125.3(±2.9)	133.8	7.6
Days to flowering	62.7(±0.4)	64.5(±0.6)	64.5(±0.7)	62.1(±1.0)	62.9(±0.6)	68.4(±0.6)	68.8(±0.8)	66.3(±1.0)	65	2.3
Panicle length	33.6(±0.3)	33.6(±0.6)	33.9(±0.7)	35.3(±0.9)	33.2(±0.5)	33.1(±0.5)	32.7(±0.8)	31.6(±0.9)	33.4	ns
Panicle width	6.6(±0.1)	6.5(±0.2)	6.4(±0.2)	7.0(±0.3)	6.4(±0.2)	6.8(±0.2)	6.6(±0.2)	6.7(±0.3)	6.6	ns
Panicle weight	144.7(±2.9)	138.3(±5.7)	151.4(±6.4)	153.4(±8.8)	135.5(±4.9)	171.4(±5.3)	136.7(±7.3)	146.4(±8.8)	147.2	16.2
Panicle yield	94.6(±2.0)	90.3(±4.1)	101.0(±4.6)	98.5(±6.3)	85.7(±3.5)	115.4(±3.7)	91.1(±5.2)	97.5(±6.4)	96.8	10.3
†KN	3579(±62)	3399(±136)	3362(±152)	3574(±212)	3038(±115)	4113(±214)	3379(±174)	3425(±212)	3484	194
‡TKW	26.1(±0.2)	26.4(±0.5)	30.1(±0.6)	27.4(±0.8)	27.8(±0.4)	27.8(±0.5)	26.4(±0.7)	28.5(±0.8)	27.6	1.6
Grain yield	4597(±168)	4581(±274)	5277(±301)	4901(±401)	5748(±242)	4692(±256)	4161(±336)	5378(±401)	4917	702

†KN = kernel number per panicle, ‡TKW = thousand kernel weight.
§LSD = Least Significant difference; ns = not significant.

Table 4.11. Pearson correlation coefficients between evaluated yield components for all hybrids tested under Experiment set I.

Parameter	Chlorophyll content	Plant height	Days to flowering	†Chl. Fluo. (Fv/Fm)	Panicle length	Panicle width	Panicle weight	Panicle yield	‡KN	§TKW
Plant height	0.134**									
Days to flowering	0.048	-0.026								
†Chl. Fluo. (Fv/Fm)	0.370**	0.126**	0.103*							
Panicle length	0.006	0.526**	0.071	0.089						
Panicle width	0.143	0.241**	-0.044	0.041	0.345**					
Panicle weight	0.008	0.281**	-0.016	0.036	0.445**	0.631**				
Panicle yield	0.138**	0.104*	-0.089*	0.154**	0.216**	0.449**	0.871**			
‡KN	0.111**	0.117**	-0.093*	0.164**	0.241**	0.506**	0.844**	0.927**		
§TKW	0.090	-0.079	-0.079	0.016	-0.002	0.059	0.395**	0.548**	0.211**	
Grain yield	0.181**	0.028**	-0.240**	0.110**	0.0290**	0.217**	0.159**	0.221**	0.194**	0.144**

†Chl. Fluo. = chlorophyll fluorescence; ‡KN = kernel number per panicle, §TKW = thousand kernel weight;
* and **statistically significant at P ≤ 0.05 and 0.01, respectively.

Table 4.12. Nutritional and physical grain quality traits of sorghum (*Sorghum bicolor* (L.) Moench) hybrids evaluated under all experimental sets.

Set I		Hybrid group				Mean	†LSD
Parameters	Unit	ALS × ALS	ALS × Regular	Regular × ALS	Checks		
Protein	%	14.9(±0.3)	14.1(±0.3)	15.9(±0.3)	13.5(±0.8)	14.6	1.80
Starch		73.6 (±0.2)	73.5 (±0.2)	72.6(±0.2)	72.8 (±0.7)	73.2	0.94
Fat		4.4 (±0.1)	4.3 (±0.1)	4.1 (±0.1)	4.6 (±0.2)	4.40	0.24
Ash		1.7 (±0.02)	1.7 (±0.02)	1.7 (±0.02)	1.5 (±0.06)	1.65	0.17
Nitrogen (N)		2.0 (±0.03)	1.8 (±0.03)	2.1 (±0.03)	1.9 (±0.09)	1.95	0.16
Phosphorus (P)		0.36 (±0.01)	0.34 (±0.01)	0.37 (±0.01)	0.29 (±0.02)	0.34	0.09
Potassium (K)		0.28 (±0.01)	0.34 (±0.01)	0.31 (±0.01)	0.28 (±0.02)	0.30	0.05
Calcium (Ca)		0.017 (±0.001)	0.019 (±0.001)	0.018 (±0.001)	0.014 (±0.002)	0.017	0.004
Magnesium (Mg)		0.16 (±0.003)	0.15 (±0.003)	0.17 (±0.003)	0.14 (±0.009)	0.16	0.02
Iron (Fe)	ppm	36.9 (±0.78)	33.1 (±0.78)	40.9 (±0.78)	34.2 (±2.5)	36.3	3.70
Zinc (Zn)		23.7 (±0.7)	21.6 (±0.7)	26.1 (±0.7)	19.9 (±2.3)	22.8	2.17
Copper (Cu)		3.30 (±0.13)	2.60 (±0.13)	3.40 (±0.13)	2.00 (±0.39)	2.80	0.65
Manganese (Mn)		14.2 (±0.4)	14.2 (±0.4)	14.5 (±0.4)	11.0 (±1.3)	13.5	3.20
Kernel hardness	‡HI	84.8 (±1.5)	74.6 (±1.5)	77.7 (±1.5)	76.5 (±3.8)	78.4	7.10
Kernel diameter	mm	2.5 (±0.02)	2.6 (± 0.02)	2.5 (±0.02)	2.7 (±0.06)	2.60	0.08

Set II	ACCase × ACCase	ACCase × Regular	Regular × ACCase	ALS × ACCase	ACCase × ALS	ALS × Regular	Regular × ALS	Checks	Mean	†LSD
Protein (%)	11.4 (±0.3)	11.6(±0.2)	12.0 (±0.2)	11.8(±0.2)	11.6(±0.3)	11.6(±0.2)	-	11.5 (±0.5)	11.6	ns
Starch (%)	74.9(±0.3)	75.1(±0.2)	75.4(±0.2)	74.9(±0.1)	75.6(±0.4)	75.1(±0.1)	-	74.9(±0.5)	75.1	ns
Fat (%)	4.9(±0.05)	5.0 (±0.03)	4.8(±0.04)	4.9(±0.02)	5.0 (±0.06)	4.8(±0.02)	-	5.0(±0.08)	4.9	0.22
Ash (%)	1.5(±0.03)	1.5(±0.02)	1.5(±0.02)	1.5(±0.01)	1.5(±0.04)	1.5(±0.01)	-	1.4(±0.05)	1.5	ns
Set III										
Protein (%)	11.8 (±0.3)	10.8(±0.2)	11.1(±0.2)	11.1(±0.1)	11.1 (±0.2)	11.1(±0.2)	11.2(±0.2)	10.7(±0.4)	11.1	ns
Starch (%)	76.6(±0.3)	75.9(±0.2)	75.3(±0.2)	75.5(±0.1)	76.7(±0.2)	75.8(±0.2)	76.7(±0.3)	75.8(±0.5)	76.0	0.91
Fat (%)	5.0 (±0.04)	5.0 (±0.02)	5.0 (±0.03)	5.0 (±0.01)	5.1(±0.03)	5.0(±0.03)	5.2(±0.03)	5.0 (±0.06)	5.0	0.20
Ash (%)	1.5(±0.03)	1.4(±0.02)	1.4(±0.02)	1.4(±0.01)	1.4(±0.02)	1.4(±0.02)	1.3(±0.03)	1.3(±0.04)	1.4	0.10

†LSD = Least Significant difference; ‡HI = Hardness index; ns = not significant.

Discussion

Resistance based weed control options stand out as one of the most viable approaches towards effective weed management in agronomic crops. In its short existence, the glyphosate resistance has revolutionized weed control where cultivars bred for resistance to the herbicide have taken over the largest share of acreage planted. Many crops including sorghum, however, have been left out from benefiting from this or similar technology. Owing to the difficulty and cost in discovering new herbicide chemistries and finding novel mode of actions being even more difficult (Gressel, 2002), it's unlikely that any new herbicide chemistries solely targeting sorghum or other relatively minor crops would be introduced to the market in the recent future. Therefore, development of novel varieties resistant to one or more of the existing herbicide chemistries is plausible and can offer growers more herbicide choices and mixtures if resistance to more than one herbicide becomes available (Green et al., 2008). Following the discovery of resistant mutants to ALS and ACCase inhibitor herbicides among wild sorghum populations, significant efforts were made to incorporate these traits into cultivated grain sorghum. These efforts are bearing fruits that several seed companies and Kansas State University have developed series of agronomically desirable parental lines into which the resistance traits were incorporated. One outstanding concern raised by growers and the industry alike is the possible yield drag that may be caused either by the unusually yellow seedling phenotype associated with ALS resistance trait or by the wild genes that may drag along the resistance gene. Industries speculate about how much yield penalty farmers are willing to accept while at the same time expect to pay a premium price for the technology at least during the initial years of its deployment. This study was aimed at answering these questions and attempts to determine how much yield drag, if any, is associated with herbicide resistance.

The result showed that hybrids resistant to ALS inhibitor herbicides tend to express seedling chlorosis which eventually disappears at later stages of growth (Table 4.5) and that photosynthetic efficiency in adult ALS resistant hybrids was the same as in the susceptible commercial checks (Table 4.6). Though the early season chlorosis may certainly reduce photosynthetic efficiency at early stage, it appears to have no or little effect on phenology and agronomic characteristics of the adult plant (Figure 4.1). In one of the experimental sets (set I), the mean grain yield of eight ALS resistant hybrids was 4999 kg ha^{-1} compared to the mean of commercial checks which was 4696 kg ha^{-1} (Table 4.6). Other yield components such as grain weight panicle yield, TKW were not affected suggesting that photosynthetic stress caused by seedling chlorosis at early growth stages has little impact on these traits. This agrees with previous studies where much of the assimilate contributing to grain yield and yield components was reported to come from current photosynthesis (Richards, 2000). Results from experimental sets II and III were also similar to set I (Tables 4.7-4.10) except that mean grain yield of commercial checks was superior to that of the herbicide resistant groups in set II Experiment though there are several herbicide resistant hybrids that had yields higher than or comparable to the checks (Table 4.7). The early season chlorosis and the associated reduced seedling vigor is obviously undesirable and the hybrids may compete poorly against early season weed flushes. However, since they are resistant to herbicides over the top chemicals can be applied to eliminate any damage that these weeds may cause.

The effect of herbicide treatment was not studied in this experiment because some of the entries are herbicide susceptible. But the result from previous chapter shows that though flowering may be delayed by 2-3 days, herbicide treatment does not have any effect on yield of inbred lines. Based on that and previous observations we expect that yield potential in ALS resistant hybrids

will not be affected by herbicide treatment. In fact in fields where pre-emergence weed control is not effective, ALS herbicide application is expected to maintain high yield by reducing weed competition. Since the herbicides do not bind with the ALS enzyme in resistant plants to compromise their function, the typical herbicide damage response seen in susceptible plants such as leaf browning or bottle brush roots (University of Missouri, 2016) are not visible in resistant sorghums.

Similar to the agronomic parameters, nutritional profiles of ALS resistant hybrids were comparable to that of susceptible commercial checks. The major nutrients such as protein, starch and fat in herbicide resistant groups was the same as that of the checks under all environments. Nevertheless, some of the mineral nutrients such as phosphorus and calcium as well as micro nutrients such as copper, zinc and manganese appear to be higher in herbicide resistant hybrids by an order of 17 to 21% for macro nutrients and 8-30% for micro nutrients compared to the checks. But the concentration of other minerals such as iron and potassium is similar to that of the checks. The difference in the mineral profiles between the herbicide resistant and susceptible hybrids may be either the result of genes that may have inadvertently introduced along with the resistance genes or is simply the background effect as the susceptible checks may be likely distant from the public herbicide resistant hybrids.

Conclusion

With concerns over yield drags possibly caused by the ALS mutation still hanging, the ALS resistant hybrids are making their way to farmers' field. The first batch of resistant seeds were sold for 2016 planting and farmers will see, for the first time not just the effects of the yield drag but also the prospect of over the top herbicide use in sorghum. This study showed that though

ALS resistance genes are associated with seedling yellowing which is the basis for the concerns, yield drag as a result of such phenomena is unlikely. Moreover, the wide difference in leaf chlorophyll content among ALS resistant hybrids shows that proper selection after and before herbicide application can reduce the occurrence of such phenotypes. Likewise, both the ALS and ACCase resistant hybrids also do not have negative effect on nutritional profile of the grains that these resistant hybrid crops can be used in all food, feed and industrial applications that sorghum has been traditionally used for.

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Appendix A - Supplemental information for chapter 2

Table A.1. The read mapping summary for yellow and normal samples at each stage of sampling.

Sample	Trimmed Reads	Mapped Reads	Mapped%	Confident Mapped Reads	Confident Mapped %
Green-S0 stage-replicate1	17100000	16717481	97.8	15489374	90.6
Green-S0 stage-replicate2	11386966	10314346	90.6	9478054	83.2
Green-S0 stage-replicate3	8138406	7919657	97.3	7302557	89.7
Green-S1 stage-replicate1	12544645	11972499	95.4	11094164	88.4
Green-S1 stage-replicate2	11304313	11009385	97.4	10188836	90.1
Green-S1 stage-replicate3	12367994	12098903	97.8	11261965	91.1
Green-S2 stage-replicate1	12715191	12151177	95.6	11236344	88.4
Green-S2 stage-replicate2	11345117	10851960	95.7	10078756	88.8
Green-S2 stage-replicate3	15031984	14694783	97.8	13570020	90.3
Green-S3 stage-replicate1	12185823	11838370	97.1	10975077	90.1
Green-S3 stage-replicate2	10549889	10096828	95.7	9352468	88.6
Green-S3 stage-replicate3	10053833	9774332	97.2	9048876	90
Yellow-S0 stage-replicate1	12932513	12585942	97.3	11656006	90.1
Yellow-S0 stage-replicate2	14913289	14551466	97.6	13512155	90.6
Yellow-S0 stage-replicate3	14578395	14244619	97.7	13222902	90.7
Yellow-S1 stage-replicate1	8850648	8598795	97.2	7995057	90.3
Yellow-S1 stage-replicate2	11699313	11428736	97.7	10566808	90.3
Yellow-S1 stage-replicate3	11271245	10928415	97	10133689	89.9
Yellow-S2 stage-replicate1	14450551	14051613	97.2	13064471	90.4
Yellow-S2 stage-replicate2	13050379	12460776	95.5	11593536	88.8
Yellow-S2 stage-replicate3	11872101	11542378	97.2	10755236	90.6
Yellow-S3 stage-replicate1	11241136	10989004	97.8	10182955	90.6
Yellow-S3 stage-replicate2	11541148	11235620	97.4	10434927	90.4
Yellow-S3 stage-replicate3	12012777	11625966	96.8	10759674	89.6

Appendix B - Supplemental information for chapter 3

Table B.1. The effect of herbicide treatment and seedling phenotype on physiological and agronomic characteristics of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment (Environment 1).

Treatment Effects	Chlorophyll content (SPAD units)		Days to Flowering	Plant height (cm)		Biomass (g)	
	Seedling	Adult Plant		Seedling	Adult plant	Seedling	Adult plant
Herbicide							
Untreated	33 (±0.31)	55.3 (±0.26)	70.4 (±0.21)	18.8 (±0.31)	129.3 (±0.25)	46.9 (±1.1)	336.0 (±6.3)
Treated	28.6 (±0.31)	53.3 (±0.26)	71.7 (±0.21)	15.9 (±0.31)	122.3 (±0.25)	32.3 (±1.1)	344.8 (±6.3)
Mean	30.8	54.3	71.1	17.4	125.8	39.6	340.4
†LSD	2.56	ns	ns	1.09	5.25	5.76	ns
Seedling Color							
Yellow	27.8 (±0.65)	54.5 (±0.69)	72.3 (±0.57)	16.7 (±0.32)	128 (±0.67)	33.8 (±1.8)	339.4 (±9.3)
Normal	37.1 (±0.92)	54 (±0.98)	68.4 (±0.80)	18.6 (±0.46)	97.2 (±0.92)	51.5 (±2.4)	342.5 (±13.2)
Mean	32.5	54.3	70.4	17.7	112.6	42.7	340.9
†LSD	2.37	ns	1.96	1.23	5.65	5.92	ns

†LSD = Least significant difference; ns = not significant.

Table B.2. The effect of herbicide treatment and seedling phenotype on physiological and agronomic characteristics of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment (Environment 2).

Treatment effect	Chlorophyll content (SPAD units)		Days to Flowering	Plant height (cm)		Biomass (g)	
	Seedling	Adult Plant		Seedling	Adult plant	Seedling	Adult plant
Herbicide							
Untreated	23.4 (±0.57)	55.4 (±0.63)	72.9 (±0.60)	18.1 (±0.24)	106.3 (±2.0)	30.3 (±0.84)	317.71 (±4.79)
Treated	29.7 (±0.57)	55.5 (±0.63)	75 (±0.60)	16.2 (±0.24)	102.8 (±2.0)	34.9 (±0.84)	260.7 (±4.79)
Mean	26.6	55.4	73.9	17.1	104.6	32.6	289.2
†LSD	2.67	ns	ns	0.61	ns	ns	26.8
Seedling Color							
Yellow	23.2 (±0.65)	55.1 (±0.56)	73.8 (±0.91)	16.8 (±0.19)	104.5 (±1.57)	31.0 (±1.11)	279.7 (±8.2)
Normal	33.4 (±0.92)	56.6 (±0.69)	74.2 (±1.29)	17.7 (±0.27)	104.4 (±1.92)	35.7 (±1.57)	308.3 (±11.6)
Mean	28.3	55.8	74.0	17.3	104.5	33.4	294.0
†LSD	2.53	1.39	ns	0.73	ns	3.86	ns

†LSD = Least significant difference; ns = not significant.

Table B.3. Phenology and growth characteristics of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment during the 2013 and 2014 seasons (Environment 1).

Genotype	Chlorophyll content (SPAD units)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
MN13-7458	38.0	40.4	51.1	55.2	67.5	64.5	21.3	18.1	129.2	116.2	83.5	55.8	376.9	256.4	G
MN07-1916	42.2	40.2	63.8	63.5	68.5	65.5	18.5	16.6	120.5	100.7	36.9	35.5	273.0	303.6	G
MN13-7450	39.7	39.3	54.5	54.7	65.0	66.0	20.3	18.0	116.2	105.5	74.0	51.0	468.2	345.1	G
PR12/13-763-2	35.2	37.8	46.5	35.9	76.5	71.5	18.5	16.3	133.7	118.7	34.5	35.6	231.2	311.5	G
PR12/13-763-4	30.5	33.5	47.1	32.4	71.5	72.0	19.5	17.2	109.0	118.2	62.2	37.6	424.4	234.6	Y
MN13-7498	38.4	36.6	53.5	57.8	64.5	62.5	21.8	19.3	123.7	108.7	79.5	43.0	293.2	389.8	G
MN11-10362	38.9	36.4	58.1	57.1	74.0	75.0	20.0	17.1	143.7	137.5	55.2	30.1	431.8	519.2	Y
PR12/13-763-1	39.8	36.3	55.1	36.5	71.5	73.0	18.6	16.5	131.5	117.0	59.7	33.9	381.0	396.5	G
MN13-7462	38.1	36.3	55.1	57.6	64.0	62.5	20.0	17.5	113.7	105.5	66.3	35.9	300.8	193.4	G
PR12/13-763-3	34.1	34.8	54.1	54.5	71.5	75.5	18.1	17.3	130.7	121.5	54.9	40.0	455.8	317.7	Y
MN13-7439	37.3	35.1	57.6	53.9	64.0	62.5	21.3	18.3	140.0	130.2	70.2	47.2	345.0	324.5	G
PR12/13-763-5	30.3	34.9	55.0	31.5	71.5	72.0	18.2	16.4	127.0	125.7	41.4	38.5	401.0	314.1	Y
PR11/12-1026	36.9	34.9	51.6	60.7	64.5	65.5	21.1	17.7	123.2	117.5	70.7	47.6	282.5	315.1	G
MN07-2118	31.0	32.9	54.7	55.0	64.5	64.5	18.0	15.6	123.2	109.2	56.4	42.0	227.4	206.2	Y
MN13-7840	31.4	31.7	57.8	58.3	61.0	62a	21.3	17.5	111.2	107.5	65.3	35.2	329.7	246.1	Y
PR11/12-852	33.2	31.3	56.0	56.4	76.5	83e	19.0	15.8	142.0	131.2	41.7	36.8	350.6	414.2	Y
MN13-7838	34.5	30.4	56.5	62.5	61.5	62.5	18.2	15.9	110.7	104.0	48.5	45.2	224.9	236.0	Y
PR11/12-873	34.0	30.0	59.6	59.5	76.0	75.5	24.0	15.5	121.2	114.5	51.5	37.5	455.3	485.1	Y
MN13-7463	32.6	30.0	51.9	58.0	64.5	65.5	20.8	18.4	120.7	118.2	53.7	37.5	265.6	306.9	Y
PR11/12-850	33.6	29.3	62.3	61.9	76.5	80.0	18.1	15.7	138.7	145.2	46.1	41.2	433.2	419.8	Y
MN13-7923	36.9	29.1	57.4	40.1	64.5	73.5	19.8	16.1	109.0	106.2	84.2	31.7	343.4	363.9	G
PR11/12-851	38.1	28.4	61.6	58.8	76.5	80.5	19.0	15.8	139.0	128.2	53.1	22.1	352.5	427.0	G
PR11/12-984	33.6	27.4	60.7	42.9	68.0	73.0	17.1	13.5	113.7	109.5	43.1	17.2	274.7	321.0	Y
MN13-7455	34.2	24.5	56.0	59.5	64.5	67.0	20.3	17.7	112.5	112.5	45.8	38.1	322.8	267.4	Y
MN13-7499	32.3	24.2	54.5	57.3	64.5	62.5	20.3	17.8	106.2	102.0	49.8	42.3	296.8	247.5	Y
MN07-2165	27.2	22.4	59.0	59.9	73.5	78.0	19.1	13.4	105.7	107.0	27.9	19.1	314.0	317.3	Y
PR12/13-764-3	24.8	19.6	45.5	53.3	71.5	75.5	18.8	14.2	150.7	157.5	27.3	19.8	358.7	354.1	Y
PR12/13-762-1	25.0	19.6	56.5	56.4	73.5	75.5	15.5	13.0	130.0	129.0	20.7	16.4	248.5	330.1	Y
PR12/13-764-2	22.6	19.3	54.0	53.1	73.0	75.0	17.0	12.5	150.2	152.5	18.9	12.1	312.6	344.8	Y
PR12/13-762-2	25.5	19.2	57.0	54.7	74.5	78.5	14.8	14.0	125.7	116.5	26.2	12.4	302.6	426.4	Y
PR9/10-4720-1	22.7	18.1	58.0	56.5	76.0	80.0	15.1	15.2	150.0	141.2	23.1	18.9	377.9	472.5	Y
MN13-7500	19.5	17.9	52.6	55.2	76.5	78.5	16.3	14.0	163.2	155.0	32.5	32.3	510.0	537.8	Y

Genotype	Chlorophyll content (SPAD units)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
PR12/13-764-4	19.2	17.1	52.0	54.0	77.0	76.0	16.3	14.7	166.7	142.5	15.3	15.0	162.9	338.6	Y
PR12/13-761	21.1	16.5	55.3	53.2	76.5	77.5	12.6	12.2	123.2	115.2	23.5	18.6	327.3	363.0	Y
PR12/13-764-6	23.3	15f	52.4	52.2	74.0	74.0	16.0	13.1	139.0	134.5	25.6	21.5	346.2	272.5	Y
PR12/13-764-1	21.1	14.4	59.0	53.3	76.5	75.5	14.8	14.3	156.2	144.5	21.4	17.4	293.7	399.1	Y
Tx430	40.3	-	57.7	-	65.3	-	18.0	-	102.5	-	32.5	-	390.9	-	G
Mean	33.2	28.6	55.4	53.4	70.4	71.7	18.6	15.9	129	122.25	47	32.3	336.0	342.0	-
‡LSD	3.38	3.95	3.35	3.17	3.4	3.09	3.4	2.81	4.7	4.5	12.8	11.7	66.1	58.29	-

†Seedling color Y= yellow G = Green;
‡LSD = Least significant difference.

Table B.4. Phenology and growth characteristics of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment during the 2013 and 2014 seasons (Environment 1).

Genotype	Chlorophyll content (SPAD)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
PR11/12-1026	32.2	42.2	58.4	56.7	72.0	71.5	18.5	16.0	115.0	100.0	53.1	36.0	229.4	289.3	G
MN13-7923	31.5	41.5	57.1	57.8	62.0	68.5	20.0	17.5	102.5	101.3	28.5	48.8	307.9	222.1	G
MN13-7450	35.3	41.5	58.0	59.7	73.0	72.0	19.5	16.5	95.0	92.5	15.1	31.7	486.0	346.2	G
MN13-7462	30.9	40.1	60.0	59.8	68.0	69.0	18.5	16.0	107.5	102.5	45.7	41.4	344.5	237.0	G
PR12/13-763-1	22.4	39.4	50.0	53.5	85.5	77.0	17.0	13.5	83.8	98.8	33.1	25.7	538.9	352.1	G
MN11-10362	23.7	34.3	54.8	55.1	75.5	84.0	18.0	18.5	118.8	126.3	44.9	36.4	403.2	409.5	Y
PR12/13-763-4	30.3	33.2	49.3	51.4	83.5	80.5	15.5	16.0	90.0	106.3	13.5	26.5	383.4	279.1	Y
PR12/13-763-5	25.8	31.7	55.6	48.5	76.0	88.5	17.5	16.0	95.0	100.0	32.7	31.4	263.5	257.5	Y
MN13-7439	21.1	38.4	54.5	57.7	71.0	71.0	21.5	16.5	117.5	116.3	31.6	40.2	368.2	271.2	G
MN07-1916	26.5	37.3	62.1	58.2	62.0	67.5	19.5	18.5	101.3	106.3	16.3	32.9	223.0	203.7	G
PR12/13-763-2	38.4	36.3	56.6	54.0	80.5	96.0	15.5	12.5	90.0	110.0	28.3	40.7	261.5	195.0	G
PR11/12-851	34.1	36.0	63.5	59.2	91.5	97.5	20.0	16.5	102.5	108.8	34.3	22.5	309.6	375.2	G
MN13-7458	33.8	35.4	49.5	54.1	69.0	69.0	20.5	18.5	92.5	108.8	34.0	53.9	279.3	195.0	G
PR12/13-763-3	18.6	34.9	51.2	55.5	83.5	86.5	17.0	15.0	85.0	103.8	26.2	24.3	321.6	195.0	Y
MN13-7463	31.0	33.2	56.7	57.3	69.5	68.5	18.5	17.5	110.0	103.8	43.3	39.7	377.3	239.0	Y
MN07-2118	28.4	33.2	55.3	52.2	65.5	66.5	18.0	16.5	106.3	107.5	37.5	65.6	262.7	221.7	Y
MN13-7498	27.2	32.5	57.1	50.4	61.5	67.5	20.0	19.0	108.8	105.0	44.6	39.5	294.0	299.8	G
MN13-7838	18.6	32.3	58.3	56.1	57.5	63.5	19.0	17.0	103.8	93.8	30.6	43.7	205.3	195.0	Y
PR11/12-852	24.6	31.5	53.7	54.3	90.0	94.0	18.0	16.5	108.8	121.3	21.3	24.5	279.7	320.1	Y
PR11/12-984	20.5	29.9	61.9	59.2	67.5	65.0	17.0	17.0	106.3	113.8	39.7	31.2	276.3	262.2	Y
PR11/12-850	19.9	29.7	54.5	55.4	77.0	88.5	19.0	16.5	111.3	123.8	29.8	32.9	347.2	290.2	Y
MN13-7840	23.9	28.8	58.5	56.1	62.0	65.0	20.0	18.5	101.3	98.8	33.9	28.3	228.8	195	Y
MN13-7455	19.3	28.3	55.9	60.7	70.0	66.5	20.5	17.5	100.0	101.3	39.4	38.0	335.7	278.3	Y
PR11/12-873	20.2	27.9	52.6	58.0	74.5	82.5	19.0	16.5	105.0	111.3	31.9	39.1	422.2	331.3	Y
MN13-7499	21.7	27.7	58.4	56.5	61.0	65.5	19.0	18.0	106.3	110.0	14.3	45.7	288.6	298.8	Y
PR12/13-762-2	20.9	23.3	55.1	55.6	74.0	78.0	16.0	14.5	92.5	103.8	32.5	19.2	375.2	233.7	Y
PR12/13-762-1	20.1	21.6	55.9	56.3	72.0	71.0	17.5	16.0	98.8	107.5	27.5	37.2	339.0	216.8	Y
MN07-2165	17.3	21.0	54.1	62.3	76.0	69.0	17.5	15.0	78.8	85.0	41.1	36.0	276.9	225.4	Y
PR12/13-764-2	15.2	18.4	50.9	53.2	72.0	71.0	16.5	15.0	118.8	106.3	21.0	24.1	278.7	208.7	Y
PR12/13-761	17.5	17.8	52.3	53.7	81.5	81.0	15.0	13.0	88.8	92.5	18.0	22.5	263.0	195.0	Y
MN13-7500	20.2	17.8	51.9	55.3	72.0	73.5	21.0	17.5	113.8	107.5	33.3	36.7	482.2	505.8	Y

Genotype	Chlorophyll content (SPAD)				Days to flowering		Plant height (cm)				Biomass (g)				†Seedling color
	Seedling		Adult plant				Seedling		Adult plant		Seedling		Adult plant		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
PR9/10-4720-1	16.1	17.4	54.0	58.5	77.0	77.5	16.0	15.0	105.0	107.5	21.4	42.2	264.4	380.6	Y
PR12/13-764-3	19.1	15.8	52.6	51.5	74.5	68.0	17.5	15.0	106.3	111.3	24.0	21.7	232.0	195.0	Y
PR12/13-764-4	17.1	14.6	55.0	52.7	73.5	75.0	17.0	15.5	118.8	115.0	30.1	23.1	338.9	195.0	Y
PR12/13-764-1	17.7	14.1	57.6	52.0	71.5	73.5	16.5	15.5	111.3	112.5	23.9	40.0	244.9	244.2	Y
PR12/13-764-6	18.0	11.8	53.9	51.6	72.0	71.5	17.5	14.5	110.0	108.8	20.7	23.1	303.7	204.8	Y
Tx430	36.4	-	57.0	-	70.3	-	17.5	-	114.3	-	35.0	-	314.4	-	G
Mean	23.3	29.7	55.5	55.6	72.9	75	18.2	16.2	102.7	106.2	30.5	34.6	317.6	266.4	-
‡LSD	4.3	4.5	3.2	3	3.6	3.7	2.9	2.5	16.2	17	10.9	9.6	57.1	69.3	-

†Seedling color Y= yellow G = Green;
‡LSD = Least significant difference.

Table B.5. Mean of the effect of herbicide treatment and seedling phenotype on agronomic parameters and yield components of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment (Environment 1).

Treatment Effects	Chlorophyll content	Adult plant Height	Days to flowering	Panicle length	Panicle diameter	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
Herbicide										
Control	55.4(±0.56)	103.3(±1.6)	73(±0.73)	25.2(±0.39)	5.6(±0.08)	76.9(±2.19)	52.8(±1.57)	2169(±57.7)	24.4(±0.29)	3713.4(±363.4)
Treated	55.9(±0.56)	106.5(±1.6)	78(±0.73)	27.3(±0.40)	5.4(±0.08)	77.3(±2.22)	52.6(±1.55)	2243(±58.5)	23.4(±0.30)	4139.1(±365.8)
Mean	55.7	104.9	76	26.3	5.5	77.1	52.7	2206	23.9	3926.3
§LSD	ns	ns	2.55	0.85	ns	ns	ns	ns	1.04	ns
Seedling color										
Yellow	55.3(±0.56)	105.0(±1.52)	75(±1.01)	26.1(±0.39)	5.4(±0.08)	73.5(±2.4)	50.1(±1.90)	2129(±71.4)	23.5(±0.36)	3907.2(±357.3)
Normal	56.0(±0.67)	104.7(±1.80)	74(±1.44)	26.4(±0.50)	5.7(±0.11)	83.5(±3.5)	57.1(±2.70)	2328(±92.50)	24.6(±0.52)	4017.4(±401.4)
Mean	55.7	104.9	75	26.3	5.6	78.5	53.6	2229	24.1	3962.3
§LSD	ns	ns	ns	ns	0.25	7.42	5.60	ns	ns	ns

†TKW = thousand kernel weight; ‡KN = kernel number per panicle.
§LSD = Least significant difference; ns = not significant.

Table B.6. The effect of herbicide treatment and seedling phenotype on agronomic parameters and yield components of ALS herbicide resistant sorghum (*Sorghum bicolor* (L.) Moench) genotypes evaluated with and without herbicide treatment (Environment 2).

Treatment Effects	Chlorophyll content	Adult plant Height	Days to flowering	Panicle length	Panicle diameter	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
Herbicide										
Control	56.3(±0.58)	125.2(±1.3)	72(±0.56)	29.1(±0.17)	6.0(±0.07)	120.6(±1.81)	77.5(±1.72)	2956(±53.7)	26.5(±0.25)	2957.0(±162.8)
Treated	57.2(±0.58)	122.9(±1.3)	73(±0.56)	29.3(±0.17)	6.2(±0.07)	124.4(±1.81)	77.6(±1.72)	2884(±53.7)	27.1(±0.25)	2954.0(±162.8)
Mean	56.8	124.1	73	29.2	6.1	122.5	77.6	2920	26.8	2955.5
§LSD	ns	ns	ns	ns	ns	ns	ns	ns	0.82	ns
Seedling Color										
Yellow	56.5(±0.47)	123.2(±1.2)	73(±0.52)	29.3(±0.20)	6.1(±0.07)	122.3(±2.3)	77.1(±1.75)	2959(±71.4)	26.3(±0.27)	3026.7(±141.3)
Normal	57.4(±0.52)	126.0(±1.6)	70(±0.73)	29.1(±0.29)	6.2(±0.11)	122.9(±3.3)	78.5(±2.47)	2842(±92.5)	27.8(±0.37)	2799.3(±199.8)
Mean	56.9	124.6	72	29.2	6.1	122.6	77.8	2901	27.1	2913.0
§LSD	ns	ns	1.78	ns	ns	ns	ns	ns	0.84	ns

†KN = kernel number per panicle; ‡TKW = thousand kernel weight;
§LSD = Least significant difference; ns = not significant.

Table B.7. Agronomic characteristics and yield components of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment (Environment 1).

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle weight		Panicle yield		†KN		‡TKW		Grain yield		§Seedling color
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
MN07-2165	80.7	83.2	74.3	69.0	22.3	25.7	5.0	5.7	54.6	77.6	35.4	56.3	2277	2286	17.6	24.5	2548	4059	Y
PR11/12-984	108.2	111.7	67.0	66.0	22.9	28.8	5.7	5.9	72.1	78.7	48.9	56.0	2056	2418	23.9	23.1	4085	4525	Y
MN13-7463	108.2	101.7	68.7	69.7	31.1	29.8	6.4	5.9	102.3	102.7	69.0	70.0	3062	3108	22.6	22.5	3231	3319	Y
MN13-7455	104.2	103.2	70.7	67.3	33.0	32.6	6.6	6.3	106.5	101.6	69.8	66.9	2979	2877	23.4	23.1	4079	4087	Y
MN13-7439	118.2	118.2	71.3	75.7	31.3	29.7	6.1	5.3	106.9	78.9	73.8	62.3	2956	2669	24.9	23.3	3620	4102	G
PR11/12-873	105.0	113.2	75.0	85.0	29.8	31.0	5.4	4.9	95.2	77.5	67.8	52.2	2488	2009	27.4	26.2	4212	4055	Y
PR11/12-1026	113.2	100.7	72.3	70.3	28.8	27.0	7.1	6.7	117.6	107.1	77.5	81.7	2694	3019	28.8	27.0	3144	4279	G
MN07-1916	100.7	109.2	61.7	65.7	22.4	24.4	5.4	5.0	56.2	61.1	39.1	38.9	1710	1661	22.8	23.4	4160	4115	G
MN13-7923	105.0	101.7	63.0	69.7	27.1	26.7	5.6	4.9	77.9	64.0	52.4	44.4	2262	1932	23.1	22.8	3248	3669	G
PR9/10-4720-1	106.7	109.2	76.0	83.7	25.9	26.1	5.3	5.0	71.6	58.5	58.8	36.8	2649	1617	22.3	22.7	2987	3775	Y
MN13-7838	104.2	94.2	59.7	65.7	22.7	23.6	4.9	4.7	55.8	57.2	38.2	42.2	1622	1567	23.8	26.8	4039	3945	Y
MN13-7462	105.7	102.5	68.3	68.7	26.9	26.8	6.8	6.0	106.1	104.	71.9	73.9	3227	3174	22.2	23.4	3120	3659	G
PR11/12-850	116.7	122.5	76.0	95.0	23.9	29.6	4.6	4.1	48.2	53.5	38.6	35.6	1419	1572	27.0	22.6	2895	3228	Y
MN13-7499	107.5	114.2	61.3	64.7	25.6	27.3	6.6	5.2	101.4	83.6	75.2	66.5	2854	2542	26.4	26.0	2599	3330	Y
PR11/12-851	99.2	108.2	81.0	98.7	25.0	28.2	5.3	4.1	82.2	45.9	59.7	26.0	2149	1190	27.7	21.9	4010	3322	G
MN13-7450	94.2	90.7	76.3	72.0	25.2	27.6	6.3	6.4	97.7	96.6	66.3	61.2	2419	2338	27.2	25.4	4157	2576	G
PR12/13-762-1	99.2	108.2	73.3	72.0	27.1	28.3	6.2	6.2	95.8	98.6	61.1	65.9	2498	2785	24.3	23.7	4578	3513	Y
MN11-10362	120.0	126.7	75.3	85.0	27.3	29.0	5.2	5.3	79.4	82.4	55.5	46.8	2213	2825	25.3	17.8	3740	3568	Y
PR12/13-762-2	90.7	102.5	76.3	81.0	22.4	25.1	5.4	4.7	62.1	52.7	43.5	34.9	1758	1638	24.7	21.6	4402	2179	Y
MN13-7840	100.0	99.2	63.3	64.3	22.9	26.2	5.2	5.4	61.1	75.2	42.5	57.4	1843	2518	23.0	22.8	3867	4356	Y
MN13-7500	117.5	114.2	71.7	74.3	28.2	32.0	6.2	5.9	109.0	96.5	75.8	73.0	2017	2739	37.1	26.4	2998	3219	Y
PR12/13-761	90.0	93.2	79.7	85.3	22.1	29.6	4.9	5.2	43.2	71.9	35.0	42.0	1258	1951	21.4	21.4	3316	2733	Y
PR12/13-764-1	110.7	112.5	72.0	73.0	25.9	25.2	6.3	5.2	103.2	83.8	69.7	62.1	2970	2628	23.5	23.7	4509	4034	Y
PR12/13-764-6	110.0	111.7	72.7	71.7	25.0	23.6	5.7	5.0	84.2	58.2	61.7	39.4	2627	1663	23.5	23.7	4346	3881	Y
PR12/13-764-4	114.25	111.7	73.3	84.7	25.9	25.2	5.8	4.8	80.4	47.3	55.8	36.7	2363	1634	23.5	22.4	4106	2612	Y
PR12/13-763-2	91.7	107.5	79.3	96.0	23.2	26.0	5.1	5.6	59.2	79.4	40.6	51.3	1835	2549	22.1	19.7	3863	2385	G
PR12/13-763-3	89.2	98.2	82.0	92.0	22.8	24.2	5.2	4.7	59.6	55.1	42.4	32.8	2000	1605	21.1	20.5	2535	4181	Y
PR11/12-852	107.5	120.7	86.7	95.3	22.0	28.7	4.0	4.5	38.6	57d	29.3	18.8	1118	1408	23.4	16.7	1419	2593	Y
MN13-7458	95.0	105.0	69.3	69.3	23.8	27.4	5.4	6.4	60.1	92.4	41.2	62.7	1497	2151	27.7	29.8	2527	3417	G
PR12/13-763-1	85.7	97.5	85.3	78.0	21.6	32.5	5.0	6.5	53.2	129.	35.6	90.5	2038	3564	18.2	25.4	4036	4383	G
MN07-2118	101.7	108.2	67.7	68.0	24.7	26.3	5.3	5.3	65.2	66.4	42.9	40.3	1554	1610	27.3	25.1	2927	4009	Y
PR12/13-763-4	95.7	105.0	81.0	85.3	24.8	26.4	5.3	5.4	71.3	65.9	47.0	43.3	1903	2105	25.9	20.5	4391	2970	Y

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle weight		Panicle yield		†KN		‡TKW		Grain yield		§Seedling color
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
PR12/13-764-2	115.7	112.5	72.3	70.7	25.8	27.1	5.9	6.0	82.8	83.4	48.9	61.3	2216	2710	22.0	22.6	2116	3499	Y
PR12/13-764-3	101.7	112.5	74.0	70.0	23.8	25.2	6.0	5.4	78.2	79.9	48.8	54.4	2122	2399	23.0	22.6	3271	3135	Y
PR12/13-763-5	94.2	102.5	80.7	95.0	22.6	26.5	5.1	5.2	52.2	64.2	37.6	42.8	1748	2021	21.5	20.9	2238	2323	Y
MN13-7498	110.7	105.7	62.7	67.0	23.4	25.2	6.1	6.0	89.8	96.4	61.2	69.8	2014	2280	30.5	30.8	4014	4018	G
Tx430	102.5	-	72.3	-	30.0	-	5.9	-	99.4	-	68.1	-	1974	-	35.0	-	3956	-	G
Mean	102.5	106.5	71.6	76.7	29.3	27.3	6.4	5.4	100.4	77.3	74.9	52.6	2250	2243	33.3	23.4	4150	3529	-
¶LSD	11.8	12.0	6.3	6.9	3.5	3.4	1.1	1.2	31.3	34.3	23.8	25.4	231.1	265.3	4.6	4.1	688.3	654.6	-

†KN = kernel number per panicle; ‡TKW = thousand kernel weight; §SC= Seedling color Y = Yellow G = Green;
¶LSD = Least significant difference; ns = not significant; Untrt.= without herbicide treatment, Trt. = Herbicide treated.

Table B.8. Agronomic characteristics and yield components of ALS herbicide resistant sorghum genotypes evaluated with and without herbicide treatment (Environment 2).

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle yield		Panicle weight		†KN		‡TKW		Grain yield		§Seedling color
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
MN13-7450	120.0	119.3	76.7	76.7	28.2	29.8	7.3	7.3	78.7	83.4	129.7	142.9	3124	3138	25.3	25.9	3401.2	4252.7	G
PR11/12-851	118.3	128.3	68.3	73.0	31.5	30	5.0	4.7	54.4	44.5	89.0	76.9	1899	1464	28.8	30.5	2107.4	3270.5	G
MN11-10362	132.5	128.3	75.7	77.3	31.8	32.8	5.3	5.6	86.2	82.8	126.3	129.5	2795	2809	30.8	29.5	3263.5	3055.6	Y
PR11/12-852	120.8	121.8	85.0	84.3	29.0	29.8	4.8	4.3	38.4	36.1	67.6	62.6	1434	1260	26.4	28.4	4345.4	4279.2	Y
PR11/12-1026	130.8	128.3	70.0	71.0	29.3	29.8	6.8	7.3	93.5	79.9	132.2	122.9	3097	3039	30.2	26.1	3017.4	3169.4	G
PR11/12-873	115.0	115.8	76.3	77.3	31.0	32.2	5.2	5.8	66.5	97.9	100.2	141.1	2140	3081	31.1	31.8	2504.9	3201.1	Y
PR12/13-763-3	125.0	115.8	74.0	75.7	30.2	29.5	6.3	5.8	94.1	88.5	145.7	139.2	3890	3563	24.2	24.9	2822.4	1566.7	Y
MN07-1916	120.0	110.0	58.0	69.7	25.5	25.3	5.2	5.3	40.9	37.2	76.0	73.3	1554	1371	26.1	27.1	2821.6	1222.3	G
PR11/12-984	119.3	113.3	68.7	73.3	26.3	26.7	5.7	6.7	61.3	71.1	96.1	113.1	2403	2788	25.5	25.5	2877.2	2071.7	Y
MN13-7840	112.5	104.3	71.0	77.7	26.3	25.2	6.2	5.8	64.3	50.6	102.9	87.7	2465	2026	25.9	25	2118.4	4328.5	Y
PR11/12-850	134.3	134.3	81.0	78.3	28.3	30.7	6.1	5.3	88.0	71.5	113.8	106.4	2900	2281	30.3	31.3	2149.8	4380.7	Y
MN07-2165	96.8	97.5	77.0	76.0	25.7	27.7	5.3	5.4	51.4	47.9	82.0	80.7	2190	2033	23.4	23.5	1603.6	3846.6	Y
PR9/10-4720-1	133.3	130.0	74.7	77.0	29.8	30.2	6.5	6.8	99.0	94.6	153.2	153.7	4004	3676	24.7	25.6	2576.3	2993.3	Y
MN13-7455	121.8	115.0	69.7	70.0	31.8	33.8	6.2	7.7	81.4	92.2	128.0	147.9	3475	3828	23.4	24.1	3806.6	1656.9	Y
MN13-7439	130.8	129.3	69.0	71.7	28.5	28.5	7.1	7.0	104.0	87.1	151.3	135.9	3936	3220	26.6	27.4	2435.2	2507.6	G
MN07-2118	118.3	120.8	71.0	71.0	28.7	28.5	5.9	6.5	63.2	70.8	112.0	113.1	2529	2596	25.0	27.6	2916.8	2105	Y
PR12/13-763-4	130.8	125.8	75.0	77.7	30.3	28.7	6.1	5.8	92.5	73.5	140.6	118.5	3638	2935	25.4	24.9	3364.3	1949.7	Y
PR12/13-763-2	135.0	134.3	69.7	73.3	32.2	30.5	6.3	6.8	99.8	104	150.6	154.4	3776	3955	27.1	26.5	3123.7	3630.4	G
PR12/13-764-1	139.3	131.8	71.7	72.0	28.8	30.0	6.4	6.7	77.7	84.7	124.6	137.2	3079	3283	25.4	25.8	2704.4	3055	Y
MN13-7500	146.8	148.3	72.3	72.3	30.3	29.3	5.2	5.9	78.3	74.1	115.1	110.8	2467	2330	31.7	31.5	2450.8	1548d	Y
PR12/13-763-1	123.3	121.8	74.3	74.7	29.7	28.2	5.7	5.8	77.5	83.5	126.6	129.9	3224	3284	24.0	25.4	3029.5	1215.5	G
MN13-7923	120.0	119.3	75.0	69.3	30.8	28.7	5.0	5.0	56.0	59.9	91.31	97.3	2246	2299	25.1	26.1	2600.3	2271	G
PR12/13-761	102.5	108.3	74.0	75.0	30.0	30.0	6.5	7.6	88.5	102.0	138.5	155.7	3600	4076	24.6	25.0	3518.9	2508.2	Y
PR12/13-764-3	135.8	133.3	72.7	72.7	27.3	30.2	6.7	7.0	74.5	88.0	122.5	142.1	2931	3442	25.3	25.5	2648.8	2230.7	Y
PR12/13-764-2	136.8	136.8	74.0	70.7	30.5	29.7	7.0	7.1	100.0	91.7	156.1	153.5	4010	3496	25.0	26.2	2905.2	2916.1	Y
MN13-7458	135.8	134.3	70.3	68.0	28.3	30.0	7.1	7.8	81.2	103	120.3	147.9	2785	2949	29.0	35.1	3818.1	2210.6	G
PR12/13-762-2	113.3	104.3	72.3	75.3	28.8	29.2	6.4	6.8	77.8	82.6	127.1	137.3	3126	3272	24.8	25.3	2926.2	2519	Y
PR12/13-763-5	125.8	121.8	82.7	75.0	28.7	27.5	5.7	5.8	78.0	71.2	121.7	116	3338	2945	23.4	24.2	3310.5	2640.5	Y
PR12/13-762-1	119.3	117.5	73.3	75.3	28.5	28.5	5.7	6.2	74.5	58.4	114.6	107.7	2949	2402	25.1	24.3	2503.5	1713.3	Y
MN13-7499	129.3	121.8	66.7	66	30.8	30.3	5.8	6.1	68.5	85.8	109.7	130.6	2214	2718	30.7	31.6	2881.2	1723.3	Y
PR12/13-764-4	139.3	142.5	73.7	73.0	29.0	31.3	6.3	6.5	81.0	99.0	130.0	153.6	3165	3846	25.6	25.7	2965.3	3195.7	Y

Genotype	Plant height		Days to flowering		Panicle length		Panicle width		Panicle yield		Panicle weight		†KN		‡TKW		Grain yield		§Seedling color
	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	Untrt	Trt	
MN13-7838	119.3	113.3	58.0	67.7	25.3	28.2	5.6	5.5	63.5	63.4	100.1	103.4	2166	2100	29.3	30.2	3041.5	4259.8	Y
MN13-7462	127.5	129.3	68.7	68.7	29.7	29.3	7.3	7.1	101.0	76.6	157.5	137.1	4170	2973	24.3	25.8	4274.9	1855.2	G
MN13-7498	125.0	125.0	68.3	57.7	25.2	26.3	5.8	6.5	80.0	87.7	114.7	130.4	2431	2679	33.1	32.7	2705.8	2024.0	G
PR12/13-764-6	117.5	113.3	73.0	74.3	29.5	28.7	6.3	7.0	85.0	80.5	135.7	135.2	3384	3117	25.1	25.5	3215.1	3849.8	Y
MN13-7463	132.5	131.8	60.3	70.0	34.2	33.2	6.1	6.2	91.9	86.4	138.5	137.7	3890	3569	23.5	24.2	3464.4	2581.6	Y
Tx430	114.0	-	73.0	-	30.6	-	5.4	-	61.3	-	98.4	-	1698	-	34.6	-	4453.8	-	G
Mean	124.8	123.0	72.0	73.0	29.2	29.4	6.0	6.3	77.2	77.6	120.0	124.0	2922	2884	26.8	27.1	2991.2	2775.1	-
¶LSD	10.0	12.0	5.9	5.0	3.2	3.5	1.0	1.2	29.8	33.2	22.4	25.4	210.2	202.1	2.5	4.2	665.3	607.5	-

†KN = kernel number per panicle; ‡TKW = thousand kernel weight; §SC= Seedling color Y = Yellow G = Green;
¶LSD = Least significant difference; ns = not significant; Untrt. = without herbicide treatment, Trt. = Herbicide treated.

Table B.9. Correlation coefficients between all tested parameters under Experiment II. Correlations for environment 1 and 2 are shown above and below diagonal separately.

	Environment 1									
	Chlorophyll content	Plant height	Days to Flowering	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
Chlorophyll content	**0.59****	0.08	-0.13**	0.18**	0.11*	0.12*	0.12*	0.08	0.12*	0.05
Plant height	0.10	**0.45****	-0.12**	0.51**	0.27**	0.55**	0.51**	0.39**	0.38**	0.40**
Days to Flowering	-0.25**	0.001	**0.44****	0.01	-0.43**	-0.36**	-0.39**	-0.28**	-0.35**	-0.15*
Panicle length	0.17*	0.38**	0.09	**0.31****	0.39**	0.61**	0.55**	0.55**	0.12	0.13
Panicle width	0.16*	0.03	-0.11	0.10	**0.26****	0.87**	0.81**	0.78**	0.27**	0.14*
Panicle weight	0.15*	0.18**	-0.09	0.40**	0.75**	**0.17***	0.95**	0.88**	0.34**	0.19**
Panicle yield	0.16*	0.17*	-0.12	0.37**	0.69**	0.95**	**0.18****	0.88**	0.44**	0.20**
†KN	0.16*	0.13	-0.05	0.37**	0.67**	0.93**	0.92**	**0.20****	-0.02	0.17*
‡TKW	0.05	0.13	-0.17*	0.04	0.08	-0.05	0.11	-0.29**	**0.46****	0.09
Grain yield	0.19**	-0.03	-0.20**	0.09	0.09	0.25**	0.27**	0.31**	0.05	**0.14***
	Environment 2									

†KN = kernel number per panicle; ‡TKW = thousand kernel weight;
* and ** statistically significant at $P \leq 0.05$, and 0.01, respectively.

Table B.10. Mean genotype performance for nutritional parameters in tested ALS resistant parental lines under environment 1.

Genotype	Protein%	Fat%	Starch%	Ash%	Seedling Color
MN07-2118	19.1	4.8	71.6	1.55	Yellow
PR11/12-851	18.7	4.7	69.9	1.84	Green
PR12/13-762-2	17.7	4.8	72.2	1.80	Yellow
MN13-7838	17.7	4.8	69.6	1.74	Yellow
PR12/13-764-4	17.2	5.0	72.7	1.71	Yellow
PR11/12-852	17.1	4.8	71.7	1.53	Yellow
MN07-1916	16.8	4.7	71.8	1.72	Green
PR11/12-1026	16.8	5.3	74.6	1.59	Green
MN13-7458	16.8	5.0	75.1	1.55	Green
PR12/13-764-2	16.3	4.8	73.1	1.53	Yellow
PR11/12-984	16.2	4.8	72.9	1.72	Yellow
MN11-10362	16.1	4.8	73.6	1.51	Yellow
MN13-7923	16.1	5.1	73.3	1.74	Green
MN13-7439	16.0	5.0	73.3	1.44	Green
PR11/12-850	16.0	5.0	72.0	1.53	Yellow
PR12/13-762-1	15.9	4.8	73.3	1.60	Yellow
MN13-7462	15.7	4.9	72.4	1.59	Green
MN13-7498	15.6	5.0	75.7	1.43	Green
PR11/12-873	15.3	5.0	72.4	1.41	Yellow
MN13-7499	15.3	4.8	74.5	1.60	Yellow
PR12/13-763-1	15.2	4.8	69.9	1.54	Green
MN13-7840	15.2	5.1	73.2	1.61	Yellow
PR12/13-764-1	15.1	4.9	73.6	1.50	Yellow
PR12/13-761	14.9	4.8	73.4	1.56	Yellow
PR12/13-763-5	14.8	4.9	70.2	1.61	Yellow
PR12/13-764-3	14.8	4.9	73.3	1.50	Yellow
PR12/13-764-6	14.8	5.2	73.5	1.46	Yellow
MN13-7500	14.8	5.1	76.3	1.31	Yellow
MN13-7455	14.7	4.6	72.9	1.41	Yellow
MN13-7450	14.7	4.8	72.0	1.49	Green
PR12/13-763-3	14.6	5.0	70.1	1.53	Yellow
MN07-2165	14.4	4.8	74.5	1.37	Yellow
MN13-7463	14.3	4.9	73.9	1.46	Yellow
PR12/13-763-4	14.1	4.8	70.7	1.55	Yellow
PR12/13-763-2	14.0	5.0	70.9	1.50	Green
PR9/10-4720-1	14.0	5.0	73.8	1.42	Yellow
Tx430	14.7	1.5	4.7	71.7	Green
Mean	15.7	4.9	72.7	1.6	-
LSD	1.27	0.32	1.36	0.1	-

[†]LSD = Least significant difference.

Table B.11. Mean genotype performance for nutritional parameters in tested ALS resistant parental lines under environment 2.

Genotype	Protein%	Fat%	Starch%	Ash%	Seedling color
PR11/12-851	15.4	5.1	73.2	1.57	Green
MN13-7838	14.6	4.8	71.1	1.63	Yellow
MN07-1916	14.4	4.9	73.1	1.60	Green
PR11/12-852	14.4	5.2	71.7	1.68	Yellow
MN07-2118	14.3	5.1	74.6	1.48	Yellow
MN11-10362	13.3	5.3	74.8	1.36	Yellow
MN13-7923	13.1	5.1	74.6	1.50	Green
MN13-7499	12.8	5.0	74.5	1.40	Yellow
PR11/12-873	12.7	4.9	74.0	1.42	Yellow
PR11/12-1026	12.7	5.2	75.6	1.47	Green
PR11/12-850	12.7	5.2	73.5	1.52	Yellow
MN13-7458	12.7	5.3	78.5	1.21	Green
PR12/13-764-2	12.6	5.2	75.4	1.51	Yellow
MN13-7500	12.4	5.2	75.8	1.36	Yellow
MN13-7439	12.4	5.1	74.4	1.46	Green
PR12/13-763-3	12.1	5.0	73.2	1.44	Yellow
PR12/13-763-1	12.0	5.0	73.3	1.40	Green
PR11/12-984	12.0	4.8	74.8	1.52	Yellow
MN13-7450	12.0	5.2	75.5	1.46	Green
MN13-7840	12.0	4.9	74.5	1.66	Yellow
PR12/13-762-2	11.9	5.2	76.0	1.55	Yellow
MN13-7462	11.9	5.1	75.7	1.46	Green
PR12/13-764-6	11.8	5.2	75.8	1.40	Yellow
MN13-7498	11.8	5.3	77.2	1.33	Green
PR12/13-762-1	11.8	5.2	76.3	1.57	Yellow
PR12/13-763-4	11.7	5.0	72.9	1.40	Yellow
PR12/13-763-2	11.7	5.0	74.1	1.42	Green
PR12/13-764-1	11.6	5.2	75.7	1.42	Yellow
PR12/13-764-3	11.5	5.2	76.0	1.44	Yellow
MN07-2165	11.3	5.3	77.4	1.34	Yellow
PR12/13-763-5	11.2	5.0	73.2	1.41	Yellow
PR12/13-764-4	11.2	5.3	76.8	1.37	Yellow
MN13-7455	11.1	5.0	75.1	1.29	Yellow
PR9/10-4720-1	10.9	5.2	76.1	1.41	Yellow
PR12/13-761	10.8	5.2	76.7	1.37	Yellow
MN13-7463	10.7	4.9	75.4	1.36	Yellow
Tx430	13.1	5.1	74.2	1.62	Green
Mean	12.3	5.1	74.9	1.5	-
†LSD	1.18	0.23	1.36	0.16	-

†LSD = Least significant difference.

Appendix C - Supplemental information for chapter 4

Table C.1. Mean performance of all hybrid groups for parameters evaluated at adult plant stage under Experiment set I. Results for each environment is presented separately.

Parameter	¶Env	Hybrid Group									
		ALS × ALS	ALS × Regular	Regular × ALS	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Regular × Regular	Checks	Mean	#LSD
Seedling chlorophyll contnet	E1	30.3(±0.6)	38.3(±0.5)	37.5(±0.5)	-	-	-	-	45.7(±1.3)	37.9	3.6
	E2	36.8(±1.0)	38.5(±0.9)	39.9(±0.9)	-	-	-	-	36.9(±1.9)	38.0	3.0
	E3	44.9(±0.7)	45.9(±0.6)	46.0(±0.6)	-	-	-	-	46.6(±1.5)	45.9	3.4
Seedlnig height	E1	17.9(±0.6)	18.6(±0.6)	19.1(±0.6)	-	-	-	-	17.3(±0.6)	18.2	ns
	E2	12.3(±0.6)	12.5(±0.5)	12.2(±0.5)	-	-	-	-	11.6(±0.8)	12.2	ns
	E3	14.5(±0.6)	14.9(±0.6)	15.4(±0.6)	-	-	-	-	16.5(±0.6)	15.3	ns
Adult plant chlorophyll contnet	E1	57.8(±0.9)	59.2(±0.8)	57.1(±0.8)	60.2(±0.8)	59.7(±0.8)	61.8(±0.8)	58.2(±0.8)	59.0(±2.1)	59.2	ns
	E2	59.7(±0.9)	61.2(±0.8)	62.6(±0.8)	60.7(±0.9)	59.8(±0.8)	60.5(±0.8)	61.6(±0.8)	59.7(±1.5)	60.7	ns
	E3	52.6(±0.8)	52.5(±0.7)	52.7(±0.7)	49.9(±0.8)	52.8(±0.7)	52.6(±0.7)	51.6(±0.7)	49.8(±1.5)	51.8	ns
†Chl. Fluo. (Fv/Fm)	E1	0.73(±0.005)	0.74(±0.005)	0.75(±0.005)	0.79(±0.005)	0.79(±0.005)	0.78(±0.005)	0.77(±0.005)	0.78(±0.01)	0.76	0.01
	E2	0.79(±0.03)	0.77(±0.01)	0.77(±0.01)	0.79(±0.03)	0.77(±0.01)	0.77(±0.01)	0.75(±0.03)	0.76(±0.05)	0.77	0.01
	E3	0.73(±0.005)	0.69(±0.005)	0.73(±0.005)	0.75(±0.005)	0.74(±0.005)	0.74(±0.005)	0.74(±0.005)	0.74(±0.01)	0.73	0.01
Adult plant height	E1	110.3(±1.4)	117.3(±1.3)	114.0(±1.3)	115.3(±1.4)	109.3(±1.3)	113.5(±1.3)	111.3(±1.3)	105.5(±1.8)	112.1	5.8
	E2	134.0(±1.1)	139.5(±1.0)	144.0(±1.0)	139.3(±1.1)	143.3(±1.0)	136.5(±1.0)	126.3(±1.0)	120.0(±2.2)	135.4	7.2
	E3	121.3(±2.0)	124.8(±1.8)	123.5(±1.8)	131.8(±2.0)	126.0(±1.8)	124.8(±1.8)	120.5(±1.8)	118.8(±1.6)	123.9	5.8
Days to floewring	E1	64(±1.2)	63(±1.2)	62(±1.2)	61(±1.2)	65(±1.2)	61(±1.2)	68(±1.2)	72(±3.0)	64.5	3.9
	E2	69(±0.8)	67(±0.8)	67(±0.8)	68(±0.8)	66(±0.7)	68(±0.8)	72(±0.8)	75(±2.2)	69.0	2.8
	E3	68(±0.6)	63(±0.5)	63(±0.5)	64(±0.6)	64(±0.5)	64(±0.5)	65(±0.5)	68(±1.6)	64.8	2.3
Panicle length	E1	30.1(±0.5)	30.0(±0.4)	28.2(±0.4)	27.5(±0.5)	28.3(±0.4)	28.9(±0.4)	28.3(±0.4)	26.5(±1.3)	28.4	1.7
	E2	34.5(±0.6)	35.1(±0.6	33.9(±0.6)	32.4(±0.6)	32.6(±0.6)	32.2(±0.6)	32.5(±0.6)	33.5(±1.8)	33.3	2.4
	E3	32.7(±0.5)	32.9(±0.5)	31.9(±0.5)	30.9(±0.5)	32.6(±0.5)	32.9(±0.5)	31.8(±0.5)	31.0(±1.5)	32.1	2.0
Panicle width	E1	5.9(±0.1)	6.1(±0.1)	6.0(±0.1)	6.1(±0.1)	5.8(±0.1)	6.5(±0.1)	5.8(±0.1)	5.9(±0.4)	6.0	0.5
	E2	6.7(±0.2)	7.0(±0.2)	6.2(±0.2)	6.4(±0.2)	6.9(±0.2)	6.5(±0.2)	6.0(±0.2)	6.3(±0.5)	6.5	0.7
	E3	6.2(±0.2)	6.3(±0.2)	6.9(±0.2)	6.2(±0.2)	6.7(±0.2)	7.5(±0.2)	7.4(±0.2)	7.4(±0.5)	6.8	0.7
Panicle weight	E1	108.7(±6.3)	118.0(±5.8)	107.7(±5.8)	102.6(±6.0)	104(±5.8)	113.9(±5.8)	98.4(±5.8)	106.7(±9.1)	107.5	18.0
	E2	144.3(±6.4)	136.6(±5.9)	107.8(±5.9)	119.8(±6.4)	117.6(±5.8)	108.6(±6.0)	111.2(±5.8)	108.5(±8.6)	119.3	21.7
	E3	117.1(±5.6)	111.8(±5.2)	118.6(±5.2)	112.1(±5.7)	121.8(±5.2)	132.2(±5.2)	111.3(±5.2)	119.5(±8.8)	118.1	19.4
Panicle yield	E1	79.5(±4.7)	87.6(±4.3)	77.5(±4.3)	71.9(±4.4)	78.3(±4.3)	81.2(±4.3)	68.7(±4.3)	74.3(±6.7)	77.4	13.7
	E2	93.4(±5.0)	86.1(±4.6)	63.0(±4.6)	75.9(±5.1)	67.0(±4.6)	65.4(±4.8)	66.1(±4.6)	55.9(±9.1)	71.6	17.0
	E3	68.9(±3.9)	63.0(±3.6)	71.6(±3.6)	60.7(±3.6)	67.8(±3.6)	74.3(±3.6)	65.8(±3.6)	69.9(±9.2)	67.7	12.6

Parameter	¶Env	Hybrid Group									
		ALS × ALS	ALS × Regular	Regular × ALS	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Regular × Regular	Checks	Mean	#LSD
‡KN	E1	2852(±150)	3083(±134)	3023(±134)	2700(±139)	3114(±134)	3107(±134)	2560(±134)	2483(±221)	2865	464
	E2	3281(±157)	3216(±146)	2590(±146)	2880(±158)	2714(±144)	2640(±151)	2555(±145)	2242(±205)	2764	541
	E3	2704(±144)	2442(±133)	2849(±133)	2527(±146)	2802(±133)	2933(±133)	2567(±133)	2528(±240)	2669	491
§TKW	E1	27.8(±0.6)	28.5(±0.5)	25.6(±0.5)	26.6(±0.5)	24.9(±0.5)	26(±0.5)	26.9(±0.5)	29.7(±1.4)	27.0	1.9
	E2	28.3(±0.6)	26.5(±0.5)	24.0(±0.5)	26.1(±0.6)	24.6(±0.5)	24.3(±0.5)	25.6(±0.5)	24.6(±1.6)	25.5	2.1
	E3	25.4(±0.3)	25.7(±0.3)	25.2(±0.3)	24.1(±0.3)	24.2(±0.3)	25.4(±0.3)	25.7(±0.3)	27.6(±1.0)	25.4	1.4
Grain yield	E1	6614(±508)	6233(±480)	5640(±480)	5210(±508)	5317(±480)	5779(±480)	3966(±480)	4880(±706)	5454	676
	E2	4807(±485)	4723(±460)	4263(±460)	5769(±484)	5714(±460)	4756(±460)	3310(±460)	3453(±818)	4599	636
	E3	3575(±408)	3826(±383)	2956(±383)	3911(±408)	4367(±383)	3553(±383)	4713(±383)	5113(±674)	4001	622

†Chl. Fluo. = chlorophyll fluorescence; ‡KN = kernel number per panicle, §TKW = thousand kernel weight;
¶Env = Environment ; E1 = Environment 1, E2 = Environment 2, E3 = Environment 3 ;
#LSD = Least Significant difference.

Table C.2. Mean performance of all hybrid groups for all parameters evaluated under Experiment set II. Results for each environment is presented separately.

Parameter	§Env	Hybrid Group							Mean	¶LSD
		ALS × ACCase	ACCase × ALS	ALS × Regular	ACCase × ACCase	ACCase × Regular	Regular × ACCase	Checks		
Adult plant height	E1	141.1(±0.6)	144.6(±1.6)	143.9(±0.6)	136.7(±1.3)	140.4(±0.9)	141.5(±1.1)	129.2(±1.6)	139.5	5.9
	E2	163.0(±1.3)	168.3(±2.9)	160.4(±1.3)	163.3(±2.4)	158.4(±1.7)	169.7(±2.0)	170.0(±2.9)	164.5	6.5
	E3	140.6(±1.0)	142.1(±3.1)	140.3(±0.9)	139.2(±2.5)	138.0(±1.5)	142.8(±1.9)	128.8(±3.1)	138.8	4.6
Days to flowering	E1	63(±0.9)	66(±1.8)	64(±0.9)	64(±1.5)	65(±1.1)	65(±1.3)	66.5(±2.4)	64.7	2.9
	E2	60(±0.4)	58(±1.3)	60(±0.4)	59(±1.1)	56(±0.6)	58(±0.8)	62.5(±1.9)	59.1	3.7
	E3	69(±0.4)	72(±1.1)	68(±0.4)	66(±0.9)	69(±0.6	70(±0.7)	69(±1.6)	69.0	2.4
Panicle length	E1	34.6(±0.3)	37.5(±1.1)	35.4(±0.3)	34.5(±0.9)	35.3(±0.5)	36.8(±0.7)	31.5 (±1.5)	35.1	3.7
	E2	32.6(±0.4)	32.2(±1.2)	33.2(±0.4)	31.6(±0.1)	31.9(±0.6)	33.3(±0.7)	30.4(±1.7)	32.2	3.5
	E3	33.3(±0.4)	33.4(±0.4)	34.7(±0.4)	33.4(±1.1)	35.2(±0.6)	34.9(±0.8)	28.5(±1.8)	33.3	2.6
Panicle width	E1	7.1(±0.1)	6.8(±0.3)	7.0(±0.1)	6.8(±0.2)	6.1(±0.1)	7.3(±0.2)	7.5(±0.4)	6.9	0.7
	E2	6.2(±0.1)	5.9(±0.4)	6.1(±0.1)	5.9(±0.3)	6.7(±0.2)	6.6(±0.2)	6.7(±0.5)	6.3	0.8
	E3	6.4(±0.6)	6.7(±1.8)	7.5(±0.6)	6.5(±1.5)	6.6(±0.9)	6.6(±1.2)	6.4(±0.5)	6.6	0.8
Panicle weigth	E1	173.7(±3.1)	176.5(±10.3)	176.7(±3.1)	172.9 (±8.4)	169.8(±5.1)	186.3(±6.5)	172.4(±14.1)	175.5	16.5
	E2	101.8(±11.3)	117.1(±27.9)	106.0(±11.2)	109.4(±23.2)	100.7(±15.7)	100.1(±18.6)	108.6(±38.8)	106.3	11.3
	E3	124.1(±3.7)	120.2(±10.1)	126.3(±3.7)	120.7(±8.4)	123.1(±5.4)	131.6(±6.6)	125.7(±14.2)	124.5	16.9
Panicle yield	E1	116.0(±6.6)	117.1(±21.2)	116.4(±6.5)	110.1(±17.3)	139.7(±10.6)	123.5(±13.5)	133.3 (±30.1)	122.3	12.5
	E2	62.5(±4.7)	70.2(±7.5)	64.3(±4.6)	65.6(±6.6)	59.7(±5.3)	60.3(±5.8)	59.5(±9.8)	63.2	10.4
	E3	78.9 (±3.1)	76.3(±7.6)	79.1(±3.1)	73.7(±6.3)	73.8(±4.2)	83.0(±5.1)	84.2(±10.6)	78.4	15.7
†KN	E1	4109(±103)	4106(±262)	4045(±102)	3831(±217)	3684(±143)	4426(±173)	3625(±365)	3975	441
	E2	2527(±149)	2749 (±259)	2567(±148)	2734(±225)	2354(±172)	2446(±193)	2372(±342)	2535	436
	E3	3372(±162)	3033(±525)	3132(±162)	2937(±428)	2830(±262)	3356 (±332)	3030(±345)	3098	482
‡TKW	E1	28.3(±2.8)	28.6(±8.7)	28.8(±2.7)	28.6(±7.1)	36.3(±0.5)	28.6 (±5.5)	32.2(±2.4)	30.2	3.2
	E2	24.6(±0.4)	25.6(±1.1)	25.0(±0.4)	23.9(±0.8)	24.9(±0.6)	24.4(±0.7)	24.9(±1.4)	24.8	2.9
	E3	24.9(±0.2)	25.2(±0.8)	25.2(±0.2)	25.0(±0.7)	26.0(±0.4)	24.7(±0.5)	27.7(±1.2)	25.5	1.7
Grain yield	E1	5907(±155)	5935(±473)	6464(±155)	6265(±388)	6777(±241)	5777(±302)	6929(±473)	6293	670
	E2	3433(±200)	3243(±522)	3696(±200)	3747(±432)	3317(±283)	4239(±344)	5451(±522)	3875	644
	E3	7204(±384)	6536(±481)	7326(±382)	8032(±413)	7964(±434)	7634(±448)	8372(±487)	7581	608

†KN = kernel number per panicle, ‡TKW = thousand kernel weight; §Env = Environment, E1 = Environment 1, E2 = Environment 2, E3 = Environment 3; ¶LSD = Least Significant difference.

Table C.3. Mean performance of all measured parameters for hybrid groups evaluated under Experiment set III. Results each environment is presented separately.

Parameter	§Env	Hybrid Group									
		ALS × ACCase	ALS × Regular	ACCase × ACCase	ACCase × ALS	ACCase × Regular	Regular × ALS	Regular × ACCase	Check	Mean	¶LSD
Adult plant height	E1	129.8(±0.9)	130.5(±2.4)	128.7(±3.6)	122.2(±2.3)	124.8(±1.9)	120.8(±2.9)	127.5(±2.1)	120.0(±3.6)	125.5	7.7
	E2	140.1(±1.2)	147.8(±3.4)	142.5(±4.5)	139.5(±2.8)	144.8(±2.4)	150.0(±3.7)	140.4(±2.6)	130.6(±4.5)	141.9	8.3
Days to flowering	E1	59(±0.4)	62(±1.1)	60(±1.5)	63(±1.0)	61(±0.8)	66(±1.2)	64(±0.9)	63(±2.2)	62.3	2.7
	E2	61(±0.5)	62(±0.9)	59(±1.3)	61(±0.9)	60(±0.8)	66 (±1.1)	67 (±0.8)	65(±1.9)	62.6	2.3
Panicle length	E1	33.9(±0.3)	34.8(±0.9)	33.3(±1.2)	32.7(±0.8)	33.4(±0.6)	34.5(±1.0	34.0(±0.7)	33.1(±1.7)	33.7	2.5
	E2	33.4(±0.8)	33.1(±1.1)	37.3(±1.6)	34.6(±1.2)	33.2(±1.1)	31.0(±1.4)	32.4(±1.1)	30.3(±2.2)	33.2	3.1
Panicle width	E1	7.0(±0.1)	6.8(±0.2)	6.6(±0.3)	6.6(±0.2)	6.6(±0.2)	6.9(±0.3)	7.1(±0.2)	6.6(±0.5)	6.7	0.6
	E2	6.1(±0.1)	5.9(±0.2)	7.2(±0.4)	6.3(±0.2)	6.0(±0.2)	6.3(±0.3)	6.3(±0.2)	6.6(±0.6)	6.3	0.8
Panicle weight	E1	167.0(±3.4)	182.8(±9.6)	154.8(±12.7)	157.4(±8.0)	154.3(±6.8)	166.1(±10.4)	185.5(±7.3)	157.5(±18.0)	165.7	28.4
	E2	122.2(±4.1)	119.6(±8.1)	151.9(±12.0)	119.0(±7.9)	116.5(±6.8)	107.0(±9.9)	147.3(±7.2)	135.1(±16.8)	127.3	25.1
Panicle yield	E1	113.4(±2.6)	123.9(±7.4)	100.1(±9.8)	107.8(±6.2)	101.4(±5.2)	115.7(±8.0)	136.2(±5.6)	106.3(±13.8)	113.1	11.9
	E2	76.5(±3.4)	78.4(±5.6)	97.5(±8.2)	73.4(±5.6)	70.5(±4.9)	67.0(±6.8)	95.3(±5.0)	89.3(±11.2)	80.9	13.7
†KN	E1	4034(±83)	3860(±233)	3428(±398)	3848(±195)	3363(±164)	4000(±251)	4528 (±178)	3508 (±436)	3821	314
	E2	3127(±95)	2863(±197)	3724(±293)	2954(±190)	2718(±163)	2763(±241)	3704 (±173)	3346(±410)	3149	354
‡TKW	E1	28.1(±0.3)	32.6(±1.0)	29.0(±1.3)	28.3(±0.8)	30.1(±0.7)	28.9(±1.1)	30.1(±0.7)	30.6(±1.9)	29.7	1.9
	E2	24.3(±0.4)	27.7(±0.6)	26.0(±0.9)	24.7(±0.6)	25.7(±0.5)	24.2(±0.7)	25.6(±0.5)	26.5(±1.2)	25.5	1.2
Grain yield	E1	5560(±241)	6185(±465)	6577(±594)	5098(±402)	6757(±354)	4982(±496)	5974(±375)	5844(±594)	5872	793
	E2	3634(±235)	4370(±387)	3226 (±540)	4064(±372)	4740(±330)	3339(±454)	3411(±348)	4913(±540)	3962	752

†KN = kernel number per panicle, ‡TKW = thousand kernel weight; §Env = Environment, E1 = Environment 1, E2 = Environment 2; ¶LSD = Least significant difference.

Table C.4. Pearson correlation coefficients between evaluated yield components for all hybrids tested under Experiment set II and III.

Parameter	Days to flowering	Adult plant height	Panicle length	Panicle width	Panicle weight	Panicle yield	†KN	‡TKW	Grain yield
	Experiment set II								
Days to flowering		-0.690**	-0.046	0.105	0.045	0.004	-0.081	0.0261	-0.417**
Adult plant height	-0.312**		0.119*	0.077	0.047	0.039	0.052	-0.052	0.220**
Panicle length	-0.168*	0.357**		0.119*	0.310**	0.270**	0.404**	-0.0182	0.164**
Panicle width	-0.172*	0.250**	0.315**		0.153**	0.137*	0.146**	0.022	0.102*
Panicle weight	-0.215**	0.513**	0.419**	0.744**		0.441**	0.483**	0.083	0.170**
Panicle yield	-0.232**	0.544**	0.371**	0.728**	0.977**		0.485**	0.804**	0.242**
†KN	-0.165*	0.462**	0.422**	0.707**	0.909**	0.908**		-0.006	0.428**
‡TKW	-0.261**	0.427**	0.12481	0.394**	0.605**	0.651**	0.287**		0.086*
Grain yield	-0.487**	0.535**	0.134*	0.345**	0.528**	0.576**	0.419**	0.590**	
	Experiment set III								

†KN = kernel number per panicle, ‡TKW = thousand kernel weight; * and ** statistically significant at $p \leq 0.05$ and 0.01, respectively.

Table C.5. ANOVA for nutritional and physical grain quality attributes measured on tested sorghum (*Sorghum bicolor* (L.) Moench) hybrids under all three experiments.

Source of variation	df	†Nutritional traits													‡Physical traits	
		Protein	Fat	Starch	Ash	N	P	K	Ca	Mg	Fe	Zn	Cu	Mn	KH	KD
Experiment I																
Block	2	0.01	0.09	0.4	0.006	0.002	8.2E-6	7.7E-4	5.0E-6	1.2E-6	9.6	5.1	0.06	1.0E-5	88.6	1.4E-3
Hybrid group	3	13.8**	0.62**	4.6**	0.05**	0.3**	0.09**	0.01*	5.5E-5**	0.002**	211.6**	87.9**	3.9**	14.5**	373.1**	3.9E-2**
Error	6	1.34	0.09	0.9	0.007	0.017	7.7E-4	6.8E-4	7.3E-6	1.7E-4	12.2	10.8	0.31	0.3	28.3	8.2E-3
Experiment II																
Environment (E)	2	71.4**	4.5**	83.3**	0.5**	-	-	-	-	-	-	-	-	-	-	-
Block/E	6	6.0	0.07	6.3	0.03	-	-	-	-	-	-	-	-	-	-	-
Hybrid group	6	1.0	0.13*	1.6	0.008	-	-	-	-	-	-	-	-	-	-	-
HG × E	12	1.4	0.05	1.0	0.01	-	-	-	-	-	-	-	-	-	-	-
Error	36	1.3	0.04	1.6	0.01	-	-	-	-	-	-	-	-	-	-	-
Experiment III						-	-	-	-	-	-	-	-	-	-	-
Environment (E)	1	323.3**	0.1	170.6**	0.06	-	-	-	-	-	-	-	-	-	-	-
Block/E	4	1.8	0.3	0.9	0.01	-	-	-	-	-	-	-	-	-	-	-
Hybrid group	7	1.0	0.1**	6.8**	0.04**	-	-	-	-	-	-	-	-	-	-	-
HG × E	7	0.6	0.2	0.7	0.006	-	-	-	-	-	-	-	-	-	-	-
Error	28	0.5	0.1	0.8	0.007	-	-	-	-	-	-	-	-	-	-	-

†Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn);
‡Kernel hardness (KH), Kernel diameter (KD); * and ** statistically significant at $p \leq 0.05$ and 0.01, respectively.

Table C.6. Pearson correlation coefficients between the tested nutritional attributes for herbicide resistant hybrids tested under Experiment set II and III.

Parameter	Protein%	Fat%	Ash%	Starch%
	Experiment II			
Protein%		-0.28**	0.35**	-0.64**
Fat%	-0.26**		-0.48**	0.43**
Ash%	0.37**	-0.28**		-0.38**
Starch%	-0.77**	0.49**	-0.39**	
	Experiment III			

* and ** statistically significant at $p \leq 0.05$ and 0.01, respectively.