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GENETIC, AGRONOMIC AND COMPOSITIONAL CHARACTERIZATION OF BROWN MIDRIB SWEET SORGHUM LIGNOCELLULOSIC BIOMASS FOR ETHANOL PRODUCTION

A Dissertation

Submitted to the Faculty

of

Purdue University

By

Luis A. Rivera-Burgos

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

May 2015

Purdue University

West Lafayette, Indiana

To my parents, Pedro and Rosa, my unconditional loves who encouraged me to pursue my dreams.

To dear sister, Liliana, she has taken care of me during all my life.

To my beautiful little niece Andrea de Guadalupe, she brought joy to our family.

To my grandmother Maria and my dear aunts Julia and Ysabel. They are like mothers to me.

Thank you for the love, prayers, encouragement, and support.

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ABSTRACT

Rivera-Burgos, Luis A. PhD., Purdue University, May 2015. Genetic, agronomic and compositional characterization of brown midrib×sweet sorghum RILs for ethanol production. Major Professor: Gebisa Ejeta.

Sorghum is a promising bioenergy crop due to its unique phenotypic and genotypic attributes. Quality (low lignin and high stem sugar concentration) and quantity (biomass yield, plant height, plant maturity, etc.) biomass traits are key contributors to ethanol yield and production. In this study, a 236 sorghum recombinant inbred line (RIL) population was subjected to genetic, agronomic and compositional characterization for ethanol yield and production. We found that the sweet mutation enhances biomass quantity traits in the RILs which translates to higher ethanol production and biomass quality which improves ethanol yield. The variance components showed from moderate to high heritability for biomass quantity and quality traits. The variability observed in most of these traits was due mainly to genetic effects. Correlations showed positive associations between biomass quantity traits and stem sugar concentration (SSC). These results indicate that selection for multiple traits could increase ethanol production. Single marker analysis showed two possible quantitative trait loci, on chromosomes 6 and 7, explaining only 2 and 7% of the variation in SSC measurements.

The brown midrib mutation in this population was previously identified in the caffeic acid-*O*-methyltransferase (*COMT*) gene resulting in reduced lignin content. A useful InDel marker for the mutant allele of *COMT* was identified for this population. Fiber detergent analysis (FDA) was performed to estimate the amount of hemicellulose, cellulose and lignin. Glucose recovery and theoretical ethanol yield and production were calculated and differences among grouped RILs analyzed. Only RILs carrying the brown midrib mutation showed significantly higher glucose recovery, those carrying both compositional mutations, showed significantly higher ethanol yields, and those with double mutations or the sweet mutation had significantly higher theoretical ethanol production. Lignin (R^2= 0.66) was identified as the most reliable predictor for glucose recovery. Lignin and SSC (R^2= 0.46 and 0.35, respectively) were identified as good predictors for ethanol yield. Dry stover and fresh stover yield (R^2= 0.89) were the most appropriate predictors for ethanol production.

Additionally, a nitrogen experiment was conducted to study the effect of four nitrogen rates on biomass traits of nine sorghum varieties, as lines and hybrids with and without brown midribs, a sweet and a photoperiod sensitive cultivar and a maize hybrid. Nitrogen application rate had significant effects on biomass components. The grain sorghum hybrid and the grain maize hybrid maximized grain yields across nitrogen rates. The photoperiod sensitive and sweet sorghums maximized stover yields across nitrogen rates. Maximum grain yield was obtained at 135kg N ha^{-1}, while maximum stover yield was 67kg N ha^{-1}. Across genotypes, grain nitrogen use efficiency (NUE) ranged from 19 to 50kg kg^{-1}, while stover NUE ranged from 31 to 125kg kg^{-1}. The dual-purpose sorghum hybrid showed the

highest grain NUE, while the sweet sorghum showed the highest stover NUE. This research suggests that targeted improvement of biomass quantity and quality traits, and nitrogen management could increase ethanol production.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Energy demand is gradually increasing and affecting the global economy and the environment. The tremendous appetite for fossil fuels not only affects energy prices but also causes intractable environmental problems. The four primary consumer sectors for energy are industrial, transportational, residential and commercial. The majority of the energy used by these four sectors is non-renewable.

Most of the energy resources utilized on a daily basis in developed and developing countries are petroleum products. Although energy is primarily dedicated to power generation and industrial processes, transportation also plays an important role. By far, petroleum products are the main input of transportation, creating a strong dependency. The dependency on non-renewable energy sources demands the attention of the scientific community. New alternative energy sources are needed in order to attenuate the energy demand and at the same time reduce its impact on prices and the environment.

Biofuels are a promising renewable energy source. Bioenergy is currently receiving international attention in politics and media. Its production is becoming more feasible and is being expanded to a large scale. Lignocellulosic biomass for ethanol production is an

attractive option but not enough to compete where petroleum products control the majority of the market. An enhanced biomass that boosts ethanol production is needed.

Compositional (quality) and agronomic (quantity) traits are main components in the process of improving biomass feedstock for ethanol production. Ethanol fuel, also known as ethyl alcohol, is the same type of alcohol found in alcoholic beverages. It is most often used as a motor fuel, mainly as a biofuel additive for gasoline. Ethanol can be produced through hydrolysis and fermentation of simple and complex carbohydrates stored in plant biomass. By improving biomass quality and quantity, ethanol could become a competitive renewable energy source worldwide.

Sorghum, a well-adapted annual crop with the ability to endure abiotic and biotic stresses, is an attractive source of biomass for ethanol production. This crop shows remarkable traits that when combined properly, could become an economically viable alternative fuel source.

After several cycles of breeding, a new population of recombinant inbred lines (RILs) was development by the Purdue University sorghum program. These 236 RILs combine desirable compositional (quality) and agronomic (quantity) traits for ethanol production. We hypothesize that the combination of quality and quantity biomass traits could increase ethanol production in the U.S.A.

1.2 Motivation

Fossil fuel dependency and increased greenhouse gases are major concerns that has caught the attention of environmentalists, economists and scientists in developed and developing countries. On one hand, fossil fuel is a non-renewable source of energy. Its production and utilization generates gases associated with environmental pollution as well as respiratory health problems. However, there is still an insatiable hunger for energy that leads to a strong dependency on fossil fuel products. This dependency creates a demand that raises petroleum product prices affecting the economy within and among countries. Fossil fuel demand has increased in the last decade, elevating gas prices substantially from $1.5 to $5.26 per gallon (Davis et al., 2013). The United States is the world's largest energy consumer. U.S. gas consumption occurs in agriculture, construction, manufacturing, transportation and public utilities, etc., making the economy of this country dependent on fossil fuels. Pollution caused by greenhouse gas generated from burning fossil fuel products is having a tremendous impact on climate change causing global warming that is expected to result in drastic changes in temperature and precipitation in the coming decades that would affect the world economy.

In Brazil, sugarcane (*Saccharum* spp.) is utilized as source of fermentable sugars. The sugarcane stems are harvested and transported to processing plants where the stem juice is removed by crushing and then fermented to produce ethanol. In the U.S., sugarcane can only be cultivated in the sub-tropic zones such as Louisiana, Florida and Texas due to the crop's sensitivity to low temperatures. In the U.S., most of ethanol is being produced by fermentation of glucose derived from the starch of corn (*Zea mays* L.) kernels. In 2012 the United States produced 52.8 billion liters of ethanol derived from corn starch

(http://www.ethanolrfa.org). In that year, 42 percent of the total maize production was utilized to produce ethanol. This is two-fold of corn grain production that was utilized to produce ethanol in 2007. This trend is expected to keep growing in the coming decades (Agricultural Outlook 2030-2050 FAO.org). The increased utilization of grain crops as fuels has prompted concerns to the global food supply (FAO 2008). Arguments that biofuels are responsible for more than 75 percent of the increase in global food prices witnessed in 2008 (Mitchell 2008). However, crop fuels are one of a vast reasons causing increments in food price. An increase economy in Asia, an increasing population that demands food, climate change, etc. are also factors that strongly impact food prices (Hubbard 2008, Tyner and Taheripour 2008). A new alternative source of crop-based ethanol production is lignocellulosic rather than from grain (Ragauskas et al., 2006). Lignocellulosic biomass, the non-grain portion of a crop, is the most abundant material in the world (Osborne et. al., 2011). Lignocellulosic biomass contains three primary constituents: cellulose, hemicellulose and lignin. Cellulose and hemicellulose are structural carbohydrates that can be broken down by enzymes, acids, or other compounds to simple sugars, and then fermented to produce ethanol in a process called stover conversion. Lignin is complex polymer of aromatic alcohols that binds the cells and vessels which constitute wood and the lignified elements of plants, as in straw. However, the feasibility of an efficient lignocellulosic biomass conversion to produce ethanol at an economically competitive price is still a concern (Zhao et. al., 2009; Vogel et. al., 2010; Han et. al., 2013). To achieve this goal, dedicated bioenergy crops need to be developed. An ideal bioenergy crop should be able to produce considerable amounts of biomass on marginal lands with

low fertilizer inputs, so as to not divert resources from food crops, and it needs to be adequate for processing.

1.3 Biomass Energy Production

Renewable energy can be produced through conversion of organic feedstock produced from agriculture every farming season. Plant biomass harbors different sources of organic compounds to produce ethanol as major output of this conversion process (Jacobsen and Wyman 2000; Badger 2002; Gírio et al., 2010). Structural and nonstructural are the two types of carbohydrates present in crop biomass. Plant structural carbohydrates are polysaccharides which function in cell wall structure (Vassilev et al., 2010; Erickson et al., 2011). These organic compounds are known as cellulose and hemicellulose, and both are found tightly attached to lignin; a complex polymer of aromatic alcohols known as monolignols. The major function of nonstructural carbohydrates is energy storage, mainly in the form of starch (Gírio et al., 2010; Vassilev et al., 2013). However, in some crops such as sugarcane and sweet sorghum, these non-structural carbohydrates can be stored in the stem juice as soluble glucose, fructose and sucrose. The nonstructural carbohydrates present in plants are monosaccharides (glucose and fructose), disaccharides (sucrose) and polysaccharides (starch). All these plant based carbohydrates are fermentable to ethanol.

Ethanol fuel has remarkable benefits over traditional fossil fuels. Ethanol adds oxygen to gasoline which helps to reduce air pollution and harmful emissions in tailpipe exhaust. Sugarcane ethanol cuts carbon dioxide emissions by 90 percent on average compared to gasoline making it an attractive alternative (Obernberger and Thek 2004). Ethanol

performance can be enhanced in high-octane mixes that help to prevent engine knocking and to generate more power in higher compression engines (Gulati et al., 1996).

1.3.1 Soluble sugar based ethanol

Sucrose, fructose and soluble glucose are simple fermentable sugars present in the stem juice of species such as sugarcane and sweet sorghums. Both crops are naturally capable of producing high concentrations of these fermentable sugars in their stalks. Sweet sorghums belong to the same species as grain sorghum, grass sorghum and broom corns (Elangovan *et al*., 2007). However, sweet sorghums have been selected to accumulate high concentrations of soluble sugars, especially sucrose (Dogget, 1988; Tarpley and Vietor, 2007). Sweet sorghum is closely related to sugarcane; indeed, they share characteristics such as tall plants with high biomass and juicy stems containing high concentrations of soluble sugars (Billa *et al.*, 1997). Both crops can yield a maximum of 22 degree Brix (Almodares and Hadi, 1996). This measurement accounts for the amount of soluble sugars in stem juice. One degree Brix is the ratio of 1 gram of sugar dissolved in 100 milliliters of water. The soluble sugars extracted from sugarcane or sweet sorghums account for around 30% of the chemical energy stored in the harvested parts of the mature plant. Around 35% of a plant's accumulated chemical energy lies in the leaves and stems, which in grain crops are left in the fields after harvest (Reddy et al., 2005). An estimated ratio of 4.8 grams of ethanol can be produced per 100 grams of fresh stalk (Mamma *et al.*, 1995; Zhao et al., 2012; Han et al., 2013). Ethanol obtained from soluble sugars is produced by the fermentation of stem juice and molasses. Stem juice ethanol is a clean, affordable and low-carbon biofuel that emerged as a leading renewable fuel source for the transportation sector

(Han et al., 2013; Rao et al., 2013; Nghiem et al., 2013). Ethanol can be used in a blend with gasoline at levels ranging from 5 to 25 percent to reduce petroleum use and also as mainly ethanol fuel made up of 85 to 100 percent ethanol depending on country's specifications (Reddy et al., 2005; Moller 2005; Nelson et al., 2011). The conversion process of stem juice starts with fermentation, followed by distillation and finally a dehydration process. Fermentation is the process of converting sugars into ethanol and carbon dioxide. This process is practiced with yeast in complete absence of oxygen (Jacobsen and Wyman 2000; Sun and Cheng 2002; Canilha et al., 2012). Ethanol obtained from yeast fermentation has a high water content and cannot be used as fuel in any form. Distillation is carried out to remove the water from the ethanol and gives 95-96% ethanol with of the remainder as water (USDA, 2006). This fuel can be used as stand-alone fuel in modified engines but cannot be blended with the gasoline. The final step is dehydration. It uses desiccants for further removal of water producing 99.7% pure ethanol (Budsberg et al., 2012).

Brazil is the largest sugarcane ethanol producer and a pioneer in using ethanol as a motor fuel. In 2009 Brazil's sugar and ethanol exports generated approximately 9.9 billion US$ and in 2012 and 2013, Brazilian ethanol production reached 23.2 billion liters (6.1 billion gallons). All gasoline sold in Brazil includes a blend of 18 to 25 percent ethanol and this has helped them achieve greater energy security. In fact, Brazil has replaced almost 40 percent of its gasoline needs with sugarcane ethanol fuel. Many observers take the Brazil experience as a case study for other nations seeking to expand the production of renewable fuels and have identified two key factors for success which are plant-based ethanol and flex fuel vehicles (especially designed for ethanol fuel usage) (Budsberg et al., 2012).

The United States of America is the second ethanol producer in the world. Sugarcane produced in Louisiana is the major input utilized in bio-refineries to produce stem juice based ethanol. During the fermentation/distillation process, the cane is crushed and squeeze and approximately 1.5 L of water are added to each kilogram of sugar cane juice (Canilha et al., 2012). In the same way sweet sorghum cane is utilized to produce ethanol by simultaneous saccharification then fermentation (Ballesteros et al., 2004). By suspended culture and immobilized yeast cells, ethanol can be obtained from sweet sorghum juice (Laopaiboon et al., 2007; Laopaiboon et al., 2009; Liu and Shen 2008; Mei et al., 2009; Wu et al., 2010).

1.3.2 Starch based ethanol

The starch fermentation process to ethanol is similar for all grains (maize, sorghum, wheat etc.). Starch and glucose polymers are converted enzymatically to glucose, followed by fermentation of glucose to ethanol (Russell 2003). Maize kernels contain approximately 64 – 78% starch on a dry weight basis, along with 9% proteins, 4% lipids and 13% fiber (Hicks et al., 2005). Similarly, sorghum grain contains 56-77% starch, 7-15% protein, 0.5-5% lipids and 10% fiber (Taylor et al., 2006). These crops can yield approximately 410 and 402 l Mg^{-1} of ethanol. However, grain composition and ethanol yield can vary significantly due to genotype and environment effects (Corredor et al., 2006; Taylor et al., 2006). The United States produces the most starch- based ethanol in the world. U.S. ethanol production reached up to 52.8 billion liters of ethanol last year (www.ethanollrfa.org). With the new plants being added, ethanol production is projected to double by the end of the decade (USDA, 2006).

1.3.3 Lignocellulosic based ethanol

The second generation of biofuels is focused on the production of ethanol by breaking down structural carbohydrates (hemicellulose and cellulose) present in the lignocellulosic biomass of crops. Lignocellulosic biomass from agricultural and forestry wastes is a highly abundant source of organic compounds that are renewable annually as a result of photosynthesis. Utilization of lignocellulosic biomass to produce ethanol will not only provide a significant fraction of fuels for use in the transportation sector, but also help reduce substantially the emission of greenhouse gases by as much as 86% (Farrell et al., 2006). However, the chemical and physical complexity of lignocellulosic substrate to be degraded to simple compounds is a long process that in nature takes weeks or longer, involving a multitude of organisms. Research efforts to understand the biological degradation of lignocellulosic materials suggests a variety of pretreatment processes to optimize cellulosic substrate reactivity (Wyman et al., 2005). Furthermore, thermostable cellulases and recombinant microorganisms capable of co-fermenting mixed streams of sugars to ethanol has been developed in the past decade (Ho et al. 1998; Himmel et al. 2007; Ingram et al. 1999; Zhang et al. 1995). Based on the current technology, the utilization of lignocellulosic plant biomass for fuel ethanol follows these steps: a) feedstock pretreatment, b) enzymatic hydrolysis, c) ethanol co-fermention using hexose and pentose, and d) ethanol recovery operations. However, current technologies are still relatively expensive, and many factors that impede efficiency of the lignocellulosic ethanol process are still poorly understood (Lu and Mosier 2007).

1.3.4 Brown midrib and sweet sorghum based ethanol

The complexity of the chemical and physical compositions of lignocellulosic biomass is challenging scientists to develop new approaches to make ethanol production feasible at a landscape scale. One area that has recently received attention is the genetic basis of plant cell wall recalcitrance in lignocellulosic biomass. There are possibilities for plant breeding to substantially improve enzymatic digestibility at lower cost to release fermentable sugars from lignocellulosic materials (Sticklen 2008). Indeed, studies of the biosynthesis of plant cell wall components have given new insights into the molecular basis for an efficient enzymatic hydrolysis of lignocellulosic biomass (Yong et al., 2005). Of particular interest has been the biosynthesis of lignin. Several mutations reducing lignin content of cell walls have been shown to improve cellulose digestibility by cellulases in maize (Marita et al., 2003) and sorghum (Bout and Vermerris 2003). This improved characteristic is an important added value of lignocellulosic biomass. Additionally, another desirable characteristic associated with more fermentable sugars in sorghum lignocellulosic feedstock is the ability to accumulate sucrose, glucose and fructose in their stalks (Zhao et al., 2009; Han et al., 2013). This extra source of sugars can be directly converted to ethanol by simultaneous saccharification fermentation. The combination of both these characteristics in an enhanced lignocellulosic biomass through traditional breeding methods is a feasible approach toward the development of a superior sorghum bioenergy crop.

1.4 Sorghum as Bioenergy Crop

During this decade, the demand for a large and sustainable supply of biomass to make biofuel generation from lignocellulosic ethanol production profitable is driving the development of specialized feedstock crops. There are many suitable crops that can be exploited through genetics, genomics and plant breeding approaches for this purpose. One of the most promising of these crops is sorghum (*Sorghum bicolor* L. Moench). Sorghum species harbor an enormous genetic variability reflected in phenotypic and morphological traits to improve lignocellulosic biomass quality and quantity (Rooney 2007). Forage sorghums are tall, fast growing and warm season grasses that provide feed for livestock. Their ability to accumulate large amounts of lignocellulosic material, make it attractive as silage, hay and direct grazing. Indeed, in 2009 over 254 000 acres of sorghum were harvested producing an average of 13.7 tons of silage per acre (USDA National Agricultural Statistics, 2008). One of the traits that enhance lignocellulosic biomass quantity in sorghum is the photoperiod sensitivity characteristic. The regulation of flowering by day length is referred to as photoperiodism. This characteristic allows sorghum genotypes to control the transition from vegetative to reproductive growth (Rooney and Aydin 1999; Morgan et al., 2002). A photoperiod sensitive sorghum plant puts all its photosynthate into vegetative growth, thereby producing huge amounts of lignocellulosic biomass.

A useful trait enhancing lignocellulosic biomass quality trait in sorghum is brown midrib. The brown midrib phenotype is fairly easy to see with an experienced eye as soon as adult leaves begin to expand in young plants. It has been established that the reddish-brown

pigmentation of the leaf midribs of sorghum plants, thus the name brown midrib, or bmr, is associated with low lignin content in cell walls (Porter et al., 1978). This trait is recessive, the causal mutant alleles denoted as *bmr*, and by multiple backcrossing cycles can be easily introduced into new genotypes. The low lignin concentration in cell walls results in high livestock digestibility and efficient recovery of fermentable sugars during enzymatic hydrolysis of lignocellulosic biomass (Sun and Cheng 2002; Dien et al., 2009; Vogel et al., 2010). Therefore, brown midrib is a desirable lignocellulosic biomass quality trait that can enhance sorghum's value as a feedstock.

The sweet trait is another useful lignocellulosic biomass quality trait in sorghum. Sweet sorghum genotypes accumulate high concentrations of fermentable sugars in their stems. The juice of sweet sorghum stalks are rich in sucrose, fructose and soluble glucose. These carbohydrates can rapidly be broken down by simultaneous saccharification fermentation to produce ethanol (Ohgren et al., 2006; Nghiem et al., 2013). Compared with other crops, sorghum is cheaper to produce, has high yields of quality lignocellulosic biomass, and is nutrient efficient. Therefore, sorghum can be designed is an excellent bioenergy feedstock to produce ethanol at a landscape scale (Oliver et al. 2005a; Oliver et al., 2005b; Rooney 2007).

1.5 Sorghum plant

The genus *Sorghum* is formed of C4 cane grasses located mainly in Africa and Asia. Cultivated sorghum (*S. bicolor* spp. *bicolor* (L.) Moench) belongs to the genus *Sorghum*, which is composed by two wild species, *S. halepense* and *S. propinquum*. The species *S. halepense* (Johnsongrass) was introduced as a forage crop to the United States, but soon became wild. The *S. bicolor* varieties are grasses with a range of 0.5 to 6 m of height (Smith and Frederiksen 2000). Each of the stems is able to produce a panicle which comes in a variety of architectures. The stems of sorghum can be juicy or dry, and the sweet sorghums accumulate soluble sugars in the stem juice, like sugarcane (Zhao et al., 2009). The forage sorghum genotypes produce massive amounts of tillers in comparison to the grain and sweet sorghum genotypes. Forage sorghum stems are thin, 0.5–3.5 meters tall, and the panicles frequently opened (Smith and Frederiksen 2000). The wild races of *S. bicolor* (*S. bicolor* ssp. *verticilliflorum* and *S. bicolor* ssp. *drummondii*) are mainly located in Africa. From the *S. bicolor* ssp. *verticilliflorum* four races are reported: *arundinaceum, virgatum, aethiopicum* and *verticilliflorum*. These races are able to intermate among each other and with the cultivated types. Because of the morphological and anatomical characteristics, and the ecology of the wild races, the *verticilliflorum* race is considered as the closer ancestor of the cultivated sorghum races. This race is widely distributed in the eastern and southern zones of Africa (Doggett 1988; Smith and Frederiksen 2000).

Sorghum is mainly cultivated for grain, forage and syrup, but lately for biofuel production. The selection in sorghum has resulted in elite lines optimized for different type of uses. From the bioenergy point of view, sorghum can be used to feed three important processes: grain starch, which has similar value as corn starch ethanol production, high- soluble sugar

concentration in stem juice that could be utilized for fermentation, and the felt over bagasse after juice extraction that could be used as biomass feedstock for fermentation or as boiler fuel.

Grain sorghums, also known as milo, have a grain to leaf-stem biomass ratio, they are short, with low number of tillers and suitable to combine harvesting. Because of the human selection pressure, most grain sorghum types produce a single compact or semi-compact panicle. The average grain yield of sorghum in the U.S. 2013 was 3.7 ton/ha (59.6 bushels per acre) in 2013, and is expected to increase to 4.0 ton/ha (64.3 bushels per acre) by the end of 2014 (USDA, 2014). Since grain sorghum ethanol production requires similar processes as the production from corn kernels, they can be used together in the same bio-refinery plants. Stover from milo after grain harvest is similar to corn (roughly 4 to 5 ton dry stover/ha). This lignocellulosic material can be harvested for animal forage or for lignocellulosic ethanol production. Therefore, in areas where grain sorghum production is important, the lignocellulosic material left after harvest could be a utilized as source of structural carbohydrates present on biomass.

Forage sorghum types are known as sorghum, sometimes as sudangrass or ultimately as sorghum-sudangrass hybrids. They produce abundant tillers and some types are perennial in tropical and sub-tropical zones. Tillering types produce multiple panicles located in the basal nodes or branches that develop from stem nodes. Lignocellulosic material is the primary product, usually harvested before physiological maturity (Hamelinck et al., 2005). Lignocellulosic material digestibility and total yield are the main reasons for cultivar selection. Forage sorghum production varies widely due to the genotype used, and ranges from 14 to 16 tons of dry biomass per ha (Corredor et al., 2009; Rocateli et al., 2012).

Sudangrass produces thin stems and considerable number of leaves. During growing season, sudangrass is harvested multiple times to produce green chop and silage. Forage sorghums possess thicker and larger stems, higher dry matter, but much reduced regrowth capacity. For this reason, this type of sorghums are use as silage, because of their thicker stems delay drying. Sorghum × sudangrass hybrids have intermediate yield potential, and could be utilized for silage. Forage sorghums possess great yield potential, and could play an important role in the production of renewable energy (Rooney 2007; Shoemaker et al., 2010). Studies on the development of biomass sorghums hybrids have showed promising results with reported yields of up to 30 ton of dry lignocellulosic biomass per hectare (Rooney et al. 2007; Vermerris et al., 2007; Vermerris 2008).

Sweet sorghums possess high concentrations of soluble carbohydrates in the stem juice. These sorghum types are mainly used for alcoholic beverages, syrup production, crystal sugar and, in some cases, the stalks is use for fresh consumption (Biradar et al., 2007). Usually, sweet sorghum types produce low grain yield, but, recently, new varieties with more balanced non-structural (grain) and structural (soluble sugars) carbohydrates production have been developed in Asia (Li et al. 2004; Reddy et al. 2007). These elite varieties could be used as a dual-purpose crops with the grain harvested for human and animal consumption, and the lignocellulosic biomass for ethanol production. After extraction of the juice, the bagasse can be used as lignocellulosic feedstock (Powell 2012). There is growing interest to production ethanol-fuel from sweet sorghum stems due to the simple approachability of promptly fermentable sugars combined with high lignocellulosic biomass yield. Sweet sorghum has been used as the preferred renewable source for ethanol production in developed countries since the first energy crisis in the 1970s (Nathan, 1978).

Fresh lignocellulosic biomass yields vary with variety and location from 25–130 ton per ha, with extractable juice ranging from 30 - 50%, and soluble sugar content, measured in degree Brix (°Brix), of 15-22% (Channappagoudar et al., 2007; Tew et al., 2008). The major non-structural soluble carbohydrate (NSSC) in the stem juice is sucrose (~90%) followed by soluble glucose and fructose (~8%) and starch (~2%) (Sherwood, 1923; Vogel et al., 2010; Han et al., 2013).

Many approaches have been proposed to produce, harvest and process sweet sorghum at a commercial scale. Usually, the key organic compound obtained and fed into the ethanol production is the saccharine juice. The stems are harvested, the panicles removed, and the stems are crushed, allowing the complete extraction of the juice. The same plants designed to process sugarcane stalks to produce ethanol could use sweet sorghum stalks as feedstock, with the soluble carbohydrates extracted from the stem juice, typically fermented by yeast to produce ethanol. Researchers and engineers have proposed new alternatives to enhance the sugarcane ethanol production model, to be adapted for sweet sorghum. They suggest a directly juice extraction and fermentation during harvesting (Li et al., 2004; Kundiyana et al., 2006). Harvesting of sweet sorghum stalks with a forage chopper produces better biomass density compared to harvesting with a sugarcane harvester. However, the chopped stems showed a quick reduction in soluble carbohydrates concentration compared to the stems harvested at once (Keating et al., 2004).

1.6 Trait Improvement of a Dedicated Bioenergy Sorghum

1.6.1 Germplasm

Sorghum germplasm harbors important traits required to breed for a dedicated bioenergy crop. Currently, ICRISAT is the major repository of sorghum world germplasm with a total of 38,675 accessions from 92 countries. This collection represents about 80% of the variability present in sorghum (Eberhart et al. 1997). Landraces constitute 85.3%, breeding material 13.2%, wild species accessions 1.2% and named cultivars 0.3% of the total collection (http://www.icrisat.org/crop-sorghum-genebank.htm). The ICRISAT germplasm bank consists of five basic sorghum races: bicolor, guinea, caudatum, kafir and durra. However, three races are predominantly represented: durra (23.5%), caudatum (20.6%) and guinea (14.8%). India, Uganda and Zimbabwe have all the five basic and ten hybrid races (Reddy et al. 2002).

Compositional and agronomic traits are major factors affecting the feasibility of designing sorghum as major bioenergy crop in the U.S. By understanding the physiology and genetics of traits associated with bioenergy production, it would be possible to exploit the vast genetic variability present in sorghum.

1.6.2 The brown midrib trait

It has been reported that the chemical composition of plant cell walls can drastically affect glucose recovery during the conversion of lignocellulosic material of plants (Pederson et al., 2008; Dien et al., 2009). Lignin is a complex polymer of aromatic alcohols strongly attached to cellulose and hemicellulose, making it difficult for enzymes to degrade complex

carbohydrates to fermentable sugars (Binder et al., 2010; Martin et al., 2013). Several studies reported that high lignin concentration is responsible of the poor yield of fermentable sugars to produce lignocellulosic ethanol (Dien et al., 2009; Vogel et al., 2010). Therefore, high lignin content has become an obstacle that dedicated bioenergy crops need to overcome. Grasses such as *Miscanthus*, switchgrass, wheatgrass, etc., have been selected as promising bioenergy crops. However, their low efficiency of releasing glucans during biomass conversion raise questions regarding whether or not these crops can offer an economically sustainable feedstock for ethanol production. The brown midrib trait enhances lignocellulosic biomass conversion in sorghum (Bucholtz et al., 1980; Palmer et al., 2008; Corredor et al., 2009; Dien et al., 2009; Vogel et al., 2010). A reddish-brown color present in the midrib of some sorghum leaves was associated to the low concentration of lignin in cell walls (Porter et al., 1978; Shoemaker and Bransby, 2010). The sorghum brown midrib trait was first reported by Porter et al. (1978) from mutagenesis aimed at improving sorghum forage quality. In this study, they identified nineteen chemically induced brown midrib mutants (*bmr*1 – *bmr*19). The compositional characterization showed variation in lignin concentration in sorghum stems and leaves. Decades later, Saballos et al. (2008) grouped the brown midrib mutants into allelic groups. By combining genetic and chemical approaches, they established the presence of at least four independent *BMR* loci, represented by BMR_{2}, BMR_{6}, BMR_{12} and BMR_{19}. Of the *bmr* mutants, bmr_{12} is the mutant allele of the gene encoding the monolignol biosynthetic enzyme caffeic acid O-methyl transferase (COMT; Bout and Vermerris 2003). Also, there is evidence that the mutant allele bmr_{6} affects the activity of the enzyme cinnamyl alcohol dehydrogenase (CAD; Vermerris et al., 2007). More recently, there is evidence that the

mutant allele *bmr*$_2$ affects the activity of the enzyme 4-coumarate coenzyme A ligase (4CL) (Saballos et al., 2012). These recessive mutations can easily be incorporated in selected sorghum lines by backcrossing (Fehr 1993).

1.6.3 Stem sugar traits

The ability of sweet sorghums to accumulate soluble sugars in their stems offers a source of genetic variability to maximize total usable energy storage in lignocellulosic biomass. In this way not only structural carbohydrates but also soluble nonstructural carbohydrates (jointly present in sorghum lignocellulosic biomass) could be converted to ethanol. The complex genetics of the stem sugar trait is not well understood. It was believed that the inheritance of high stem sugar concentration is due to a single recessive gene that confers the sweet character (Ayyangar et al., 1936). Later studies showed evidence of several genomic regions associated to stem sugar concentration. Quantitative trait loci (QTL) on chromosomes 03, 05, 06, and 07 have been reported to be significantly associated to Brix measurements (1 °Brix represents 1 gram of soluble sugars in 100 ml of water) indicating that multiple genes with additive effects determine stalk sweetness (Li et al., 2004; Murray et al., 2008; 2009; Ritter et al., 2008). In fact, several genes controlling this trait open the chance that superior genotypes with beneficial combinations of genes can be improved via crossing and selection (Murray et al., 2008a and 2008b).

1.6.4 Lignocellulosic biomass traits

The distinguishing feature of lignocellulosic biomass production in sorghum is attributed to effects of combined traits such as plant height, stem thickness, leaf size, tillering capacity, photoperiod sensitivity and maturity. These traits can all be considered sorghum biomass yield components (Vermerris et al., 2011). Photoperiod sensitivity is a novel feature that increases lignocellulosic biomass quantity of sorghum. Photoperiod-sensitive sorghums do not flower in temperate latitudes which in turn will avoid the decline in late season forage quality providing flexibility in harvest management (McCollum et al., 2004). However, photoperiod-sensitive sorghums have relatively high lignin content in the stalks, which minimizes lodging but decreases biomass conversion efficiency for ethanol production. Recessive mutations in the photoperiod pathway have been discovered and selected by farmers in temperate latitudes. These early selections rapidly displaced the original photoperiod sensitive cultivars, resulting in increased acreages of sorghum, and providing genetic material for the development of modern cultivars by plant breeders (Smith and Frederiksen, 2000).

Tropical cultivars require shorts days to flower, so they will not flower during summer days of temperate regions. However, the substitution of one locus from dominant "*Ma*" to recessive *"ma"* have converted the tropical sorghum to a temperate one that will flower in high latitudes (Quinby, 1974). Several maturity loci has been identified to be responsible for flowering time, and their effects are related to the production of sorghum lignocellulosic biomass (Rooney et al., 2007). Genetic studies determined the presence of four loci influencing flowering time in sorghum (Quinby, 1966; Major et al., 1990). These genes were designated as maturity genes because they influenced the duration of growth, and

were respectively named as *Ma_1*, *Ma_2*, *Ma_3*, *Ma_4*, *Ma_5*, and *Ma_6* (Ellis et al., 1997; Morgan et al., 2002). There is evidence that the effect of the maturity genes on sorghum plant developmental traits, such as number of leaves and their area, were not strictly pleiotropic (Pao and Morgan, 1986a; 1986b). Indeed, the variability observed in plant development was probably the consequence of field stress conditions (Blum, 1996; Maas et al., 1987). The transition from vegetative to reproductive phases decreases biomass accumulation, so delayed flowering is desirable in order to maximize lignocellulosic biomass yield (Rooney et al., 2007). The discovery of multiple maturity genes that induce photoperiod insensitivity enables a scenario where two early-maturing lines can be hybridized to create photoperiod-sensitive, late maturing hybrids. This method is currently being used to create high-biomass lines for biofuel production (Rooney et al., 2007; Mullet et al., 2010).

Plant height is also a trait related to high lignocellulosic biomass production. Four different mutations, named as *dw_1*, *dw_2*, *dw_3*, and *dw_4,* have been reported at loci controling sorghum stem internode length (Quinby and Karper, 1954). Among these loci, there is evidence that *dw_3* is responsible for high levels of peroxidase production in stem internode, thereby inhibiting growth promoting substance activities in the stem (Schertz et al., 1971; Multani et al., 2003). In wheat, the dwarfing gene effects were mediated largely by gibberellin metabolism, to the extent that the height genotype could be identified by the phenotypic response to exogenous gibberellin application (Gale and Youssefian, 1985). Perhaps, this should be expected also for sorghum. Although other height mutants have been recognized, only the four brachytic mutations (which affect only internode length) are utilized for breeding purposes.

The tillering capacity of some sorghum genotypes is the main reason why this plant if often referred to as "perennial grass" in tropical zones (Hart et al., 2001; Hae-koo et al., 2010). Sorghum plants can regrow from basal tillers after grain is harvested, and the tillers are able to produce new panicles. Perhaps this characteristic would be beneficial in locations where sorghum is manually harvested. For grain production, basal tillering ability could be useful for grain yield stability (Heinrich et al., 1983; Garcia del Moral et al., 2003). Besides, more basal tillers could increase stem yield; however, the effect of the increased number of stems per plant in stem juice sugar concentration is still not well understood. For instance, when sweet sorghum is grown for syrup production, high plant density decreases syrup yield per hectare (Doggett, 1988). Therefore, the utility of tillering ability might depend of the production system (Hae-koo et al., 2010). Two QTL controlling basal tillers number have been mapped in a parental population of recombinant inbred lines derived from contrasting parent phenotypes. Across environments, these QTL explained 49 – 66 % of the variation in tillering capacity (Hart et al., 2001). Similarly, evidence of four genomic regions controlling number of tillers were reported by Paterson et al. (1995). These QTL showed very low environmental effects; therefore, they should be fairly easy to introgress high tillering ability in sorghum (Paterson et al., 1995; Jordan et al., 2004; Jang, et al., 2006). A decade later, evidence that lignocellulosic biomass traits QTL are located in similar locations as nonstructural carbohydrate QTL was reported by Murray et al. (2008b). Because both types of carbohydrates are strongly correlated with plant height, physiological maturity, and stand density–tillering, this result was expected. Indeed, the co-localization between traits was probably due to pleiotropic effects of a single gene; this

means that taller plants would produce more stem biomass given thicker stem diameters and high density–tillering ability (Murray et al., 2009).

The measurement of the maximum rate at which leaves are able to fix carbon during photosynthesis is known as photosynthetic capacity or performance (Nguyen and Blum, 2004). It is known that sorghum can fix more carbon than many other crops, so dry matter production efficiency would be reached with relatively low water usage (Dercas and Liakatas, 2007). Photosynthetic rates between sorghum races are variable and dependent on their natural habit. For instance sweet sorghum cultivars have higher photosynthetic rates than grain sorghums (Steduto et al., 1997). Also, drought resistant sorghums maintain a higher photosynthetic rate under late season water stress conditions (Saneoka et al., 1995). Selection for dry matter accumulation in lignocellulosic biomass could be an indirect way to select for increased photosynthetic activity in potential bioenergy sorghum varieties. Genetic variation for photosynthetic capacity was reported by Hubick (1990). They suggested that photosynthetic capacity and/or water-use efficiency genetic variation may result from bundle-sheath cells variable ‘‘leakiness’’ or from variable ratios of assimilation rate to stomatal conductance. Therefore, genetic variation and even heterosis exists in sorghum for the ratio of carbon exchange rate to stomatal conductance (Blum, 1989) and the increase in this ratio expressed very well the effect of heat hardening on the photochemical component of sorghum assimilation under very high temperatures (Blum, 2004).

Drought tolerance is an important feature displayed by some sorghum cultivars. The genetic mechanism of drought tolerance is very complex due to its inconsistency in testing environments and interaction between stages of plant growth and environment (Paterson

et al., 2009; Besufekad and Bantte, 2013). Some genetic studies reported polygenic inheritance of root characters that confer the ability to endure low soil moisture in cultivated grasses (Aharoni et al., 2004; Zhang et al., 2005). Indeed, drought tolerance is controlled by many genes and depends on timing and severity of the moisture stress. Physiological traits have been proposed to enhance drought tolerance. However, only a few mechanisms have been demonstrated to be associated to the expression of tolerance to drought under stable environmental conditions (Ejeta and Knoll, 2007). The genetic improvement of adaptation to drought stress has been addressed through the conventional breeding approaches by selecting for yield performance over locations and years (Pathan et al., 2004). However, selection for drought tolerance while maintaining maximum productivity under optimal condition is very difficult because of the complexity of the drought tolerance trait. Then, gains from selection to improve drought tolerance are quite low. This approach remains slow because of the difficulty in finding optimal environments for evaluation (phenotyping). Thus, molecular marker techniques could offer a good chance to develop drought tolerant crops through understanding the tolerance genetic components (Zavala-Garcia et al., 1992).

To increase crop yields across drought and non-drought environments, conventional breeding strategies and marker assisted selection have been very successful (Witcombe et al., 2008); however, some traits selected for stressful climates may be genetically drained in some crops (Duvick, 2005). The current availability of several crop genomic sequences are helpful tools for comparing genomes and evaluating transcriptome response to abiotic stress. Therefore, it is possible to consider comprehensive libraries of abiotic stress genes. Approaches related to gene discovery and plant transformation have helped to increase the

effectiveness of physiological and cellular mechanisms involved with stress tolerance. These approaches have focused in moving tolerance genes between species, and have been successful in developing new useful combinations of genes. In addition, traits like drought tolerance, governed by multiple genes, can now be manipulated as systems rather than only one gene at a time (Umezawa et al., 2006). These genetic approaches hold great potential for combining genes to meet the future stress tolerant crop needs (Shinozaki and Yamaguchi - Shinozaki, 2007). A drought tolerant sorghum cultivar is seen as one of the most promising biomass crops for the coming decades.

Sorghum nutrient use efficiency, and adaptability to a variety of environments, are reasons why sorghum would be an excellent candidate as a bioenergy crop for marginal lands (Rooney et al., 2007; Murray et al., 2008a; 2008b). Indeed, a growing interest to improve plant nitrogen use efficiency (NUE) in many crops, including sorghum, have led scientists to search for genes associated with nitrogen uptake and utilization. Traditional breeding approaches to improve NUE in crop plants have reached a plateau, where increases of nitrogen do not result in increases in grain yield (Masclaux-Daubresse et al., 2010). Therefore, new strategies and technologies may be useful to identify genes related not only to physiological processes but also biochemical pathways contributing to plant NUE. Nitrogen uptake, assimilation, remobilization and storage candidate genes have been reported in the last decade in several crops (McAllister et al., 2012). They offer a new source of genetic variability to improve NUE crop plants. However, issues identifying the correct gene variant, proper gene expression and how and why NUE phenotypes occur under stress and non-stress conditions are topics that require a deep understanding. It seems obvious that the most likely candidates to produce a NUE phenotype are those gene

products involved in primary N metabolism. However, there is very little evidence of how NUE phenotypes perform consistently well, specifically from field trials (Coque et al., 2008; Gelli et al., 2014). At the same time as geneticists and breeders focus on finding NUE genes, the understanding not only of N metabolism but also of C metabolism has increased. Insights of C/N ratios changes as well as possible interaction between pathways has both broadened and complicated the range of NUE targets. Moreover, due to the NUE gene complexity, molecular geneticists and biotechnologists may need to explore pyramiding candidate genes to obtain stable NUE phenotypes across environments.

The United States grows approximately 20 million acres of sorghum, which could provide 25 percent of the country's long term goal for biofuels. In fact, traits related to sorghum agronomic performance that enhance biomass quantity and lignocellulosic biomass quality, both exploited and imagined are present in sorghum germoplasm. The combination of such useful traits could boost the bio-refinery industry. The prospects for accelerated development of sorghum as a premier source of biofuels are therefore excellent.

1.7 Reproduction and Breeding Methods

Although sorghum originally belongs to tropical zones of Africa and Asia, it has positively adapted to temperate zones and agricultural systems allowing selection to be applied annually. Sorghum crop cycle length ranges from 14 to 16 weeks (Doggett, 1988; Smith and Frederiksen, 2000). However, in tropical and subtropical zones, it is possible to produce multiple crops per year. The sorghum inflorescence has hermaphrodite flowers, thus most of flowers will self-pollinate; however, a low degree of outcrossing occurs and it sometimes ranges from 5 to 30% (Smith and Frederiksen, 2000). Sorghum hybrid

production is based on male sterility systems. Cytoplasmic and genetic male sterility are widely utilized to produce commercial hybrids (Rooney, 2000). Male sterility results from homozygosity at one of six male sterility (*ms*) loci (Ayyangar, 1942; Ayyangar and Ponnaiya, 1937; Barabas, 1962; Stephens, 1937). Depending on the locus, male sterility is expressed differently, sometimes from no pollen production to complete anther absence. The ms_3 sterility system is utilized in research and plant breeding programs. Cultivated sorghum is able to produce fertile hybrids only when intercrossed to species within the *Sorghum* subgenus. The genus *Sorghum* basic chromosome number is five. Species within the *Sorghum* genus have multiples of that basic number. Genome duplication in *Sorghum* subgenus ancestor was reported by Gomez et al. (1997). Indeed, the species *S. bicolor* and *S. propinquum* were reported as ancient tetraploids; however, genetically they behave like diploids, with n = 10.

Because sorghum is mainly self-pollinated, pure lines selection from outstanding plants in the field is the ancient and most used plant breeding method. Mutations, crosses between different varieties and mutants, and crosses between wild relatives has increased the genetic diversity within the cultivated sorghum. In most developed countries, sorghum was genetically improved to be short, photoperiod insensitive, and adapted to mechanical harvesting (Rosenow and Dahlberg, 2000). Such a need of very specific characteristics has restricted the use of exotic sorghum germoplasm in most of commercial breeding programs. Conversion programs in the private sector, universities and international research centers of agriculture have been of great help to increase the use of tropical lines. However, the genetic diversity of the improved lines is lower in comparison with improved lines from the world collection (Menz et al., 2004; Rooney, 2007). It is known that exotic

sorghums possess desirable set of genes that can improve the resistance to abiotic and biotic stress, lignocellulosic biomass yield and grain yield (Murray et al., 2008b). The development of sorghum lines for lignocellulosic biomass production is less restricted by some of the factors that have prevented their use in grain sorghum improvement (Murray et al., 2008a). For instance, tall photoperiod-sensitive sorghums are probable to play a major role in increasing lignocellulosic biomass yield of sorghum. Information on QTL, genomics and genome wide association studies (GWAS) f these traits could facilitate the introgression of specific traits into promising lines. Sorghum breeders focused in the production of enhanced lignocellulosic biomass lines are in an advantageous position to use the existent genetic variability in the crop.

Several plant breeding methodologies can be used to successfully improve sorghum. Due to the self-pollinated nature of the crop, pure lines are easily selected by the pedigree method. In breeding programs, to create a genetically diverse population, it is necessary to cross several diverse lines. Manual emasculation and plastic bag methods can be used to produce specific crosses. When the female flower is receptive, pollen from the male parent is harvested and applied to the female panicle. The female panicle is covered to avoid contamination from undesirable pollen (Rooney, 2000). The pedigree method is only used to produce either open-pollinated cultivars or inbred lines for hybrid production. The ms_3 male sterility system allows for population improvements, mass selection and recurrent selection methods, regardless those methods are more suitable to cross-pollinated species (Doggett and Eberhart 1968). Therefore, it is important that one of the parents in the initial cross must carry the male sterility gene. The F_1 selfed progeny will be then grown, and the seeds from male sterile plants will be bulk harvested. This process must be repeated for

several generations before selection begins. At this time, fertile plants are selfed to begin the production of lines. Importantly, the period of obligate outcrossing allows for more recombination of the parental genes, useful to break linkage blocks and to produce novel combinations of genes (Fehr 1991).

Sorghum hybrid production can be achieved by using a male sterility system or by manual emasculation. Commercial seed production is possible by using the genetic-cytoplasmic sterility system. A-lines (male-sterile) result when the plant carries the male-sterile cytoplasm gene and lacks a restorer fertility gene in the nucleus. The A-lines are maintained by the crossing them with B-lines that carry a fertile cytoplasm, therefore able to produce pollen. The progeny given, it will still have the male sterile cytoplasm and no restorer gene will be present in A-lines. Hybrid production is achieved by crossing the A-line with another line carrying the fertility restoring gene (R-line). The progeny will be fertile heterotic hybrids (Doggett 1988). For an effective utilization of the genetic-cytoplasmic system in a breeding program, pure lines in the program need to be assessed for its B or R reaction by crossing it with a known A-line. New A-lines and B-lines can be created from B-lines by backcrossing (House 1985). R-lines can be developed by any of the methods used to develop pure lines. Since the development of new A-lines is time and resource-consuming, most commercial programs maintain stocks of A-lines and focus on R-lines improvement (Smith and Frederiksen 2000). Promising R-lines are test-crossed to the A-lines to test for their combining ability (Fehr 1991).

1.8 Genomics of Sorghum

The first published sorghum genetic linkage genetic map was constructed by Chittenden et al. (1994) by using more than 270 restriction fragment length polymorphism (RFLP) loci mapped on a bi-parental population (Chittenden et al., 1994). Conserved genome regions among cereal species (Moore et al., 1995) allows the use of molecular markers in the sorghum genome. RFLP, amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and morphological markers have been successfully utilized to build several genetic sorghum maps in bi-parental populations (Berhan et al., 1993; Woo et al., 1994; Boivin et al., 1999; Bhattramakki et al., 2000; Klein et al., 2000; Menz et al., 2002). As a result, high-density sorghum genetic maps are available for genetic diversity studies, genetic architecture studies and QTL studies (Bowers et al., 2003; Menz et al., 2004; Feltus et al., 2006). Additionally, useful information on the chloroplast and mitochondrial genomes is also available for sorghum genetic and breeding studies (Chase and Pring, 1985; Dang and Pring, 1986). Due to its importance as staple crop in many parts of the world (mainly Africa and Asia), sorghum has been the subject of genetic and genomic studies for almost ten decades (Smith and Frederiksen, 2000). Because of the combined work and effort of thousands of scientist, nowadays we have plenty of information to enhance sorghum germoplasm. Sorghum genetic and physical maps can be browsed and compared with other cereal crops via the phytozome website (http:/www.phytozome.net). Moreover, the complete sorghum genome (inbred line BTx623) has been successfully sequenced as part of a Community Sequencing Program (CSP) by the Department of Energy Joint Genome Institute (JGI). The sorghum genome sequence is publicly available (Paterson et al., 2009, http:/www.phytozome.net/sorghum). This website offers

information linked to other databases, thus providing a useful genomic tool to discover and manipulate genes.

1.9 Nitrogen Use Efficiency

Nitrogen (N) is one of the most limiting nutrients for grain production in many areas of developed and developing countries where sorghum is cultivated. One of the strategies to improve yields is to select sorghum lines with high N use efficiency (NUE) that can produce economic yield under limited N and water supply (Cassman et al., 1998; Sowers et al., 1994). Nitrogen use efficiency (NUE) can be defined as the percent of N fertilizer which is recovered and then utilized by a fertilized crop. The average NUE estimates are 33% for grain production, and about 45% for forage production in the U.S. (Raun and Johnson, 1999). According to Johnston et al. (2000) and Stewart et al. (2005), N fertilizer consumption has increased yield more in the past decades than any other agricultural input. Smith et al. (1990) reported that corn and sorghum yields dropped by 41 and 19%, respectively, without N fertilizer application.

In crop production systems, nitrogen use efficiency can be calculated by different methodologies (Pandey et al., 2001; Doberman 2005; 2007). Nitrogen use efficiency can be divided into several components that identify soil and plant processes contributing to overall nitrogen use (Moll et al., 1982). Nitrogen use efficiency components include the ability of the aboveground plant to uptake (N_t/N_s) N from fertilizer, and the efficiency with which N is transform to produce grain (G_w/N_t), where N_t is the total N in the plant at maturity (grain + stover), N_s is the nitrogen supply or rate of fertilizer N, and G_w is the

grain weight (Doberman, 2007). For simplicity NUE is calculated as the total N uptake in sorghum/corn from unfertilized plots is subtracted from the total N uptake in sorghum/corn from the N fertilized plots, and then divided by the rate of fertilizer N applied. Cassman et al. (2002) discusses these components as well, however, he raises the issue of applying adequate N to maintain a soil N pool for sustainable production. Regardless of how NUE is measured, utilization of applied fertilizer N is generally low (Novoa and Loomis, 1981). Agricultural inputs have to be managed efficiently, especially during periods of high dry matter production in the crop to maximize yield and profit, and to minimize environmental consequences (Feinerman et al., 1990). Pathways for N losses from agricultural ecosystems include gaseous plant emissions of ammonia, soil denitrification, surface runoff, volatilization of ammonia, and leaching of nitrates (Raun and Johnson, 1999). With the exception of N denitrified to N_2, the remaining pathways all can lead to an increased load of biologically reactive N in the environment (Cassman et al., 2002). Continued low NUE in crops could have a drastic impact on land-use and food supplies worldwide (Frink et al. 1999).

There are several causes for low NUE in crops. One of the most important is the inability to predict the amount of N fertilizer that should be applied to a crop, particularly crops such as corn and sorghum grown in a high risk environment (Ciampitti and Vyn, 2010). With the current management practices that emphasize pre-plant N application, poor synchrony between crop demand and soil N supply is critical (Raun and Johnson, 1999; Cassman et al., 2002; Fageria and Baligar, 2005). Poor synchronization is affected by many factors including: a) Applications of N made after the primary uptake periods of the crop, b) Loss of fertilizer N from the soil applied long before the plant was capable of utilizing it through

leaching or denitrification, particularly during fall or spring pre-plant applications of fertilizer, c) Immobilization and volatilization losses of pre-plant, surface applied N fertilizers, particularly in high residue management systems (William et al., 1999). To increase NUE in crops, several approaches have been proposed. These include: a) Appropriate N timing applications to synchronize with crop needs but avoid potential periods of high N loss; b) Proper fertilizer placement to minimize potential loss from immobilization and volatilization; c) The use of specific additives to minimize loss through leaching, denitrification or volatilization; d) The use of crop sensors during growing season to better estimate soil contributions to the crop and efficiently determine supplemental N fertilizer need (Dobermann 2005; 2007).

1.10 References

Almodares A, Hadi MR (1996) Production of bioethanol from sweet sorghum: A review. African J. Agric. Res. 4(9):772-780.

Badger PC (2002) Ethanol from cellulose : A general review. Trends new crop new uses. Janick hipkey (eds) ASHS Press Alexandria, VA 17–21.

Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I. (2004) Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochemistry, *39*(12):1843-1848.

Blum A, Arkin GF, Jordan WR (1977a) Sorghum root morphogenesis and growth I. Effect of maturity genes. Crop Sci. 17:149–153.

Blum A, Jordan WR, Arkin GF (1977b) Sorghum root morphogenesis and growth: II. Manifestation of heterosis. Crop Sci. 17:153–157.

Ciampitti I, Vyn TJ (2010) A comprehensive study of plant density consequences on nitrogen uptake dynamics of maize plants from vegetative to reproductive stages. Field Crop Research 121:2–18.

Davis, S. C., Diegel, S. W., & Boundy, R. G. (2003) Transportation energy data book: Edition 23 (No. ORNL 6970). United States. Department of Energy.

Dien BS, Sarath G, Pedersen JF, et al. (2009) Improved sugar conversion and ethanol yield for forage sorghum (Sorghum bicolor L. Moench) lines with reduced lignin contents. BioEnergy Res. 2:153–164.

Dobermann AR (2005) Nitrogen use efficiency – State of the art. Agronomy and Horticulture, University of Nebraska-Lincoln. Faculty publications paper 316.

Dobermann AR (2007) Nitrogen use efficiency measurement and management. Fertilizer best management practices, 1:1-28.

Doggett, H. (1988) Sorghum. 2nd edition. Wiley, New York.

Ellis RH, Qi A, Craufurd PQ, Summerfield RJ, Roberts EH (1997) Effects of photoperiod, temperature and asynchrony between thermo-period and photoperiod on development to panicle initiation in sorghum. Ann Bot 79:169–178.

Erickson JE, Woodard KR, Sollenberger LE (2011) Optimizing sweet sorghum production for biofuel in the southeastern USA through nitrogen fertilization and top removal. BioEnergy Res. 5:86–94.

García del Moral, L. F., Belén García del Moral, M., Molina-Cano, J. L., & Slafer, G. A. (2003). Yield stability and development in two-and six-rowed winter barleys under mediterranean conditions. Field crops research, 81(2):109-119.

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques SR (2010) Hemicelluloses for fuel ethanol: A review. Bioresource Technology 101:4775–800.

Hart GE, Schertz KF, Peng Y, Syed NH (2001) Genetic mapping of Sorghum bicolor (L.) Moench QTLs that control variation in tillering and other morphological characters. TAG Theor Appl Genet 103:1232–1242.

Heinrich, G. M., Francis, C. A., & Eastin, J. D. (1983) Stability of grain sorghum yield components across diverse environments. Crop Science 23(2):209-212.

Jacobsen SE, Wyman CE (2000) Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Appl. Biochem. Biotechnol. 84(86):81–96.

Jang C, Kamps T, Skinner D, Schulze S, Vencill W, Paterson A (2006) Functional classification, genomic organization, putatively *cis*-acting regulatory elements, and relationship to quantitative trait loci, of sorghum genes with rhizome-enriched expression. Plant Physiol. 1148–1159.

Jordan DR, Casu RE, Besse P, et al. (2004) Markers associated with stalk number and suckering in sugarcane colocate with tillering and rhizomatousness QTLs in sorghum. (993):988–993.

Johnston AE, Trust L, Fellow S. (2000) Efficient use of nutrients in agricultural production systems. Communications in Soil Science & Plant Analysis 31(11-14): 1599-1620.

Kim HK, Luquet D, van Oosterom E, et al. (2010) Regulation of tillering in sorghum: genotypic effects. Ann Bot 106:69–78.

Li G, Gu W, Chapman K (2004) Sweet Sorghum. China Agricultural Science and Technology Press, Beijing, China.

Maas SJ, Arkin GF, Rosenthal WD (1987). Relationship between the area of successive leaves on grain sorghum. Agron. J. (79):739–745.

Major DJ, Rood SB, Miller FR (1990) Temperature and photoperiod effects mediated by the sorghum maturity genes. Crop Sci. 30:305–310.

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron. J. 74:562 – 564.

Multani DS, Briggs SP, Chamberlin MA, Blakeslee JJ, Murphy AS, & Johal GS (2003) Loss of an MDR transporter in compact stalks of maize br2 and sorghum dw3 mutants. Science, 302(5642), 81-84.

Murray SC, Rooney WL, Mitchell SE, Sharma A, Klein PE, Mullet JE, Kresovich S (2008) Genetic improvement of sorghum as a biofuel feedstock: II. QTL for stem and leaf structural carbohydrates. Crop Science 48(6):2180-2193.

Murray SC, Rooney WL, Hamblin MT, Mitchell SE, and Kresovich S (2009) Sweet sorghum genetic diversity and association mapping for brix and height. Plant Genome 2: 48–62.

Nghiem NP, Nguyen CM, Drapcho CM, Walker TH (2013) Sweet sorghum biorefinery for production of fuel ethanol and value-added co-products. 6:143–155.

Novoa R, Loomis RS (1981) Nitrogen and plant production. Plant Soil 58:177- 204.

Obernberger I, Thek G (2004) Physical characterisation and chemical composition of densified biomass fuels with regard to their combustion behaviour. Biomass and Bioenergy 27:653–669.

Ohgren K, Bura R, Saddler J, Zacchi G (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresource Technology 98:2503–10.

Pao CI, Morgan PW (1986a) Genetic regulation of development in Sorghum bicolor: I. Role of the maturity genes. Plant Physiol. (82):575–580.

Pao CI, Morgan PW (1986b) Genetic regulation of development in Sorghum bicolor: II. Effect of the ma3R allele mimicked by GA3. Plant Physiol. (82):584–584.

Paterson AH, Schertz K. F, Lin Y, Liu S, and Chang Y (1995) The weediness of wild plants: Molecular analysis of genes influencing dispersal and persistence of Johnsongrass, *Sorghum halepense* (L.). Proc. Natl. Acad. Sci. USA. 92, 6127–6131.

Porter KS, Axtell JD, Lechtenberg VL, Colenbrander VF (1978) Phenotype, fiber composition , and in vitro dry matter disappearance of chemically induced brown midrib (bmr) x mutants of sorghum. Crop Sci. (18):205–208.

Quinby JR (1966) Fourth maturity gene locus in sorghum1. Crop Sci. 6(6):516.

Russel I (2003) Understanding yeast fundamentals. In: The alcohol textbook: A reference for the beverage, fuel and industrial alcohol industries, 4th Ed. K. A. Jacques, T. P.

Lyons, and D. R. Kelsall, eds. Nottingham University Press: Nottingham, UK. Pages 103-110.

Saballos A, Vermerris W, Rivera L (2008) Allelic association , chemical characterization and saccharification properties of brown midrib mutants of sorghum (*Sorghum bicolor* (L .) Moench). Bioenergy Res. 1:193–204.

Saballos A, Sattler SE, Sanchez E, et al. (2012) Brown midrib2 (Bmr2) encodes the major 4-coumarate:coenzyme A ligase involved in lignin biosynthesis in sorghum (*Sorghum bicolor* (L.) Moench). Plant J. 70:818–30.

Schertz KF, Sumpter NA, Sarkssian IV, Hart GE (1971) Per-oxidase regulation by the 3-dwarf height locus in sorghum. J. Hered. 62:235-238

Shoemaker CE, Bransby DI, Hall F (2010) The role of Sorghum as a bioenergy feedstock. Chapter 9:149–159.

Smith EG, Knutson RD, Taylor CR, Penson JB (1990) Impact of chemical use reduction on crop yields and costs. Texas A&M Univ., Dep. of Agric. Economics, Agric. and Food Policy Center, College Station.

Stewart WM, Dibb DW, Johnston AE, T.J. Smyth TJ (2005) The contribution of commercial fertilizer nutrients to food production. Agron. J. 97, 1-6.

USDA (2006) The economic feasibility of ethanol production from sugar in the united states.

USDA (2014) U.S. Acreage, production, yield and farm price. Yearbook tables. Data set table 1.

Vassilev S V., Baxter D, Andersen LK, et al. (2012) An overview of the organic and inorganic phase composition of biomass. Fuel 94:1–33.

Vassilev S V, Baxter D, Andersen LK, Vassileva CG (2010) An overview of the chemical composition of biomass. Fuel 89:913–933.

Vogel KP, Dien BS, Jung HG, et al. (2010) Quantifying actual and theoretical ethanol yields for switchgrass Strains Using NIRS Analyses. BioEnergy Res. 4:96–110.

William R, Gordon VJ (1999) Improving N use efficiency in cereal production. Agron. J. 93:358 – 363.

CHAPTER 2. GENETIC AND AGRONOMIC CHARACTERIZATION OF BROWN MIDRIB×SWEET SORGHUM RECOMBINANT INBRED LINES.

2.1 Abstract

With growing influent in next generation biofuels, sorghum stover has emerged as a promising feedstock for ethanol production due to its rich genetic diversity in its genome that could be exploited. In this study, a population of 236 recombinant inbred lines derived from a cross between a brown midrib (low lignin) sorghum mutant (bmr12) and a sweet sorghum (high stem sugar concentration) line (Brown County) was evaluated over two years for potential improvement of biomass quality and quantity, to estimate heritability and genetic effects in biomass sugar related traits. Molecular markers associated with high stem sugar concentration (sweet) and the caffeic acid-*O*-methyltransferase (*COMT*) gene responsible for the low lignin trait contributed by the brown midrib parent (bmr12) were also identified. Seven biomass related traits were compared among RILs grouped according to whether they carried the brown midrib or sweet mutations, both or none. The brown midrib × sweet sorghum population showed high genetic variability for biomass quality and quantity. We found evidence that the sweet mutation enhances stover yield, plant height, stem thickness and stem sugar concentration in “brown-sweet” RILs. The study showed that selection was feasible for improving ethanol yield in sorghum. Genetic

analysis showed high heritability for plant height and stem sugar concentration, and moderate heritability for biomass yield, stover yield, stem thickness, grain yield and plant maturity. The variability observed in most of these traits was due mainly to genetic effects. Additionally, phenotypic and genotypic correlations showed positive associations between lignocellulosic biomass related traits and stem sugar concentration. However, both groups were negative associated with starch related traits (grain yield). Interestingly, the principal component analyses (PCA) grouped the seven measured traits based on the type of carbohydrates produced in plant biomass, indicating that selection for multiple traits could increase ethanol production. The results of single marker analysis showed two possible quantitative trait loci, on chromosomes 6 and 7, each explaining 2 and 7% of the variation in stem sugar concentration measurements. A useful InDel marker that can be used for the selection of the mutant allele of *COMT* was identified for this population.

2.2 Introduction

Sorghum (*Sorghum bicolor* [L.] Moench), a crop capable of growing on marginal lands under adverse environmental conditions, is the fifth most economically important cereal cultivated in the world (FAO 2012). Its potential to produce high yields of grain, lignocellulosic biomass and sugar in stems are among its desirable qualities. The importance of sorghum as food, feed, fiber and fuel warrants further investigation of the underlying genetic components contributing to these qualities.

The over-reliance on crude oil and a global desire to reduce greenhouse gas emissions have triggered an interest in renewable sources of fuel. Lignocellulosic biomass, known as stover, of row crops is generally left unharvested but could be utilized to produce ethanol through (process that encompasses hydrolysis and fermentation) bioconversion (Dien et al., 2006; Sticklen, 2008; Canilha et al., 2012). Ethanol is a renewable eco-friendly source of energy (Canilha et al., 2012; Nghiem et al., 2013). The potential ethanol yield of stover depends on the quality and quantity of lignocellulosic biomass from which it is produced. Forage sorghums are coarse, fast growing and warm season grasses that provide livestock feed in mid- summer. In recent years, gains in lignocellulosic biomass production have been demonstrated and could be applied to enhance ethanol production (Oliver et al. 2005a; 2005b).

Several crop traits are associated with lignocellulosic biomass production and productivity in forage sorghums. Photoperiod sensitivity is a characteristic that dramatically increases lignocellulosic biomass of sorghum through increased vegetative growth. Photoperiod-sensitive sorghums delay flowering which in turn delays the decline in forage quality

providing flexibility in harvest management (McCollum et al., 2004). However, photoperiod-sensitive sorghums generally have high lignin content in the stalks, which offers stalk strength and minimizes lodging but decreases biomass conversion efficiency for ethanol production. Recessive mutations in photoperiodism have been discovered and selected by farmers to allow the crop to reach physiological maturity even in temperate latitudes. In sorghum, such early selections rapidly displaced the original photoperiod sensitive cultivars, resulting in increased acreages of grain sorghum, providing the genetic material for the development of modern cultivars by plant breeders (Rooney et al., 1999; Smith and Frederiksen, 2000).

Plant height is another desirable trait related to high yield biomass production. Four different mutations named as dw_1, dw_2, dw_3, and dw_4 have been reported to reduce sorghum height (Quinby and Karper 1953). Although other height mutants have been recognized, only these four brachytic mutations (which affect only internode length) are utilized in selection for breeding purposes. Reduced plant height makes sorghum more amenable to mechanical harvesting as a grain crop, but generally results in a decrease in lignocellulosic biomass production (Multani el at., 2003; Brown et al., 2003).

The manipulation of maturity loci within photoperiod insensitive genotypes has been of fundamental importance to the production of high-biomass sorghum for bioenergy (Rooney et al., 2007). Genetic studies determined that four loci influenced flowering time in sorghum. These genes were designated as maturity genes because they influenced the duration of growth (days to maturity) and were named as Ma_1, Ma_2, Ma_3, Ma_4, Ma_5, and Ma_6 (Morgan et al., 2002). The transition from vegetative to reproductive phases curtails

biomass accumulation, so delayed flowering is desirable in order to obtain maximum biomass yield. The discovery of multiple maturity genes that induce photoperiod insensitivity enables a scenario where two early- maturing lines can be hybridized to create photoperiod-sensitive, late maturing hybrids. This method is currently being used to create high-biomass lines for biofuel production (Rooney et al., 1999; Mullet et al., 2010).

The brown midrib and sweet traits are important characteristics in the production of sorghum with efficient bioconversion of high quality lignocellulosic biomass. The reddish-brown pigmentation in the midrib of sorghum leaves is referred to as brown midrib (*bmr*). The *bmr* trait is recessive, and when present in the homozygous state, the *bmr* mutation is associated with reduced lignin content (Porter et al., 1978, Pedersen, 1996; Casler et al., 2003). The genetics of brown midrib have been studied well in the last decade (Bout and Vermerris, 2003;Vermerris et al., 2007; Saballos et al., 2008; Vogler et al., 2009; Sattler et al., 2012; Srinivasa et al., 2012; Gorthy et al., 2013). Four allelic groups have been reported by Saballos et al. (2008) among known brown midrib mutations. These allelic groups represent four independent *BMR* loci i.e. BMR_2, BMR_6, BMR_{12} and BMR_{19}. Two of these genes, BMR_6 and BMR_{12}, have been characterized and found to encode cinnamyl alcohol dehydrogenase (CAD) and caffeic acid-*O*-methyltransferase (COMT) enzymes, respectively. These enzymes are involved in the last two steps of monolignol biosynthesis (Bout and Vermerris, 2003; Palmer et al., 2008). The bmr_6 mutation contains a C-to-T transition at position 2800 of the $SbCAD_2$ genomic sequence, while the bmr_{12} mutation leads to a C-to-T transition at position 486 relative to the transcription start site. Both mutations introduce a premature stop codon that prevents translation to a functional biosynthetic enzyme (Bout and Vermerris, 2003; Saballos et al., 2009).

The inheritance of the sweet (high stem sugar concentration) trait is more complex and less understood than the brown midrib pehonotype. In the last century, it was thought that a single dominant gene confers the non-sweet character (Ayyangar et al., 1936). However, the genetics of high stem sugar concentration appears to vary depending on the particular cross in which its inheritance is studied, having been shown to be either additive or dominant (Schluhuber, 1945; Clark, 1981). Gene mapping studies have identified several loci controlling the sweet character in sorghum (Natoli et al., 2002; Ming et al., 2002; Bian et al., 2006; Ritter 2007). The QTL have been mapped to four different sorghum chromosomes, but the small variance explained by these QTL suggests that additional loci with complex interactions may also be involved (Murray et al., 2008; 2009; Ritter et al., 2008).

The objectives of this study were as follows:

1. To characterize biomass yield and quality among sorghum genotypes that vary for component traits.
2. To estimate genetic variability and heritability among traits important for the production of lignocellulosic biomass in sorghum.
3. To identify quantitative trait loci (QTL) and marker(s) associated with stem sugar and a specific allele that determines a brown midrib phenotype in sorghum.

2.3 Material and Methods

2.3.1 Genetic material

A bi-parental population consisting of 236 recombinant inbred lines (RILs) was selected as our breeding population for a two year study (2008 and 2009). This population was developed through seven generations of single seed descent selection from the original F2 population of a cross between two lines, bmr12 (a brown midrib, low lignin sorghum) and Brown County (a sweet sorghum) as parents (Appendix A.1). The experiment was planted on May 29th, both in 2008 and 2009 at the Agronomy Center for Research and Education (ACRE) in West Lafayette, Indiana. A randomized complete block design with two replicates was utilized in both years. All RILs, both parents, and a sweet brown midrib line used as check (bmrAtlas) were each planted in two row plots. Dimensions of each plot were 6.10m long with 0.76m spacing between the two rows. Approximately 2.5 grams per row of sorghum seed was planted at a depth of 5 cm. The seeds were treated with a fungicide (Captan at 48.9%) prior to planting to ensure better seedling emergence and stand establishment. Three weeks after planting, plots were thinned to 6 plants per foot for an approximate plant population of 250,000 plants per hectare. Nitrogen fertilizer was applied and incorporated at a rate of 150 kilograms per hectare. In both years, the experiment was managed following standard cultural practices recommended for commercial sorghum production.

2.3.2 Agronomic data and sample collection

Two year data sets of biomass yield and quality traits were collected from the RIL population. These traits were plant height (cm), plant maturity (days), stem thickness (cm),

dry grain yield (t/ha), dry stover yield (t/ha), dry total biomass yield (t/ha), and stem sugar concentration (Brix).

In both years and replicates, data recording was done as follows: The length of the plant from the ground to the panicle tip was measured to obtain plant height (in cm). Plant maturity (PM) was considered as 45 days after flowering date. It is at plant maturity that the sweet trait is maximally expressed. Based on the flowering date for each RIL, three plant maturity groups were defined. In this way, this study managed any bias caused by differences in plant maturity among RILs of the brown midrib × sweet sorghum population. Measurements of stem thickness of each recombinant inbred line, parent and the check were collected. For each plot, two plants located in the middle part of each row were collected randomly as plot samples (a total of four plants per plot). Stem cylinders were cut between the fourth and the fifth node of each plant sampled. Stem thickness (ST) was recorded from each stem cylinder by using a digital caliper, and the average of the four measurements per plot was used for further analysis. At harvesting time of each maturity group, a sample plot of 10 plants (5 from each row) were randomly selected from the middle part of each plot to record biomass components. The panicles of the 10 plants were cut at the flag leaf and saved in paper bags. The paper bags containing panicles of each plot were dried for 3 to 4 days at 45ºC. The weight of leaves and stems of the same 10 plants (without panicles) was recorded as fresh stover weight per sample plot. Fresh stover weight per sample plot was used only in the calculation of dried stover (leaves-stems) weight per sample plot (see below). Next, the ten plants (without panicles) of each plot were chopped in a tractor driver mechanical chopper, the chopped leaves and stems mixed, and a subsample of roughly one and a half fistfuls was weighed and saved in a paper bag (fresh

stover subsample weight). The paper bags containing chopped subsamples of fresh stover were dried for 3 – 4 days at 60ºC, and dried stover subsample weight was recorded. Dried stover weight per sample plot was calculated by dividing the dried stover subsamples weight by fresh stover subsample weight and multiplying by fresh stover weight per sample plot.

Before threshing, dry panicle weight per sample plot was recorded. Then, the panicles were threshed when sorghum grain had approximately 12-14% of moisture. After threshing, dry grain weight per sample plot was recorded. Following, dry rachis-branches weight per sample plot was calculated by subtracting dry grain weight per sample plot from dry panicle weight per sample plot. Then, the dry rachis branches weight per sample plot was added to the dry leaves and stems weight per sample plot to finally obtain dry stover per sample plot. Therefore, dry stover weight per sample plot was calculated as the sum of leaves and stems dry weight and panicle rachis branches dry weight. These measurements were later converted to yield per hectare to obtain yield estimates of biomass components in tons per hectare.

2.3.3 Stem sugar analysis

Phenotypic data set (Brix) measured on 236 RILs was used in this analysis. At plant maturity, measurements of sugar concentration in degrees Brix (ºBrix, or simply, Brix) of each recombinant inbred line, parent and the check were collected. For each plot, two plants located in the middle part of each row were collected randomly as plot samples (a total of four plants per plot). Stem cylinders were cut between the fourth and the fifth node of each plant sampled. Following, a garlic press was used to squeeze stem juice from each of the

four cylinders sampled. A digital refractometer (ATAGO Model PAL-1) was utilized to measure the percentage of soluble sugars present in the stem juice (Brix) of each sampled cylinder. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. The average of the four measurements of Brix per plot was utilized for further calculation and analysis in this chapter and in Chapter 3.

2.3.4 Molecular analysis

2.3.4.1 Genotyping

At growth stage two, leaf samples were collected and lyophilized from each of the 236 RILs and the two parents i.e. bmr12 and Brown County. Genomic DNA was extracted using the high throughput method described by Xin et al., 2003 (Appendix A). The genomic DNA was utilized to generate genotypic data.

A set of 38 simple sequence repeat (SSR) markers were selected based on their physical proximity to QTL markers reported to be associated with high stem sugar concentration for chromosomes 3, 5, 6 and 7 (Ritter et al., 2008; Murray et al., 2008a; 2009) (Table 2.1). Each marker was screened for polymorphism between the two parents. At the end, only ten of the 38 markers where polymorphic and used to genotype the entire brown midrib × sweet sorghum population (Table 2.2).

The PCR conditions for amplifying the polymorphic markers for the RIL population were carried out according to Xin et al. (2003) modified protocol (Appendix A). A total of 20µl of PCR product was obtain for each sample after these procedures. Only 4.2ul of the

amplification product was loaded in each well of a 3% percent high resolution agarose gel containing the nucleic acid stain, GelRed (Biotium, Inc.). The agarose gel was run for a minimum of 24 hours at 45 volts to allow proper separation of bands and reliable scoring. The visualization of amplification products was achieved by using a Bio-Rad UV camera. Bands corresponding to the allele from the sweet parent Brown County were checked for co-segregation the sweet phenotype (Brix ≥ 12) among the RILs.

2.3.4.2 QTL analysis, mapping, and COMT gene sequencing

Genotypic data from 10 polymorphic markers and phenotypic data from Brix measurements of 236 RILs were used to carry out single marker analysis.

QTL Cartographer 2.5 was used to carry out single marker analysis to identify regions in the genome associated to the sweet trait. Significant associations were determined and R^2 were reported as the amount of genetic variation that is explained by a specific molecular marker (Doerge 2002).

Table 2.1. List of SSR markers selected based on reported QTL associations to the sweet trait.

QTL - Reference	Chr	SSR associated to Brix	SSR position cM	R^2 (%)	Sequence of forward primer	Sequence of reverse primer	Size	Tm (°C)	Physical Position (Phytozome)	Selected SSR markers (Yonemaru et al., 2009)
Murray et al., 2008a & Murray et al., 2009	3	*CIR276*	140.4	25	CCCCAATCTAACTATTTGGT	GAGGCTGAGATGCTCTGT	228	52	55,567,937 - 55,567,956	*SB1979, SB1980, SB1983, SB1984, SB1986, SB1987*
		Xtxp31	143.4		TGCGAGGCTGCCCTACTAG	TGGACGTACCTATTGGTGC	222	62	55,224,665 - 55,224,683	
Ritter et al., 2008	5	*Xtxp65*	9.2	11	CACGTCGTCACCAACCAA	GTTAAACGAAAGGGAAATGGC	128	55	1,907,527 - 1,907,547	*SB3005, SB3008, SB3012, SB3015, SB3019, SB3022, SB3027, SB3031, SB3034, SB3038, SB3041, SB3047, SB3052*
Ritter et al., 2008	6	*Xtxp547*	15.4	23	GAGAGAGAGCGCGATGAGAC	ATCCATCGCAAACCGATAAA	190	60	42,754,922 - 42,754,941	*SB3504, SB3505, SB3506, SB3507, SB3508, SB3509, SB3510*

Table 2.2. Polymorphic markers used to genotype the brown midrib × sweet sorghum population.

Marker name	Chr	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	T_m	Product size (bp)	Start position (bp)	End position (bp)
*Xtpx31**	3	TGCGAGGCTGCCCTACTAG	TGGACGTACCTATTGGTGC	62	222	55224665	55224683
SB1986		AACTGACCTGCCACTTGAACGAG	CAACCCAACTCAGGCAGACACTC	65	244	55930737	55930980
SB3019	5	GCTTCGCCCTTAAATAAAACCTCG	ATTCTACCACCCCGGTCCTACTGT	60	216	527049	527264
SB3027		GTACGTACGGTGCTTCCATTCCAT	ACAAAGGCATGAGCTAGCAAGACC	60	171	860017	860187
SB3047		CCAAACAAAGAAACCCACATGTCA	AGACGACAGCTTTCCGTCAGAACT	60	259	1595068	1595326
SB3508	6	CACACTAGCCCCTTCCTAGCAGAA	TCCAATGATTCCGAACCAGGATAC	60	171	42782211	42782381
SB3509		GCAAGCAGCGTCTACTCGATTATGT	GTCCGATCCAACACATGTGCTAAC	60	252	42797751	42798002
*Xtxp295**	7	AAATCATGCATCCATGTTCGTCTTC	CTCCCGCTACAAGAGTACATTCATAGCTTA	57	165	61119146	61119168
SB4197		CGATCGAGTTTTTCTTGTGGTGTTC	CATGCATCCATGTTCGTCTTCTCT	65	251	61171882	61172132
SB4199		AGCGATTCCTTCAGGTGAGAACC	TCCCCTACACTGCACATGAAGCTA	65	239	61193324	61193562

*QTL-marker reported (Murray et al., 2008, Ritter et al., 2008)

Ten SSR primers were designed based on the genomic sequence of the sorghum *COMT* gene (obtained from Phytozome, locus name: *Sobic.007G047300*, Table 2.3). These PCR primers cover the entire *COMT* gene with a minimum overlap of 50bp between the pieces. The sequence of bmr12 for this gene was already published (Bout and Vermerris, 2003). The sequence for Brown County was unknown, but assumed to be similar to the reference genome since it codes for a functional enzyme. All reactions were performed in a PTC-200 thermocycler fitted with a gradient block and a heated lid. Each 20µl reaction contained 30ng of genomic DNA from either bmr12 and Brown County, 10µl of MyTaq Red 2× Mix DNA polymerase (Bioline), 0.1µl of 20% BSA, 1µl of 20% PVP, and 50ng of each of the primers (Appendix A). A three-step program was used, consisting of an initial denaturation for 2min at 94ºC, followed by 35 cycles of 10s denaturation at 94ºC, 20s annealing at 62ºC, and 1.5min elongation at 72ºC, and followed by a final extension step of 5min at 72ºC. PCR products were purified with a *QIAquick* PCR Purification kit. Finally, PCR products and primers were sent to Purdue Genomics Core Facility for high throughput sequencing by LTL Sanger Sequencing protocol from both ends. Overlap sequences were aligned to the reference genome.

Table 2.3. Overlapping primer designed to amplify Sobic07g003860 in sequencable pieces.

Primer name	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	Expected product size (bp)	
			WT	bmr12
A2	CTCTACGCACTTGACACTCACGCT	GAGCATGCGGTCCACCATGT	742	738
B2	TGCTGGAGGTGCTTCAGAAGGA	CAAGTGGTCCGTCCTTTGCTTAC	645	645
C	GATATGATGCTGGCGTGCTA	ACCCACTTCACACACACCAA	548	548
D2	CTGACGGCTCACATGGATCATG	CAAGGCCCATGTGTCTGAACTCTG	337	337
E2	GACCGGACAGTGACTTCAGAG	GGACTGTTACTGCTGCCATGGC	643	294
F2	GTCGGAATTGACGAGACGAATC	CAGCACTGATCGATCGACATGG	395	395
G	TCCGAAGTGCTCAAGCCTAT	CAGTCGTGGAGGATCCACTT	615	607
H	ACCTTACACGCCATCACCTC	CACCATGTATGGATCGGACA	684	684
I	AAGTGGATCCTCCACGACTG	TACTGGTACATGGCGCAGAG	622	622
J	TTGCTGCTGCTACTGCTGTC	TTAAGGCAATGGAGGAGAGG	508	508

2.3.5 Statistical analyses

2.3.5.1 Analysis of variance

Data on several variables collected over the two year study were subjected to statistical analyses. Analysis of variance was conducted using a mixed model, and source of variation were Year, RIL (Genotype), three orthogonal contrasts and Year × RIL interaction. The RILs of the population were treated as fixed effects, and Replications, Years and Year × RIL interactions were treated as random effects to determine differences in means and to generate corrected trait means (Least Squared Means). The population was grouped into four unbiased phenotypic groups, for a better comparison among RILs. The 236 RILs were grouped based on results of the genetic recombination of expressed through the two phenotypes of brown midrib (low lignin) and high Brix reading (sweet stalk) mutations they carried. The "normal" (non-brown; non-sweet) group was formed by 43 RILs without brown midribs or high stem sugar concentrations (Brix < 12). The "sweet" (non-brown; high stem sugar) group was formed by 108 RILs that carried a mutation for high stem sugar concentration (Brix ≥ 12), but did not have brown midribs. The "brown" (non-sweet; low lignin) group contained those RILs that had brown midribs but were not sweet (10 RILs). The fourth group named "brown-sweet" (recombinants of low-lignin and high stem sugar) were 75 RILs that carried both mutations, one for low lignin (brown midrib) and sweet, having a relatively high stem sugar concentration (Brix ≥ 12). We dubbed this group the double mutant group because of the two mutations its members carry. This grouping allowed us to obtain three orthogonal contrasts. The first linear combination compared the double mutant group ("brown-sweet") against the "normal" RIL group. The second linear

combination compared the double mutant group against the “sweet” group. The last linear combination compared the double mutant group against the “brown” group. Analysis of variance (ANOVA) was performed by using the PROC MIXED procedure from the SAS 9.3 statistical package. Restricted maximum likelihood (REML) with and without the GROUP statement, and the TYPE III test of fixed effect methods were used for a preliminary analysis of the seven traits evaluated in this study. The best method was selected based on Bayesian and Akaike’s information criterion (BIC and AIC), which measure the goodness of fit for each. Therefore, the methodology that showed the lowest BIC and AIC was chosen as the best, because it gives the correct balance between the fit to the data and model complexity. In our study, the TYPE III test of fixed effect was the best method to determine differences in means of RILs. Adjusted means were obtain with the command LSMean from SAS. The corrected trait means (Least Squares Means) were used for mean comparison within RILs and among RIL groups.

2.3.5.2 Phenotypic and genotypic correlation

Corrected trait means (Least Squared Means) generated after performing the analysis of variance were used to estimate possible correlation among biomass components and sugar related-traits. Phenotypic correlations (Pearson’s correlation) among traits were estimated by using the PROC CORR procedure from SAS 9.3.

Based on the great flexibility, the ability of handling unbalanced data as well as complex experimental designs, multivariate mixed-model analysis based on REML were used to estimate genetic correlations according Holland (2006) and Piepho and Mohring (2011). A

SAS code macro was adapted for our data analysis (Littell et al., 2006; Kumar, 2013). The complete code is shown in Appendix A.

2.3.5.3 Heritability estimates

The PROC MIXED procedure from SAS 9.3 statistical package was used for the estimation of variance components and heritability of the seven traits evaluated in this study. All variance parameters such as recombinant inbred lines (RIL), year (Y) and recombinant inbred lines × year (RIL × Y) were treated as random. There were no significant differences between the replications, therefore replications and the interactions between genotypes and replication were omitted during the whole analysis. The REML method was used to estimate variance components of each of the seven traits evaluated in this study. The COVTEST option from PROC MIXED procedure was specified to determine variance component significance. As reported by Gravois and Bernhardt (2000), Littell et al. (2006), and Yang (2002), the general model to estimate the variance components in a mixed model was defined as:

$$Trait_{ijk} = \mu + Y_i + RIL_j + RIL \times Y_{ij} + b_{k(i)} + e_{ijk}$$

Where $Trait_{ijk}$ was trait of the jth recombinant inbred line (RIL) in the kth replicate (b) within the ith year (Y), the μ was the overall mean and e_{ijk} was the residual error.

For this experimental design, broad-sense heritability for each trait was calculated as follows:

$$H = [\sigma^2_{RIL} / (\sigma^2_{RIL} + \sigma^2_{Y}/y + \sigma^2_{RIL \times Y}/y + \sigma^2_{b}/ry + \sigma^2_{e}/ry)]$$ (Littell et al., 2006)

Where "r" and "y" are replicates and years respectively.

2.3.5.4 Principal component analysis (PCA)

Selection for favorable biomass components and sugar-related traits of sorghum lines with high yield potential is the main objective of our breeding program. Many researchers (Rooney et al., 2007) believe that genetic improvement of biomass components and sugar-related traits must be done via genetic improvement of agronomic traits. In order to determine the potential of genetically different sorghum lines of the brown midrib × sweet sorghum population, it is necessary to observe many different characters that influence biomass yield and stem sugar concentration. In general, a series of univariate analyses carried out separately for each of the variables is not adequate as it ignores the correlation among variables. Principal component analysis (PCA) helps researchers to distinguish significant relationships between traits. This multivariate analysis method aims to explain the correlation between a large set of variables in terms of a small number of underlying independent factors. PCA of all phenotypic traits was performed for a graphic representation of phenotypic correlations. PROC PRINCOMP from SAS 9.3 statistical package was used to carry out PCA.

2.4 Results

2.4.1 Molecular analysis for stem sugar

After performing single marker analysis, our results showed three possible regions associated with Brix measurements in our brown midrib × sweet sorghum mapping population. Two of these genomic regions are located on chromosome 6 and one on chromosome 7. SSR markers *SB3508* and *SB3509* located on chromosome 6 explained 7% and 4% of the variation, respectively. SSR marker *SB4199* explained only 2% of the variation (Table 2.4).

Table 2.4. Single marker analysis of ten SSR markers in four genomic regions

Chr	Marker	b_0	b_1	R^2	
3	*Xtpx31*	14.2	-0.069	0.0	
	SB1986	14.2	0.041	0.0	
5	*SB3019*	14.2	0.118	0.0	
	SB3027	14.2	-0.144	0.0	
	SB3047	14.2	-0.186	1.0	
6	*SB3508*	14.1	-0.645	7.0	***
	SB3509	14.1	-0.547	4.0	**
7	*Xtxp295*	14.2	0.003	0.0	
	SB4197	14.2	0.039	0.0	
	SB4199	14.2	0.359	2.0	*

Significance at the 5%, 1%, and 0.1% levels are indicated by *, **, and ***, respectively.

2.4.2 Molecular analysis for *COMT* gene

The mutation responsible for the brown midrib phenotype in bmr12 was already known to be a C-T transition at position 745 in the first exon of *COMT* where it introduced a premature stop codon, thereby destroying the function of this critical enzyme in lignin biosynthesis. We amplified the *COMT* gene of Brown County using a combination of PCR primers designed against the sequence of this gene in the sorghum reference genome available on Phytozome. The alignment of bmr12 with the reference BTx623 sequence is shown in Figure 2.1. The sequence amplified from Brown County was identical to BTx623. The alignment shows the critical point mutation identified by Bout and Vermerris (2003) at position 745 that prematurely ends transcription thereby destroying the function of *COMT* and causing deficiency in lignin biosynthesis visible as brown midrib, highlighted in bright green in Figure 2.1. Other polymorphisms between bmr12 and the reference sequence in the annotated *COMT* gene are highlighted in yellow in Figure 2.1, none of which would be expected to destroy the function of the enzyme. What the authors who characterized this mutation did not mention was a gross size polymorphism between the reference genome and bmr12, a 348bp deletion in the intron of *COMT* with respect to the wild type BTx623. Brown County did not share this deletion, looking like the reference genome. Therefore, the primer pair “E2” which flanked the polymorphic region (marked in red in Figure 2.1) gave products by PCR that differed by 348bp between bmr12 and Brown County (Figure 2.2). This InDel marker cosegregated 100% with the brown midrib phenotype in our brown midrib × sweet sorghum population.

```
0001 TTAGCATGCA TATATAGGAG ATTAGCAGTA TAGCTTTTTC TTAGTGCCAT GCATCTTTCA TGCTACCTTT TTTCTTCCCA AAATTTCAAT CCATTGTTAA 0100 BTx623
                GenBank accession AY217766 "bmr12-ref" cat gcatctttca tgctaccttt tttcttccca aaatttcaat ccattgttaa 0053 bmr12

0101 ATAAAATGCA AAAAAAAAGA AAAGAAAAGA AAACAGTTAG TAATTAATTG ACTAATTGGT AAGCTAGTGC GTGATTTGGT GTGGTGGTTG GTGAGCTCTC 0200 BTx623
0054 ataaaatgca aaagaaaaga aaagaaaaaa aaacagttgg taactaattg actaattggt aagctagtgc gtgatttggt gtggtggttg gtgagctctc 0153 bmr12

0201 CGGCCCCATA TAACCCCCCT CCCTGCTCCT CCTTCCTCCT CGCAGCAGCA GCACACGCCA ACACTTGCCA AGCTCTCGCG TCGCTCAGCG CTAGCTCCTA 0300 BTx623
0154 cggccccata taacccc_t ccctgctcct ccttcctcct cgcagcagca gcacacgcca acacttgcca agctctcgcg tcgctcagc_ _tagctccta 0250 bmr12

                                                            MetGlySerT hrAlaGluAs pValAlaAla ValAlaAspG protein
0301 GCTAGTATCT TCTTCCACCG GGCACCGGCC GGCCAGCCGT CGTCAGCTAG CTAGCTAGCC ATGGGGTCGA CGGCGGAGGA CGTGGCGGCG GTGGCGGACG 0400 BTx623
0251 g____tatct tcttccaccg ggcaccagcc ggccagccgt cgtcagctag ctagctagcc atggggtcga cggcggagga cgtggcggcg gtggcggacg 0346 bmr12

     luGluAlaCy sMetTyrAla MetGlnLeuA laSerSerSe rIleLeuPro MetThrLeuL ysAsnAlaLe uGluLeuGly LeuLeuGluV alLeuGlnLy
0401 AGGAGGCGTG CATGTACGCG ATGCAGCTGG CGTCGTCGTC GATCCTCCCC ATGACGCTGA AGAACGCGCT GGAGCTGGGC CTGCTGGAGG TGCTTCAGAA 0500 BTx623
0347 aggaggcgtg catgtacgcg atgcagctgg cgtcgtcgtc gatcctcccc atgacgctga agaacgcgct ggagctgggc ctgctggagg tgcttcagaa 0446 bmr12

     sAspAlaGly LysAlaLeuA laAlaGluGl uValValAla ArgLeuProV alAlaProTh rAsnProAla AlaAlaAspM etValAspAr gMetLeuArg
0501 GGACGCCGGC AAGGCGCTGG CGGCGGAGGA GGTGGTGGCG CGGCTGCCCG TGGCGCCGAC GAACCCCGCC GCGGCGGACA TGGTGGACCG CATGCTCCGC 0600 BTx623
0447 ggacgccggc aaggcgctgg cggcggagga ggtggtggcg cggctgcccg tggcgccgac gaaccccgcc gcggcggaca tggtggaccg catgctccgc 0546 bmr12

                                Arg
     LeuLeuAlaS erTyrAspVa lValLysCys GlnMetGluA spLysAspGl yLysTyrGlu ArgArgTyrS erAlaAlaPr oValGlyLys TrpLeuThrP
0601 CTCCTCGCCT CCTACGACGT CGTGAAGTGC CAGATGGAGG ACAAGGACGG CAAGTACGAG CGTCGGTACT CCGCCGCCCC CGTCGGCAAG TGGCTCACCC 0700 BTx623
0547 ctcctcgcct cctacgacgt cgtgaggtgc cagatggagg acaaggacgg caagtacgag cgtcggtact ccgccgcccc cgtcggcaag tggctcaccc 0646 bmr12

                                                   ***
     roAsnGluAs pGlyValSer MetAlaAlaL euAlaLeuMe tAsnGlnAsp LysValLeuM etGluSerTr
0701 CTAACGAGGA CGGCGTCTCC ATGGCCGCCC TCGCGCTCAT GAACCAGGAC AAGGTCCTCA TGGAGAGCTG GTGAGTAGTC GTCGTCAGAG CACATCTCGC 0800 BTx623
0647 ctaacgagga cggcgtctcc atggccgccc tcgcgctcat gaactaggac aaggtcctca tggagagctg gtgagtagtc gtcgtcagag cacatctcgc 0746 bmr12

0801 CCCACCTCAC CATTTCATCT GTAGATCAGT TGTTGCTTTG CTGTTGATAT GATGCTGGCG TGCTAGCTGC ATGATGATGA GCTCGCTCAT CATTAGTACT 0900 BTx623
0747 cccacctcac catttcatct gtagatcagt tgttgctttg ctgttgatat gatgctggcg tgctagctgc atgatgatga gctcgctcat cattagtact 0846 bmr12

0901 AGCTAGTGAT TTATTTTGTC ATTTAATTTT TTCCAAGTAA AATTGATTGA GGTGCACTAC TAGTACTAGC TGCTAGTACA AAGCTGGCAG TAGTTAAGTT 1000 BTx623
0847 agctagtgat ttattttgtc atttaatttt ttccaagtaa aattgattga ggtgcactac tagtactagc tgctagtaca aagctggcag tagttaagtt 0946 bmr12

1001 ATCCATGATA TAATATTTGA CTAAAACAAA AAAAATATTT _TTTTACAAAA AAAGGGAAGT AAGCTCAAGT TCTTCCTAAA AAAATGTAGA GTAGGATGGA 1100 BTx623
0947 atccatgata taatatttga ctaaaacaaa aaaaatattt ttttacaaaa aaagggaagt aagctcaagt tcttcctaaa aaaatgtaga gtaggatgga 1047 bmr12

1101 AAAGTAAGCA AAGGACGGAC CACTTGTCAT CTCCACTATC CAGTGGGCGA GACTTCGGCG AACCTTGGAG AAGGAGAGCA TTATTGGCCA ACTCTCTCTC 1200 BTx623
1048 aaagtaagca aaggacggac cacttgtcat ctccactatc cagtgggcga gacttcggcg aaccttggag aaggagagca ttattggcca actctctctc 1147 bmr12

1201 TAATTTTTTT TTCCTGGATT CGCAAAACTG GAGCCGTCGA TCGCCGGACT TATTACTGAC GGCTCACATG GATCATGGAA TTCTGCGAAA TTCCTGATCT 1300 BTx623
1148 taattttttt ttcctggatt cgcaaaactg gagccgtcga tcgccggact tattactgac ggctcacatg gatcatggaa ttctgcgaaa ttcctgatct 1247 bmr12

1301 AGACTTTTGC GAAACTCCGT TCAGTCATTC ACCAACTGAT GGTGAATCTT CAGACTCTCA AATTGTTTGG TGTTTGGTGT GTGTGAAGTG GGTGTAGAAA 1400 BTx623
1248 agacttttgc gaaactccgt tcagtcattc accaactgat ggtgaatctt cagactctca aattgtttgg tgtttggtgt gtgtgaagtg ggtgtagaaa 1347 bmr12

1401 AGAGGCAGTT GGACCACAGG CGACTGACTG ACCCATTACC ATGTCACTGA TGCTGATAGA TTCTTGCCCT GTTCCTTTTA GAAACTTTTG CACAGATCGA 1500 BTx623
1348 agaggcagtt ggaccacagg cgactgactg acccattacc atgtcactga tgctgataga ttcttgccct gttcctttta gaaacttttg cacagatcga 1447 bmr12

                                                           Sb07g003860_E2-F
                                                     5'-GACCGGAC AGTGACTTCA GAG->3
1501 TATCTGTAGC AGTTTTCCTT TCATGCAATT TTTGACTAGT TTAAAATGTT CAGACCGGAC AGTGACTTCA GAGTTCAGAC ACATGGGCCT TGTTTAGTTA 1600 BTx623
1448 tatctgtagc agttttcctt tcatgcaatt tttgactagt ttaaaatgtt cagaccggac agtgacttca gagttcagac acatgggcct tgtttagtta 1547 bmr12

1601 GGCCCTGTTT AGTTCCCCAC AAAAAAAAAT TTCATCCATC CCATCGAATC TTTGAACACA TGCATGGAAC ATTAAATGTA AATAAAAAAT AAACTAATTA 1700 BTx623
     __________ __________ __________ __________ __________ __________ __________ __________ __________ __________      bmr12

1701 CACAGTTTGG TTGAAAATCG CGAGACGAAT CTTTTAAGCC TAGTTAGTCC ATGATTAGCC TTAAGTGCTA CAGTAACCTA CATGTGCTAA TGACAGATTA 1800 BTx623
     __________ __________ __________ __________ __________ __________ __________ __________ __________ __________      bmr12

1801 ATTATAGTTA ATAGATTTGT CTTGCAGTTT CCTGATGAGC TATGTAATTT GTTTTTTTAT TAGTTTTTAA AAACCCCTCC CGACATCATT CTGACATATC 1900 BTx623
     __________ __________ __________ __________ __________ __________ __________ __________ __________ __________      bmr12

1901 CGATGTGACA TCCAAAAATT TTTCATTCAC AATCTAAACA GATCCTTACC AAAAAAATTT TGCAAAATCT TTCAGATTCT CCGTCACATC AAATCTTTAG 2000 BTx623
1548 __________ __________ __________ __________ ________cc aaaaaa_ttt tgcaaaatct ttcagattct ccgtcacatc aaatctttag 1598 bmr12
```

Figure 2.1. Sequence alignment of *COMT* of brown midrib mutant bmr12 with the reference genome, BTx623. Mutations highlighted in yellow are not predicted to cause loss of function of *COMT*. The causal mutation is a premature stop codon resulting from a C-T transition (highlighted bright green). Primer pair used to amplify E2 InDel marker is indicated in red.

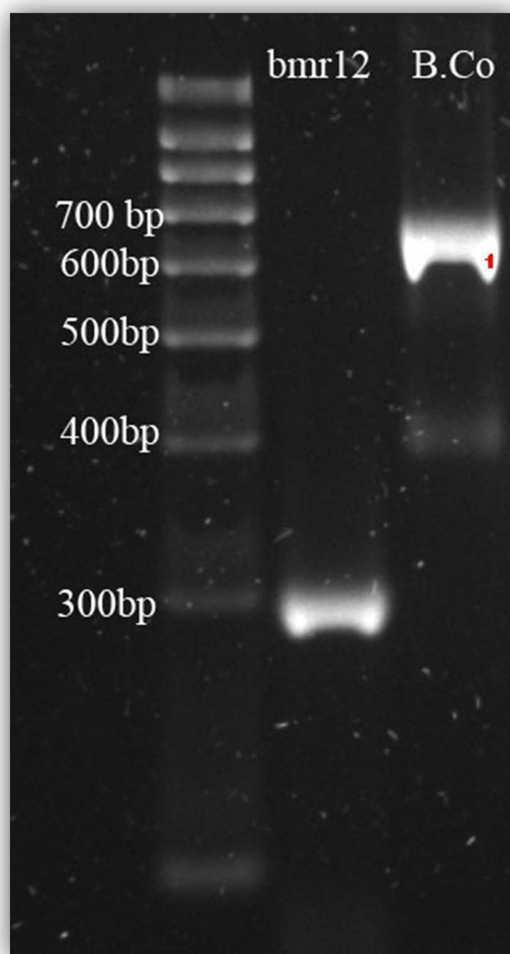


Figure 2.2. Gel image of InDel marker “E2” distinguishing *COMT* allele from bmr12 and Brown County.

2.4.3 Evidence for improving biomass quantity and quality through recombination with brown midrib and sweet mutations

Table 2.5 presents the analysis of variance (ANOVA) of combined years for seven traits evaluated in this study, plant height (cm), plant maturity (days), stem thickness (cm), dry grain yield (t/ha), dry stover yield (t/ha), dry total biomass yield (t/ha), and stem sugar concentration (Brix). Year showed significant difference for all traits except dry total biomass yield and stem sugar concentration. RIL (Genotype) showed very high significant differences for all seven traits. Year effects were highly significant for maturity, significant for grain yield, stover yield and plant height but were not significant for total biomass, stem thickness and stem sugar concentration. The orthogonal contrast between the recombinant (“brown-sweet”) and “normal” were significant for only two traits, stover yield and stem sugar concentration. When compared to the sweet types, the recombinant showed very high significant differences only for stem thickness. The contrast between recombinant and brown types showed highly significant differences in plant height and stem thickness; significant differences in stover yield, total biomass, and stem sugar concentration; and no significant difference was shown for plant maturity and grain yield. The Year × RIL interaction were significant for all traits except for stover yield. Differences were significant for grain yield and total biomass; and highly significant for plant height, plant maturity, stem thickness, and stem sugar concentration.

Table 2.5. Combined Years ANOVA of biomass components and sugar-related traits analyzed by TYPE III test of fixed effects mixed model

		Mean Square													
Source of variation	df	PH		PM		ST		DGY		DSY		DTBY		SSC	
Year	1	66077	*	22632.0	***	0.14		2124.7	*	185.3	*	1.29		0.6	
RIL	235	4990	***	63.2	***	0.10	***	21.0	***	1.7	***	0.04	***	25.9	***
brown-sweet vs normal	1	79588		2305.2		0.06		121.7		19.5	*	0.16		2648.6	*
brown-sweet vs sweet	1	1045		1334.3		0.54	***	274.6		8.8		0.33		116.8	
brown-sweet vs brown	1	91695	**	348.5		0.28	***	0.8		17.7	*	0.47	*	722.8	*
Year×RIL	235	386	***	19.1	***	0.05	***	8.4	*	0.5		0.01	*	6.7	***
Error	469	267		10.7		0.04		6.7		0.4		0.01		4.2	

PH=plant height (cm), PM=plant maturity (days), ST=stem thickness (cm), DGY=Dry Grain Yield (t/ha), DSY=dry stover yield (t/ha), DTBY=dry total biomass yield (t/ha), SSC=stem sugar concentration (Brix). *significant at the 0.05 probability level, ** significant at the 0.01 probability level, *** significant at the 0.001 probability level.

Results of mean analysis of biomass components and sugar-related traits for all RILs evaluated over two years are presented in Table 2.6. This table presents results in each year and combined over the two years means of the RIL population and the commercial check, bmrAtlas. Overall, RIL dry grain yield (t/ha) was 7 and 10 t/ha in 2008 and 2009 respectively, 9 t/ha in combined years. In contrast, the commercial check, bmrAtlas, produced 7 and 9 t/ha of grain in 2008 and 2009, respectively, and 8 t/ha in combined years. RILs showed a maximum grain yield of 16.8 and 20.4 t/ha in 2008 and 2009, respectively; and a combined year maximum grain yield of 17.9 t/ha. This is double that of the check. Based on the ANOVA, the variation in dry grain yield mean performance within the RILs is mainly attributable to genotype effects. However, some environmental and genotype×environment interaction effects could also have influenced this trait's mean performance (Table 2.5).

Dry stover yield (t/ha) performance showed significant differences between RILs and bmrAtlas within each year. In 2008, RIL dry stover yield reached an average amount of 32 t/ha, while bmrAtlas, produced only 22 t/ha. The opposite happened for 2009; the check, bmrAtlas, obtained a higher dry stover yield of 30 t/ha and the average of the RILs was only 23 t/ha of dry stover in 2009. Maximum dry stover yields of 80.8 in 2008, 55.1 in 2009 and 48.6 t/ha combined over years was recorded for all RILs. This is roughly double that of the check. Based on the ANOVA, variation in stover yield mean performance within RILs was mainly due to genotype effects. However, some of the observed variation in this trait could be also attributed to environmental effects during growing season (Table 2.5).

Total biomass yield performance had a similar variation pattern to stover yield, within years and combined years for RILs and bmrAtlas. RIL maximum dry total biomass yield was at least twice that of bmrAtlas within each year and combined over years. As shown in the ANOVA, the observed variation in total biomass mean performance within RILs was mainly due to genotypic effects (Table 2.5).

Other traits related to biomass performance are also presented in Table 2.6. On average, bmrAtlas produced taller plants than most RILs, its average plant height of 240 in 2008 and 235 in 2009 was higher than the mean height of RILs. This was also observed in combined year performance, where bmrAtlas reached 238 cm and the average of the RILs reached only 218 cm in plant height. However, the RILs had maximums of 315 and 285 cm for plant height for 2008 and 2009, respectively and 297.5 cm in combined years. Once again, it was observed that The RILs showed a wide range for plant height. Some RILs were significantly taller than the control contributing to increased biomass production. The variation observed within RILs for this trait was mainly due to genotype effects; however, some genotype×environment interactions could also have influenced plant height mean performance within the RILs (Table 2.5).

The average stem thickness (ST) between RILs and bmrAtlas was similar within years and combined years. However, there was great variation in stem thickness among the RILs, with maximum thick stem of 2.0 and 2.3 cm recorded for 2008 and 2009, respectively, and 1.9 cm in combined years. Stem thickness variation was mainly attributed to genotype effect and genotype×environment interactions (Table 2.5).

RILs showed high variation for flowering days with a minimum of 42 days in 2008 and a maximum of 86 days in 2009. The mean plant maturity for RILs was similar with bmrAtlas, however, the variation observed in plant maturity for RILs was due to genotype, environment and genotype×environment effects (Table 2.5).

RILs stem sugar concentration (SSC) measurements in ºBrix were pretty similar to the bmrAtlas check, i.e., around 14. However, some of the RILs could reach higher stem sugar concentration measurements of 19.5 ºBrix within years and 18.6 ºBrix in combined years. The variation in stem sugar concentration RILs was mainly caused by genotype and genotype×environment effects (Table 2.5).

Table 2.6. Mean analysis for biomass components and sugar related traits evaluated over two years.

	ACRE 2008					ACRE 2009					ACRE Combined Years				
	Control	RILs				Control	RILs				Control	RILs			
Trait	bmrAtlas	Mean	SD	Min.	Max.	bmrAtlas	Mean	SD	Min.	Max.	bmrAtlas	Mean	SD	Min.	Max.
Plant height (cm)	240.0	226.7	±35.8	122.5	315.0	235.0	209.9	±34.1	110.0	285.0	237.5	218.0	±35.3	126.3	297.5
Plant maturity (days)	75.0	72.0	±3.8	42.0	85.0	85.0	82.0	±4.6	73.0	86.0	80.0	77.0	±4.0	62.3	85.3
Stem thickness (cm)	1.2	1.4	±0.2	0.9	2.0	1.5	1.5	±0.2	1.0	2.3	1.3	1.4	±0.2	0.9	1.9
Grain yield (t ha^{-1})	7.5	7.5	±2.0	2.2	16.8	9.4	10.5	±2.5	4.0	20.4	8.4	9.0	±2.3	3.9	17.9
Stover yield (t ha^{-1})	22.3	31.9	±6.8	13.7	80.8	30.3	22.7	±5.8	10.8	55.1	26.1	27.1	±6.3	13.2	48.6
Total biomass (t ha^{-1})	28.6	39.3	±8.1	16.3	67.7	38.6	33.3	±7.2	16.8	64.4	33.1	35.2	±7.8	19.7	57.5
Stem sugar concentration (°Brix)	12.9	14.3	±2.3	0.0	19.0	12.7	14.3	±2.3	0.0	19.9	13.9	14.3	±2.3	0.0	18.6

Based on Figure 2.3 and Table 2.5, the double mutant RILs ("brown-sweet") were significantly taller as a group than the "normal" and "brown" RILs. Additionally, no significant differences were observed between the "brown-sweet" and "sweet" RIL groups. Similarly to stover yield, it seems that the introduction of the sweet mutation could enhance not only stover yield but also plant height of sorghum as is evident in this RIL population.

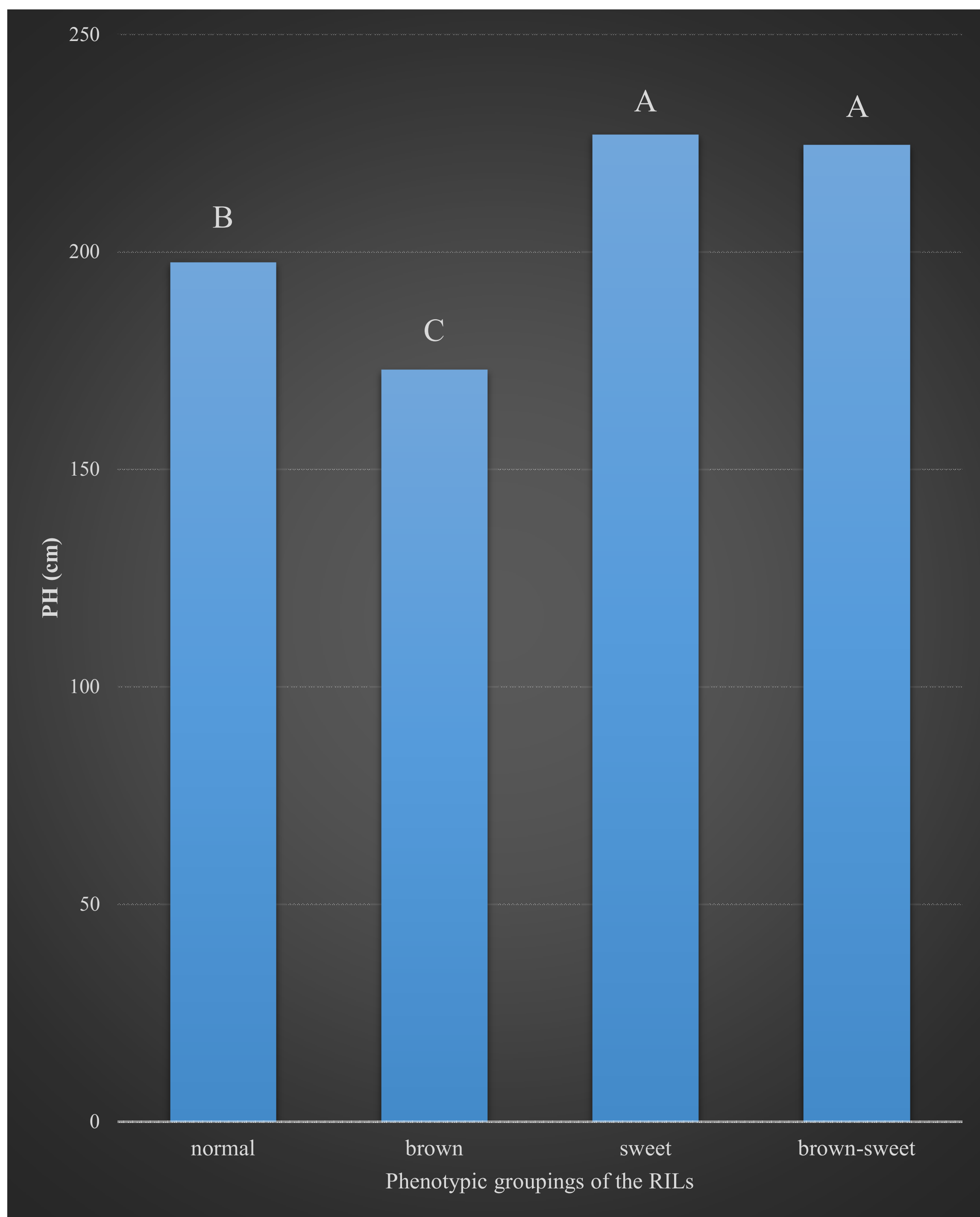


Figure 2.3. Mean plant height (PH) among four different RIL phenotypic classes. LSD (P<.05).

Based on results presented in Figure 2.4 and Table 2.5, the "sweet" RILs group had significantly thicker stems than the "brown-sweet" RILs group. Further, the "brown-sweet" RILs group generally had thicker stems than those RILs in the "brown" group. Since the low lignin mutation (present in the "brown" RILs group) is associated with a high percentage of lodged plants (data not shown) in the measured plots, perhaps, the problem of lodging could be mitigated by combining the brown midrib trait with the sweet mutation as evident by the relatively thicker stems among the "brown-sweet" RILs.

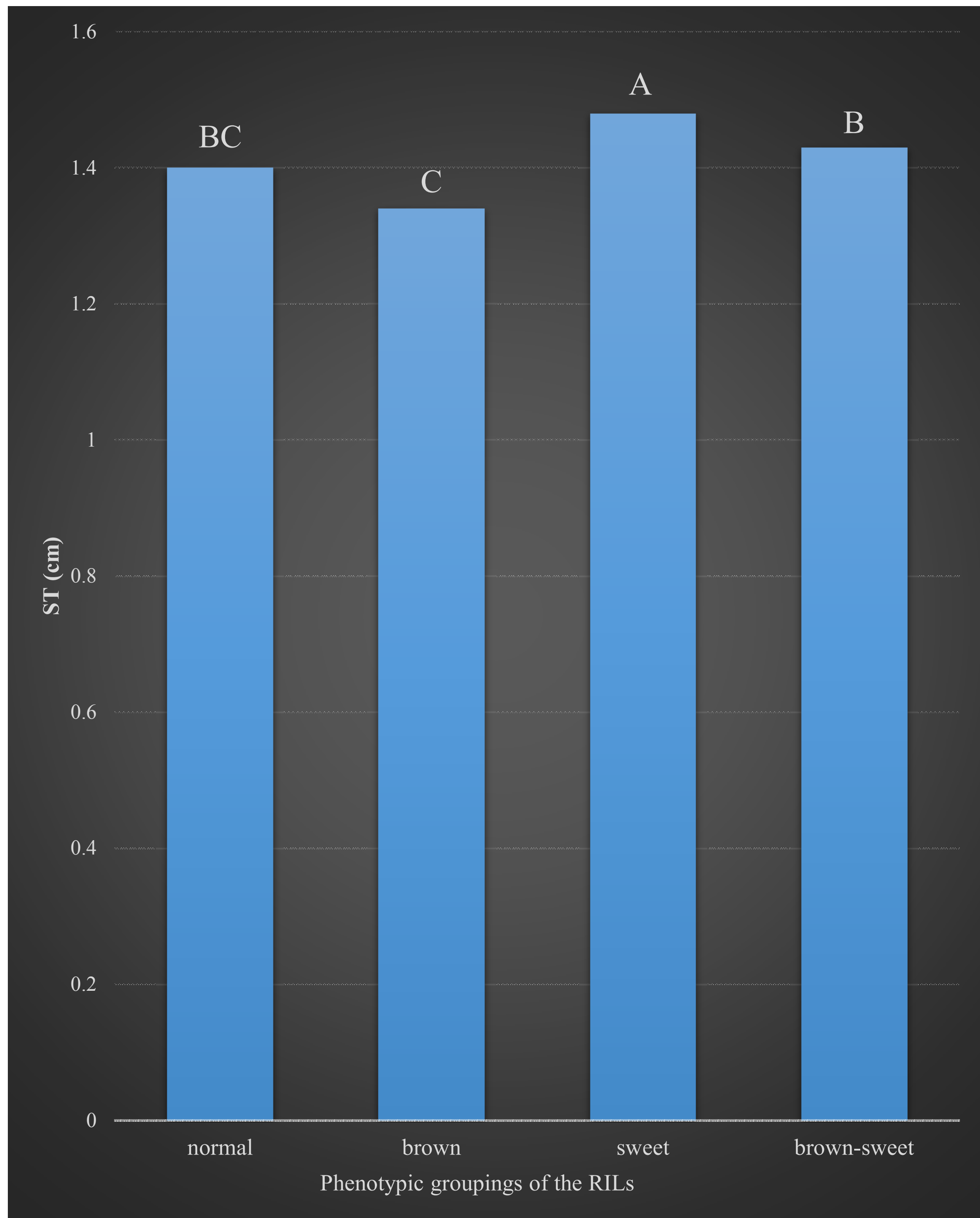


Figure 2.4. Mean stem thickness (ST) among four different RIL phenotypic classes. LSD (P<.05).

Dry grain yield mean performance (t/ha) among the four different RIL phenotypic classes were not significantly different (Figure 2.5 and Table 2.5). These results suggest that there are no grain yield penalties associated with low lignin content (brown midrib) and sweet stems in this brown midrib × sweet sorghum population.

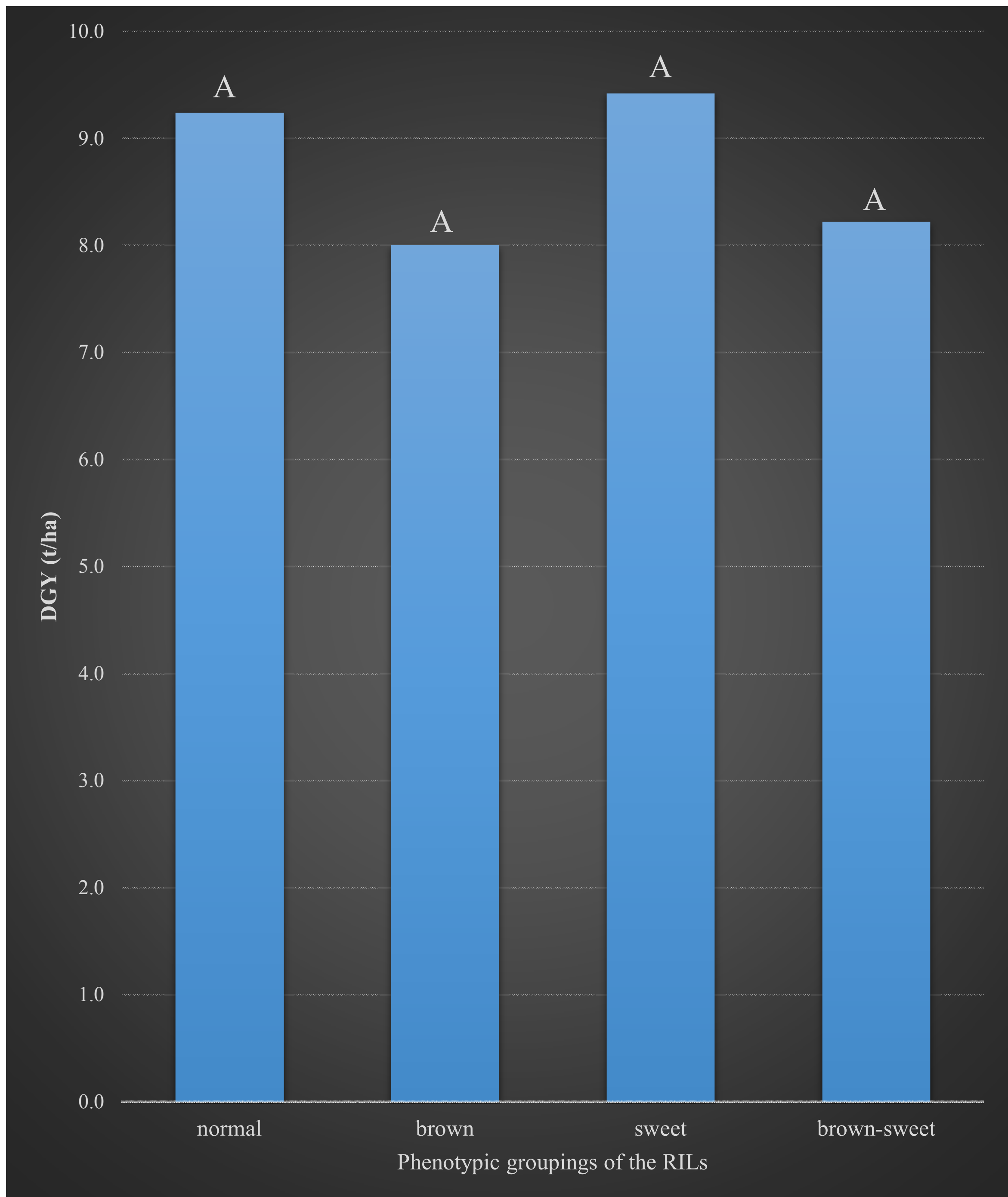


Figure 2.5.Mean dry grain yield (DGY) among four different RIL phenotypic classes. LSD (P<.05).

In contrast, dry stover yield mean performance (t/ha) among the four different RIL phenotypic classes (Figure 2.6 and Table 2.5), does vary. Significant differences were observed between the single mutant "sweet" group and the single mutant "brown" group, and between the double mutant "brown sweet" group and the single mutant "brown" group. The "sweet" and "brown-sweet" RILs, on average, actually obtained higher estimated stover yield than the "brown" RILs (24.7, 23 and 14.88 t/ha, respectively). This result suggests the absence of a possible trade-off affecting stover yield performance when low lignin and the stem sugar mutations are combined in sorghum inbred lines, perhaps even compensating for any penalty in terms of stover yield associated with the brown midrib mutation. It seems that the introduction of the stem sugar mutation could enhance dry total stover yield performance. This is because traits such as plant height and stems thickness associated to stem sugar mutation were introduced simultaneously in "brown-sweet" RILs.

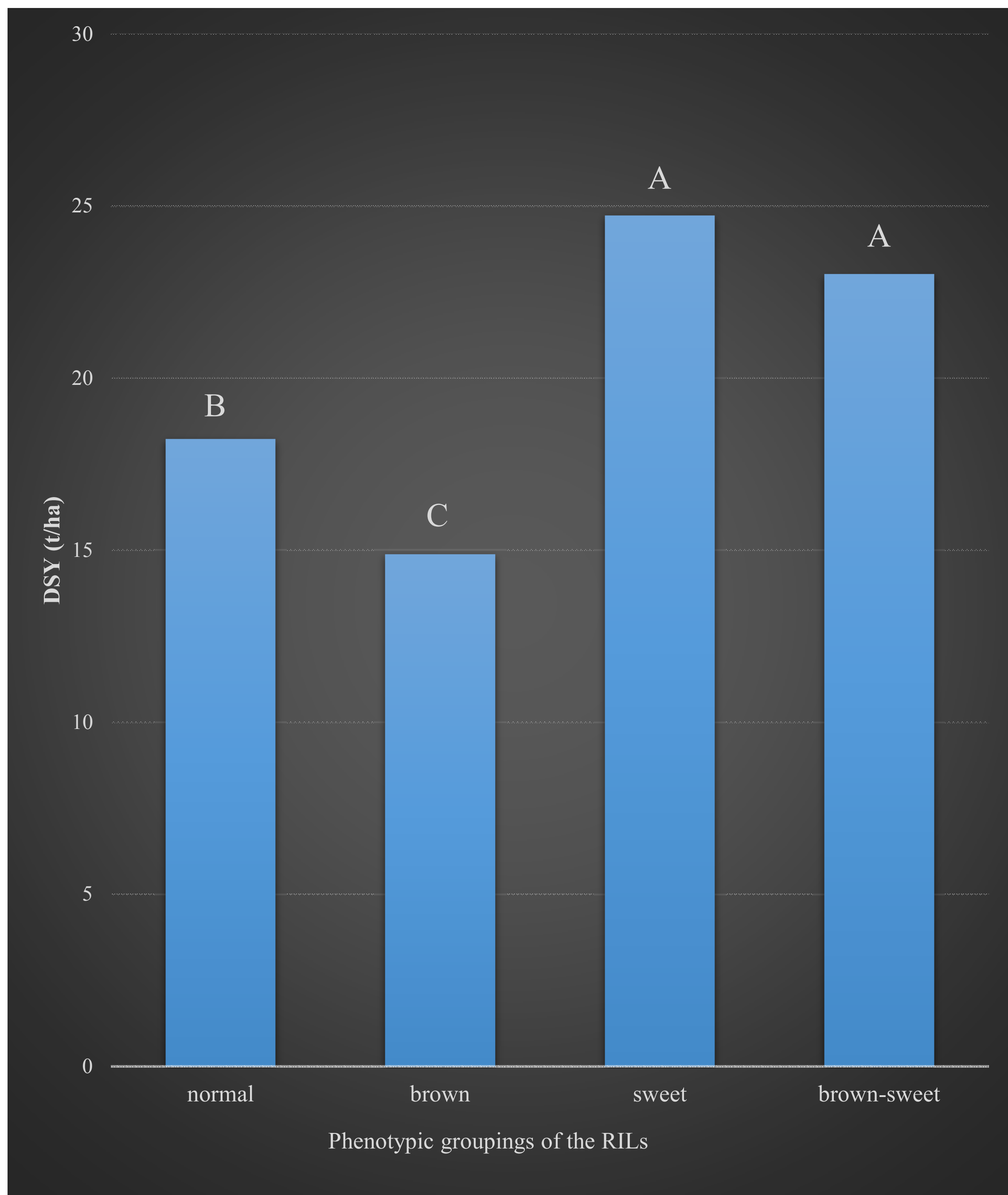


Figure 2.6. Mean dry stover yield (DSY) among four different RIL phenotypic classes. LSD (P<.05).

Figure 2.7 shows dry total biomass yield mean performance (t/ha) among four different RIL phenotypic classes. All groups showed a similar performance for total biomass yield (t/ha), except the “brown” RILs group. This result is consistent with the comparisons made in Table 2.5, where only significant differences were observed for the linear combination brown sweet vs brown. These results suggest possible trade-offs due to the low lignin mutation affecting total biomass yield performance in the “brown” RILs group. However, when both mutations are combined, total biomass yield of the brown sweet RILs group increased. This is an indication of gains in total biomass yield when both mutations are present.

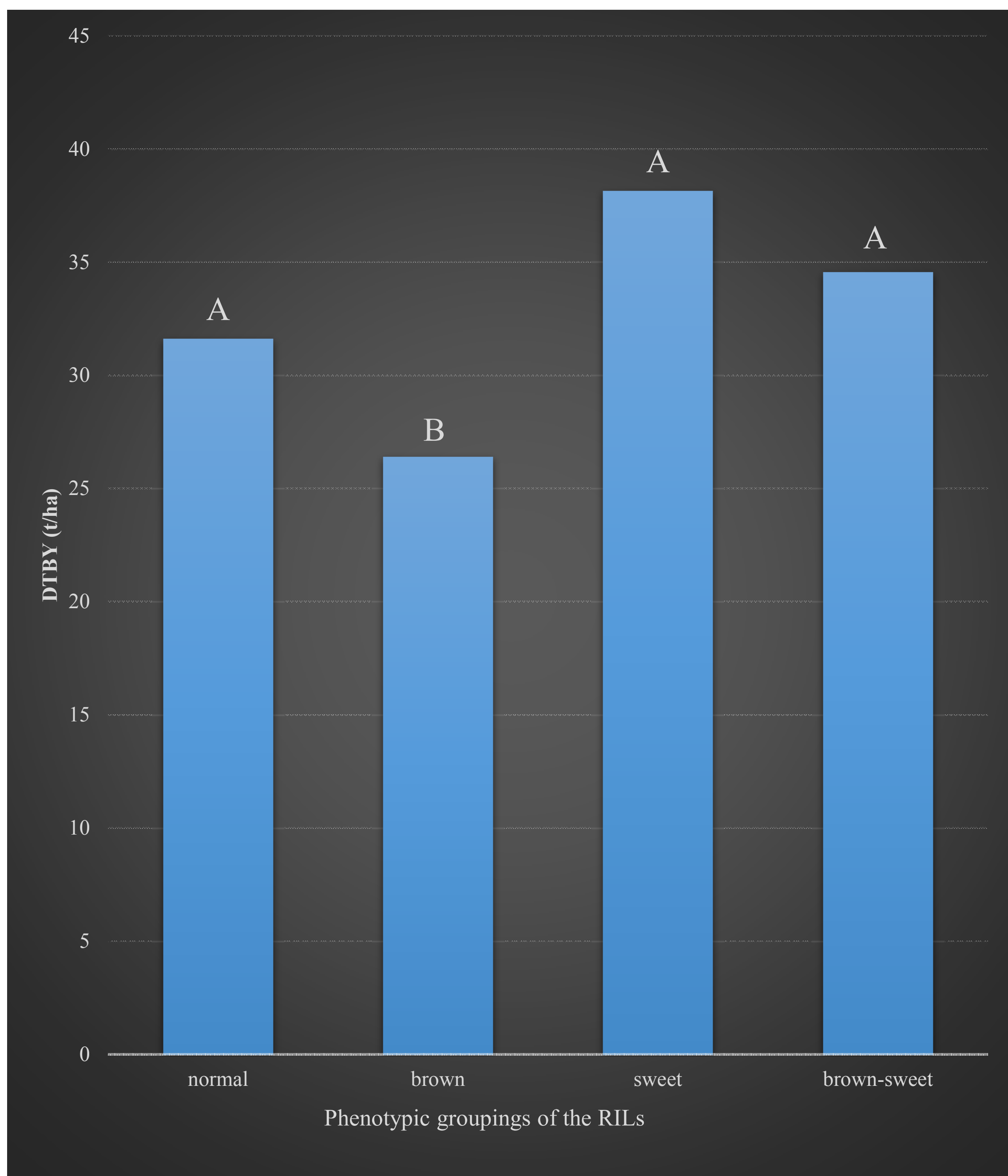


Figure 2.7. Mean dry total biomass yield (DTBY) among four different RIL phenotypic classes. LSD (P<.05).

Not surprisingly, Figure 2.8, showed that the “brown-sweet” RILs group and the “sweet” RILs group showed on average the highest stem sugar concentrations. And, significant differences between these two groups were not observed for this trait (Table 2.5). The stem sugar concentration of the “brown-sweet” RILs group was significantly higher than the “normal” RILs group and the “brown” RILs group. This is evidence of the positive gains of soluble sugar concentration by “brown-sweet” recombinants.

So it seems that in these inbreds, soley by the virtue of having inherited the presumed biomass quality boosters of brown midrib and sweet stalks, when taken as a group, the combination is generally a favorable one resulting in bigger, taller plants with thicker stalks that contain more sugar, and no price paid in loss of grain yield.

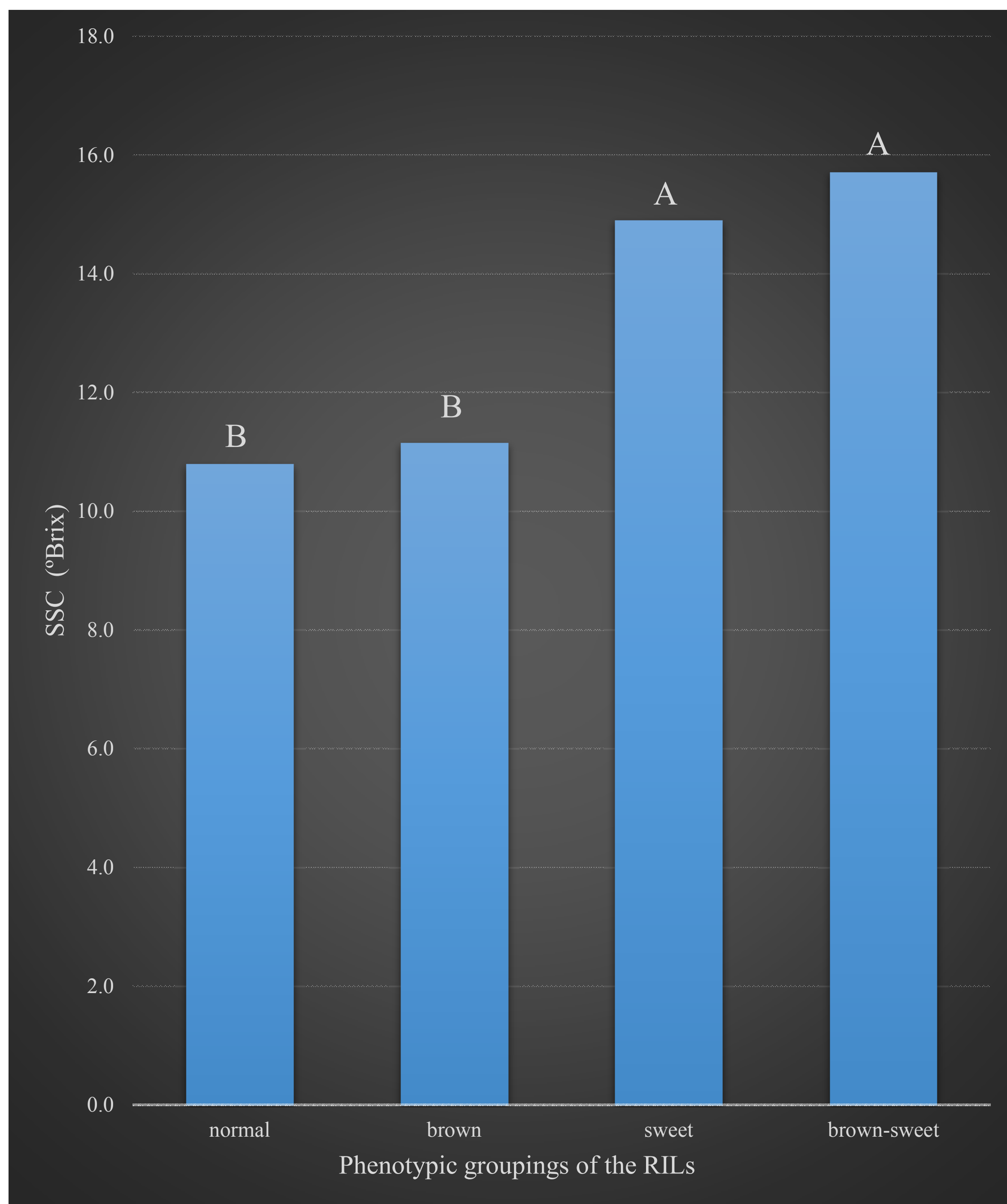


Figure 2.8. Mean stem sugar concentration (SSC) among four different RIL phenotypic classes. LSD (P<.05).

2.4.4 Correlations among traits

High, moderate and low phenotypic correlations were observed among traits (Table 2.7). Most traits showed low but significant correlations (below 40%). The highest significant correlations were observed between stover yield and total biomass yield (r_P=0.95), stover yield and plant height (r_P =0.75), and total biomass yield and plant height (r_P=0.72). The lowest significant correlation was between grain yield and plant maturity (r_P=0.13). No significant correlation were observed for stem thickness and stem sugar concentration (r_P =0.08). A negative but non-significant correlation was observed between grain yield and stem sugar concentration (r_P=-0.10).

Table 2.7. Phenotypic correlation of coefficient (r_P) of biomass components and sugar related traits.

	DSY	**DTBY**	**PH**	**ST**	**PM**	**SSC**
DGY	0.38***	0.63***	0.25***	0.24***	0.13*	-0.10
DSY		0.95***	0.75***	0.46***	0.53***	0.34***
DTBY			0.72***	0.46***	0.48***	0.27***
PH				0.30***	0.42***	0.37***
ST					0.34***	0.08
PM						0.30***

DGY=Dry Grain Yield (t/ha), DSY=Dry Stover Yield (t/ha), DTBY=Dry Total Biomass Yield (t/ha), PH=Plant Height (cm), PM=Plant Maturity (days), SSC=Stem Sugar Concentration (ºBrix). *significant at the 0.05 probability level, *** significant at the 0.001 probability level.

Table 2.8 presents genetic correlation coefficients of biomass components and sugar-related traits. High positive correlations were observed between stover yield and total biomass yield (r_G=0.97), stover yield and plant height (r_G=0.89), total biomass yield and plant height (r_G=0.87), total biomass yield and stem thickness (r_G=0.78), stover yield and stem thickness (r_G=0.77), stover yield and plant maturity (r_G=0.69), and total biomass yield and plant maturity (r_G=0.62). Moderate correlations were observed between grain yield and total biomass yield (r_G=0.56), plant height and plant maturity (r_G=0.47), stover yield and stem sugar concentration (r_G=0.45), plant height and stem sugar concentration (r_G =0.44), and grain yield and stem thickness (r_G=0.43). Low correlations were observed between total biomass yield and stem sugar concentration (r_G =0.37), grain yield and stover yield (r_G=0.33), plant maturity and stem sugar concentration (r_G=0.33), grain yield and plant height (r_G=0.31), stem thickness and stem sugar concentration (r_G=0.15), and grain yield and plant maturity (r_G=0.11). Finally, significant negative correlation was observed only between grain yield and stem sugar concentration (r_G=-0.16). This is consistent with previous reports that stem sugar concentration was negatively correlated with sink organ related traits like grain yield (Ritter et al., 2008).

Table 2.8. Genotypic correlation coefficient (r_G) of biomass components and sugar related traits.

	DSY	DTBY	PH	ST	PM	SSC
DGY	0.33^{++}	0.56^{++}	0.31^{++}	0.43^{++}	0.11^{+}	-0.16^{+}
DSY		0.97^{++}	0.89^{++}	0.77^{++}	0.69^{++}	0.45^{++}
DTBY			0.87^{++}	0.78^{++}	0.62^{++}	0.37^{++}
PH				0.42	0.47^{++}	0.44^{++}
ST					0.57	0.15^{+}
PM						0.33^{++}

DGY=Dry Grain Yield (t/ha), DSY=Dry Stover Yield (t/ha), DTBY=Dry Total Biomass Yield (t/ha), PH=Plant Height (cm), PM=Plant Maturity (days), SSC=Stem Sugar Concentration (Brixº). +, ++ Estimate exceeds its standard error once or twice, respectively.

2.4.5 Estimation of components of variance and heritability.

Estimates of variance components analyzed by restricted maximum likelihood (REML, Table 2.9) indicated that year was not a significant source of variation for the traits studied except for plant maturity (days), dry stover yield (t/ha) and dry grain yield (t/ha). Year effect accounted for 65% of the total variation for plant maturity, 34% of the total variation for dry stover yield and 29% of the total variation for dry grain yield. The year effect was zero for stem thickness and stem sugar concentration.

The genetic effect for all traits was high, accounting for 71% of the total variation for plant height, 47% for stem sugar concentration, 33% for dry total biomass yield, 25% for dry stover yield, 21% for dry grain yield and stem thickness, and 15% for plant maturity. The G × Y effect contributed a significant variation of 12% of the total variation for stem sugar concentration and stem thickness.

Results of broad sense heritability are presented in Table 2.9. Heritability was high for plant height (87%) and stem sugar concentration (74%). This suggest that improvement for this two traits can be undertaken readily, as they are highly heritable. It also confirms that year and year-genotype interaction contributions were smaller than that of genetic contribution in these traits. Moderate heritability estimates were obtained for dry stover yield (57%), dry total biomass yield (60%), stem thickness (48%), dry grain yield (42%) and plant maturity (40%). Most of the traits in this study had similar heritability estimates to those reported by others (Brown et al. 2006; Murray et al. 2008; Ritter et al. 2007). The heritability estimate for plant maturity in our study, however, was relatively lower than in their studies. The lower plant maturity heritability could be due to differences in

temperature and precipitation between 2008 and 2009. Quinby and Karper (1945) reported that the major flowering gene in sorghum is regulated by photoperiod while minor genes are influenced by temperature and precipitation. Thus, breeding for plant maturity (PM) could be challenged when seasonal fluctuation in temperature and precipitation affect crop maturity.

Genetic components and heritability estimates for traits contributing biomass production generally range from moderate to large in recombinant inbred lines populations. Thus, we can successfully breed and select for biomass traits.

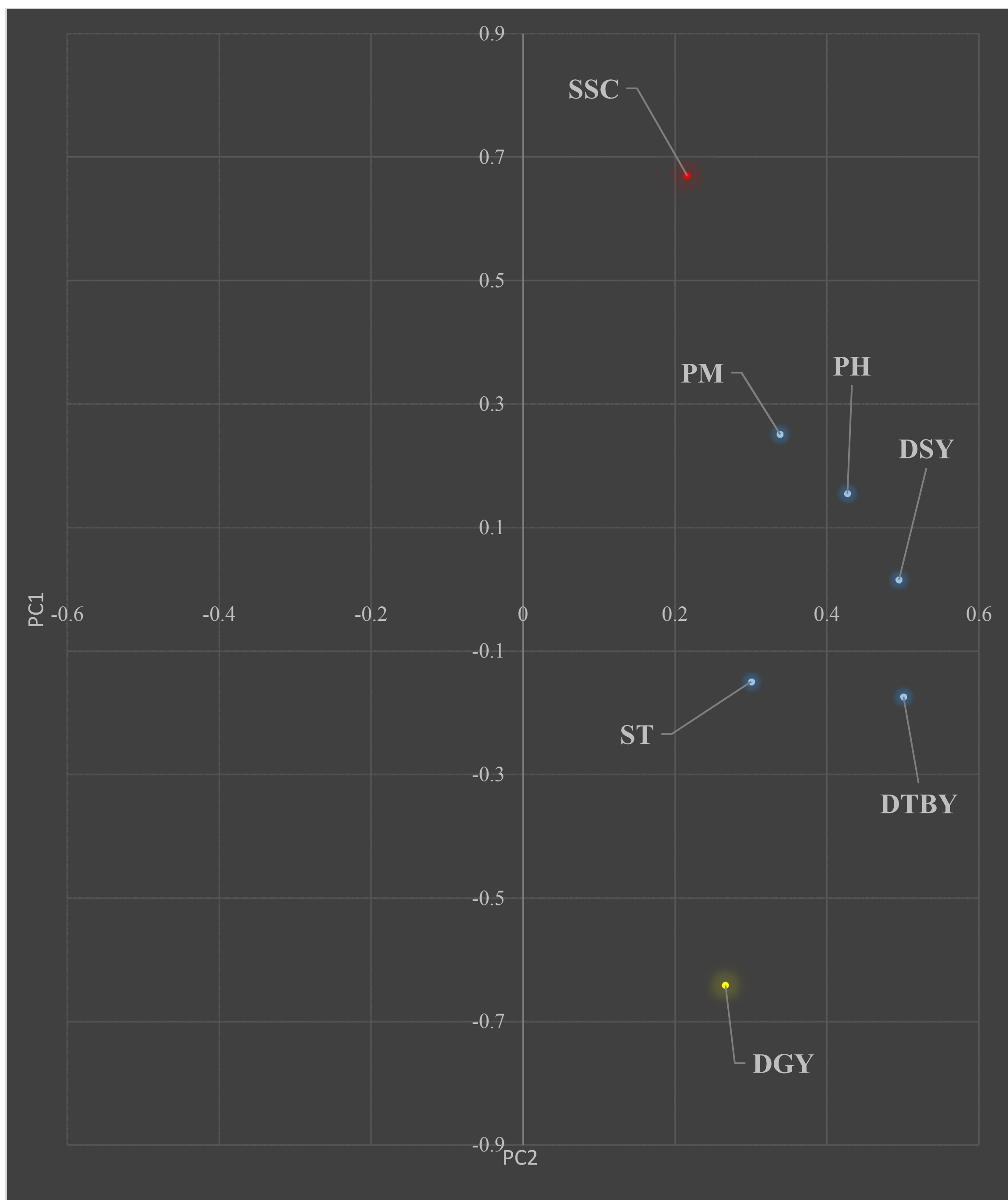
Table 2.9. Variance components and heritability estimates for biomass components and sugar-related traits analyzed by REML.

Trait	Heritability	Variance component+				
		G	Y	G×Y	B(Y)++	Residual
Plant height, cm	0.87	0.71 **	0.08	0.04 **	0.01	0.16
Plant maturity, days	0.40	0.15 **	0.65	0.06 **	0.00	0.14
Stem thickness, cm	0.48	0.21 **	0.00	0.12 **	0.00	0.67
Grain yield, t ha^{-1}	0.42	0.21 **	0.29	0.06 *	0.01	0.44
Stover yield, t ha^{-1}	0.57	0.25 **	0.34	0.03	0.01	0.37
Total biomass yield, t ha^{-1}	0.60	0.33 **	0.13	0.06 *	0.02	0.46
Stem sugar concentration, °Brix	0.74	0.47 **	0.00	0.12 **	0.01	0.41

* Significant at 0.05 probability level; ** significant at 0.01 probability level. +Variance component of each significant effect divided by the total variance components.
++Block effect which is nested in Year (Y).

2.4.6 Principal components analysis (PCA)

Figure 2.9, the PCA analysis showed five of seven traits together. These traits were stover yield, total biomass yield, plant height, stem thickness and plant maturity. A strong association among traits involved in production of structural carbohydrates, cellulose and hemicellulose, in lignocellulosic biomass has also been reported by others (Rooney 2007; Murray et al., 2008b). Stem sugar concentration and grain yield were isolated from the other traits by PCA. Unlike the other traits, stem sugar is associated with the concentration of non-structural carbohydrates, soluble sugar, in lignocellulosic biomass. Based on the location of the traits and their proximity to each other in the Cartesian plane, the PCA showed all the lignocellulosic traits close to each other in quadrant I and the reproductive plant trait in quadrant IV. The opposite direction and distance between stem sugar concentration and grain yield, suggests a negative correlation, and hence a possible physiological tradeoff between these two traits.



DGY=Dry Grain Yield (t/ha), DSY=Dry Stover Yield (t/ha), DTBY=Dry Total Biomass Yield (t/ha), PH=Plant Height (cm), PM=Plant Maturity (days), SSC=Stem Sugar Concentration (ºBrix).

Figure 2.9. PCA of biomass components and sugar related traits.

2.5 Discussion

Mean comparisons among the four RILs groups indicated that the sweet mutation enhances desirable agronomic traits such as stover yield, plant height and stem thickness, as well as biomass quality traits such as stem sugar concentration. Brown midrib mutation generally reduces plant fitness, resulting in shorter plants, delayed maturity, and increased tendency to lodge (Pederson et al., 2005). The sweet mutation, at least among the lines of this population, appears to positively influence these same fitness traits resulting in taller plants with thicker stems that are less likely to lodge. When both mutations are combined, as in the RILs of the “brown-sweet” group, the reduced fitness caused by the brown midrib mutation is compensated for by the introduction of the sweet mutation, with some individuals even exceeding the performance of RILs carrying only one of the mutations. These positive attributes of sweet sorghums are generally reflected in the phenotypic and genotypic correlations, where stem sugar concentration shows a significant positive correlation with plant height (taller plants) and stover yield (bigger plants). Strangely, the positive correlation was not significant between stem sugar concentration and stem thickness in the phenotypic comparison, and only weakly associated in the genotypic correlation. However, derivative traits like plant height and dry stover yield, both positively correlated with stem thickness, showed highly significant correlations, both phenotypically and genotypically, with stem sugar concentration.

Because the results of the analysis of variance obtained by TYPE III mixed model method only indicate which of the source of variation are significantly influencing mean trait performances, an estimation of heritability and the contributions of its determinants Year

(the environmental component, “E”), RIL (the genetic component, “G”) and Year×RIL (the interaction between environment and genetics, “G×E”) were also performed for the seven traits measured in this study. These estimates of variance components and narrow sense heritability based on the REML method from PROC MIXED procedure are shown in Table 2.9. In this population, the highest heritability was observed for plant height and stem sugar concentration measures with 87% and 74%, respectively. The results showed that the major contributor to plant height and stem sugar concentration heritability was the genetic effect (71% and 47%, respectively). This is consistent with similar studies (Ritter et al., 2008; Murray et al., 2008). Dry stover and dry total biomass yields were much more influenced by the environmental effect of year, though the genetic component of the heritabilities of these traits were major and significant. Dry stover yield with a moderate heritability estimate of 57% showed a genetic effect contribution of 25% and dry total biomass yield showing 60% heritability had a 33% variance due to genotype effect. Major effects of growing season on lignocellulostic biomass have been reported in other studies (Chaudari et al., 1993; Abubakar and Bubuche 2013). Interestingly, dry grain yield major contributors were Year (28%) and Genotype (21%) effects. Although grain yield was only considered in this study in as much as it contributed to total biomass yield, it is interesting to note the relatively low contribution of genotype as a variance component (21%). This reflects what has been in countless other studies, that grain yield is a complex trait with strong environmental influences and hence a challenging breeding objective.

Stem thickness, with moderate heritability of 48% showed positive correlations with plant height and all the yield parameters, making it an attractive target for biomass quantity improvement. That it has a strong genetic component (21%) suggests that selection for

this trait in a breeding objective to increase biomass yield would be fruitful. Plant maturity showed a moderate heritability estimate (40%) in this study, mainly explained by year effect (65%). Plant maturity can have profound effects on biomass production as the transition from the vegetative to reproductive phase negatively impacts lignocellulosic biomass accumulation. This study showed significant positive (phenotypic and genotypic) associations of plant maturity to all yield components and plant characteristics predicted to improve ethanol productivity. Very late maturity or photoperiod sensitivity have been breeding targets for maximizing dry stover yield (Jakob et al., 2009). In another study it was shown that a photoperiod sensitive sorghum was by far the highest dry stover yielder (see Chapter 4). Unfortunately, of all the measured traits, plant maturity was most sensitive to the environmental component of Year (65%). Similarly large seasonal variations in plant maturity that impact biomass production have been reported by others (Vermerris et al., 1999; Bhosale et al., 2012).

From these results it appears that both qualitative and quantitative gains in biomass for ethanol production can be achieved by creating recombinant genotypes with sweet stalk and brown midrib mutations. The moderate to high heritabilities with a strong genetic component of most of the traits measured in this study indicate that this is feasible. Increased soluble nonstructural carbohydrates were evident among the sweet RILs with a high stem sugar concentration (Brix $\geq$ 12). This desirable quality trait for ethanol production was generally associated with increased biomass quantity parameters of dry stover yield, plant height and stem thickness representing increased structural carbohydrates for ethanol production. While all these traits varied with year

(environmental effect) they all showed a significantly high genetic component indicating these traits would be useful in breeding for improved sorghum feedstock.

The linear association between breeding values of individuals for two traits (genetic correlations) of biomass components and sugar related traits are presented in Table 2.8. Based on the types of carbohydrate plants produce, the seven traits measured in this study can be associated with three types of carbohydrate produced by brown midrib sweet sorghum lines. Grain yield is a trait associated with starch production in plant biomass. Grain storage starches are non-structural carbohydrates easily hydrolyzed and fermented, but practically, at least in the context of this study, represent a minor contribution to overall plant biomass. Though they are included in the dry total biomass yield, starches are not considered a part of lignocellulosic biomass. It is clear from measurements undertaken in this study that the bulk of the sorghum plant biomass comes from the non-grain portion of the plant, that is, the stover. Stover yield, total biomass yield, plant height, stem thickness and plant maturity are traits mainly involving the structural carbohydrates of plant biomass, hemicellulose and cellulose. Qualitative gains in these traits are major determinants of lignocellulosic biomass productivity.

Stem sugar concentration represents the nonstructural soluble carbohydrate portion of the biomass for ethanol production. The results of genetic correlations showed that, stover yield was strongly correlated with total biomass yield (r_G=0.97), plant height (r_G=0.89) and stem thickness (r_G=0.77) and moderately correlated with plant maturity (r_G=0.69). Similarly, total biomass yield was strongly correlated to plant height (r_G=0.87) and stem thickness (r_G=0.78); and moderately correlated with plant maturity (r_G=0.62). These results

were expected based on previous studies reported in the literature (Murray et al., 2008; Ritter et al., 2008). Interestingly, plant height, stem thickness and plant maturity showed a consistent moderate to strong correlation only with the lignocellulosic biomass related traits (dry stover yield and dry total biomass yield). This suggests that while breeding and selecting for plant height, stem thickness and plant maturity, the lignocellulosic biomass traits are indirectly improved (Bernardo 2010; Vermerris 2008; Jakob et al., 2009).

Stem sugar concentration showed moderate correlation with dry stover yield (r_G=0.45) and plant height (r_G=0.44). Perhaps, the lack of strong correlation between stem sugar concentration and other traits is attributable to the complexity of Brix measurements. Brix is an average measurement of total soluble carbohydrates present in stem juice. Three major soluble carbohydrates are present in stem juice, soluble glucose, sucrose and fructose. If it were possible to measure these individually, then one may have found a stronger association of a particular sugar to stover yield and plant height (Han et al., 2013). Stem sugar concentration also showed negative correlations with dry grain yield (r_G=-0.16). This makes sense in physiological terms, based on whether the plant is going to produce more soluble sugars in the stems (sucrose) or more grain yield (starch). Plants must maintain a balance between sink and source. Therefore, some tradeoff can be obtained from this physiological adjustment (Slewinski 2012). Those RILs with a high concentration of soluble carbohydrates in stem juice may not be able to maximize grain yield (Heiz 1987). It follows that maximal lignocellulosic biomass yield might be attained with no grain set in dedicated biofuel crops (Jakob et al., 2009). However, this may not be the best use of sorghum, which is generally considered a grain crop. In sorghum, there exists the option of harvesting the grain for food and feed use and using the stover as feedstock for ethanol

production. The end use of a crop, of course, will determine the optimal partitioning of plant carbon. It is encouraging, however that a total trade-off is not necessary, as even grain yield showed a moderate genetic correlation with total biomass yield (r_G=0.56) and stem thickness (r_G=0.43) and a weak correlation with dry stover yield (r_G=0.33) and plant height (r_G=0.31).

For centuries, plant breeders have relied on phenotypic selection as major tool of genetic gain and improvement in crops (Bernardo 2010). Selection criteria, particularly when trying to improve several traits at once, depend on the degree to which the selection phenotype is correlated to the target phenotype. With the ultimate goal of improving ethanol productivity of a sorghum, a trait not discernable during the time when field selection is exercised, other more tangible traits highly and positively correlated to the ethanol productivity are needed. Overall, most of the traits measured in this study showed significant correlation between each other. These correlations were sometimes quite high, for instance, a significant correlation of (r=0.75) was observed between plant height and dry stover yield and one of (r=0.72) between plant height and dry total biomass yield. This suggests that selecting for plant height would also improve dry stover yield and total dry biomass yield, and ultimately ethanol yields. Other traits would be less useful as selection criteria. Stem thickness and stem sugar concentration, with a small non-significant correlation, showed that stem thickness would not be a useful selection criterion for increasing stem sugar concentration.

This concept is corroborated by results of principal components analysis, which showed that plant height, plant maturity, stem thickness, stover yield, and total biomass were highly

correlated. Grain yield was shown to be least correlated with these same traits. Indeed, the far distance of grain yield from the other traits could indicate possible trade-offs against the lignocellulosic biomass related traits (stover yield, total biomass yield, plant height, stem thickness and plant maturity) and the non-structural soluble carbohydrate trait (stem sugar concentration). Stem sugar concentration was also separated from the lignocellulosic biomass traits; thus, a lower but positive correlation will be expected between them. This all suggests that lignocellulosic biomass traits can be selected simultaneously, but tradeoffs with grain yield will be expected. (Murray et al., 2008 and Ritter et al., 2007).

Ultimately, for a trait so complex as ethanol production that is not realized until post-harvest processing, it would be useful if molecular markers were available. Marker assisted selection could speed the process of improving ethanol production in sorghum. For the two mutations associated with improved sorghum feedstock quality, brown midrib and sweet, and additional objective was added to this study to identify genomic regions in which mutant alleles could be marked and tracked in a segregating population. Among the few polymorphic SSR markers distinguishing the sweet sorghum parent, Brown County, and the non-sweet parent of this RIL population, bmr12 used to genotype representative sweet and non-sweet RILs, three possible regions associated with stem sugar concentration measurements were identified in this study. We targeted our marker search to genomic regions associated with high stem sugar concentration by others (Murray et al, 2008a; 2009; Ritter et al., 2008). We found by single marker analysis that chromosomes six and seven harbor significant QTL explaining from 2 to 7% of the variation in Brix measurements. The low R^2, perhaps, is attributed to the low number of polymorphic markers (ten) assessed in the brown midrib × sweet sorghum population. Indeed, increasing

the number of markers increases the chances of finding regions associated to Brix measurements (Murray et al, 2008a; 2009; Ritter et al., 2008). Saturating these regions with additional markers could also lead to useful selection tools for alleles determining high stem sugar concentration. Another explanation of low R^2 in this study could be the genetic complexity of the mixtures of sugars (sucrose, fructose and glucose) contributing to the Brix measurements (Han et al., 2013). It is known that the relative and total amounts of these sugars, and thereby the Brix measurement, are highly affected by the environment (Shiringani et al., 2010, Chen et al., 2014). The results of mapping conducted in this study have shown indication of two QTL associated with the sweet mutation in this population. The markers we have in hand for genotyping the favorable alleles at these loci would not adequately replace phenotyping by Brix measurements for selecting sweet sorghums in segregating populations for the mutation donated by Brown County.

In contrast, we have a robust genotypic marker, for tracking the bmr12 brown midrib mutation. When aligned to the BTx623 reference genome, bmr12 showed in addition to the base pair (C to T) change at position 745 of gene model *Sobic.007G047300* (the *COMT* gene), the causal mutation, bmr12 carries a 348bp deletion relative to the reference genome in an intron beginning at position 1601. The sweet sorghum (without brown midrib) Brown County, does not have this deletion. The primer pair targeting this portion of *COMT* (E2) in a PCR gives an amplicon size of 643bp in the wild type Brown County, but in the brown midrib mutant bmr12, the amplicon size is only 295bp. Therefore, primer pair E2 is an InDel marker easily scorable on an agarose gel. In genotypes of this population, it clearly distinguished brown midrib RILs from those without brown midribs. The association was 100% since the marker is within the mutant *COMT* gene. Although the brown midrib trait

is fairly easy to phenotype, simply by looking at the midribs of plants, having a molecular marker for the trait allows one to select for the mutant phenotype at a seedling stage, before the brown color of the mutant midribs are apparent, or even in seed chips by genotyping. This would be very useful in backcrosses aimed at introgressing this powerful mutation into a genotype with desired background, provided that the recurrent parent does not share the deleted region.

This InDel marker may not work to follow the segregation of other brown midrib mutations, for instance, those of involving *CAD* (bmr_6) and the other two allelic groups of brown midrib mutations (Saballos et al., 2008). The deletion in the intron of *COMT*, with respect to the reference genome, is shared by the wild type counterpart of bmr12, N12 (Sattler et al., 2012) and indeed is shared by the original sorghum lines, P945104 and P954114, used in the chemical mutagenesis by Porter et al. (1978) that generated all the sorghum brown midrib mutants described in the literature. It is useful in our population because it is within the mutant *COMT* locus and does not occur in Brown County.

2.6 Conclusion

The biofuel industry faces challenges to develop economically viable and sustainable biorefineries for ethanol production in the USA. Using the stover of crops like sorghum as a feedstock could help meet that challenge. By carefully tracking seven traits related to biomass quality and quantity over two years, this study has shown that in a RIL population containing mutations for improved lignocellulosic biomass quality traits, brown midrib and sweet, that favorable combinations of these traits can result in superior feedstock. Key lignocellulosic biomass yield indicators (stover yield, total biomass yield, plant height, plant maturity and stem thickness) were found to be highly correlated and could be improved together. They showed moderate to high heritability estimates showing that selection for these traits would be successful. Also, genetic and phenotypic correlations clustered the seven evaluated traits into three groups, allowing assessment of possible tradeoffs among traits. Interestingly, each of groups represented the three different source of carbohydrates available in plant biomass for ethanol production: the non-structural carbohydrate (starch) in the grain, the non-structural carbohydrates (sugars) in the stem, and the structural carbohydrates (cellulose and hemicellulose) in all vegetative plant parts. Further, the introduction of the sweet mutation generally enhanced biomass quantity traits as well as offset some of the negative aspects associated with lines carrying the brown midrib mutation (smaller plants prone to lodging) in the “brown-sweet” RILs containing both mutations. Including these traits in sorghum biomass improvement for lignocellulosic ethanol production is therefore recommended based on our study. Useful molecular markers for speedy introgression of these traits were identified for the brown midrib trait, but not for the sweet mutation particular to this population.

2.7 References

Abubakar L, Bubuche TS (2013) Genotype × environment interaction on biomass production in sorghum (*Sorghum bicolor* L. Moench). African J Agric Res 8:4460–4465.

Amrit Kumar Paul (2007) SAS for statistical genetics. ICRISAT. I.A.S.R.I., Library Avenue, Pusa, New Delhi – 110 012

Ayyangar G, Ayyar M, Rao V, and Nambiar A (1936) Mendelian segregation for juiciness and sweetness in sorghum stalk. Madras Agric. J. 24: 247-256.

Bian YL, Seiji Y, Maiko I, Cai H-W (2006) QTLs for sugar content of stalk in sweet sorghum (*Sorghum bicolor* L. Moench). Agric. Sci. China 5:736–744.

Bout S, Vermerris W (2003) A candidate-gene approach to clone the sorghum brown midrib gene encoding caffeic acid O-methyltransferase. Mol. Genet. Genomics 269:205–214.

Canilha L, Kumar Chandel A, dos Santos Milessi TS, et al. (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. J Biomed. Biotechnol. Volume 2012, Article ID 989572, 15 pages.

Casler MD, Pedersen JF, Undersander DJ (2003). Forage yield and economic losses associated with the brown-midrib trait in sudangrass. Crop Sci. 43:782–789.

Chaudari, SD, Sreenivas B and Seetharama N (1993) Multilocational evaluation of sweet-stalk sorghum genotypes for alternate uses. Annals of Plant Physiology, 7 (2). pp. 256-262.

Chen S-F, Danao M-GC, Singh V, Brown PJ (2014) Determining sucrose and glucose levels in dual-purpose sorghum stalks by Fourier transform near infrared (FT-NIR) spectroscopy. J Sci. Food Agric. 8pages.

Clark JW (1981) The inheritance of fermentable carbohydrates in stems of *Sorghum bicolor* (L.) Moench. Ph.D. diss., Texas A&M Univ., College Station.

Dien B, Jung H, Vogel K, et al. (2006) Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass and Bioenergy 30:880–891.

Gorthy S, Mayandi K, Faldu D, Dalal M (2013) Molecular characterization of allelic variation in spontaneous brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). Mol. Breed. 31:795–803.

Gravois KA, Bernhardt JL (2000) Heritability and genotype x environment interactions for discolored rice kernels. Crop Sci. 40:314-318.

Han KJ, Pitman WD, Kim M, Day DF, Alison W, McCormick ME, Aita G (2013) Ethanol production potential of sweet sorghum assessed using forage fiber analysis procedures. GCB Bioenergy 5:358–366.

Heinz D (1987) Introduction. p. 1–5. In D.J. Heinz (ed.) Sugarcane improvement through breeding. Elsevier, New York.

Holland JB (2006) Estimating genotypic correlations and their standard errors using multivariate restricted maximum likelihood estimation with SAS PROC MIXED. Crop Sci. 46:642.

McCollum TM, Mccuistion K, Bean B (2004) Brown mid-rib and photoperiod-sensitive forage sorghums. The Texas A&M University System. The Agriculture Program. 11 pages.

Liang GHL, Walter TL (1968) Heritability estimates and gene effects for agronomic traits in grain sorghum, *Sorghum vulgare* Pers. Crop Littell RC, Milliken GA, Stroup WW, Wolfinger R (1996) SAS System for Mixed Models. Illustrated Edition, SAS Institute Inc., Cary, NC, USA, ISBN: 10.

Ming R, Liu SC, Moore PH, Irvine JE, Paterson AH (2001) QTL analysis in a complex autopolyploid: Genetic control of sugar content in sugarcane. Genome Res. 11:2075–2084.

Morgan PW, Finlayson SA, Childs KL, Mullet JE, Rooney WL (2002) Opportunities to improve adaptability and yield in grasses: Lessons from sorghum. Crop Sci. 42:1791-1799.

Mullet JE, Rooney WL, Klein PE, Morishige D, Murphy R, Brady JA (2010) Discovery and utilization of sorghum genes (ma5/ma6). The Texas A&M University System. The Agriculture Program.

Murray SC, Rooney WL, Hamblin MT, et al. (2009) Sweet sorghum genetic diversity and association mapping for brix and height. Plant Genome Journal 2(1):48-62.

Murray SC, Sharma A, Rooney WL, et al. (2008a) Genetic improvement of sorghum as a biofuel feedstock: I. QTL for stem sugar and grain nonstructural carbohydrates. Crop Sci. 48:2165-2179.

Natoli A, Gorni C., Chegdani F, Ajmone-Marsan P, Colombi C., Lorenzoni C, Marocco A (2002) Identification of QTLs associated with sweet sorghum quality. Maydica 47:311–322.

Nghiem NP, Nguyen CM, Drapcho CM, Walker TH (2013) Sweet sorghum biorefinery for production of fuel ethanol and value-added co-products. Biological Engineering Transaction 6:143–155.

Oliver AL, Pedersen JF, Grant RJ, et al. (2005a) Comparative effects of the sorghum *bmr*6 and *bmr*12 Genes: I. Forage sorghum yield and quality. Crop Sc. 25:2234–2239.

Oliver a. L, Pedersen JF, Grant RJ, Klopfenstein TJ (2005b) Comparative effects of the sorghum *bmr*6 and *bmr*12 Genes: II. Grain yield, stover and stover quality in grain sorghum. Crop Sc. 45:2240- 2245.

Palmer N a, Sattler SE, Saathoff AJ, et al. (2008) Genetic background impacts soluble and cell wall-bound aromatics in brown midrib mutants of sorghum. Planta 229:115–27.

Pedersen JF (1996). Annual forages: New approaches for C-4 forages. p. 246-251. In: J. Janick (ed.), Progress in new crops. ASHS Press, Alexandria, VA.

Pederson, JF, Vogel KP, and Funnell DL (2005) Impact of reduced lignin on plant fitness. Crop Sci. 45:812-819.

Pedersen JF, Toy JJ, Funnell DL, Sattler SE, Oliver AL, Grant RA (2008) Registration of BN611, AN612, BN612, and RN613 sorghum genetic stocks with stacked -*bmr*6 and *bmr*2 Genes. J Plant Regist. 2:258.

Piepho H-P, Möhring J (2007) Computing heritability and selection response from unbalanced plant breeding trials. Genetics 177:1881–8.

Porter KS, Axtell JD, Lechtenberg VL, Colenbrander VF (1978) Phenotype , fiber composition , and in vitro dry matter disappearance of chemically induced brown midrib (bmr) x mutants of sorghum. Dep. Of Animal Science. Purdue University, West Lafayette, IN.

Quinby JR and Karper RE (1954) Inheritance of height in sorghum. Agron. J. 46:211–216

Ritter KB (2007) An investigation into the genetics and physiology of sugar accumulation in sweet sorghum as a potential model for sugarcane. Ph.D. diss., Univ. of Queensland, St. Lucia, Australia.

Rooney WL, Blumenthal J, Bean B, Mullet JE (2007) Designing sorghum as a dedicated bioenergy feed stock. Biofuels Bioprod. Biorefin. 1:147–157.

Saballos A, Ejeta G, Sanchez E, et al. (2009) A genomewide analysis of the cinnamyl alcohol dehydrogenase family in sorghum (*Sorghum bicolor* (L.) Moench) identifies SbCAD2 as the brown midrib6 gene. Genetics 181:783–95.

Saballos A, Vermerris W, Rivera L (2008) Allelic association , chemical characterization and saccharification properties of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). 193–204.

Sattler SE, Palmer NA, Saballos A, et al. (2012) Identification and characterization of four missense mutations in brown midrib 12 (bmr12), the Caffeic O-Methyltranferase (*COMT*) of Sorghum. Bio. Energy Res. 5:855–865.

Schluhuber, AM 1945. Inheritance of stem characters: In certain sorghum varieties and their hybrids. J. Hered. 36:219–222.

Shiringani AL, Frisch M, Friedt W (2010) Genetic mapping of QTLs for sugar-related traits in a RIL population of *Sorghum bicolor* L. Moench. Theor. Appl. Genet. 121:323–36.

Slewinski TL (2012) Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and methylation and chromatin patterning biofuel production. J. Exp. Bot. 63:695–709.

Smith CW, Frederiksen RA (2000) Sorghum: origin, history, technology and production. Vol. 2. John Wiley & Sons, New York.

Srinivasa P, Santosh R, Blümmel M, et al. (2012) Characterization of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). The European Journal of Plant Science and Biotechnology. 6(1):71-75.

Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9:433–43.

Vermerris W, McIntyre LM (1999) Time to flowering in brown midrib mutants of maize: an alternative approach to the analysis of developmental traits. Heredity 83 (2):171–8.

Vermerris W, Saballos A, Ejeta G, et al. (2007) Molecular breeding to enhance ethanol production from corn and sorghum stover. Crop Sci. 47(S3):142-153.

Vermerris W (2008) Genetic improvement of bioenergy crops. University of Florida. Springer 472 pages.

Vogler RK, Tesso TT, Johnson KD, Ejeta G (2009) The effect of allelic variation on forage quality of brown midrib sorghum mutants with reduced caffeic acid O - methyl transferase activity. African Journal of Biochemistry Research. 3(3):70–76.

Xin Z, Velten JP, Oliver MJ, Burke JJ (2003) High-throughput DNA extraction method suitable for PCR. Biotechniques 34(4):820-827.

CHAPTER 3. COMPOSITIONAL CHARACTERIZATION AND ESTIMATION OF BIOMASS CONVERSION IN A BROWN MIDRIB SWEET STALK SORGHUM POPULATION

3.1 Abstract

Ethanol production from lignocellulosic biomass of crop residues offers a sustainable alternative to fossil fuels without diverting arable land from food and feed production. Sorghum is an excellent crop capable of producing high lignocellulosic biomass as a source of fermentable energy source, in addition to the grain it produces for food and feed. There is rich genetic diversity in sorghum including mutants that enhance sorghum biomass quality, such as brown midrib mutants with low lignin concentration and sweet stem sorghums with increased sugar concentration, which produce biomass high in fermentable carbohydrates. Some of these traits could be recombined in a single genotype to increase the quantity of biomass and enhance its quality. A genetically enhanced sorghum lignocellulosic biomass could yield more ethanol per ton and per hectare. A two year field and laboratory study was completed using 236 recombinant inbred lines (RILs) derived from a sorghum population synthesized from two contrasting parents, a brown midrib (low lignin, low sugar) and sweet stem (high sugar, high lignin) lines to assess the effect of lignocellulosic biomass production of higher yield and quality. The experimental lines were grown in two row plots replicated twice over the two years, 2008 and 2009 at the

Purdue Agronomy Research and Education Farm, in West Lafayette, IN. Stover yield, and stem sugar concentration (SSC) were measured on each line. Fiber detergent analysis (FDA) was performed to estimate the amount of hemicellulose, cellulose and lignin in the lignocellulosic biomass (stover). From the measured FDA, glucose recovery and theoretical ethanol yield and production were calculated, and differences among grouped RILs were analyzed. Results showed that only RILs carrying the brown midrib mutation showed significantly higher glucose recovery. Those carrying both compositional mutations, showed even higher ethanol yields. Genotypes with only the sweet stem mutation also gave higher theoretical ethanol production. Lignin (R^2= 0.66) was identified as the most reliable predictor for glucose recovery. Lignin and SSC (R^2= 0.46 and 0.35, respectively) were identified as good predictors for ethanol yield. Dry stover or fresh stover yield (R^2= 0.89) were the most appropriate predictors for ethanol production. Based on our results, genetic enhancement of biomass quality (through brown midrib and sweet traits) and quantity (through agronomic qualities that increased plant size) could double lignocellulosic ethanol yields. This study also identified superior RI lines in the study population that could be advanced as genotypes that can be used lignocellulosic biomass crops.

3.2 Introduction

A large volume of lignocellulosic biomass is produced from a number of major and minor crops around the world every year. Row crops produce considerable amounts of both grain and stover. Grain is harvested and used for human (food) and animal consumption (feed), while stover (lignocellulosic biomass) is often left unharvested on farm every farming season (Nelson et al., 2011). Though some may consider the stover left on farm, a waste, agronomists recognize the value of crop residue for reducing soil erosion and building soil organic matter.

Sorghum research conducted over the last several years has generated interest in sorghum as a potential biomass crop for lignocellulosic feedstock and energy production. Knowledge on the genetics of several of the lignocellulosic traits in sorghum is also emerging, though at varied levels. The sweet stalk trait appears to be an inherited as a quantitative trait, controlled by multiple loci. Recent genetic analysis have placed quantitative trait loci (QTL) on four chromosomes (3, 5, 6 and 7). These QTL generally explain from 11 to 21 percent of the total variation for stem sugar content (Murray et al., 2008a; Murray et al., 2009; Ritter et al., 2008). It is suggested that environmental factors as well as additional unidentified QTL likely affect the expression of this trait. The brown midrib trait in sorghum is generally caused by single point mutations in genes involved in plant cell wall composition. From a chemical mutagenesis aimed at improving sorghum forage quality, several brown midrib (bmr) mutant lines were identified at Purdue University (Porter et al., 1978). Recent work has shown that four brown midrib allelic groups are responsible for reduced lignin concentration in sorghum lignocellulosic

biomass. A series of allelism tests were conducted among a collection of bmr mutants determining the four allelic groups, tentatively named as group 1, containing sorghum lines bmr3, bmr4, bmr6, bmr27 and bmr28, group 2, containing lines bmr7, bmr12, bmr18, bmr25 and bmr26, group 3, containing bmr19 and allelic group 4, containing bmr2, bmr5 and bmr14 (Saballos et al., 2008). From these groups, two genes have been identified to be involved in the lignin biosynthetic pathway. One identified gene, that belongs to allelic group #1, is located on chromosome 3, and affects cinnamyl alcohol dehydrogenase (CAD) activity during lignin biosynthesis in cell walls. CAD is encoded by a multi-gene family consisting of members thought to have distinct roles (Saballos et al., 2008, Palmer et al., 2008). Another locus on chromosome 7, belongs to allelic group #2, is responsible for low activity of the enzyme caffeic acid o-methyltransferase (COMT) (Bout and Vermerris 2003). This enzyme also plays an important role during lignin biosynthesis in sorghum. Stover lignin concentration plays an important role during enzymatic hydrolysis of cellulose. During this process, increased amount of lignin prevents the attachment of the hydrolytic enzyme to cellulose, and leads to a low yield of fermentable sugars. Therefore, high concentration of lignin in stover could lead to low ethanol yields (Sun and Cheng 2002; Ohgren et al., 2007).

Commercial lignocellulosic ethanol production is based on soluble and structural carbohydrates. Soluble carbohydrates are generally sugars and these accumulate in the stem of crops such as sugarcane and sweet sorghum. The sugars in these stalks can be transformed into ethanol by the process called biomass conversion. Biomass conversion of soluble carbohydrates has two major steps, namely enzymatic hydrolysis, and fermentation. However, due to the simple biochemical structure of these sugars, they can

be hydrolyzed and fermented in one single step known as simultaneous saccharification-fermentation (Saballos et al., 2008). Structural carbohydrates, on the other hand, are polysaccharides that form part of the plant cell wall, with hemicellulose and cellulose as complex sugar components that are tightly linked to lignin in plant cell walls. During biomass conversion, these complex sugars undergo three different processes to produce ethanol as their final product. In the first process, lignin is separated from the complex carbohydrates with a pretreatment of hot sulfuric acid. The function of lignin, therefore, is to cause the complex carbohydrates to be less assessable to fermentation and reducing its presence. The brown midrib trait of sorghum, generally facilitates ultimate conversion to ethanol. These complex carbohydrates (cellulose and hemicellulose) undergo enzymatic hydrolysis and finally fermentation to produce ethanol (Sun and Cheng, 2002; Dien et al., 2006; Sticklen, 2008; Canilha et al., 2012). In addition to this three step process of conversion of structural carbohydrates, ethanol can also be produced more directly from soluble carbohydrates. The ethanol from soluble carbohydrates is therefore cheaper to produce, so having a larger proportion of soluble carbohydrate in the feedstock, such as occurs in sweet sorghum, reduces the cost of ethanol production.

A genetically enhanced biomass that combines both sources of carbohydrates, maximizing the soluble, but also making the structural more accessible, could reduce energy demand and mitigate greenhouse gas emissions. A new bioconversion approach is proposed in order to improve glucose recovery, ethanol yield and ethanol production (Figure 2.1). This new approach combines two major processes. The first process is the simultaneous saccharification fermentation of soluble carbohydrates to ethanol. In parallel, the second process also happens, with reduced lignin bagasse (fibrous matter that remains after

sorghum stalks are crushed to extract their juice) undergoing hydrolysis and fermentation to also produce ethanol. Maximizing both the quantity, in terms of biomass produced per unit land, and quality, having a higher proportion of soluble to structural carbohydrate and making that structural more accessible, increases the value of a feedstock (Vogler et al., 2009; Oliver et al., 2005; Vogel et al., 2010; Gírio et al., 2010). Therefore both crop genetics and agronomic practice are important contributors to ethanol production

The objectives of this study were as follows: 1) To test the potential of a genetically enhanced brown midrib sweet sorghum lignocellulosic biomass as feedstock for lignocellulosic ethanol production; 2) To estimate glucose recovery, theoretical ethanol yield, and theoretical ethanol production as parameters to assess the value of genetic improvement in sorghum as a new feedstock; and 3) To determine suitable predictors associated with estimation of these parameters.

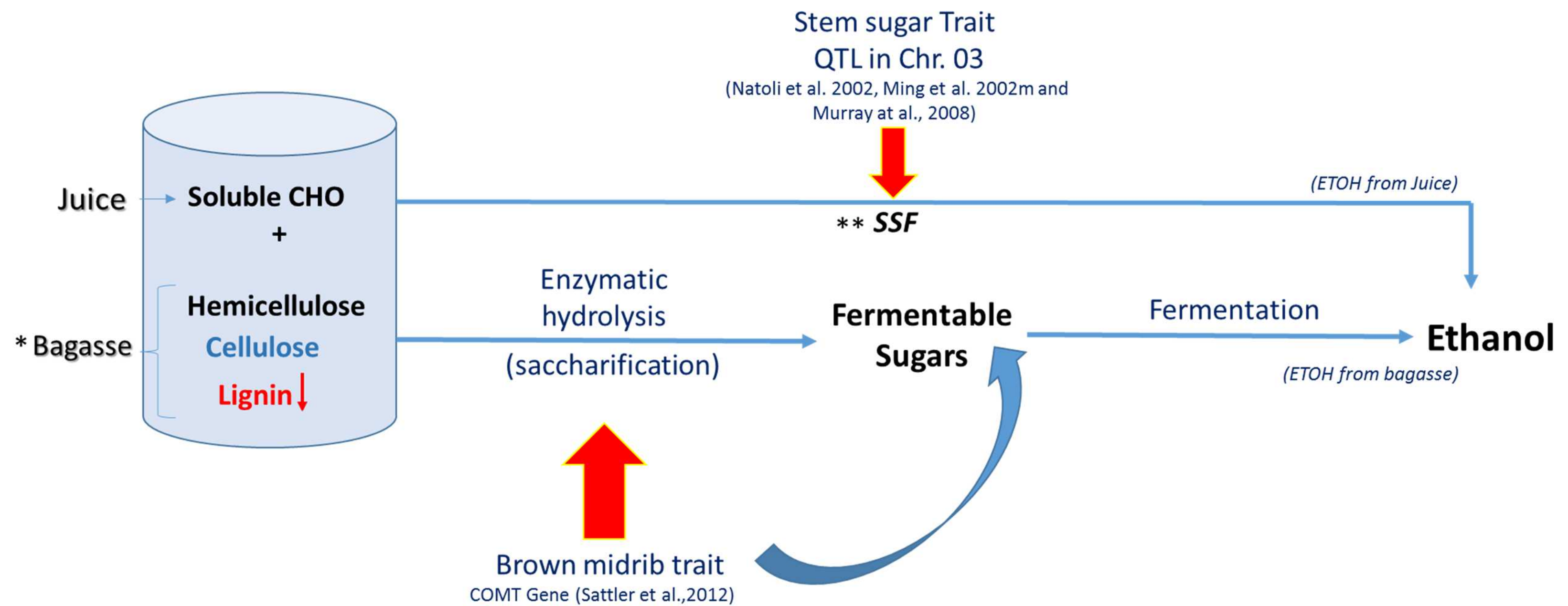


Figure 3.1. Bioconversion for an enhanced brown midrib sweet sorghum biomass feedstock.

[*Bagasse is the fibrous matter that remains after sorghum stalks are crushed to extract their juice. **Simultaneous saccharification fermentation (SSF) refers to the simultaneous enzymatic hydrolysis and fermentation of glucose and xylose to produce ethanol]

3.3 Materials and Methods

3.3.1 Plant material

A bi-parental population consisting of 236 recombinant inbred lines (RILs) was selected as our breeding population for a two year study (2008 and 2009). This population was developed through seven generations of single seed descent selection from the original F_2 population of a cross between two lines, bmr12 (a brown midrib, low lignin sorghum) and Brown County (a sweet sorghum) as parents (Appendix A.1).

3.3.2 Experimental design and field experiment

The randomized complete block design was selected for two year assessments of the brown midrib × sweet sorghum population. Two replications were conducted each year, and the RILs were randomized within each replication.

The experiment was planted on May 29th in 2008 and 2009 at the Agronomy Center for Research and Education (ACRE) in West Lafayette, Indiana. A randomized complete block design with two replicates was utilized in both years. All RILs, both parents, and a sweet brown midrib line used as check (bmrAtlas) were each planted in two row plots. Dimensions of each plot were 6.10m long with 0.76m spacing between the two rows. Approximately 2.5 grams sorghum seed row^{-1} was planted at a depth of 5 cm. The seeds were treated with a fungicide prior to planting to ensure better seedling emergence and stand establishment. Three weeks after planting, plots were thinned in to 6 plants per foot

for an approximate plant population of 250,000 plants per hectare. Urea ammonium nitrate was applied and incorporated at a rate of 150 kilograms per hectare. In both years, the experiment was managed following standard cultural practices recommended for commercial sorghum production.

3.3.3 Biomass and stem sugar measurements

Data on fresh stover yield (measured in t/ha), dry stover yield (measured in t/ha) and stem sugar concentration (ºBrix) were collected from the RIL population. In both years and replicates, data recording was done as follows: Plant maturity was considered as 45 days after flowering date. It is at plant maturity that the sweet trait is maximally expressed. Based on the flowering date for each RIL, three plant maturity groups were defined. In this way, this study managed any bias caused by differences in plant maturity among RILs of the brown midrib × sweet sorghum population. Measurements of sugar concentration in degrees Brix (ºBrix, or simply, Brix) of each recombinant inbred line and parent were collected. For each plot, two plants located in the middle part of each row were collected randomly as plot samples (a total of four plants per plot). Stem cylinders were cut between the fourth and the fifth node of each plant sampled. Following, a garlic press was used to squeeze stem juice from each of the four cylinders sampled. A digital refractometer (ATAGO Model PAL-1) was utilized to measure the percentage of soluble sugars present in the stem juice (Brix) of each sampled cylinder. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. The average of the four measurements of Brix per plot was utilized for calculation of

ethanol from soluble carbohydrates. At harvesting time of each maturity group, a sample plot of 10 plants (5 from each row) were randomly selected from the middle part of each plot to record fresh stover yield and dry stover yield. The panicles of the 10 plants were cut at the flag leaf and saved in paper bags. The paper bags containing panicles of each plot were dried for 3 to 4 days at 110ºC. The weight of leaves and stems of the same 10 plants (without panicles) was recorded as fresh stover weight per sample plot. Next, the ten plants (without panicles) of each plot were chopped in a tractor driver mechanical chopper, the chopped leaves and stems mixed, and a subsample of roughly one and a half fistfuls was weighed and saved in a paper bag (fresh stover subsample weight). The paper bags containing chopped subsamples of fresh stover were dried for 3 – 4 days at 60ºC, and dried stover subsample weight was recorded. Dried stover weight per sample plot was calculated by dividing the dried stover subsamples weight by fresh stover subsample weight and multiplying by fresh stover weight per sample plot.

Dry stover samples were ground to perform the fiber detergent analysis (FDA). Fresh stover yield and sugar concentration measurements were used as factors in the estimation of theoretical ethanol yield and production from sugars harbored in sorghum stems (sucrose, fructose and soluble glucose). Similarly, dry stover yield (DSY) was used in the estimation of theoretical production from structural sugars (cellulose and hemicellulose).

3.3.4 Fiber detergent analysis (FDA)

Fiber detergent analysis (FDA) of two replicates for each of the 236 RILs for two years trials was performed using an ANKOM2000 instrument (Vogel et al., 1999; Dien et al., 2006; Wu 2006; Lemus et al., 2008; Anderson et al., 2009). The FDA method encompasses three major steps: the neutral detergent fiber (NDF) analysis, the acid detergent fiber (ADF) analysis, and the acid detergent lignin (ADL) analysis. In order to estimate lignin content in our samples, a final procedure, the ash assay (Ash Assay) was also performed. The NDF analysis is the incomplete digestion fraction and results in an almost complete recovery of grass cell walls where fiber residues are predominantly hemicellulose, cellulose and lignin (Vogel et al., 1999; Kong et al., 2005). NDF is a joint measure of hemicellulose, cellulose, lignin and inorganic material in grass green biomass (lingocellulosic biomass). The ADF analysis is a procedure to jointly measure cellulose recovery, lignin and inorganic material (Kong et al., 2005; Wolfrum, 2009). The ADL analysis is used to estimate the amount of lignin and inorganic material in the samples (Dien et al., 2006). Finally, the ash assay, is used to determine the amount of lignin and inorganic material separately. By subtraction among the various analyses we were able to determine the amount of hemicellulose, cellulose, lignin and inorganic material in each sample from the brown midrib × sweet RIL population (Jung et al., 1999; Murray et al., 2008; Dien et al., 2009).

NDF analysis was performed following adjusted modified ANKOM technology protocol (Vogel et al., 1999). During the process, the weight of each empty filter bag (W_1) was recorded. Sorghum ground samples were pre-dried overnight at 45°C. An amount between 0.45 to 0.5g of a finely ground lignocellulosic biomass (leaves and stems) were weighed

out (W_2) in groups of 22 samples per run. The samples were spread uniformly inside the filter bag by shaking and flicking the bag to eliminate clumping. Two empty bags were included in each run to determine the blank bag correction (C_1 and C_2). Each filter bag was completely sealed and a total of 22 samples and two empty bags were placed on the suspender trays. One of the empty bags was placed in the first tray, and the other in last tray to determine the blank bag correction (C_1 and C_2). The trays were inserted into the vessel and a suspender was placed on top of the empty trays to keep the trays submerged. On the ANKOM instrument, NDF analysis START button was pressed. After the NDA solution was automatically inserted and agitation began, 4.0 ml of alpha-amylase was manually added. Later, extra 8.0 ml of alpha-amylase diluted to a volume of 250ml distilled deionizedH_2O was automatically added between the first and second rinse. After the analysis was completed (2 hours), samples were removed and the excess of water was gently pressed out. The bags were placed in a 250ml beaker and acetone was added to stop the reaction. The filter bags were dried out at room temperature for 5 minutes. After this, the bags were placed in an oven at 102 °C overnight for a complete dry and weights were recorded as W_3.

The percentage of NDF was estimated as follows:

$$\% \, NDF = \frac{100 \; x \; (\, W3 - (W1 \; x \; (\frac{C1 + C2}{2}))}{W2}$$

Where, W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with fiber after the extraction process; and C1 and C2 = Blank bag correction.

ADF analysis on the ANKOM2000 equipment uses sulfuric acid and CTAB to digest samples that already went through NDF analysis. The procedures for ADF analysis were similar to the ones described for the NDF analysis only this time with the acid specific detergent and without amylase at the ADF setting on the instrument.

The percentage of ADF was estimated as follows:

$$\% \, ADF = \frac{100 \text{ x } (W3 - (W1 \text{ x } (\frac{C1 + C2}{2})}{W2}$$

Where, W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with fiber after extraction process; and C1 and C2 = Blank bag corrections.

The ADL analysis was performed by adding 72% (by weight) sulfuric acid to completely solubilize the organic matter present in the samples (Milne et al., 1990). Only samples that previously went through NDF and ADF analysis were used for ADL analysis. The overnight dried filter bags were placed into the incubator jar containing 600 ml of sulfuric acid, enough volume to soak the bags. Four jars at a time were placed into the incubator and rotated for 3 to 4 hours. After acid digestion, each jar containing filter bags were rinsed several times with boiling water followed by water at room temperature to wash away the acid (confirmed by checking the pH). The samples were then rinsed in acetone and dried overnight at 102 ºC before reweighing.

The percentage of ADL was estimated as follow:

$$\% \, ADL = \frac{100 \text{ x } (W3 - (W1 \text{ x } (\frac{C1 + C2}{2})}{W2}$$

Where W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with fiber after extraction process; and C1 and C2 = Blank bag correction.

Hemicellulose was calculated by subtracting ADF% from NDF%, while cellulose was calculated by subtracting ADL% from ADF%.

The Ash assays were performed in porcelain crucibles where they were burned in an oven overnight at 500 ºC. From the remaining organic matter, the following determinations were made:

$$OM = (Cw. + Sample\ w.\ after\ ADL) - (Crucible + ash\ w.)$$

Where OM = organic matter, Cw. = Crucible weight and w. = weight.

$$\%\ Lignin = \frac{100 \text{ x } (\ W3 - (W1 \text{ x } (\frac{C1 + C2}{2})}{W2}$$

Where W1 = Bag tare weight, W2 = Sample weight, W3 = OM; and C1 and C2 = Blank bag correction

From these, the amount of lignin in gram per kilogram of dry stover was estimated as follow:

$$Lignin\ \left(\frac{g}{kg}\right) = \%\ Lignin \text{ x } (10)$$

Where 10 is the conversion factor from % to g/kg.

3.3.4.1 Estimation of glucose recovery

Glucose recovery formulas reported in the literature were inadequate for the RILs in this brown midrib × sweet population since they did not account for the advantage of reduced lignin. We therefore modified the estimation of glucose recovery following the estimate used by Vogel et al. (2011):

$$\text{Glucose recovery (g/kg)} = [\mathit{Cell}] \times \mathit{GRE}\ \% \times 1.1176$$

Where, *GRE* % is the glucose recovery efficiency, [*Cell*] is the amount of cellulose present in one kilogram of lignocellulosic biomass and 1.1176 is the glucan hydrolysis coefficient (Vogel et al., 2011).

The *GRE* % was calculated as follows (Dien et al., 2009):

$$\text{GRE}\ \% = (-0.825 \times \mathit{lig}) + 92.296$$

Where, *lig* is the amount of lignin in grams present in one kilogram of lignocellulosic biomass. These modifications adjusted for the differences in lignin content among the RILs of this unique population.

3.3.4.2 Estimation of xylan recovery

The amount of five carbon sugars coming from the hemicellulose portion of the dry lignocellulosic biomass was estimated as total xylan or xylan recovery. The formula used to estimate the total amount of xylan coming from hemicellulose was:

$$\text{Xylan recovery (g/kg)} = [\mathit{Hemicel}] \times [1.1353]$$

Where, [*Hemicel*] is the amount of hemicellulose in grams present in one kilogram of lignocellulosic biomass, 1.1353 is the hydrolysis coefficient for xylan (Anderson et al., 2010 and Vogel et al. 2011).

3.3.4.3 Estimation of theoretical ethanol yield (ETOHY)

Ethanol yield from glucans (L/T) was estimated based on formulas used by Vogel et al. (2010):

$$ETOHY_{(Glucan)} = \text{Glucose recovery} \times 0.51 \times 1.2674$$

Where 0.51 is the fermentation coefficient of glucans and 1.2674 is the ethanol specific volume in ml g^{-1}.

Ethanol yield from xylans (L/T) was estimated based on the following formula (Vogel et al., 2010):

$$ETOHY_{(Xylan)} = \text{Xylan recovery} \times 0.51 \times 1.2674$$

Where 0.51 is the fermentation coefficient of xylans and 1.2674 is the ethanol specific volume in ml g^{-1}.

By simultaneous saccharification fermentation (SSF), theoretical ethanol yield (L T^{-1}) from soluble sugars (SS) was estimated based on an adjusted version of formulas used by Han *et al* (2013). The adjusted formula is presented below:

$$ETOHY_{(SS)} = (FSY \times Brix\% \times 0.90 \times 0.51 \times 1.2674) \times 1/\ (FSY/1000)$$

Where, FSY is fresh stover yield (in kg ha^{-1}), Brix is the concentration (%) of soluble sugars in stem juice, 0.90 is hydrolysis efficiency of soluble sugars and 1/ (FSY/100) is a metric conversion factor to L/ T. Finally, total theoretical ethanol yield from two source of carbohydrates was calculated as:

$$\text{Total ETOHY} = ETOHY_{(Glucan)} + ETOHY_{(Xylan)} + ETOHY_{(SS)}$$

2.3.4.4 Estimation of theoretical ethanol production (ETOHP)

Theoretical ethanol production (L/Ha) from glucan and xylan were estimated as follows:

$$ETOHP_{(Glucan)} = ETOHY_{(Glucan)} \times DSY$$

$$ETOHP_{(Xylan)} = (ETOHY_{(Xylan)}) \times DSY$$

Where DSY is dry stover yield in kg/ha in both formulas.

Theoretical ethanol production from soluble sugars (SS) was estimated as follows:

$$ETOHP_{(SS)} = FSY \times Brix\% \times 0.90 \times 0.51 \times 1.2674$$

Finally, theoretical ethanol production (L/ha) was estimated by adding theoretical ethanol yield from all biomass sugar sources:

$$ETOHP = ETOHP_{(Glucan)} + ETOHP_{(Xylan)} + ETOHP_{(SS)}$$

3.3.5 Statistical analyses

TYPE III method from PROC MIXED procedure from SAS 9.3 was used in statistical analysis to determine genetic variation and mean differences among RILs for each of the variables, including fresh stover yield (t/ha), dry stover yield (t/ha), stem sugar concentration (Brix), hemicellulose concentration, cellulose concentration, lignin concentration, glucose recovery, theoretical ethanol yield and theoretical ethanol production in the 236 RILs. RILs were considered as a fixed effect, Year and Year × RIL interactions were considered random effect. Furthermore, comprehensive analysis of RILs were clustered into the following four phenotypic groups to allow a more detailed analysis among contrasting genotypes that share a common genomic background: Lines were dubbed "normal", "sweet", "brown" and "brown-sweet" based on their genetic recombination status for the two major traits of stem sugar, and low lignin that they exhibited phenotypically. The "normal" (non-brown; non-sweet) group was formed by 43 RILs without brown midribs or high stem sugar concentrations (Brix < 12). The "sweet" (non-brown; high stem sugar) group was formed by 108 RILs that carried a mutation for high stem sugar concentration (Brix ≥ 12), but did not have brown midribs. The "brown" (non-sweet; low lignin) group contained those RILs that had brown midribs but were not sweet (10 RILs). The fourth group named "brown-sweet" (recombinants of low-lignin and high stem sugar) were 75 RILs that carried both mutations, one for low lignin (brown midrib) and sweet, having a relatively high stem sugar concentration (Brix ≥ 12). We dubbed this group the double mutant group because of the two mutations its members carry. This grouping allowed us to obtain three orthogonal contrasts. The first linear combination compared the double mutant group ("brown-sweet") against the "normal" RIL group. The

second linear combination compared the double mutant group against the "sweet" group. The last linear combination compared the double mutant group against the "brown" group.

3.3.6 Predictors of glucose recovery and theoretical ethanol

Regression analysis was used to develop prediction equations for glucose recovery, theoretical ethanol yield and theoretical ethanol production. The following formula was used to estimate the predictors:

$$Y = b_0 \pm b_1 \times X$$

Where X is the explanatory variable and Y is the dependent variable. X was represented by NDF (g/kg), ADF (g/kg), cellulose (g/kg), hemicellulose (g/kg), lignin (g/kg), stem sugar concentration (Brix), dry stover yield (t/ha) and dry stover yield (t/ha). The slope of the line is b_1 and b_0 is the intercept. The PROC REG procedure from SAS was used to determine a good estimator associated with glucose recovery, theoretical ethanol yield and production.

3.4 Results

3.4.1 Biomass components traits

The results of the analysis of variance (ANOVA) for fresh stover yield, dry stover yield, and stem sugar concentration are presented in Table 3.1. Genotypes (RILs) showed highly significant differences for each of these. Years were only significant for fresh stover yield. The RIL x Year interaction was highly significant for both fresh stover yield, and stem sugar concentration, and significant for dry stover yield. Mean contrasts among the phenotypic groups are presented starting with Figure 3.2, which shows the differences in fresh stover yield. Interestingly, the “sweet” and “brown-sweet” RIL groups produced significantly higher fresh stover yield than the “normal” and “brown” RIL groups. This trend was also observed for dry stover yield, where again the “sweet” and “brown-sweet” RIL groups significantly out-yielded the other two RIL groups in dry stover production (Figure 3.3). This is also consistent with the high association reported in the literature between dry and fresh stover yield (Murray et al 2008a; Ritter et al., 2008).

Not surprisingly, stem sugar concentrations of the “sweet” and “brown-sweet” RIL groups were significantly higher than the other two RIL groups (Figure 3.4). This high stover quality parameter taken together with the superiority of these RIL groups to the others in measures of stover quantity (fresh stover yield, dry stover yield), all factors in the estimation of ethanol yield, suggests superior ethanol production from these types of sorghum.

Table 3.1. Combined year ANOVA for biomass component traits of brown midrib × sweet RIL population.

		Mean Square					
Source of variation	df	FSY		DSY		SSC	
Year	1	568.7	**	185.3	*	0.6	
RIL	235	5.4	***	1.7	***	25.9	***
brown-sweet vs normal	1	122.5		19.5	*	2648.6	*
brown-sweet vs sweet	1	5.8	***	8.8		116.8	
brown-sweet vs brown	1	88.7		17.7	*	722.8	*
Year×RIL	235	0.9	***	0.5		6.7	***
Error	469	0.6		0.4		4.2	

FSY=fresh stover yield (t/ha), DSY=dry stover yield (t/ha), SSC=stem sugar concentration (Brix); *significant at the 0.05 probability level, ** significant at the 0.01 probability level, *** significant at the 0.001 probability level.

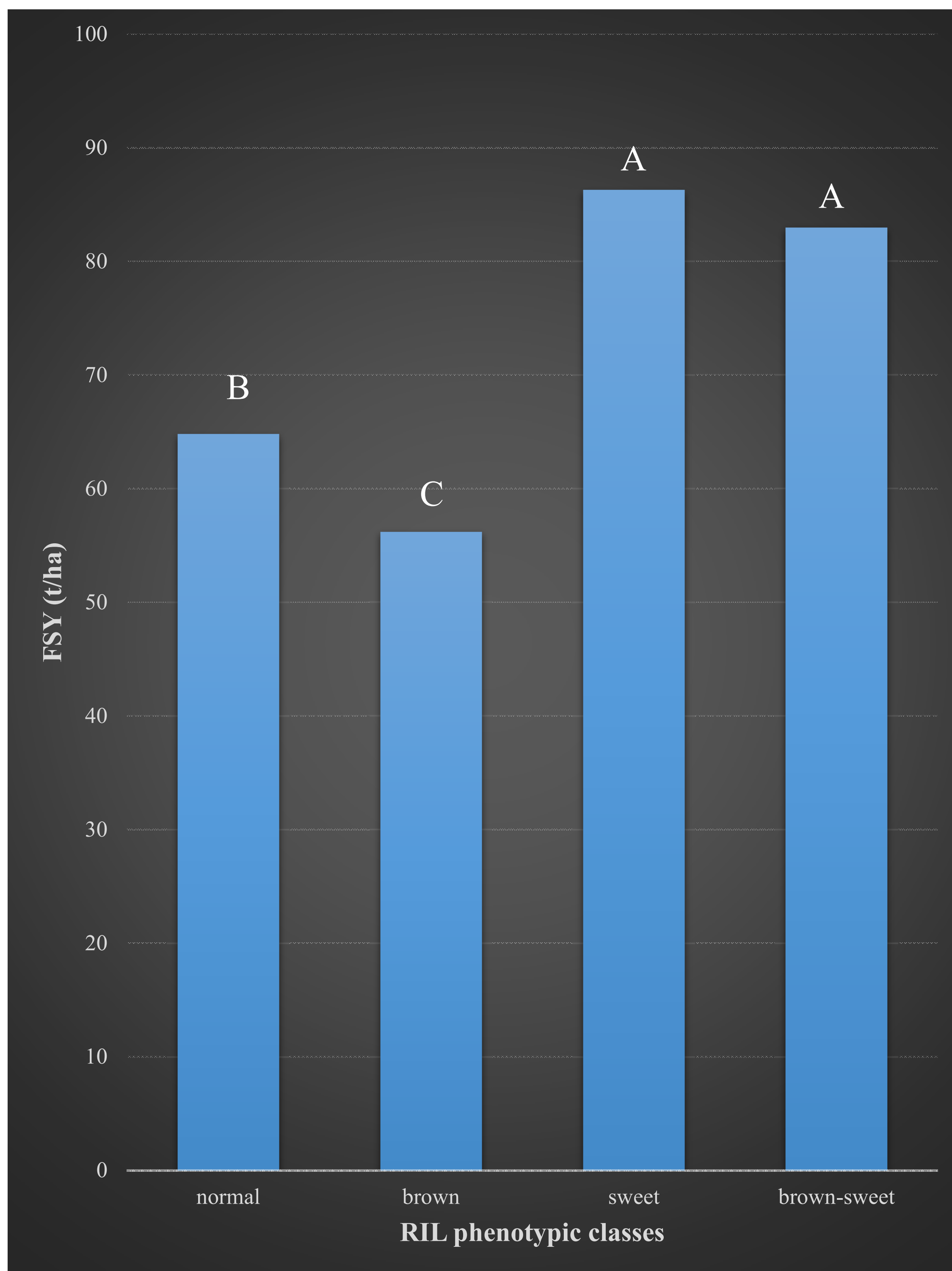


Figure 3.2. Mean fresh stover yield (FSY) among four different RIL phenotypic classes. LSD (P<.05).

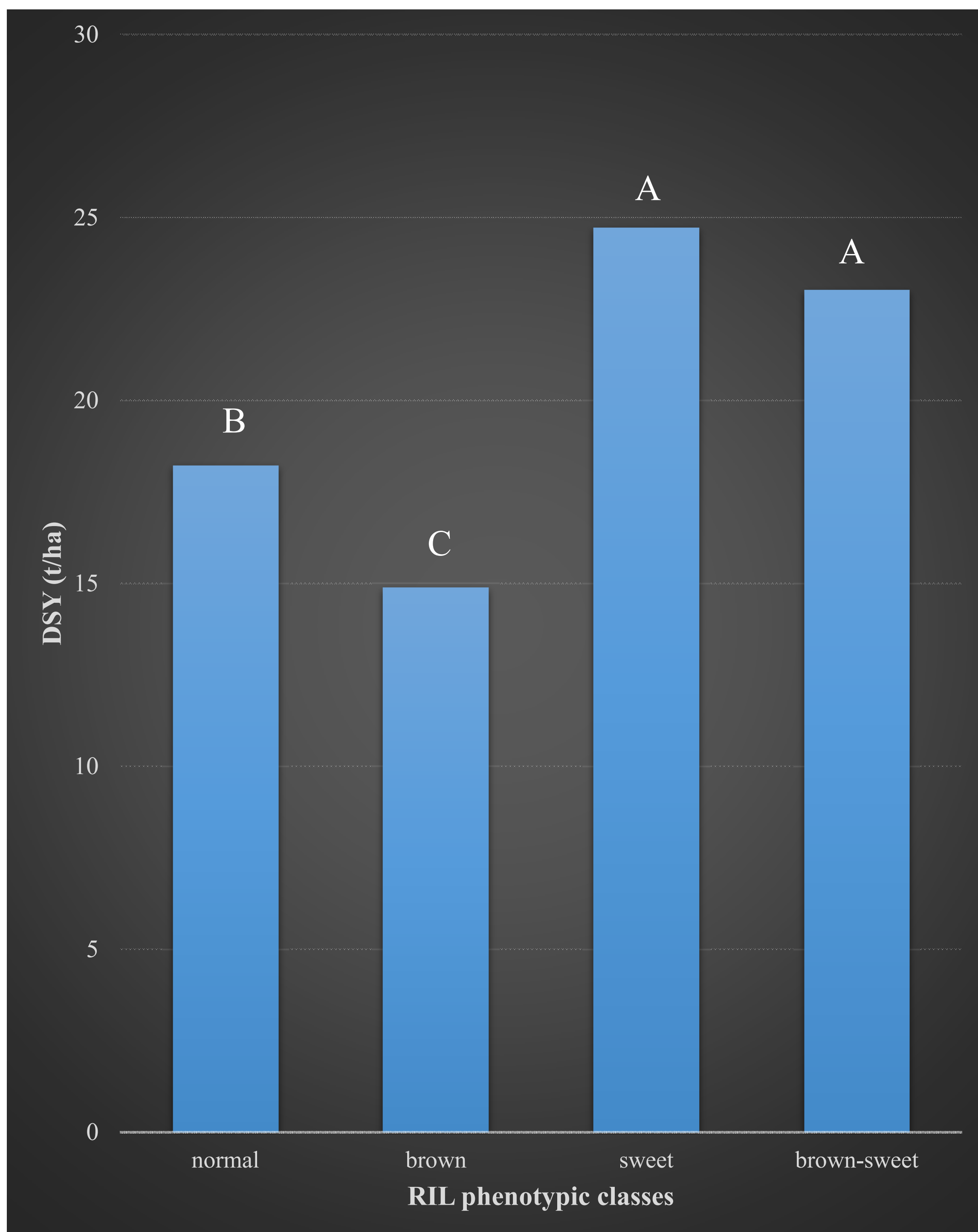


Figure 3.3. Mean dry stover yield (DSY) among four different RIL phenotypic classes. LSD (P<.05).

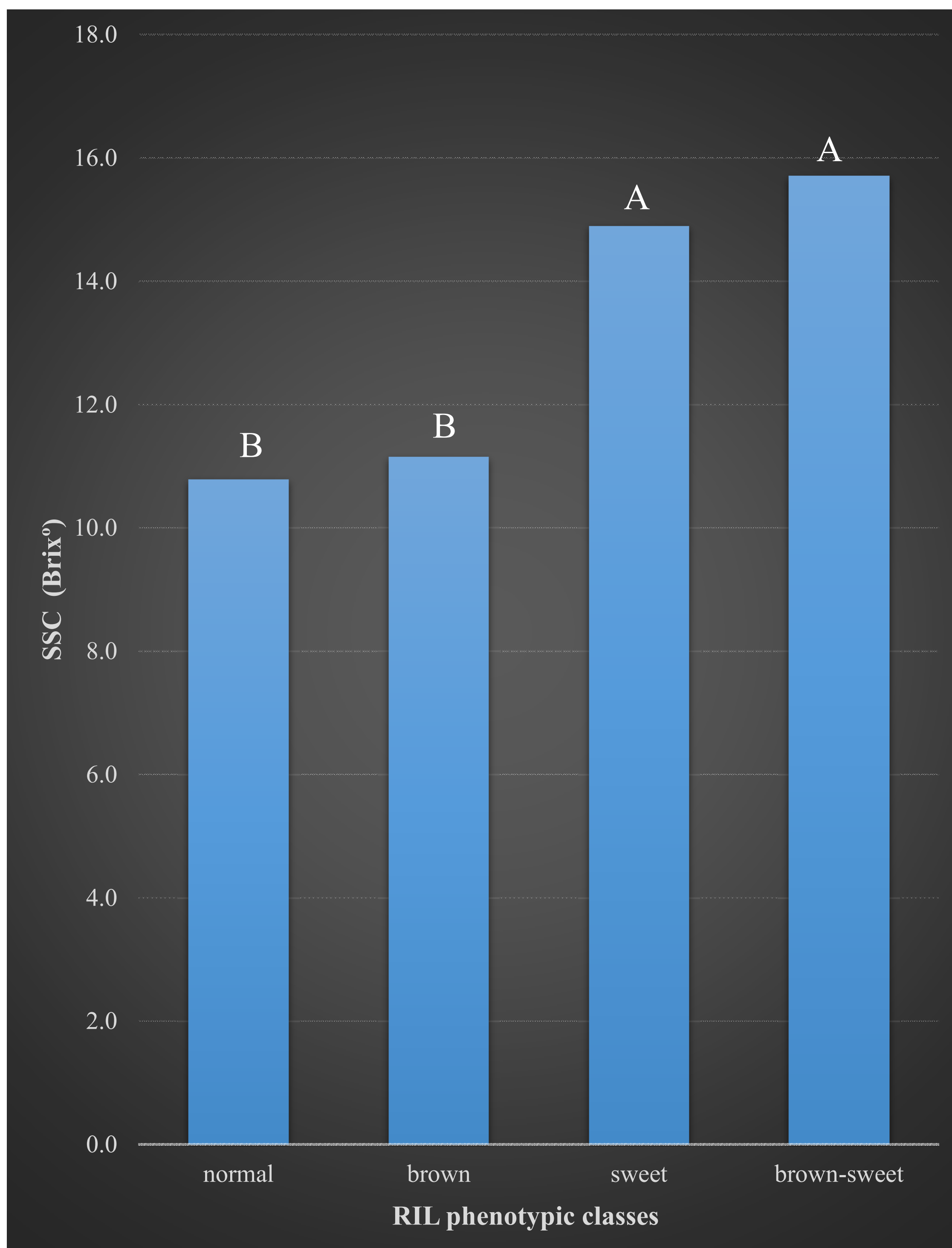


Figure 3.4 Mean stem sugar concentration (SSC) among the four different RIL phenotypic classes. LSD (P<.05).

3.4.2 Structural carbohydrates and lignin

Mean squares for cellulose, hemicellulose and lignin concentrations showed significant variation among RILs. Variation due to Year and the Year×RIL interaction were significant only for cellulose and lignin content. Hemicellulose content variation was not significantly affected by environmental factors (Year and Year×RIL).

The mean comparison among phenotypic groups for cellulose content (Table 3.5) showed that the "brown" and the "normal" groups produced significantly higher amounts of cellulose in comparison to the "sweet" and the "brown-sweet" groups. This suggests that more soluble stem sugars came at a cost of decreased cellulose. Hemicellulose was less affected, significantly lower only in the "sweet" group relative to the others (Figure 3.6). The most striking difference among the groups was observed in lignin concentration (Figure 3.7). Here, the brown midrib members of the RIL population, those in the "brown" and the "brown-sweet" RIL groups, showed significantly lower stover lignin concentrations (around 25g/kg) than the other two groups (around 40 g/kg). This represents 1.6 times less lignin in the stover of the brown midrib RILs relative to the others, presumably making the other structural carbohydrates more available to enzymatic hydrolysis.

3.4.3 Glucose recovery

The combined ANOVA for glucose recovery among the brown midrib × sweet sorghum RILs are presented in Table 3.2. RILs showed significant effects on glucose recovery estimates. This means that glucose recovered from at least one RILs after enzymatic hydrolysis was significantly higher than the others. Figure 3.8 shows mean comparisons

among RILs, and the parents of the RIL population, bmr12 and Brown County. To consider the effects of reduced lignin on glucose recovery specifically, RILs were grouped according to whether they had brown midribs (those previously grouped in the "brown" and "brown-sweet" RIL groups) and those without brown midribs (the "normal" and "sweet" RILs). The brown midrib parent of the population, line bmr12, and the brown midrib RILs showed the greatest glucose recovery. This supports the assumption that lower lignin content exposes more structural carbohydrates, like cellulose and hemicellulose, to the process of enzymatic hydrolysis, where they are converted to fermentable sugars. When averaged together, the brown midrib RILs appear to yield less glucose than the donor of the low lignin mutation they carry (bmr12), suggesting that background differences among recombinant lines that received the gene for low lignin through genetic segregation. Hence, there were individual RILs in this grouping that would be expected to yield more glucose after enzymatic hydrolysis than even bmr12. Brown County (the sweet sorghum parent without brown midribs) and the normal (non-brown) RIL group showed significantly less glucose recovery than those lines carrying the brown midrib mutation.

Table 3.2. - Combined year ANOVA for compositional traits and glucose recovery of brown midrib sweet RILs population.

Source of variation	df	Mean Square			
		Cellulose	Hemicellulose	Lignin	Glucose Recovery
Year	1	774561 *	128096	46428 *	8904 *
RIL	234	1348 **	703 **	264 **	1715 **
brown-sweet vs normal	1	6203 *	79	25934	-
browns-weet vs sweet	1	4216	11238	29021	-
brown-sweet vs brown	1	4221 *	1506	65 **	-
Year×RIL	234	613 *	326	67 **	500 **
Error	467	487	321	36	265

Cellulose = Cellulose concentration (g/kg), Hemicellulose = Hemicellulose concentration (g/kg), Lignin = Lignin concentration (g/kg) and Glucose recovery = theoretical glucose concentration after enzymatic hydrolysis (g/kg). * Significant at 0.05 probability level; ** significant at 0.01 probability level.

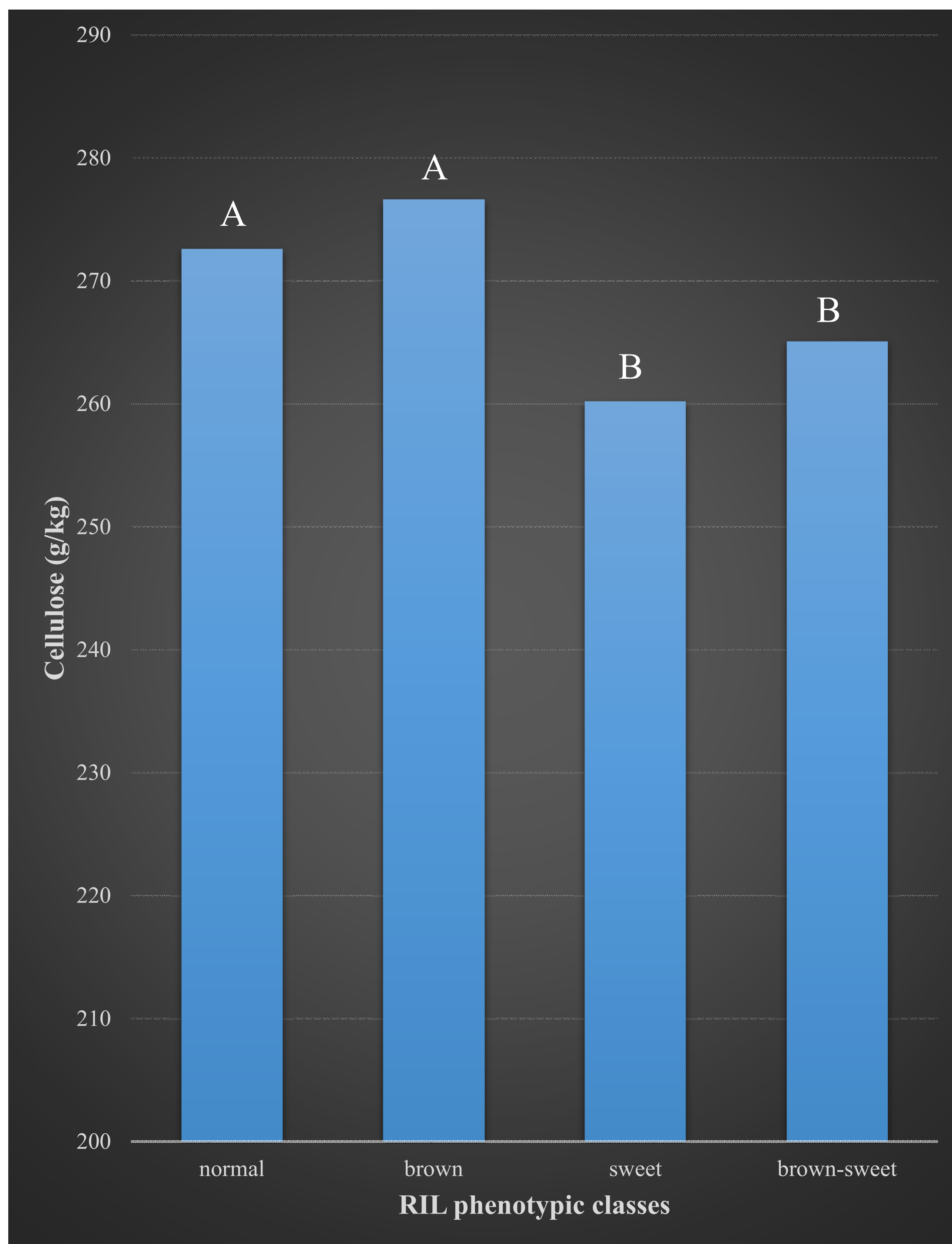


Figure 3.5. Mean cellulose concentration among four different RIL phenotypic classes. LSD (P<.05).

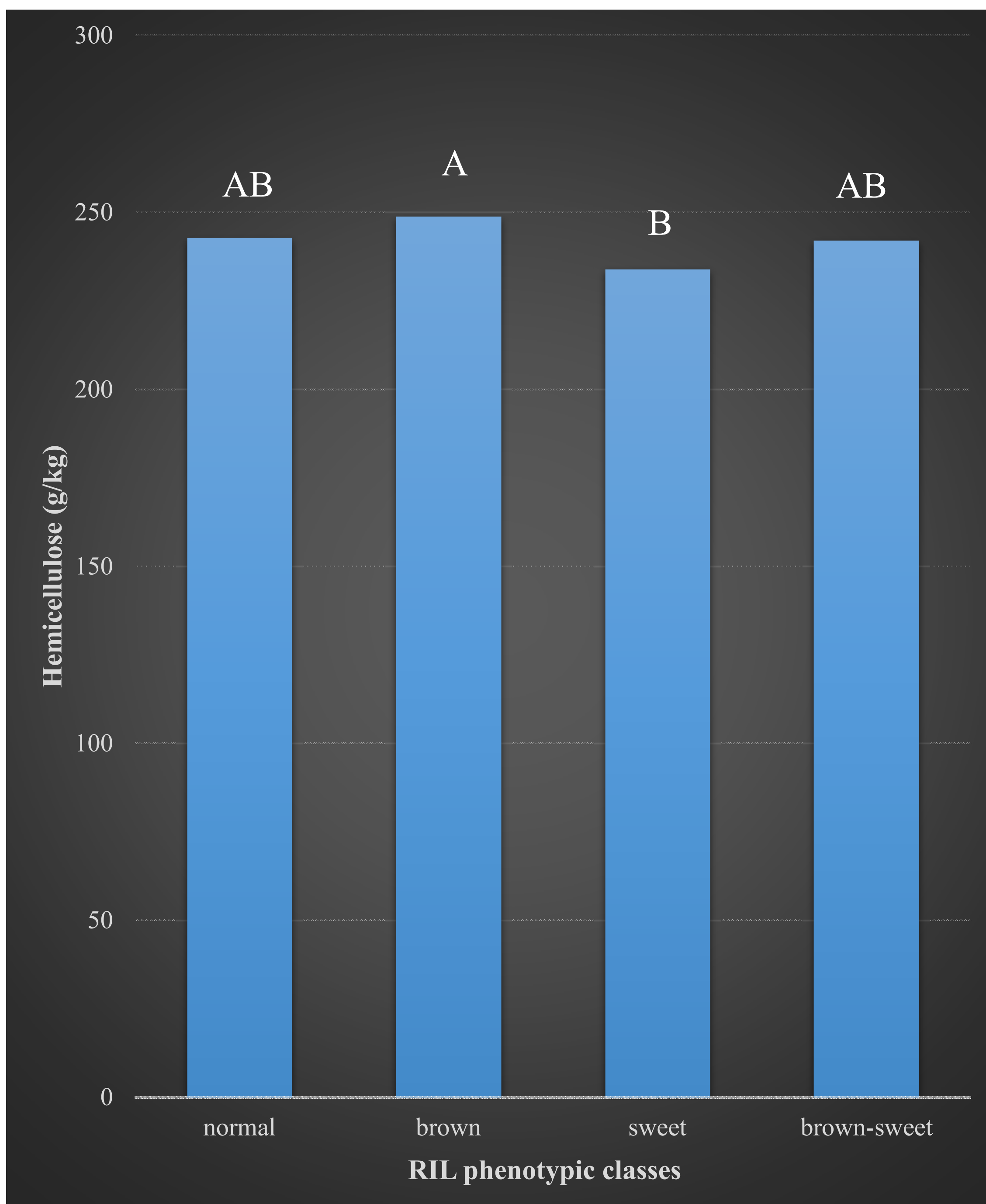


Figure 3.6. Mean hemicellulose concentration among four different RIL phenotypic classes. LSD (P<.05).

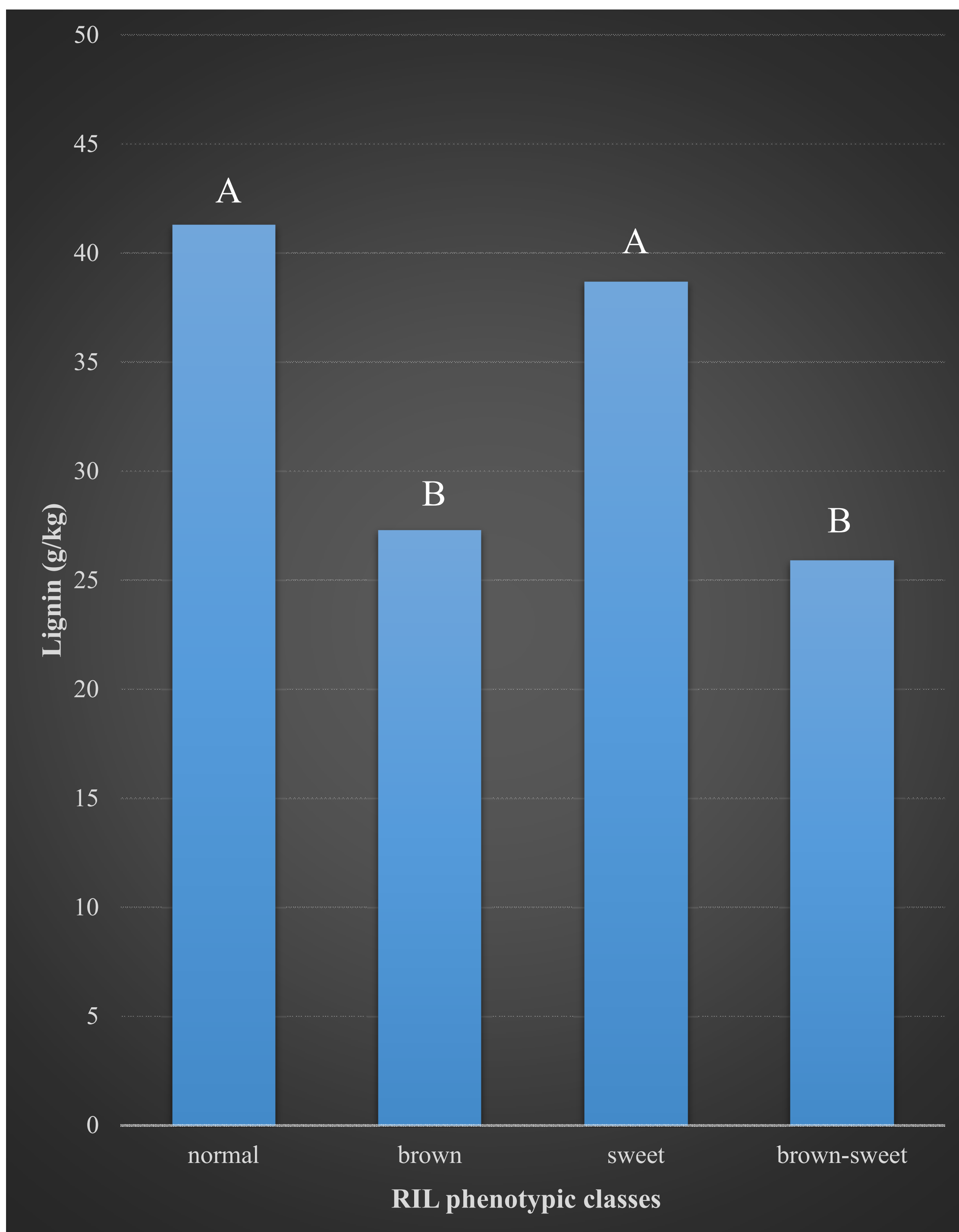


Figure 3.7. Mean lignin concentration among four RIL phenotypic classes. LSD (P<.05).

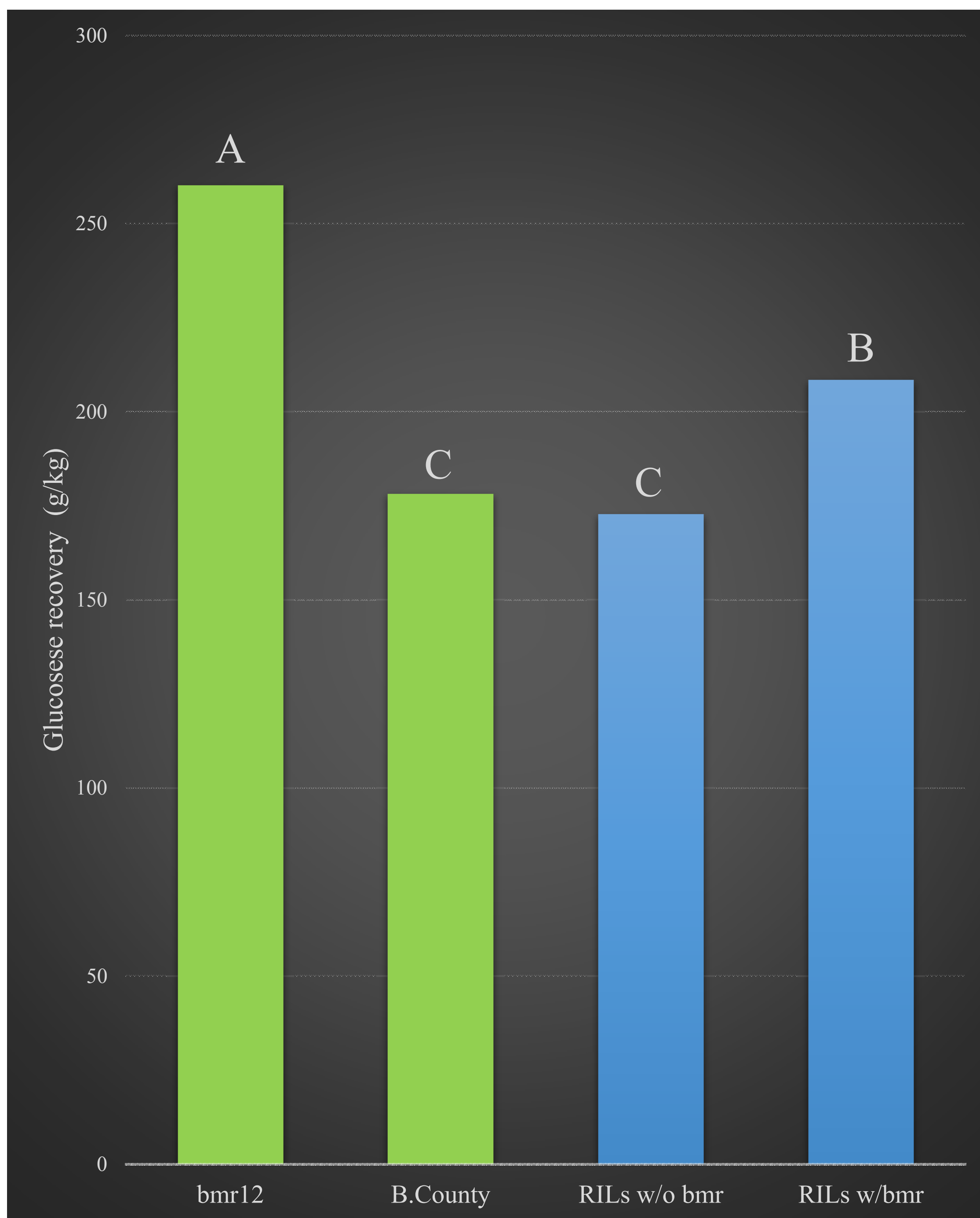


Figure 3.8. Mean glucose recovery among the brown midrib parent, bmr12, the sweet parent, Brown County (green bars) and the RILs with or without brown midribs (blue bars) RILs w/o bmr = RILs without brown midribs, RILs w/bmr = RILs with brown midribs. LSD (P<.05).

Glucose recovery of individual RILs of the brown midrib × sweet population plotted against lignin content of individual RILs shows that cell wall lignin content is negatively associated with glucose recovery (Figure 3.9). Sixty-seven percent of the variability in glucose recovery could be explained by this linear correlation with lignin concentration of the stover among the RILs of this population. This also supports the hypothesis that structural carbohydrates, like cellulose, are more readily available to digestive processes when less lignin is there to bind them (Dien et al., 2009).

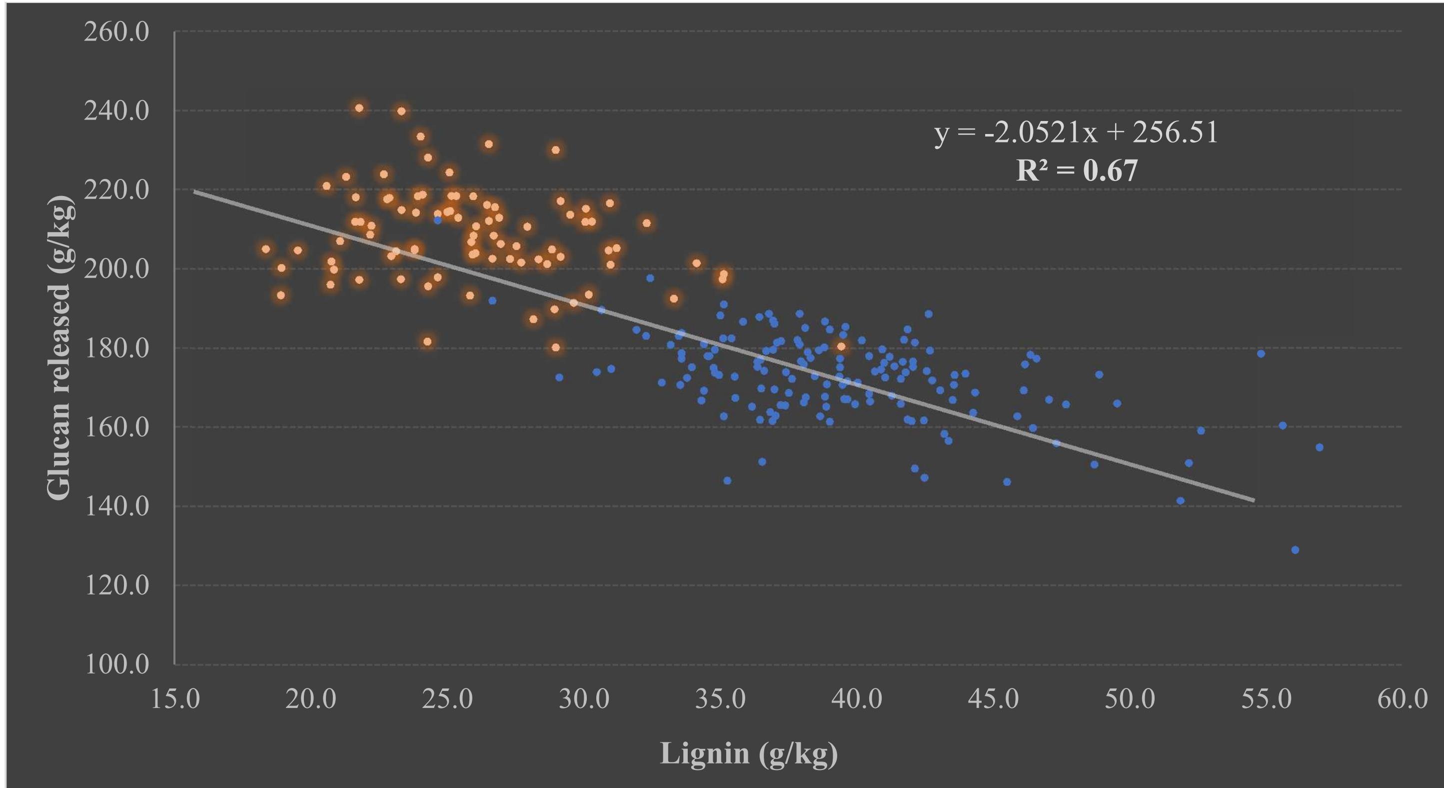


Figure 3.9. Scatter plot of glucose recovery against lignin content measured from stover samples of individual RILs averaged over both years. Data points appearing as orange circles represent RILs with brown midribs (individuals earlier assigned to the “brown” and “brown-sweet” groups), those as blue dots are RILs without brown midribs (“normal” + “sweet” groups).

3.4.4 Theoretical ethanol yield

Theoretical ethanol yield is a function of the amount of structural carbohydrates (cellulose and hemicellulose) and non-structural sugars (sucrose, fructose and soluble glucose) present per unit of lignocellulosic biomass that is ultimately available for fermentation. Table 3.3 shows the combined ANOVA of theoretical ethanol yield estimated for RILs in the three linear comparisons of the "brown-sweet" group, comprised of those RILs having both brown midribs and a high stem sugar concentration (Brix $\geq$ 12) with each of the other groups ("brown", "sweet" and "normal"). Significant differences were observed between the RIL groups in these comparisons, indicating that at least one RIL group is capable of producing significantly higher amounts of ethanol than the other in the pair, based on stover compositional traits. Within these pairwise comparisons, only two linear combinations were also significant for theoretical ethanol yield estimates. The "brown-sweet" RIL group is capable to yielding significant higher amounts of ethanol than the "normal" and "sweet" RILs, but not significantly more than the "brown" group of RILs.

Figure 3.10 shows theoretical ethanol yield mean comparison among four RILs groups. The Brown County and bmr12 lines were included in the mean comparison analysis as checks. The double mutant RIL group ("brown-sweet") ranked first, yielding an average amount of 403 liters of ethanol per ton sorghum lignocellulosic biomass. This high ethanol yield was possible because theoretically these types of sorghums not only have more sugars in their stems, carbohydrates that do not require hydrolysis before being fermented, but they also have increased availability to hydrolysis of the structural carbohydrates, cellulose and hemicellulose, due to reductions in lignin relative to non-brown midrib RILs. No

significant differences were observed between theoretical ethanol yields of the single mutant RIL groups ("brown" and "sweet"). Enhanced ethanol yields would be expected from brown midrib sorghums because their reduced lignin content exposes the structural carbohydrates to the processes of hydrolysis that break the polymers cellulose and hemicellulose into easily fermentable residues. On the other hand, sweet sorghums, by virtue of having more ready-to-ferment sugars already present at increased amounts, at least in the stem portion of the stover, would have higher theoretical ethanol yields relative to non-sweet sorghums. In fact, both biomass quality mutations (sweet, and low lignin) do give significantly higher theoretical ethanol yields than the sorghum lines without either mutation ("normal" RILs, 355L/T), but the "brown" group (383L/T) more so than the "sweet" (370L/T). This is perhaps not too surprising considering the effects of each mutation on overall availability of fermentable carbohydrates in the plant. While the sweet mutation causes more sugars to accumulate, carbohydrates which are immediately available to fermentation, this accumulation only occurs in one part of the plant, the stem. The brown midrib mutation affects every plant part, the reduced lignin exposing the greater structural carbohydrates, cellulose and hemicellulose, components of every cell wall, to the processes of hydrolysis. While these structural carbohydrates require the extra step of the cellulosic polymers being broken into sugar residues before fermentation can occur, there are so many more of these per plant than what sugars accumulated in the stem of a sweet sorghum, that the ethanol yield of the overall process is more benefitted by the mutation more generally expressed throughout the plant. Each mutant group carries only one mutation, whether low lignin or stem sugar; therefore, while the "brown" RIL group had better glucose recovery, the "sweet" RIL group produced considerable amounts of soluble

sugars in stems. Although the “brown” RIL group yielded a similar amount of ethanol in comparison to the “sweet” RIL group, the “brown” RIL group was capable of yielding higher amounts of ethanol than the “normal” RIL group (383 and 355L/T, respectively). Then, when we compare independently single mutations for lignocellulosic biomass enhancement, the low lignin mutation had a more significant effect on ethanol yield than stem sugar mutation because no significant differences were observed between “sweet” and “normal” RIL groups (370 and 355L/T, respectively). Finally, bmr12, the brown midrib low lignin mutant parent line, yielded a similar amount of ethanol as the “brown” and “sweet” RIL groups. Brown County, the sweet sorghum mutant parent line, yielded a similar amount of ethanol as the “sweet” and “normal” RIL groups. Clearly, the combination of both mutations tends to maximize biomass ethanol yield. However, when considered separately, the low lignin mutation enhances biomass conversion even more than the stem sugar mutation, a result also previously reported in other studies (Badger, 2002).

3.4.5 Theoretical ethanol production

Similar to theoretical ethanol yield, theoretical ethanol production is also a function of structural and non-structural carbohydrates present in lignocellulosic biomass. However, this variable also accounts for biomass productivity, reflected in dry stover and fresh stover yields. In the combined ANOVA of theoretical ethanol production (Table 3.3), there were significant differences among the RILs. Within RILs, the linear combinations “brown-sweet” vs. “normal” and “brown-sweet” vs. “brown” showed significant differences. This

shows that, on average the "brown-sweet" RIL groups produce significantly higher amounts of ethanol than the "normal" and the "brown" RIL plots.

Figure 3.11 shows mean comparisons among four different RILs groups and two checks, bmr12 and Brown County for theoretical ethanol production. The "brown-sweet" RIL group and the "sweet" RIL group produced 14,325 and 14,048 liters of ethanol per hectare, respectively, and not significantly different from each other. The parental check, Brown County with high sugar, but normal lignin, produced 11,996 L/Ha ethanol, significantly less than the "brown-sweet" and "sweet" RILs groups. These values were significantly higher than those yields of bmr12 and the "normal" RIL group (9,333 and 9,043L/Ha, respectively). Differences in ethanol production between the "normal" and "brown" RIL groups were not significantly (9,043 and 8,022L/Ha, respectively) from each other, and these two RIL groups did not produce as much ethanol as the "brown-sweet" and the "sweet" groups. The "brown-sweet" and the "sweet" RIL lines are therefore the superior yielders within this RIL sorghum population.

Table 3.3. Combined ANOVA of theoretical ethanol yield and theoretical ethanol production.

		Mean Square			
SOV	DF	ETOH Yield		ETOH production	
Year	1	102467		3.663	*
RIL	235	2316	**	0.080	**
brown-sweet vs Normal	1	272193	*	4.343	*
browns-sweet vs sweet	1	195583	*	0.013	
brown-sweet vs brown	1	12956		2.036	**
Year×RIL	235	667	**	0.012	**
Error	470	442		0.009	

ETOH yield = theoretical ethanol yield (L/T) and ETOH production = theoretical ethanol production (L/Ha). *P-value is less than 0.05 and **P-value is less than 0.01

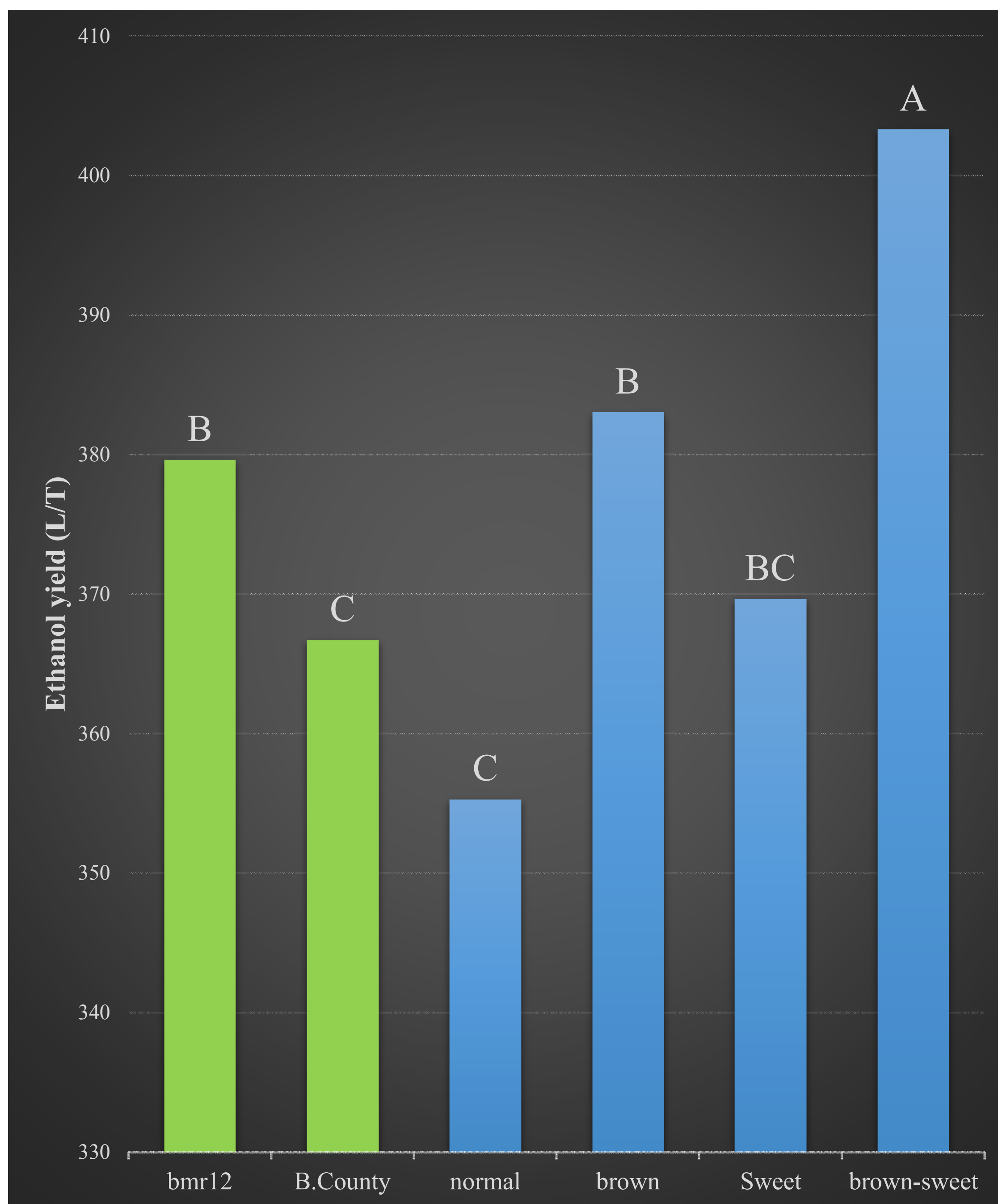


Figure 3.10. Mean theoretical ethanol yield (L/T) among the brown midrib, bmr12, and sweet, Brown County, parents (green bars) and four RIL phenotypic classes (blue bars). LSD (P<.05).

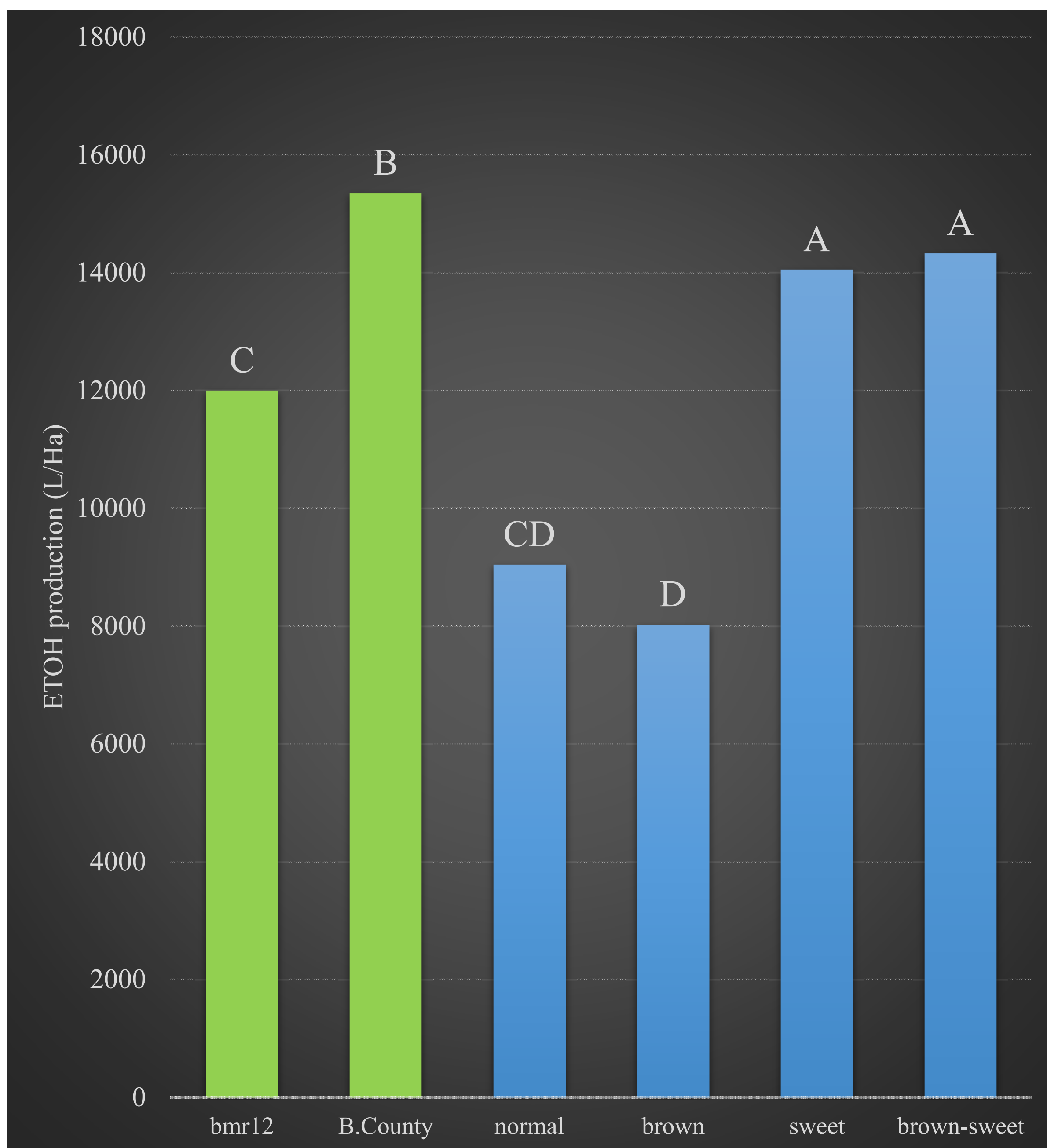


Figure 3.11. Mean theoretical ethanol production (L/Ha) among the brown midrib, bmr12, and sweet, Brown County, parents (green bars) and four RIL phenotypic classes (blue bars). LSD (P<.05).

3.4.6 Ethanol predictors

3.4.6.1 Glucose recovery predictors

After performing simple linear regression analysis, three biomass chemical components were found significantly associated to glucose recovery. Table 3.4 shows these three possible predictors for glucose recovery in the brown-midrib × sweet sorghum population as a whole (all 236 RILs). Lignin, hemicellulose and cellulose explained 66, 11 and 8% of the variation in glucose recovery, respectively. Lignin content, therefore represented the best predictor of glucose recovery in our brown-midrib × sweet sorghum population overall.

When the same analysis was applied to the RILs grouped according to whether or not they carried the two quality mutations (Table 3.4), the predictive power of other components for glucose recovery became apparent. Within the "brown-sweet" RILs group and the "brown" RILs group, cellulose explained 42% and 77% of the total variation in glucose recovery, respectively. Lignin content within these groups, of course, did not vary greatly since they all contained the brown midrib mutation and so all had generally reduced lignin with respect the non-brown members of the population. Therefore, the contributions of the other predictors in these two groups are unmasked. Acid detergent fiber (ADF), a compound measure of lignin and cellulose, and neutral detergent fiber (NDF), a compound measure of cellulose, hemicellulose and lignin, also explained some of the variation in glucose recovery for these two groups carrying the low lignin mutation, explaining 24 and 20% of the variation in glucose recovery in the "brown-sweet" group, respectively and 67 and 54% in the "brown" group. Within the "sweet" and "normal" RIL groups, lignin

explained 32% of the variation in glucose recovery in both groups. This reflects the presence of background variation in lignin content among “normal” lines not carrying the brown midrib mutation, although, this variation was not as great as when comparing to the brown midrib lines that carry a mutation for lignin production. The variation in lignin content within these groups was enough, however, to show even here that lignin content is an excellent predictor of glucose recovery, the only significant one within the “normal” group and the major one within the “sweet” group. In the latter group, cellulose and hemicellulose were also highly significant predictors of glucose recovery at 15 and 13%, respectively, with less significant determinants being NDF (8%) and ADF (4%).

Table 3.4. – Glucose recovery predictors (*y*)

Entire sorghum RIL population

Equation	*R^2*	*Probability*
$y = 256.9 – 2.06$ lignin	**0.66**	**<0.0001**
$y = 64.8 + 0.51$ hemicellulose	0.11	<0.0001
$y = 102.2 + 0.32$ cellulose	0.08	<0.0001

"brown-sweet" group

Equation	*R^2*	
$y = 82.45 + 0.47$ cellulose	**0.42**	**
$y = 116.9 + 0.31$ ADF	0.24	**
$y = 104.9 + 0.19$ NDF	0.20	**
$y = 234.0 - 1.00$ lignin	0.12	*
$y = 140.03 + 0.28$ hemicellulose	0.07	*

"sweet" group

Equation	*R^2*	
$y = 223.0 - 1.31$ lignin	**0.32**	**
y = 104.3 + 0.26 cellulose	0.15	**
y = 94.5 + 0.33 hemicellulose	0.13	**
y = 111.7 + 0.11 NDF	0.08	*
y = 139.6 + 0.11 ADF	0.04	*

"brown" group

Equation	*R^2*	
y = 68.76 + 0.52 cellulose	**0.77**	**
y = 78.8 + 0.44 ADF	0.67	**
y = 55.5 + 0.29 NDF	0.54	*

"normal" group

Equation	*R^2*	
y = 214.3 - 0.99 lignin	**0.32**	**

NDF=neutral detergent fiber, ADF=acid detergent fiber, ** Significance at 0.01; * significance at 0.05

3.4.6.2 Ethanol yield predictors

Ethanol yield shows a slightly different trend when compared to glucose recovery, though lignin content still emerges as a major predictor. Included here were plot Brix measurements that indicate stem sugar concentration (SSC) representing the contribution from the juice (Figure 3.1) and not just the glucose that is recovered from digestion of the bagasse. As in glucose recovery, considered over all RILs and regardless of whether they carry either of the quality mutations, lignin (46%), hemicellulose (17%) and cellulose (4%) content are all significant predictors of ethanol yield (Table 3.5). Stem sugar content also emerges as a major predictor of ethanol yield at 35% when considered over the entire population.

When the linear relationships between lignocellulosic biomass components and ethanol yield are considered within each RIL group (Table 3.5), other determinants become apparent. Since both lignin content and stem sugar concentration are co-confounded in the "brown-sweet" RIL group, that is, all member lines having relatively low lignin and a high stem sugar content, many suitable predictors were observed. Neutral detergent fiber (NDF), hemicellulose, cellulose, and acid detergent fiber (ADF) explained 48, 46, 45 and 37% of the variation in ethanol yield in this group, respectively. Interestingly, even stem sugar concentration explained 34% of the variation in ethanol yield, reflecting the high variation of Brix measurements among these "brown-sweet" lines grouped here because their stem sugar concentrations exceeded 12ºBrix. This reflects the more complex genetics of the sweet mutation compared to that of the brown midrib mutation. Ethanol yields in this group carrying both biomass quality mutations was highest in the population, its yields

enhanced by both increased ready-to-ferment sugars in the stems and structural carbohydrate (cellulose and hemicellulose) more available to hydrolysis, and ultimately to fermentation, due to reduced lignin content.

Within the “sweet” and “normal” RIL groups, stem sugar concentration (18% and 24%, respectively) emerges as a significant predictor of ethanol yield. Here again, there was enough variation among the members lines in Brix measurements to see associations with ethanol yield. This was also true for lignin content, even though neither group contained individuals with brown midribs. Lignin content was still a significant determinant, its variation predicting 6% of the ethanol yield among the “sweet” RILs and 22% among the “normal” RILs. The major predictor of ethanol yield in the “sweet” group was hemicellulose content at 47%. Other significant predictors within the “sweet” group were NDF, cellulose and ADF explaining 27, 21 and 11% of the variation in ethanol yield, respectively. No significant associations were obtain within the “brown” RIL group.

Table 3.5 Ethanol yield predictors (*y*)

Entire sorghum RIL population

Equation	R^2	*Probability*
y = 447.6 - 2.01 lignin	**0.46**	**<0.0001**
y = 291.2 + 6.04 SSC	**0.35**	**<0.0001**
y = 199.61 + 0.75 hemicellulose	0.17	<0.0001
y = 309.5 + 0.26 cellulose	0.04	0.0024

"brown-sweet" group

Equation	R^2	
y = 192.6 + 0.40 NDF	**0.48**	**
y = 177.17 + 0.93 hemicellulose	**0.46**	**
y = 233.5 + 0.64 cellulose	**0.45**	**
y = 255.23 + 0.51 ADF	**0.37**	**
y = 310 + 5.9 SSC	**0.34**	**

"sweet" group

Equation	R^2	
y = 176.5 + 0.83 hemicellulose	**0.47**	**
y = 227.8 + 0.27 NDF	0.27	**
y = 266.81 + 0.40 cellulose	0.21	**
y = 299.4 + 4.7 SSC	0.18	**
y = 298.3 + 0.23 ADF	0.11	**
y = 399.75 - 0.78 lignin	0.06	*

"brown" group

Equation	R^2
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"normal" group

Equation	R^2	
y **= 314.34 + 3.45 SSC**	**0.24**	**
y **= 406.34 - 1.29 lignin**	**0.22**	**

NDF=neutral detergent fiber, ADF=acid detergent fiber, SSC=stem sugar concentration (Brix), **significance at 0.01; * significance at 0.05

3.4.6.3 Ethanol production predictors

Ethanol production is highly dependent on the quantity of biomass that is used as feedstock. In the previous linear associations (Tables 3.3, 3.4 and 3.5) quality parameters were primarily considered, based on fiber analysis which were highly influenced by whether the brown midrib and sweet mutations were present. Here, the quantity parameters of fresh and dry stover yield were considered along with the quality parameters and how variations in those were associated with ethanol production. Table 3.6 shows significant linear associations of ethanol production with dry stover yield (DSY) in t/ha, fresh stover yield (FSY) in t/ha and stem sugar concentration (SSC) measured in ºBrix for all 236 RILs of the brown-midrib × sweet sorghum population. When considered together, without grouping based on presence or absence of the brown midrib and sweet mutations, dry stover yield and fresh stover yield, explained most of the total variation for ethanol production, each accounting for 89%. This means that biomass quantity is the most important determinant of ethanol production. There is also a strong association, though less than half of the stover yield measures, of the biomass quality factor, stem sugar concentration which explained 38% of ethanol production variation over the entire population.

The biomass quality parameters (dry and fresh stover yield) were also the major predictors of ethanol yield when the population was analyzed in groups based on presence or absence of the sweet and brown midrib mutations. In all groups, these two quantity measures predicted 82 to 95% of the ethanol yield. In all but the “brown” group, variation in stem sugar concentration was significantly and positively correlated with ethanol production.

One other quality parameter, hemicellulose, showed up as a predictor for ethanol production in the “normal” group, accounting for 10% of the variation.

Table 3.6. - Ethanol production predictors (y)

Entire sorghum brown midrib × sweet RIL population

Equation	R^2	*Probability*
y = -353.75 + 605.58 DSY	**0.89**	**<0.0001**
y = -1722.29 + 188.51 FSY	**0.89**	**<0.0001**
y = -1736.25 + 1082.08 SSC	**0.38**	**<0.0001**

“brown-sweet group

Equation	R^2	
y = -294.3 + 653.5 DSY	**0.94**	**
y = -310.8 + 181.94 FSY	**0.91**	**
y = -4547.2 + 1258.8 SSC	**0.24**	**

“sweet” group

Equation	R^2	
y = 860.10 + 547.5 DSY	**0.93**	**
y = -869.2 + 177.67 FSY	**0.88**	**
y = 692.24 + 949.3 SSC	0.12	**

“brown” group

Equation	R^2	
y = -490.3 + 592.6 DSY	**0.95**	**
y = 648.9 + 139.2 FSY	**0.87**	**

“normal” group

Equation	R^2	
y = -855 + 158.9 FSY	**0.84**	**
y = 690.9 + 479 DSY	**0.82**	**
y = 4347 + 480.8 SSC	**0.18**	**
y = 23252 - 55.7 hemicellulose	0.10	*

FSY=fresh stover yield (t/ha), DSY=dry stover yield (t/ha), SSC=stem sugar concentration (Brix); **significance at 0.01; * significance at 0.05

3.5 Discussion

Biomass conversion is the key process required to produce ethanol as source of renewable energy. Over the last decade, the industrial sector has focused on improving this process by designing new methodologies to efficiently hydrolyze and ferment lignocellulosic biomass (Ragauskas et al., 2006, Wang and Shengdong 2010). However, to reach significant bioconversion efficiency, products such as sulfuric acid and genetically engineered microbes capable of breaking down hemicellulose and cellulose to fermentable sugars are required in large amounts (Chung et al., 2014). These extra inputs can lead to an incremental increase in ethanol price as well as generate chemical and biohazardous pollutants. The landscape production of a genetically enhanced lignocellulosic biomass would help to improve bioconversion efficiency required by the bio-refineries. Traits that enhance biomass quality such as the brown midrib and sweet mutations, as well as traits contributing to increased biomass quantity per unit land can improve ethanol production, driving down cost without harmful environmental effects. In this study, glucose recovery estimates, theoretical ethanol production and theoretical ethanol yield of an improved lignocellulosic biomass were assessed in a genetically enhanced sorghum population. The biomass conversion approach of an enhanced brown midrib sweet sorghum feedstock offers higher levels of two sources of carbohydrates, soluble carbohydrates and structural carbohydrates, to increase ethanol yield.

Based on the results, fresh stover yield, dry stover yield and stem sugar concentration showed significant variation among genotypes and the interaction between genotype and environment (genotype×environment). Mean comparisons of grouped RILs showed significant differences in fresh stover yield, dry stover yield and stem sugar concentration

between the RIL groups with the sweet mutation (“sweet” and “brown-sweet”) and the RIL groups without the sweet mutation (“brown” and “normal”). In addition to their high stem sugar concentrations, RILs with the sweet mutation showed the highest fresh and dry stover yields in the population. Furthermore, while brown midrib plants (“brown” group) are typically smaller than those of other RILs, when the brown midrib mutation is combined with the sweet mutation, as in the “brown-sweet” group, the biomass quantity deficiencies associated with the brown midrib mutation appear to be compensated for.

Variation observed in cellulose, hemicellulose, lignin and glucose recovery estimates was mainly due to RIL and the interaction Year×RIL. Therefore, ethanol yield of the enhanced lignocellulosic biomass is going to depend on genotype of the feedstock and its interaction with its growing season (Year). Those RILs lacking the sweet mutation, those in the “normal” and the “brown” groups, had significantly higher cellulose contents than RILs with the sweet mutation, those in the “sweet” and the “brown-sweet” groups. Similarly, the “sweet” RIL group showed significantly less hemicellulose than the “brown” RIL group, though this deficiency was not significant against the “normal” and the “brown-sweet” RIL groups. In contrast, the RILs without the brown midrib mutation, “normal” and “sweet” groups, showed significantly higher lignin contents in comparison to the RILs with the brown midrib mutation (“brown” and “brown-sweet”). The groups were clearly separated based on lignin content confirming the lignin reducing effect of the brown midrib mutation. Based on the negative effect of high concentration of lignin on biomass conversion to ethanol, it is expected that the “brown” and the “brown-sweet” sorghum will yield more ethanol. Although the average glucose recovery of the RILs with the brown midrib mutation was not significantly higher than the donor of the brown midrib trait, bmr12, this

group showed significantly higher estimates of glucose recovery in comparison to the sweet parent, Brown County and the RILs without the brown midrib mutation. The ability of the *COMT* gene mutation to reduce lignin concentration in lignocellulosic biomass showed positive effects towards the improvement of biomass conversion efficiency at the population level, consistent with similar studies (Dien et al., 2006; Anderson et al., 2009; Vogler et al., 2009). This result is even more evident when glucose recovery is expressed as a function of lignin content in lignocellulosic biomass of the brown midrib × sweet sorghum population. Almost 70% of the variation in glucose recovery was explained by the variation in lignin content, a strong negative association reported by others investigating brown midrib sorghums (Dien et al., 2009). On average, the RILs with the brown midrib mutation averaged 26g of lignin per kg of lignocellulosic biomass with a glucose recovery of 208.4g. Those RILs without brown midribs averaged 39.5g lignin per kg lignocellulosic biomass and from that 172.7g of glucose were recovered (Appendix Figure B.3). Brown midrib RILs SSD#16-7130 and SSD#16-7093 showed the highest glucose recovery estimates of 240.6 and 239.8, respectively. In this population, 67 RILs, all carrying the brown midrib mutation, were capable of yielding above 200g/kg glucose upon hydrolysis of their lignocellulosic biomass. This trait then clearly improves the quality of lignocellulosic biomass in terms of glucose recovery per unit biomass in this population.

Estimates of theoretical ethanol yield (volume of ethanol per unit biomass) varied mainly due to RIL (genotype) and the interaction Year×RIL effects. Keeping in mind that ethanol yield is the volume of ethanol expressed per unit of biomass, the introduction of quality traits such as sweet and brown midrib would be expected to show their effect in this

parameter. In the orthogonal contrasts , variation in ethanol yields of “brown-sweet” vs “normal” and “brown-sweet” vs “sweet” groups, showed significant differences, though the contrast “brown-sweet” vs. “brown” did not show significant differences. However, in the mean comparisons these latter groups were clearly and significantly different. Consistent with our hypothesis, by combining the sweet and brown midrib traits into a single line, thereby increasing the soluble non-structural carbohydrates (sugars in the stems) and exposing the structural carbohydrates through reduced lignin in the entire biomass to hydrolysis, ethanol yields are maximized. This study showed that the “brown-sweet” RIL group, obtained significantly higher estimates of theoretical ethanol yield in comparison to the other RIL groups. Double mutant RILs are enhanced through two sources of carbohydrates for conversion to ethanol. Hydrolysis of the structural carbohydrates, such as hemicellulose and cellulose into fermentable sugars were enhanced by the presence of the brown midrib mutation that encodes caffeic acid-*O*-methyltransferase (*COMT*) in this RIL group (Bout and Vermerris 2003; Saballos et al., 2008; Sattler et al., 2012). The *COMT* gene has a major impact during the biosynthesis of lignin; therefore, the mutation of this gene reduces the concentration of lignin in stover, making cellulose more available to be hydrolyzed to glucose (Palmer et al., 2008; Saballos et al., 2009). The other source of carbohydrates is present in the stem juice of these RILs. Sucrose, a soluble non-structural carbohydrate, is a disaccharide produced in high concentrations in the stem juice of “brown-sweet” and “sweet” RIL groups. The presence of this second source of carbohydrates happened by the introduction of the stem sugar mutation in these RILs (Ritter et al., 2008; Murray et al., 2008). Then, by simultaneous saccharification –fermentation, sucrose is converted to ethanol. Theoretical ethanol yields

of the single mutant RIL groups “brown” and “sweet” were generally higher than the “normal” group (RILs without mutations). This is because, the “brown” RILs have a lower lignin concentration that enhance biomass conversion and the sweet mutation causes higher concentrations of soluble carbohydrates which are immediately available to simultaneous saccharification-fermentation. In our study, as in others, combination of these quality traits significantly enhance ethanol yields (Dien at al., 2009; Han et al., 2013).

The ability to produce large amounts of lignocellulosic biomass per unit area is an additional desirable agronomic characteristic that is reflected in measures of ethanol production. While ethanol production, measured in volume of ethanol per unit area, may be impacted by biomass quality traits like the sweet and brown midrib mutations, it is mainly influenced by biomass quantity (Vogler et al., 2009; Han et al., 2013). Similar to theoretical ethanol yield, theoretical ethanol production analysis of variance revealed significant effects in theoretical ethanol production due to RIL (genotype) and the interaction Year×RIL. In mean comparisons of grouped RILs for ethanol production the “brown-sweet” RILs group and the “sweet” RIL groups were superior to the others, producing roughly 14 thousand liters of lignocellulosic ethanol per hectare. This is likely much more due to the superior biomass quantity characteristic of the sweet sorghums which tend to be tall plants with thick stems and more leaves than non-sweet sorghums (Pederson et al., 2005). This was true of the sweet members of our RIL population as well, which can be seen in the higher values for plant height, stem thickness, dry stover yield and dry total biomass yield from the RILs of the “sweet” and “brown-sweet” groups presented in Chapter 2 and from the significantly higher fresh stover yield (Figure 2.2) and dry stover yield (Figure 2.3) of these RILs than those of the other groups.

RILs in the "brown" group showed the lowest mean theoretical ethanol production. This contrasts to their superior ranking in terms of theoretical ethanol yield. As we saw in Chapter 2, the "brown" group tended to have the shortest plants, with the thinnest stems of all the RILs. This translated into the lowest dry stover yields, dry total biomass yields and fresh stover yields among all the other groups of RILs in the population.

This demonstrates a very key point when considering biomass traits and improvement of feedstock for ethanol. Based on data from this study, one can argue that the most important factor in determining ethanol *production* is biomass quantity. So traits that contribute to plant size, such as tall leafy plants with thicker stems, that contribute to production of more total biomass per plot, are the most likely to increase ethanol production. Biomass quality traits, like the brown midrib mutation that exposes structural carbohydrates to hydrolysis, or the sweet mutation that increases the ready-to-ferment sugar concentration of the raw plants, traits that yield more ethanol per unit biomass, will show their contribution to feedstock improvement at the level of ethanol *yield*. From a breeding perspective initially, selection for biomass quantity traits would tend to contribute to improved ethanol productivity the most. However, as one reaches the upper genetic and agronomic limits of biomass production for a crop like sorghum, quality traits that improve the efficiency by which the biomass is converted to ethanol become important.

This point is illustrated in the various predictors for glucose recovery, ethanol yield and ethanol production. The biomass quality mutation brown midrib, causing ubiquitous low lignin content, can have a huge impact on glucose recovery from the bagasse and, mainly as a result of this, on ethanol yield. When considered over the entire population, lignin concentration emerged as the best predictor of these parameters. That lignin content as

highly negatively correlated with glucose recovery after hydrolysis is evident. The biomass quality mutation sweet causing high stem sugar concentrations, impacted both ethanol yield and production. Along with lignin concentration, stem sugar concentration was a good predictor of ethanol yield because, when considered per unit of biomass, these qualities determine that portion of the biomass that through the processes of digestion and fermentation become ethanol.

Considering ethanol production, that is, the volume of alcohol produced per unit of crop area, the predictive power of the biomass quality parameter lignin concentration drops away completely. Not only does this support the hypothesis that biomass quantity is the major determinant for ethanol production, it also reflects the positive association of lignin concentration with biomass quantity traits. The brown midrib RILs, at least the non-sweet ones carrying only this mutation, had the lowest fresh and dry stover yields. Although lignin interferes with hydrolysis of structural carbohydrates during the first step of biomass conversion, lignin also serves critical physiological functions for vascular plants and lower amounts of it affect agronomic performance. Lignin is required for vascular elements to transport water under negative pressures and in severely lignin deficient plants, vascular collapse has been observed (Piquemal et al., 1998; Jones et al., 1998; Ruel et al., 2009; Shoemaker and Bransby 2010).

The biomass quality sweet mutation, by contrast, is positively associated with biomass quantity traits. RILs carrying the sweet mutation had the highest dry and fresh stover yields. Also, as we saw in Chapter 1, plants carrying the sweet mutation (those of the "sweet" and "brown-sweet" groups) were among the tallest), had the thickest stems, with the highest dry stover and dry total biomass yields. Because of this positive association between stem

sugar concentration and biomass quantity traits, stem sugar concentration showed as a fairly good predictor of ethanol production in the population as a whole, and even, to a lesser extent, in all group comparisons, except the "brown" RIL group. The positive effect of the sweet mutation on biomass quantity parameters that it even compensated for the deleterious effects of the brown midrib mutation is evident by the superior biomass quantities measured in the "brown-sweet" RILs relative to other groupings.

Compared to other lignocellulosic and stem juice bioenergy crops, the brown midrib-sweet sorghum lignocellulosic biomass had twice the amount of ethanol production. Miscanthus, sugar beet, maize, rice, wheat, sugar cane, sweet sorghum, and forage sorghum produce no more than 6500 liters of ethanol per ha (FAO 2008). This ethanol is produced from both structural carbohydrates (bagasse) or soluble carbohydrates (stem juice) (Anderson et al., 2009; Nelson et al., 2011). The efficient utilization of two sources of carbohydrates to produce ethanol from this genetically improved lignocellulosic biomass offers an attractive added value to farmers and industry (Badger 2002; Masarin et al., 2011). The results of this study showed evidence of the importance of the brown midrib and sweet sorghum lignocellulosic biomass quality and quantity factors influencing ethanol production at an industrial scale (Moller at al., 2005; Wu 2008; Wang and Zhu 2010).

3.6 Conclusion

With the eventual target of this study of decreasing the cost of producing lignocellulosic based ethanol such that it is competitive with gasoline and starch-based ethanol, this work shows promising results. Sorghum can be a highly productive feedstock provided that selection is exercised on biomass quantity traits during the breeding process. Once this is maximized in a sorghum field plot, biomass quality traits like brown midrib and sweet mutations can push the upper limits of ethanol productivity by making that biomass yield more ethanol per unit. As we saw in Chapter 1, gains from selection for improved biomass quantity traits can be achieved simultaneously since biomass yield parameters are positively correlated with certain plant traits like height and stem thickness. Even the sweet biomass quality trait is positively associated with most of these favorable quantity traits. The deleterious effects of the brown midrib biomass quality trait (smaller plants that tend to lodge) can be overcome in favorable combinations with the sweet mutation and those genes determining biomass quantity. This brown midrib × sweet sorghum RIL population has shown that those combinations exist and can result in a superior feedstock for maximal ethanol production.

3.7 References

Anderson WF, Dien BS, Jung H-JG, et al. (2009) Effects of forage quality and cell wall constituents of Bermuda grass on biochemical conversion to ethanol. BioEnergy Res. 3:225–237.

Badger PC (2002) Ethanol from cellulose : A general review: trends new crop new uses, Janick hipkey (eds) ASHS Press Alexandria, VA 17–21.

Canilha L, Chandel A, dos Santos-Milessi TS, Fernandes FA, da Costa Freitas WL, Almeida M, Silverio da Silva S (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 989572, 15 pages.

Chung D, Farkas J, Huddleston JR, Olivar E, Westpheling J (2012) Methylation by a unique α-class N4-cytosine methyltransferase is required for DNA transformation of Caldicellulosiruptor bescii DSM6725. PLoS One, *7*(8): e43844.

Dien B, Jung H, Vogel K, Casler M, Lamb J, Iten L, Sarath G (2006) Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass and Bioenergy 30(10):880–891.

Dien B, Jung H, Vogel K, et al. (2006) Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass and Bioenergy 30:880–891.

Dien BS, Sarath G, Pedersen JF, et al. (2009) Improved sugar conversion and ethanol yield for forage sorghum (Sorghum bicolor L. Moench) lines with reduced lignin contents. BioEnergy Res 2:153–164.

FAO (2008) The state of food and agriculture. Rome ISSN 0081-4539.

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R (2010) Hemicelluloses for fuel ethanol: A review. Bioresource Technol. 101(13):4775-800.

Han K J, Pitman W D, Kim M, Day D F, Alison W, McCormick ME, Aita G (2013) Ethanol production potential of sweet sorghum assessed using forage fiber analysis procedures. GCB Bioenergy 5:358–366.

Jančík F, Homolka P, Čermák B, Lád F (2008) Determination of indigestible neutral detergent fiber contents of grasses and its prediction from chemical composition. 2008:128–135.

Jones L, Ennos AR, Turner SR (2001) Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of Arabidopsis. Plant J 26(2):205–21.

Jung HJ, Varel VH, Weimer PJ, Ralph J (1999) Accuracy of klason lignin and acid detergent lignin methods as assessed by bomb calorimetry. J Agric. Food Chem. 47:2005–8.

Kong X, Xie J, Wu X, et al. (2005) Rapid prediction of acid detergent fiber, neutral detergent fiber, and acid detergent lignin of rice materials by near-infrared spectroscopy. J Agric. Food Chem. 53:2843–8.

Lemusa R, Brummerb EC, Burrasc CL, Moore KJ, Barkerc MF, Molstad NE (2008) Effects of nitrogen fertilization on biomass yield and quality in large fields of established switchgrass in southern Iowa, USA. Biomass and Bioenergy. Vol. 32(12):1187-1194.

Lorenz AJ, Anex RP, Isci A, et al. (2009) Forage quality and composition measurements as predictors of ethanol yield from maize (Zea mays L.) stover. Biotechnol. Biofuels 2:5.

Masarin F, Gurpilhares DB, Baffa DC, et al. (2011) Chemical composition and enzymatic digestibility of sugarcane clones selected for varied lignin content. Biotechnol. Biofuels 4:55.

Milne TA, Brennan AH; Glenn BH (1990) Sourcebook of Methods of Analysis for Biomass Conversion and Biomass Conversion Processes. SERI/SP-220-3548. Golden, CO: Solar Energy Research Institute.

Moller RM (2005) Brief on Biomass and Cellulosic Ethanol. California Research Bureau.

Murray SC, Sharma A, Rooney WL, et al. (2008) Genetic Improvement of Sorghum as a Biofuel Feedstock: I. QTL for Stem Sugar and Grain Nonstructural Carbohydrates. Crop Sci. 48:2165.

Nelson P, Hood EE, and Powell R (2011) The bioeconomy: A New Era of Products Derived from Renewable Plant-Based Feedstocks. Plant Biomass Conversion, First Edition. C John Wiley & Sons Inc.

Oliver AL, Pedersen JF, Grant RJ, Klopfenstein TJ, Jose HD (2005b) Comparative Effects of the Sorghum bmr-6 and bmr-12 Genes: II. Grain Yield, Stover Yield, and Stover Quality in Grain Sorghum. Crop Sci. 45:2240-2245.

Palmer NA, Sattler SE, Saathoff AJ, Funnell D, Pedersen JF, Sarath G (2008) Genetic background impacts soluble and cell wall-bound aromatics in brown midrib mutants of sorghum. Planta 229:115–27.

Pederson, JF, Vogel KP, and Funnell DL (2005) Impact of reduced lignin on plant fitness. Crop Sci. 45:812-819.

Piquemal J, Lapierre C, Myton K, O'Connell A, Schuch W, Grima-Pettenati J, Boudet AM (1998) Down-regulation of cinnamoyl- CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. Plant J. 13(1):71–83.

Ruel K, Berrio-Sierra J, Derikvand MM, Pollet B, Thévenin J, Lapierre C, Jouanin L, Joseleau JP (2009) Impact of CCR1 silenc- ing on the assembly of lignified secondary walls in Arabidopsis thaliana. New Phytol. 184(1):99–113.

Sattler SE, Funnell-Harris DL, Pedersen JF (2010) Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. Plant Sci. 178:229–238.

Shoemaker CA, Bransby DI (2010) The role of sorghum as a bioenergy feedstock. Sustainable alternative fuel feedstock opportunities, challenges and roadmaps for six US regions. Soil and Water Conserv. Soc., Ankeny, IA, 149-159.

Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nature reviews. Genetics.9 (6):433-443

Sun Y, Cheng J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology. 83(1):1-11.

Vogel KP, Dien BS, Jung HG, Casler MD, Masterson SD, Mitchell RB (2010) Quantifying Actual and Theoretical Ethanol Yields for Switchgrass Strains Using NIRS Analyses. BioEnergy Res. 4(2):96-110.

Vogel KP, Pedersen JF, Masterson SD, Toy JJ (1999) Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. Crop Sci. 39:276–279.

Vogler RK, Tesso TT, Johnson KD, Ejeta G (2009) The effect of allelic variation on forage quality of brown midrib sorghum mutants with reduced caffeic acid O - methyl transferase activity. African Journal of Biochemistry Research 3(3):70-76.

Wolfrum EJ, Lorenz AJ, de-Leon N (2009) Correlating detergent fiber analysis and dietary fiber analysis data for corn stover collected by NIRS. Cellulose 16:577–585.

Wang Q SZ (2010) Genetically modified lignocellulosic biomass for improvement of ethanol production. 5:3–4.

Wu M (2008) Analysis of the Efficiency of the U.S. Ethanol Industry 2007. AUSD Energy Lab - Chicago Illlinois 1–7.

Zegada-Lizarazu W, Monti A (2012) Are we ready to cultivate sweet sorghum as a bioenergy feedstock? A review on field management practices. Biomass and Bioenergy 40:1–12.

CHAPTER 4. EFFECT OF NITROGEN ON BIOMASS PERFORMANCE OF SORGHUM GENOTYPES AS POTENTIAL BIOENERGY FEEDSTOCK

4.1 Abstract

Nitrogen is one of the most limiting nutrients for sustainable cropping. The positive effect of nitrogen on biomass performance of row crops is widely acknowledged. However, the cost of nitrogen impacts the net energy value of the crop, and when applied in excess, nitrogen can lead to soil and water contamination. Sorghum is a hardy crop with great persistence in marginal environments. This crop is generally able to endure harsh environmental conditions including heat, drought, as well as low soil nutrients. Knowledge of the nitrogen use efficiency (NUE) of sorghum in biomass production is limited. The objectives of this research were to determine the response of different sorghum genotypes to nitrogen application and assess its NUE in contrast with that of a grain sorghum hybrid. Field experiments were conducted over two years with ten diverse genotypes (nine sorghums and corn) grown at four nitrogen rates (0, 67, 135 and 202 kg/ha). Data on biomass performance (grain and stover kg/ha), nitrogen concentration and carbon concentration (vegetative parts and grain) were determined. Nitrogen showed a significant effect on biomass components of all cultivars. There were significant effects of genotype ($P< 0.05$), nitrogen ($P<0.05$) and nitrogen by genotype interaction ($P< 0.05$) on grain and

lignocellulosic biomass yield. Performance of sorghum and maize hybrids was generally superior to inbred lines for dry grain yield, although some inbred lines gave high yield for dry stover yield. Grain sorghum hybrid and grain maize hybrid produced maximum grain yields across nitrogen rates. A photoperiod sensitive sorghum and sweet sorghum produced maximum dry stover yields across nitrogen rates relative to the other genotypes. Maximum grain yield was obtained at 135kg N ha^{-1}, while maximum stover yield was obtained at 67kg N ha^{-1}. Across genotypes, grain NUE ranged from 19 to 50 kg kg^{-1}, while stover NUE ranged from 31 to 125 kg kg^{-1}. Among genotypes, dual-purpose sorghum was high in grain NUE, while a sweet sorghum inbred line was high in stover NUE. In grain yield, maximum yield was again obtained by hybrid sorghums, one even exceeding the yield of the maize hybrid check. Agronomic optimum nitrogen rate (AONR) for stover was half that of grain AONR. The dual-purpose sorghum hybrid was the most consistent for biomass performance traits in AONR and NUE. In all measured traits, except grain yield, sorghum out-performed maize. This study suggests that using sorghum as feedstock for ethanol production would be more economical and more environmentally friendly than starch based ethanol production from maize.

4.2 Introduction

Nitrogen (N), an essential and very often yield limiting nutrient, has an important impact in crop growth and plant development. Total biomass yield, grain and stover yield, is responsive to N supplied during the growing season. The global reliance on N fertilizer has resulted in an estimated use of 68.7 million metric tons of N fertilizer annually on arable and permanent crop areas of developed countries (FAOSTAT 2014). Modern crop production and productivity have relied on N fertilizers as an indispensable input of cropping systems everywhere. This fact places N as one of the most important production inputs, and among major economic factors in modern agriculture worldwide. N fertilizers are costly and when they are applied in excess can lead to soil contamination.

A rapidly growing world population is generating more demand for food and energy production (United Nations, 2011). Food and energy are factors of great importance and impact on the economies of both developed and developing countries. With growing interest in bioenergy to reduce overdependence on fossil fuels, food and energy are developing deep and intricate relationships in farming and from businesses, as growth in bioenergy production represents both opportunity and risk for food security (FAO, 2012). Linking these two demands to achieve an economical and environmental equilibrium will continue to be. There is great need for increased food production in much of the developing world, even as research and pilot production of bioenergy crops intensify in the developed world that is seeking relief from reliance on non-renewable fuel sources. Yet, it has been argued that sustainable and economical bioenergy production could revitalize the agriculture sector, enhance forest and rural development and alleviate poverty by

generating jobs if proper policies are developed (Lipinsky 1978; Shoemaker and Bransby 2010; Somerville et al., 2010). Currently, bioenergy production represents 74.3% of total renewable energy production in the modern world (FAOSTAT 2014).

Sorghum (*Sorghum bicolor* L. Moench) is adapted to varied environmental and nutrient conditions. The crop has its origin in the tropic and subtropic latitudes of the world (De Wet et al., 1967; Aldrich et al., 1992; Ayana et al., 1998). The genus *Sorghum* is very diverse and all cultivated sorghums belong to *Sorghum bicolor* ssp. *bicolor,* which is divided, based on morphology, into five races (bicolor, caudatum, guinea, durra, and kafir), along with the ten intermediate races resulting from all possible inter–race crosses (Harlan and de Wet, 1972). Sorghum is the fifth most important cereal crop and is the dietary staple of more than 500 million people in 30 countries (ICRISAT, 2010). It is grown on 40 million ha in 105 countries of Africa, Asia, Oceania and the Americas. The USA, India, México, Nigeria, Sudan and Ethiopia are the major producers. Over decades, sorghum cultivars have been bred to produce high grain and high stover yields. Depending on the geographic conditions, grain sorghum yields between three and six tons of grain per hectare (ICRISAT 2010; USDA-NASS 2014; Rajulapudi 2014). By exploiting the heterosis phenomenon, breeders have been able to increase grain yield with sorghum hybrids (Jordan et al, 2003; Ben-Israel et al., 2012; Hayes and Rooney 2014). Dual-purpose sorghums are used as food for people and feed for livestock. These sorghums are specialized varieties able to produce high grain yield along with high ligno-cellulosic biomass (Brummmel and Belum 2006). By incorporating traits for stover fodder quality and quantity into high yielding grain sorghums, dual-purpose sorghums are useful for meeting multiple cropping goals (Reddy et. al., 2010; Ganesamurthy et al., 2012). Forage sorghums generally produce considerable

amounts of lignocellulosic biomass for animal consumption and for lignocellulosic ethanol production. These sorghums have been improved over decades by plant breeding focused on high stover yield. Low lignin sorghums carry a single point mutation called brown midrib (*bmr*) that leads to reduction in cell wall lignin concentration in the plant lignocellulosic biomass (McCollum, et al., 2005). This mutation improves the digestibility of cellulose and hemicellulose by ruminant mycobacteria. As we saw in Chapter 2, the *bmr* mutation has a positive impact on glucose recovery and theoretical ethanol yield (Dien et al., 2009). A low lignin sorghum hybrid carries the best characteristics of a high yielding forage sorghum with improved nutrition and digestibility. The combination of high agronomic performance to produce stover and reduced lignin in the cell wall makes these types of hybrids superior feedstock for lignocellulosic ethanol production (Dien et al., 2009). Photoperiod sensitive sorghums can produce much more biomass yield than conventional forage sorghums. The ability of these sorghums to produce enormous amounts of biomass results from their inability to flower during the long days of summer in temperate latitudes (Rooney and Aydin 1999; Morgan et al., 2002). These sorghums can reach a height of 4.6 meters and yield between 25 and 30 tons of stover per hectare (USDA-NASS, 2013). The ability to produce stem juice rich in sucrose, a readily fermentable carbohydrate, is a fantasticly attractive characteristic for lignocellulosic feedstock in sweet sorghums. These sorghums are capable of producing cellulose, hemicellulose, soluble glucose, fructose and sucrose as major organic components that could be used to produce ethanol (Martin et al., 2013; Rao et al., 2013). This pool of traits, collectively make sorghum an attractive potential bioenergy crop (Rooney et al., 2007).

Generally, dedicated bioenergy sorghums are grown in dry land conditions, where precipitation and stored soil water is inadequate for other crops (Kort et al., 1998). Similarly, the majority of soils where sorghum is planted lack essential nutrients, areas where other crops such as corn, wheat or soybean produce poorly (Cai et al., 2011). In the U.S., sorghum represents the second largest feed crop; providing an important income to farmers. Its production is primarily focused in the dry states of Nebraska, Kansas, Oklahoma and Texas (USDA-NASS, 2013). Sorghum produced in these states is mainly utilized as livestock feed and some in ethanol production. Currently, a rough estimation of 12 percent of sorghum production in the U.S. is intended for ethanol production (USDA, 2007). Bio-refineries in Florida and Louisiana prefer sweet and forage sorghums as feedstock for ethanol production. Generally sorghum has the ability to produce considerable amounts of structural and non-structural carbohydrates making the bioconversion of lingocellulosic biomass an eco-friendly process (Rooney at al., 2007; Vogel et al., 2011).

Nutrient efficiency is a key concept that could help us to create a stable link between food production, energy demand and nitrogen fertilizer dependency (Cassman et al., 2012). When plants are able to recover a considerable percentage of nitrogen fertilizer supplied (43 to 55%), the plant is said to be nitrogen use efficient (Moll et al., 1981). Nitrogen use efficiency (NUE) is defined as the ratio of grain yield to nitrogen fertilizer supplied; or as yield of grain per unit of available nitrogen in the soil (Moll et al. 1981; Good et al. 2004; Lea and Azevedo 2006; Dawson et al. 2008; Moose and Below 2008; Buah and Mwinkaara 2009). NUE has two main components: (a) nitrogen recovery efficiency (NRE), and (b) nitrogen internal efficiency (NIE) (Moll et al., 1981; Dobermann 2005; Coque and Gallais

2007). NRE represents the ability of above ground plant parts to recover nitrogen from the applied fertilizer. NRE depends on plant demands, nutrient release from nitrogen fertilizer and N available from soil organic matter. This component is affected by the fertilizer application method as well as factors that determine the size of the crop nutrient sink such as genotype, climate, plant density and abiotic/biotic stresses. NIE measures the capability of plants to transform nitrogen taken up from fertilizer into grain. Similarly to NRE, changes in NIE are attributed to factors such as genotypes, environment and management (Novoa and Loomis 1981; Henry and Raper 1989; Pandey et al. 2001, Ciampitti and Vyn 2011; Cassman et al., 2002; Dobermann 2007). The typical values of NUE in cultivated crops are no more than 50%, with an average of 30% worldwide (Moll et al., 1981; Johnson and Raun, 2003). This indicates that around half of the fertilizer N applied in crop production is partially or totally lost to the environment (Nielsen, 2006). In most cereals crops, only 33% of N fertilizer applied is recovered and utilized by plants to produce grain (Johnson and Raun, 2003). Even maize and sorghum typically show low NUE, averaging 25% (Johnson and Raun, 2003; Doberman 2007). Other cereal crops like wheat, average 40% of NUE (Raun and Johnson, 1995; 1999). These values depend on factors directly or indirectly affecting physiological processes such as nitrogen uptake and nitrogen utilization during the growing season.

Genotypic (G), environmental (E) and management (M) practices are factors responsible for changes in values of NUE in crops. Plant morphology, anatomy (e.g., leaf size, leaf thickness, chlorophyll content, internal leaf anatomy, root morphology, etc.) and physiology (e.g., gas exchange, stomatal conductance, photosynthesis, respiration rates, etc.) are features that can increase or decrease values of NUE of a crop (Novoa and Loomis,

1981; Pavlik, 1983; Field, 1983; Gardner *et al.* 1994; Muchow and Sinclair, 1994; Moose and Below, 2009). Similarly, environmental factors like low soil moisture, high temperatures, low precipitation, etc. are factors affecting root uptake and assimilation of N. When less than optimal, these environmental factors can lead to low NUE values (Dawson et al., 2008).

Moreover, management practices such as cropping system, N source, method of fertilizing, plant density, etc. play an important role during the growing season. These factors also affect NUE values (Ciampitti and Vyn, 2012). In addition to their individual effects, interactions among genotypic, environmental and management factors affect NUE, making improvement complicated.

The agronomic optimum nitrogen rate (AONR) is the nitrogen rate that will produce maximum grain yield, regardless of the cost of supplied nitrogen fertilizer. This practical concept is closely linked to maximization of total biomass (whether is grain or stover) during a regular farming season (Sawyer at al., 2006; Hoben et al., 2011; Gentry et al., 2013). However, the efficiency concept of maximizing biomass yield needs special attention because yield response to nitrogen is usually not a straight forward relationship (Hoben et al., 2011; Thomason et al., 2011). It is a common misperception among farmers that by applying more nitrogen fertilizer, grain yields increase. This rule does not work in reality. Actually, it is the first kilograms of applied nitrogen that return the best and at some level of applied nitrogen fertilizer, grain yield stops increasing. Consequently, applying more nitrogen than a plant can use wastes money and is environmentally unfriendly. Agronomic optimum nitrogen rate (AONR) and nitrogen use efficiency (NUE) are

important agronomical and physiological concepts for an effective production of renewable energy.

The objectives of this research were to (a) determine the response of sorghum and maize (hybrids and inbred lines) to nitrogen fertilizer, (b) quantify phenotypic differences in AONR and NUE between sorghum and maize and (c) report variation in plant nitrogen and carbon concentration and uptake.

4.3 Materials and Methods

4.3.1 Plant material

For this experiment, ten genotypes, nine sorghums and one maize, were selected. Four sorghum lines were used including a forage sorghum line (FSL), a low lignin sorghum line (LLSL), and two lines with potential use as dual-purpose sorghum. Three sorghum hybrids, derived from some of these lines were also used, including a low lignin sorghum hybrid (LLSH), a dual-purpose sorghum hybrid (DPSH) and a grain sorghum hybrid (GSH). One sweet sorghum (SS) and one photoperiod sensitive sorghum (PSS) were also included. The maize hybrid (GMH) used was a commercial grain hybrid (Table 4.1).

Table 4.1 Genotypic and phenotypic characteristics of nine sorghums and one corn cultivar selected for this experiment.

Genotype	**Phenotype**	**Main source of carbohydrate for ethanol production**
P915B	Forage sorghum line	Hemicellulose and cellulose
bmr27	Low lignin line	
P915A × bmr27	Low lignin hybrid	
PU216B	Dual-purpose sorghum line	Hemicellulose, cellulose and Starch
P90344	Dual-purpose sorghum line	
PU216A × P90344	Dual-purpose sorghum hybrid	
Sugar Drip	Sweet sorghum line	Hemicellulose, cellulose, soluble glucose, sucrose and fructose
Is7777	Photoperiod sensitive line	Hemicellulose and cellulose
CrosbytonA747×R50	Grain sorghum hybrid	Starch
AgriGoldAG585RR	Grain maize hybrid	Starch

4.3.2 Field experiment

The experiment was conducted at the Agronomy Center for Research and Education (ACRE) at Purdue University in West Lafayette, Indiana in 2008 and 2010 (Latitude 40.47°, Longitude -86.9912°). The selected planting area of the experiment followed a soybean rotation in both years. The total area of the experiment was 1486.4m^2 (16000ft^2) and included 160 plots. Each experimental unit (plot) had an area of 9.3 m^2 (100 ft^2). The experimental units (plots) consisted of four rows, where each row had a length of 3.048m (10ft) with a row spacing of 0.762m (2.5ft). Within each experimental unit, the two middle rows were harvested and the two lateral rows were used as borders to prevent nitrogen treatment overlapping between adjacent plots. Therefore, a harvesting area of 4.7 m^2 (50 ft^2) was used as the source of data in this experiment.

A rate of 2.5 grams per row of sorghum seed was planted at a depth of 5 cm. The seeds were previously treated with a fungicide (Captan at 0.1%) to ensure seedling emergence. The treated seeds were packaged and ordered based on the experimental design randomization which was a split plot design. Three weeks after planting, the plots were thinned to six plants per 0.31m (around 60 plants per row).

4.3.3 Experimental design

A split plot design was employed for the experiments in 2008 as well as in 2010. Two factors were considered in this design. The first factor was nitrogen application in kilograms per hectare and the second factor was genotype. Each treatment in the experiment was replicated four times. N rate was considered as main plot treatment, while genotype was considered as sub-plot splits within the N rate main plot treatments. Randomization was carried out among N rates and genotypes, within nitrogen rates, in each replication for a total number of 160 experimental units in this study (Appendix C).

4.3.4 Nitrogen treatment

Based on data available in the literature, treatments consisting of four post-seeding nitrogen rates were selected with the intention of measuring changes in biomass yield and stover chemical composition. Rates of 0, 67, 135 and 202 kg per ha of nitrogen were applied after 15 days of the planting date. Urea ammonium nitrate (28%) fertilizer solution was used as the N source in both years. Single side-dressed treatments were applied 5cm below and to the side of each row after crop emergence (15 days after planting) with a carbon dioxide pressurized system mounted on a John Deere Max Emerge 2 Conservation tillage planter. Phosphorus fertilizer solution was also placed with the starter-band-N. Potassium chloride was broadcast at each site, at a rate high enough to ensure adequate K availability.

4.3.5 Climatic conditions

A normal growing season for sorghum in Indiana, with planting generally in May and harvest in October was followed. This experiment was planted during the last week of May in both 2008 and 2010. Weather data obtained from the ACRE meteorology center at Purdue University is given in Appendix C. Average precipitation and temperature of 97.7 mm and 18.3ºC were reported for the entire growing season in 2008. In that year, maximum monthly precipitation of 151.13mm was reported in May and minimum precipitation of 45.5mm was reported in October. Additionally, maximum and minimum temperatures of 29ºC and 3.3ºC were reported for July and October, respectively. In 2010, an average precipitation and temperature of 105mm and 20ºC were reported during the growing season. The maximum monthly precipitation was 251.5mm and received in June and the minimum precipitation was 22.6mm in October. Daily maximum temperature reported in that year was 31ºC between July to August and a minimum of 3ºC in October.

4.3.6 Phenotypic data collection

Data used in this study were collected through direct field measurements, laboratory analyses, or derived through calculation from some of these same measurements. Flowering date was recorded when 50 percent of the plants in the plot had their panicles in half-bloom (Kirby and Atkins 1968). This was used to estimate plant maturity as 45 days after the flowering date; for convenience, lines were clustered into three different harvest groups: early, medium and late. At harvesting time, the number of plants from the middle two rows were counted in each plot (4.7 m^2) and this count was used to determine plant

density. From these harvested rows of each plot ten random plants were sampled, five from each row, by cutting 1cm above the soil surface. Then the panicles of these ten plants were removed and added to the total grain harvest of the two middle rows. All panicles of these two middle rows (4.7m^2) were hand harvested, and placed in dryers at 60ºC for three days and hung on racks until threshing. The panicles of the 10 sampled plants were cut at the flag leaf and saved in paper bags. The paper bags containing panicles of each plot were dried for 3 to 4 days at 45ºC. The weight of leaves and stems of the same 10 plants (without panicles) was recorded as fresh stover weight per sample plot. Fresh stover weight per sample plot was used only in the calculation of dried stover (leaves-stems) weight per sample plot (see below). Next, the ten plants (without panicles) of each plot were chopped in a tractor driven mechanical chopper, the chopped leaves and stems mixed, and a subsample of roughly one and a half fistfuls was weighed and saved in a paper bag (fresh stover subsample weight). The paper bags containing chopped subsamples of fresh stover were dried for 3 – 4 days at 60ºC, after which the dried stover subsample weight was recorded. Dried stover weight per sample plot was calculated by dividing the dried stover subsamples weight by fresh stover subsample weight and multiplying by fresh stover weight per sample plot (Murray et al., 2008b). Then, the panicles were threshed when the sorghum grain had approximately 12-14 % of moisture (McKenzie and Richey 1914). Finally, dry grain weight (kg) per sample plot data set was recorded from each plot.

Agronomical optimum nitrogen rate was determined as the maximum nitrogen rate that maximized grain, stover and total biomass yield. The estimation of the AONR was based on yield mean comparisons among the N rates (LSD $P<0.05$). When non-significant differences in yield measures were found among all of the N rates, the AONR was

estimated as zero kg of nitrogen fertilizer supplied during growing season per hectare. When significant differences in yield among the N rates were found, a threshold based on equal letter (non-significant differences among means), was used to determine the AONR. Above the threshold, the lower N rate was selected as the N rate that maximized yield; therefore, the AONR.

Change in nitrogen use efficiency (ΔNUE) was estimated as a ratio of incremental biomass yield response (biomass yield $_{\text{fertilized}}$ – biomass yield $_{\text{unfertilized}}$) to change in applied N rate from that of the control (Maranville and Madhavan 2002; Cassman et al. 2003; Nielsen, 2006; Snyder and Bruulsema, 2007; Ciampitti and Vyn 2011; Wang et al., 2014). The following equation was used for ΔNUE calculation:

$$\Delta NUE = \frac{\text{Yield}_{\text{Fert}} - \text{Yield}_{\text{UnFert}}}{\Delta\text{N applied}}$$

Where $\text{Yield}_{\text{Fert}}$ is yield per unit area (kg ha^{-1}) of a treatment at AONR, $\text{Yield}_{\text{UnFert}}$ is yield per unit area of the 0 N treatment, and ΔN applied (kg ha-1) is the quantity of N applied through N fertilizers that maximize yield (AONR). Following the same reasoning, nitrogen recovery efficiency (NRE) was calculated as:

$$\text{NRE} = \frac{\text{Nupt}_{\text{Fert}} - \text{Nupt}_{\text{UnFert}}}{\Delta\text{N applied}}$$

Where $Nupt_{fert}$ is nitrogen uptake at AONR and $Nupt_{Unfert}$ is nitrogen uptake in the corresponding unfertilized plot. The nitrogen internal efficiency was calculated as:

$$NIE = \frac{Yield_{Fert} - Yield_{UnFert}}{Nupt_{Fert} - Nupt_{UnFert}}$$

Dried grain and leaf-stem subsamples were ground to obtain a fine powder material required for the nitrogen and carbon analysis (N/C analysis). The grinding process had three critical parts. In the first part, the entire subsample from each paper bag was ground using a 6.0 mm screen. In the second part, the ground subsample was ground again but this time with a 1.0 mm screen. Finally, the twice ground subsample was mixed, and saved in small containers for further analysis. Thorough sample homogenization in the grinder stage was required to make certain that the tiny subsample taken for analysis was representative of the total sample. Poor precision can often be traced to visible granules in the sample.

The nitrogen/carbon analysis was carried out on a flash combustion elemental analyzer (Flash EA 1112 series, Thermo-Fisher Scientific, The Netherlands). Flash EA 1112 is based on the well-known Flash Dynamic Combustion method, which produces complete combustion of the sample within a high temperature reactor, followed by an accurate and precise determination of the elemental gases produced. The analytical procedure started by drying ground grain and leaf-stem subsamples at 80°C for at least 12 hours (overnight). Roughly 28-32 milligrams of plant tissue (grain or leaf-stem) subsamples were weighed into pure tin capsules using an analytical balance (Metter AE166). The ground dried subsamples were sealed into 5 x 9 mm tin capsules. In addition to the dried subsamples, a

bypass (Atropin), a blank, four calibration standards (Atropin), a certified standard reference (383 B-Corn) and two checks (Atropin and 383B-Corn) every twenty subsamples were also analyzed in each run. All samples, bypass, standards and blanks were loaded in a 50-slot auto-changer carousel. Automated analyses were controlled by Windows-based EAS Software with a multichannel 24 bit A/D interface connected to the electronic detection system in the ECA. The ECS software compares the elemental peak to the calibration standard data, and generates a report for each element on a weight basis. Total nitrogen and carbon concentration (g/ha) reports obtained from N/C analysis were used to estimate biomass nitrogen and carbon uptake (kg/ha).

4.3.7 Statistical Analysis

Three biomass performance variables, six nitrogen compositional related variables and six carbon compositional related variables were evaluated in this study. Dry grain yield, dry stover yield (leaf+stem) and dry total biomass yield (grain+stover) were estimated in kilograms per hectare after harvesting time. For N/C allocation and uptake, ground dry grain and leaf-stem subsamples were used to generate estimates of grain nitrogen concentration (g/kg), stover nitrogen concentration (g/kg), total biomass nitrogen concentration (g/kg), grain nitrogen uptake (kg/ha), stover nitrogen uptake (kg/ha), total biomass nitrogen uptake (kg/ha), grain carbon concentration (g/kg), stover carbon concentration (g/kg), total biomass carbon concentration (g/kg), grain carbon uptake (kg/ha), stover carbon uptake (kg/ha), total biomass carbon uptake (kg/ha).

The split plot designs for this experiment were analyzed using the MIXED procedure in SAS statistical software package (SAS Institute, Inc.). N rate and genotype were considered whole-plot treatments. Genotype by N rate was considered sub-plot treatments. N rate, genotype and N rate×genotype were considered fixed effects. Block and years were deemed random effects (Sweeny and Moyer, 2007).

4.4 Results

4.4.1 Biomass performance

Table 4.2 shows combined analysis of variance for dry grain yield (DGY), dry stover yield (DSY) and dry total biomass yield (DTBY). Significant differences were observed between nitrogen rates (NRate) in all biomass performance traits (DGY, DSY and DTBY). Significant differences were observed between genotypes for DGY and DSY. Interactions were also significant. The two way interaction NRate×Genotype showed significant differences for DGY and DSY, while the two way interaction Year×Genotype showed significant differences in all biomass performance traits. The year source of variation was significant for dry grain yield. Finally, the three-way interaction Year×NRate×Genotype showed significant differences only in DGY. Figure 4.1 shows evidence of NRate×Genotype interaction in dry grain yield. Within nitrogen rates, significant effects among the eight sorghums (the photoperiod sensitive sorghum was not included since it yielded no grain) and the maize were observed in dry grain yield (DGY) at all nitrogen rates (0, 67, 135 and 202 kg/ha [Appendix C.2]). Within genotypes, significant effects among the four nitrogen rates were observed in dry grain yield (DGY) for most sorghum genotypes and maize (Appendix C.3). Overall, GSH and GMH were most responsive to different nitrogen rates, producing grain yields of 9664 and 9399 kg/ha, respectively, at the maximum N rate of 202 kg/ha. The GSH had over twice the grain yield of the other varieties, even GMH at 0 N rate. This is good evidence of the grain yield stability of sorghum hybrids even under nitrogen stress conditions. The GMH drastically increased grain yield up to 135kg of nitrogen but the incremental increases were negligible in grain

yield at 202kg of nitrogen. Figure 4.2 shows Nrate×Genotype interaction in dry stover yield. Within N rates, significant effects of N among the sorghum and maize varieties were observed in dry stover yield (DSY) at four N rates (0, 67, 135 and 202 kg/ha; Appendix C.2). Within genotypes, significant effects among the four N rates were observed in dry stover yield (DSY) for all genotypes except for GSH, DPSH, DPSL1 and DPSL2 (Appendix C.3). Overall, PSS and SS showed positive responses to different N rates, producing maximum stover yields of 30505 and 23096kg/ha, respectively. The other genotypes showed low responses to N rate for this trait.

Figure 4.3 shows mean comparisons of dry grain yield (kg/ha) in eight sorghum varieties and the maize genotypes. Over all, maximum grain yield value was recorded by GSH (8371kg/ha). The minimum grain yield value was recorded by sweet sorghum (SS) (2885kg/ha). No significant differences in grain yield were observed between GSH and GMH. Similarly, no significant differences were observed among GMH, LLSH, DPSL2, DPSL1, FSL and DPSH for grain yield when averaged over all N rates. Finally, no significant differences were observed between the lowest grain yielders, LLSL and SS.

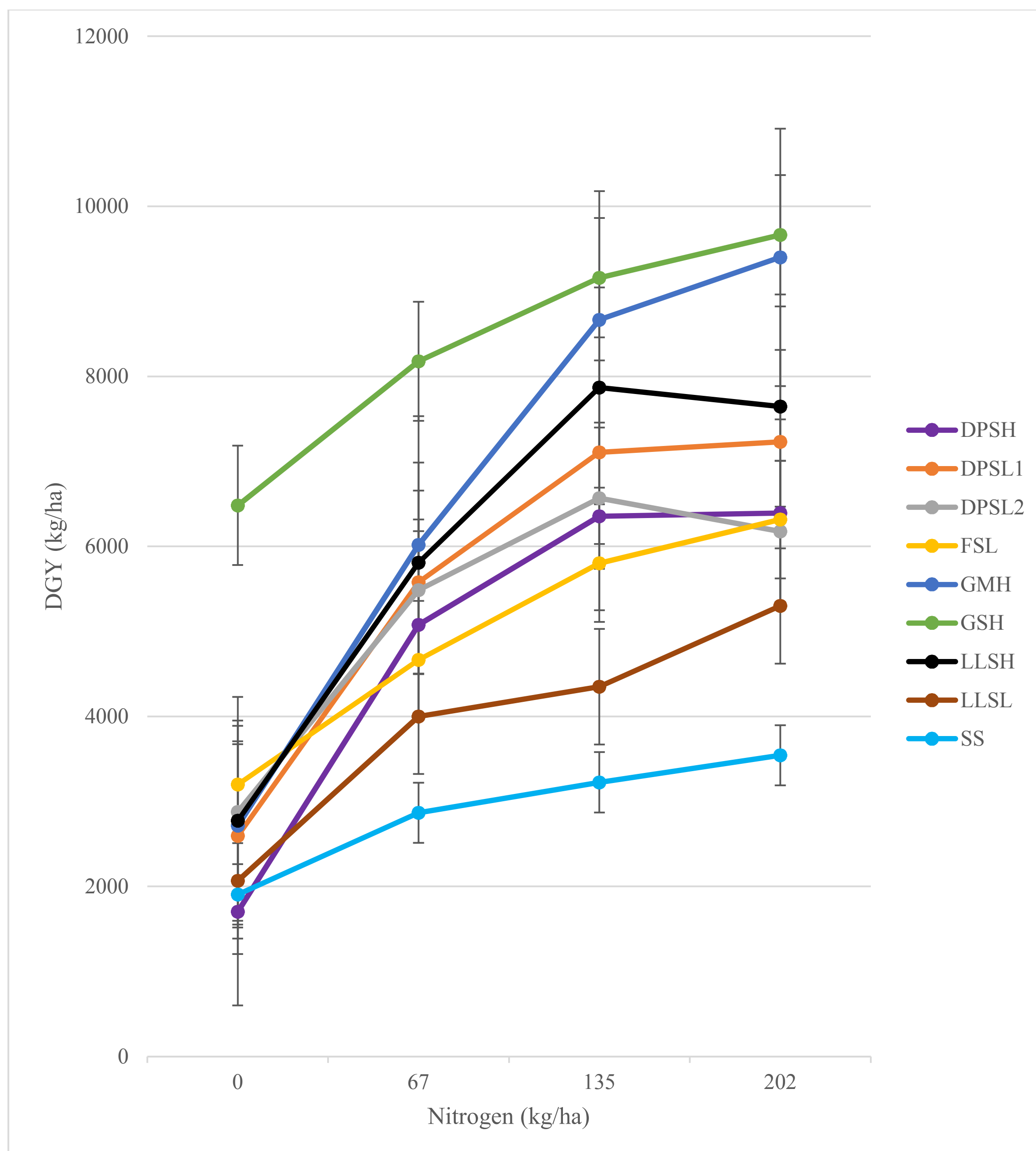
Dry stover yield (DSY) means in the nine sorghum varieties and the maize hybrid are presented in Figure 4.4. When averaged over all N rates, the maximum DSY value was achieved by PSS (25408kg/ha). The minimum DSY value was observed in GSH (8944kg/ha). No significant differences were observed between PSS and SS, however, PSS showed significant difference when compared with the other genotypes. Though the SS was the lowest grain yielder, it ranks near the top in terms of stover yield. Finally, no

significant differences were observed among DPSL2, DSPH, FSL, LLSH, DPSL1, LLSL, GMH and GSH.

Table 4.2 Combined analysis of variance of dry grain yield, dry stover yield and dry total biomass yield in nine sorghums and maize genotypes.

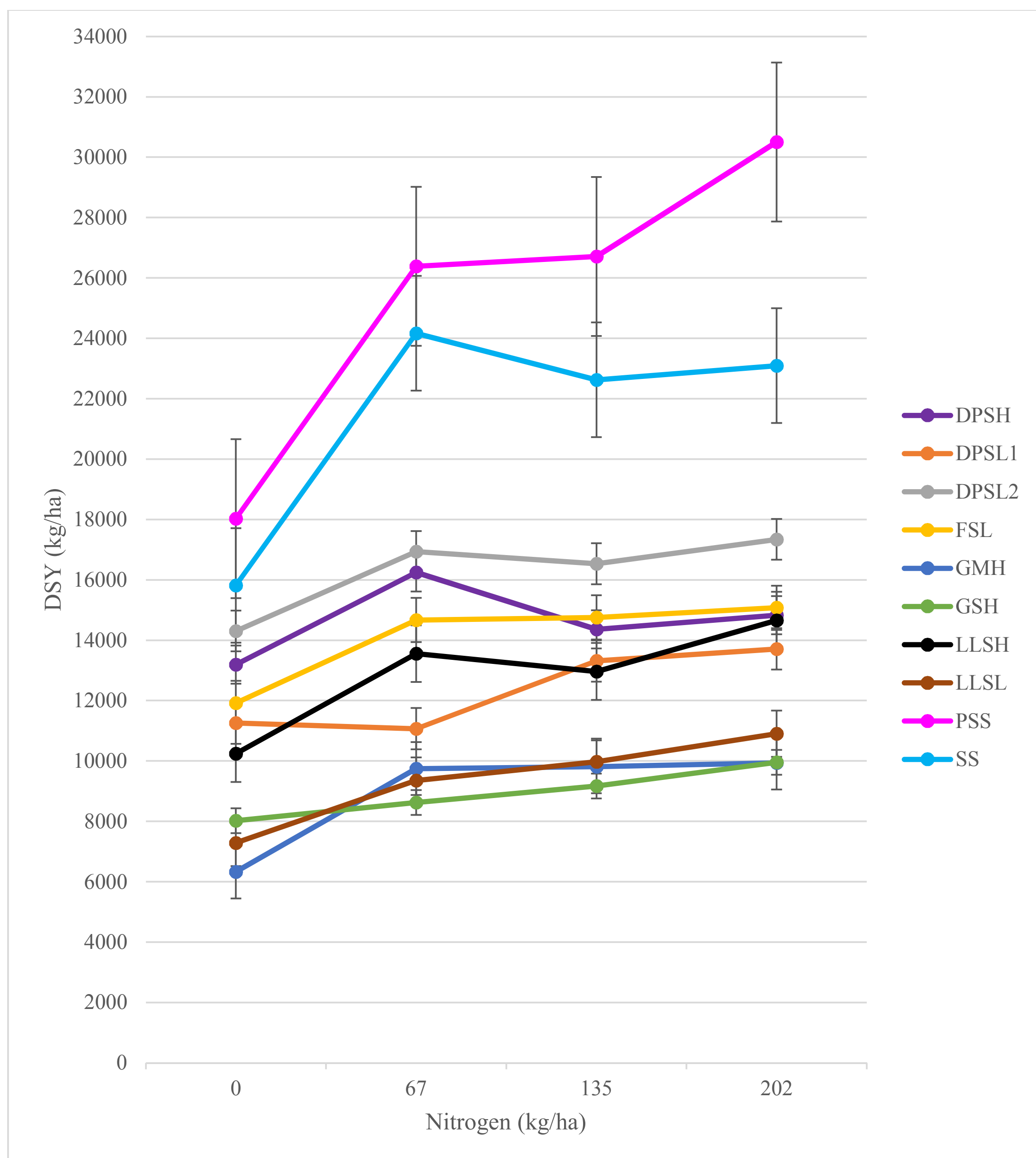
		Mean Square					
Source of variation	df	DSY		DTBY		DGY^{+}	
NRate	3	294113709	*	972748047	**	230552415	*
Genotype	9	944072981	*	436418610		79569944	**
NRate×Genotype	27	19976307	*	13012446		4381690	*
Year	1	456389803		152820179		90024717	*
Year×NRate	3	15190260		22845785		1626531	
Year×Genotype	9	225761944	***	217054225	***	11673196	***
Year×NRate×Genotype	27	8491132		11336872		2003576	***
Residual	216	12447456		13304638		576943	

DGY=dry grain yield (kg/ha), DSY=dry stover yield (kg/ha), DTBY=dry total biomass yield (kg/ha). * P-value is less than 0.05, **P-value is less than 0.01 and *** P-value is less than 0.001. $^{+}$Only 9 genotypes were evaluated for grain yield as the photoperiod sensitive sorghum did not produce grain.



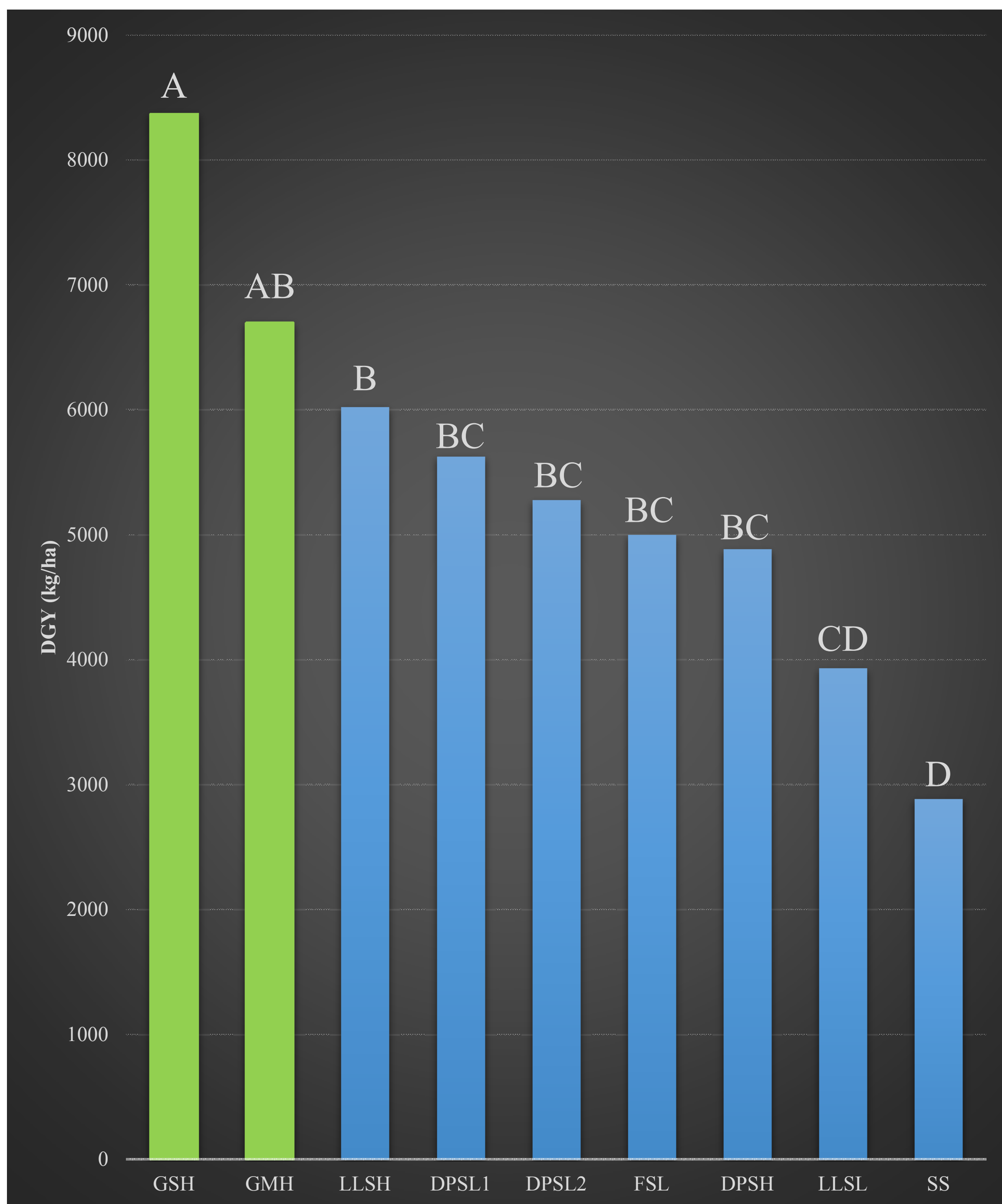
DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum. Bars represent SE.

Figure 4.1 Nitrogen by genotype interaction of dry grain yield (DGY) in 9 sorghums and maize genotypes.



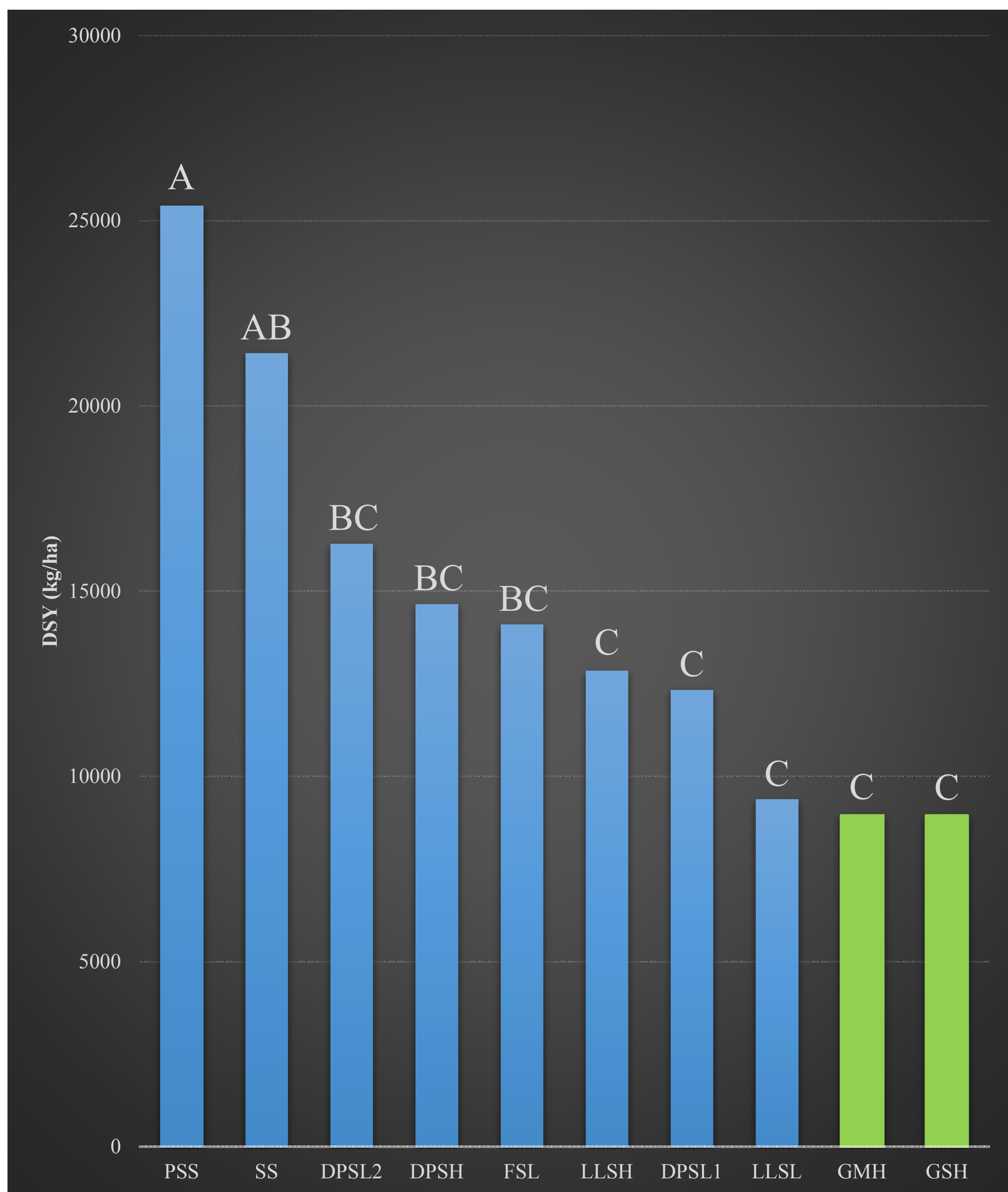
PSS=photoperiod sensitive sorghum DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum. Bars represent SE.

Figure 4.2 Nitrogen by genotype interaction of dry stover yield (DSY) in 9 sorghums and maize genotypes.



Blue bar=sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05).

Figure 4.3 Means of dry grain yield (DGY) of nine sorghums and maize genotypes.



Blue bar=sorghums genotypes, Green bar=commercial grain hybrids, PSS=photoperiod sensitive sorghum, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05).

Figure 4.4 Means of dry stover yield (DSY) of nine sorghums and maize genotypes.

4.4.2 Estimation of AONR and NUE

Estimates of grain agronomic optimum nitrogen rate (AONR) for all genotypes evaluated in this study are presented in Table 4.3. Grain AONR ranged from 67 to 135kg of nitrogen per hectare over all genotypes. Those varieties reaching an AONR of 67kg of nitrogen per hectare were LLSL, DPSL2, DPSH and SS. Dry grain yield at AONR of these genotypes ranged from 2867 to 5486kg/ha. For these genotypes, the estimated nitrogen use efficiency (NUE) ranged from 14 to 50 kg grain kg^{-1} N applied, nitrogen recovery efficiency (NRE) ranged from 0.21 to 0.59 g N uptake kg^{-1} N applied, and nitrogen internal efficiency (NIE) ranged from 67 to 88 kg grain kg^{-1} N uptake. Those varieties for which the AONR was at 135 kg of nitrogen per hectare were FSL, LLSH, DPSL1, GMH and GSH. Dry grain yield at AONR of these genotypes ranged from 5803 to 9160 kg per hectare. For these genotypes, the estimated NUE ranged from 19 to 44 kg grain kg^{-1} N applied, NRE ranged from 0.36 to 0.56 g N uptake kg^{-1} N applied, and NIE ranged from 53 to 79 kg grain kg^{-1} N uptake.

Though usually considered in terms of grain yield, it is possible to calculate AONR and NUE for stover yield as well. Estimates of stover AONR for all genotypes evaluated in this study are presented in Table 4.4. Stover AONR ranged from 0 to 202 kg of nitrogen per hectare over all genotypes. Some of the sorghum varieties, including DPSL1, DPSL2, DPSH and GSH showed an AONR of 0 kg of nitrogen per hectare because significant gains in stover yield were not observed when nitrogen was applied. With no applied nitrogen, estimates of NUE, NRE and NIE were not applicable. An AONR of 67 kg of nitrogen per hectare was obtained by FSL, LLSL, LLSH, GMH and SS. Stover yield at AONR of these genotypes ranged from 9350 to 24167kg/ha. For these genotypes, NUE estimates ranged

from 31 to 125 kg grain kg^{-1} N applied, NRE ranged from 0.19 to 0.66 g N uptake kg^{-1} N applied, and NIE ranged from 144 to 189 kg grain kg^{-1} N uptake. The AONR of the PSS was 202 kg of nitrogen per hectare because its DSY continued to show significant increases with each increase in fertilizer rate. Dry stover yield (kg/ha) at AONR of this genotype was 30505kg/ha. For this genotype, NUE estimated was 62 kg grain kg^{-1} N applied, NRE was 0.68 g N uptake kg^{-1} N applied, and NIE was 91 kg grain kg^{-1} N uptake.

When considered together, grain + stover yield measured as dry total biomass yield (DTBY), estimates of AONR for total biomass ranged from 67 to 202 kg of nitrogen per hectare over all genotypes (Table 4.5). An AONR of 67 kg of nitrogen per hectare was obtained by FSL, LLSL, LLSH, DPSL2, DPSH, SS, GMH and GSH. Total biomass yield at AONR of these genotypes ranged from 13351 to 27034 kg/ha. For these genotypes, estimated NUE ranged from 34 to 139 kg grain kg^{-1} N applied, NRE ranged from 0.55 to 0.88 g N uptake kg^{-1} N applied, and NIE ranged from 63 to 158 kg grain kg^{-1} N uptake. An AONR of 135 kg of nitrogen per hectare was obtained by DPSL1. Total biomass yield at AONR of this genotype was 20423 kg/ha. For this genotype, estimated NUE was 49 kg grain kg^{-1} N applied, NRE was 0.65 g N uptake kg^{-1} N applied, and was 75 kg grain kg^{-1} N uptake. The PSS, though it had no grain yield contributing to its DTBY, showed an AONR of 202 kg/ha of nitrogen. Total biomass yield at AONR of this genotype was 30505kg/ha. For this genotype, NUE was estimated at 62 kg grain kg^{-1} N applied, NRE was 0.68 g N uptake kg^{-1} N applied, and NIE was 91 kg grain kg^{-1} N uptake.

Table 4.3 NUE estimates of dry grain yield (kg/ha) at AONR (kg/ha) in nine sorghums and maize genotypes

Genotype	***DGY [N=0]***	***AONR***	***DGY [AONR]***	**Δ*NUE***	**S.E.**	***NRE***	**S.E.**	***NIE***	**S.E.**
Forage Sorghum Line (P915B)	3200	135	5803	19	±2.7	0.37	±0.05	53	±3.3
Low Lignin Sorghum Line (*bmr*27)	2066	67	4000	29	±4.4	0.38	±0.06	77	±3.2
Low Lignin Sorghum Hybrid (P915Ax*bmr*27)	2774	135	7868	38	±4.6	0.56	±0.05	68	±4.8
Dual-purpose Sorghum Line (PU216B)	2595	135	7107	33	±4.3	0.50	±0.07	67	±5.5
Dual-purpose Sorghum Line (P90344)	2875	67	5486	39	±8.3	0.44	±0.11	88	±9.1
Dual-purpose Sorghum Hybrid (PU216AxP90344)	1704	67	5077	50	±6.4	0.59	±0.07	86	±2.3
Sweet Sorghum (Sugar Drip)	1906	67	2867	14	±4.7	0.21	±0.06	67	±1.3
Grain Maize Hybrid (AgriGoldAG585RR)	2717	135	8665	44	±6.5	0.56	±0.05	79	±3.8
Grain Sorghum Hybrid (CrosbytonA747xR50)	6483	135	9160	20	±1.9	0.36	±0.04	55	±2.1

DGY=dry grain yield (kg/ha), AONR=agronomic optimum nitrogen rate (kg/ha), NUE=nitrogen use efficiency (kg grain kg^{-1} N applied), NRE=nitrogen recovery efficiency (kg N uptake kg^{-1} N applied) and NIR=nitrogen internal efficiency (kg grain kg^{-1} N uptake), S.E. =standard error.

Table 4.4 NUE estimates of dry stover yield (kg/ha) at AONR (kg/ha) in nine sorghums and maize genotypes.

Genotype	***DSY*** ***[N=0]***	***AONR***	***DSY*** ***[AONR]***	**Δ*NUE***	**S.E.**	***NRE***	**S.E.**	***NIE***	**S.E.**
Forage Sorghum Line (P915B)	11919	67	14670	41	±5.9	0.27	±0.03	153	±20.0
Low Lignin Sorghum Line (*bmr27*)	7288	67	9350	31	±9.6	0.19	±0.05	160	±31.3
Low Lignin Sorghum Hybrid (P915Ax*bmr27*)	10240	67	13558	50	±13.9	0.34	±0.08	144	±23.7
Dual-purpose Sorghum Line (PU216B)	11256	0	11256	-	-	-	-	-	-
Dual-purpose Sorghum Line (P90344)	14307	0	14307	-	-	-	-	-	-
Dual-purpose Sorghum Hybrid (PU216AxP90344)	13190	0	13190	-	-	-	-	-	-
Sweet Sorghum (Sugar Drip)	15816	67	24167	125	±36.9	0.66	±0.18	189	±67.5
Photoperiod Sensitive Sorghum (IS7777)	18023	202	30505	62	±12.8	0.68	±0.14	91	±27.6
Grain Maize Hybrid (AgriGoldAG585RR)	6323	67	9748	51	±9.3	0.31	±0.06	163	±20.3
Grain Sorghum Hybrid (CrosbytonA747xR50)	8020	0	8020	-	-	-	-	-	-

DGY=dry grain yield (kg/ha), AONR=agronomic optimum nitrogen rate (kg/ha), NUE=nitrogen use efficiency (kg grain kg^{-1} N applied), NRE=nitrogen recovery efficiency (kg N uptake kg^{-1} N applied) and NIR=nitrogen internal efficiency (kg grain kg^{-1} N uptake), S.E. =standard error.

Table 4.5 NUE estimates of dry total biomass yield (kg/ha) at AONR (kg/ha) in nine sorghums and maize genotypes.

Genotype	***DSY*** ***[N=0]***	***AONR***	***DSY*** ***[AONR]***	**Δ*NUE***	**S.E.**	***NRE***	**S.E.**	***NIE***	**S.E.**
Forage Sorghum Line (P915B)	15119	67	19336	63	±8.6	0.63	±0.09	100	±7.4
Low Lignin Sorghum Line (*bmr27*)	9353	67	13351	60	±11.7	0.57	±0.09	105	±16.3
Low Lignin Sorghum Hybrid (P915Ax*bmr27*)	13014	67	19366	95	±16.5	0.85	±0.11	112	±10.7
Dual-purpose Sorghum Line (PU216B)	13851	135	20423	49	±12.8	0.65	±0.11	75	±21.5
Dual-purpose Sorghum Line (P90344)	17183	67	22425	78	±25.0	0.64	±0.15	123	±24.3
Dual-purpose Sorghum Hybrid (PU216AxP90344)	14894	67	21325	96	±33.3	0.76	±0.15	126	±29.0
Sweet Sorghum (Sugar Drip)	17723	67	27034	139	±39.3	0.88	±0.23	158	±39.9
Photoperiod Sensitive Sorghum (IS7777)	18023	202	30505	62	±12.8	0.68	±0.14	91	±27.6
Grain Maize Hybrid (AgriGoldAG585RR)	9040	67	15764	100	±11.8	0.81	±0.10	124	±7.5
Grain Sorghum Hybrid (CrosbytonA747xR50)	14503	67	16805	34	±14.1	0.55	±0.13	63	±13.5

DGY=dry grain yield (kg/ha), AONR=agronomic optimum nitrogen rate (kg/ha), NUE=nitrogen use efficiency (kg grain kg^{-1} N applied), NRE=nitrogen recovery efficiency (kg N uptake kg^{-1} N applied) and NIR=nitrogen internal efficiency (kg grain kg^{-1} N uptake), S.E. =standard error.

4.4.3 Biomass nitrogen concentration and biomass nitrogen uptake

Estimates of grain nitrogen concentration (GNC), stover nitrogen concentration (SNC), and total biomass nitrogen concentration (TBNC) in gram per kilogram of grain/stover were obtained and used to determine differences in means. Similarly, estimates of grain nitrogen uptake (GNU), stover nitrogen uptake (SNU) and total biomass nitrogen uptake (TBNU) in kilograms per hectare of grain+stover were estimated and used to determine differences in means. Table 4.6 shows the combined analysis of variance for GNC, SNC, TBNC, GNU, SNU, and TBNU. Significant differences for Nrates were observed for all biomass nitrogen concentration and biomass nitrogen uptake traits. Also, significant differences for Genotypes were found in SNC, TBNC, GNU and SNU traits. Year source of variation only showed significant differences for GNU. The NRate×Genotype interaction showed significant differences in GNU, while Year×Genotype interaction showed significant differences for GNC, TBNC, SNU and TBNU, and Year×NRate showed significant difference for GNC and TBNC. The three-way interaction showed significant differences for TBNC and GNU.

Figure 4.5 shows NRate×Genotype interaction of GNU. Within each nitrogen rate, significant effects among the grain yielding sorghums and maize were observed for GNU at four different supplied N rates (0, 67, 135 and 202 kilograms of nitrogen per hectare; Appendix C.4). Within each genotype, significant effects over four N rates were observed in GNU (Appendix C.5). Overall, a positive trend in NRate×Genotype interaction was observed for GNU in all grain bearing sorghums and maize genotypes except for DPSL2. This genotype appeared to decrease GNU at rates > 135 kg/ha; however, this reduction in

GNU (kg/ha) was not significant (Appendix C.5). GSH performed consistently higher in terms of GNU than the other genotypes at all Nrates. The GSH obtained the highest estimates of GNU (128kg/ha) when 202kg of nitrogen were supplied, while the GMH obtained GNU estimates of 120 kg/ha at the same Nrate.

Figure 4.6 shows means of stover nitrogen concentration (SNC) in all nine sorghum and the maize genotypes. A maximum of 7.3g/kg and a minimum of 4.9g/kg of stover nitrogen concentration estimates were observed in the grain sorghum hybrid (GSH) and the sweet sorghum (SS), respectively. No significant differences were obtained between GSH, PSS, GMH and LLSL. These genotypes had the highest estimates of SNC of the study (7.3, 6.7, 6.5 and 6.3 grams of nitrogen per kilogram of lignocellulosic biomass, respectively). Similarly, no significant differences were observed among PSS, GMH, LLSL and LLSH. These sorghums accumulated 6.7, 6.5, 6.3 and 5.8 grams of nitrogen per kilogram of ligno-cellulosic biomass, respectively. No significant differences were observed among LLSH, DPSL1, FSL, DPSL2, DPSH and SS. These sorghums accumulated from 4.9 to 5.8 grams of nitrogen per kilogram of stover (lignocellulosic biomass). Overall, GSH, PSS and GMH, accumulated significantly more nitrogen in their lignocellulosic biomass than the other sorghum genotypes. The two commercial grain hybrids (GSH and GMH) accumulated more nitrogen (g/kg) in their stover than the other two sorghum hybrids (LLSH and DPSH).

Figure 4.7 shows means of total biomass nitrogen concentration (TBNC) from the nine sorghum and the maize genotypes. A maximum of 10.3 and a minimum of 6.7 of total biomass nitrogen concentration estimates (g/kg) were obtained by FSL and PSS, respectively. No significant differences were observed over all genotypes, except for

photoperiod sensitive sorghum (PSS). This sorghum genotype accumulated one and a half times less nitrogen in total plant biomass (6.7 g/kg) because it does not produce grain.

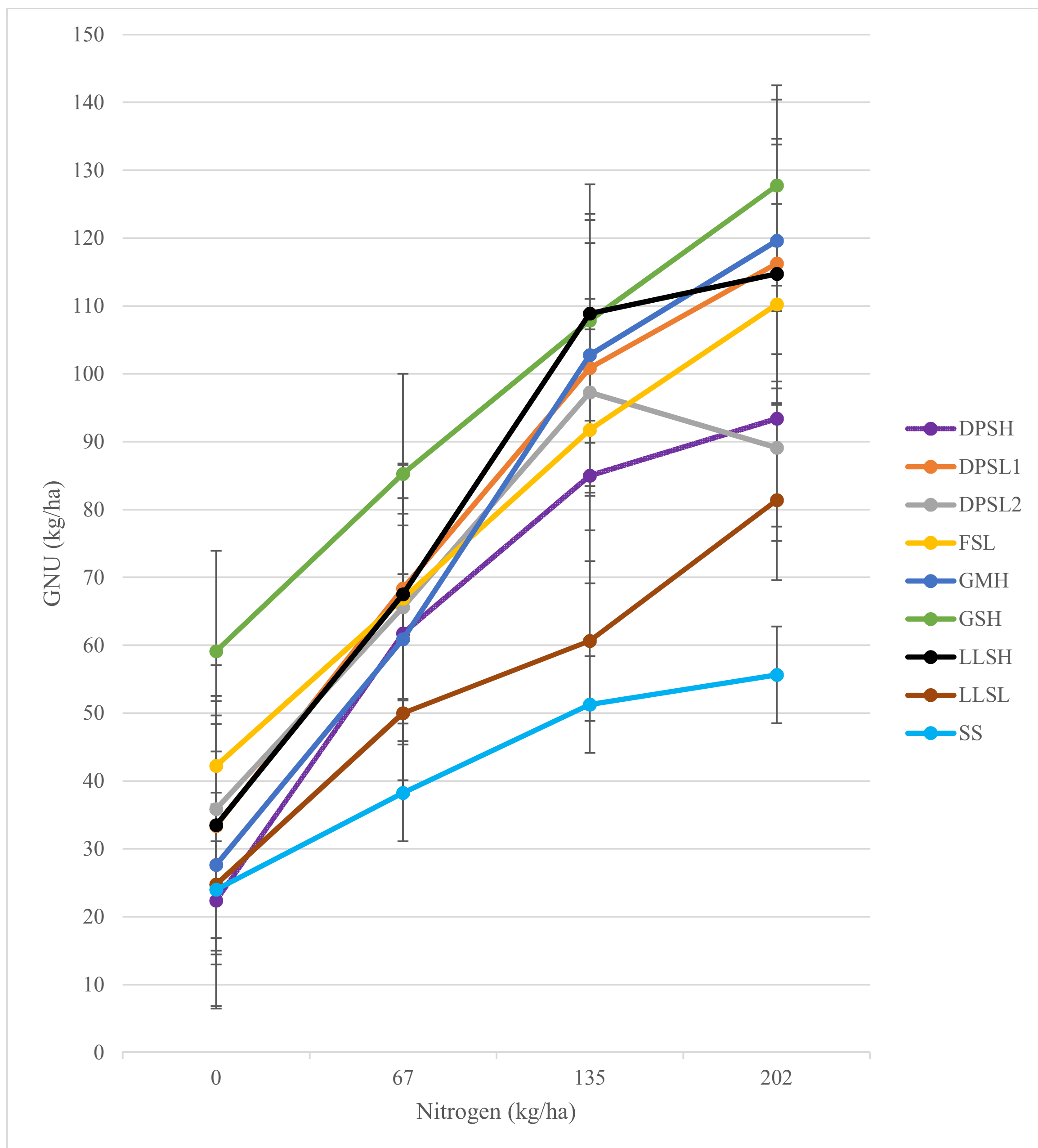
Figure 4.8 shows means of grain nitrogen uptake (GNU) in the eight grain producing sorghum and the maize genotypes. A maximum of 95kg/ha and a minimum of 42kg/ha of grain nitrogen uptake estimates were obtained by GSH and SS, respectively. Significant differences were observed between GSH and the other genotypes, except with LLSH that had a GNU of 81kg/ha. The SS, with a GNU of 42kg/ha was significantly lower than the maize hybrid and all the other sorghum varieties, except the LLSL.

Figure 4.9 shows means of stover nitrogen uptake (SNU) in the nine sorghums and the maize. A maximum of 172 kg/ha and a minimum of 59 kg/ha of stover nitrogen uptake estimates were observed in PSS and GMH, respectively. Significant differences between PSS and the other genotypes were observed. A significant difference was also observed between SS and GMH in SNU. Overall, PSS took up twice the nitrogen into stover than the other genotypes except for SS. Both sweet and photoperiod sensitive sorghums had significantly higher SNU estimates than the maize hybrid.

Table 4.6 Combined analysis of variance of biomass nitrogen concentration (g/kg) and biomass nitrogen uptake (kg/ha) in nine sorghums and maize genotypes.

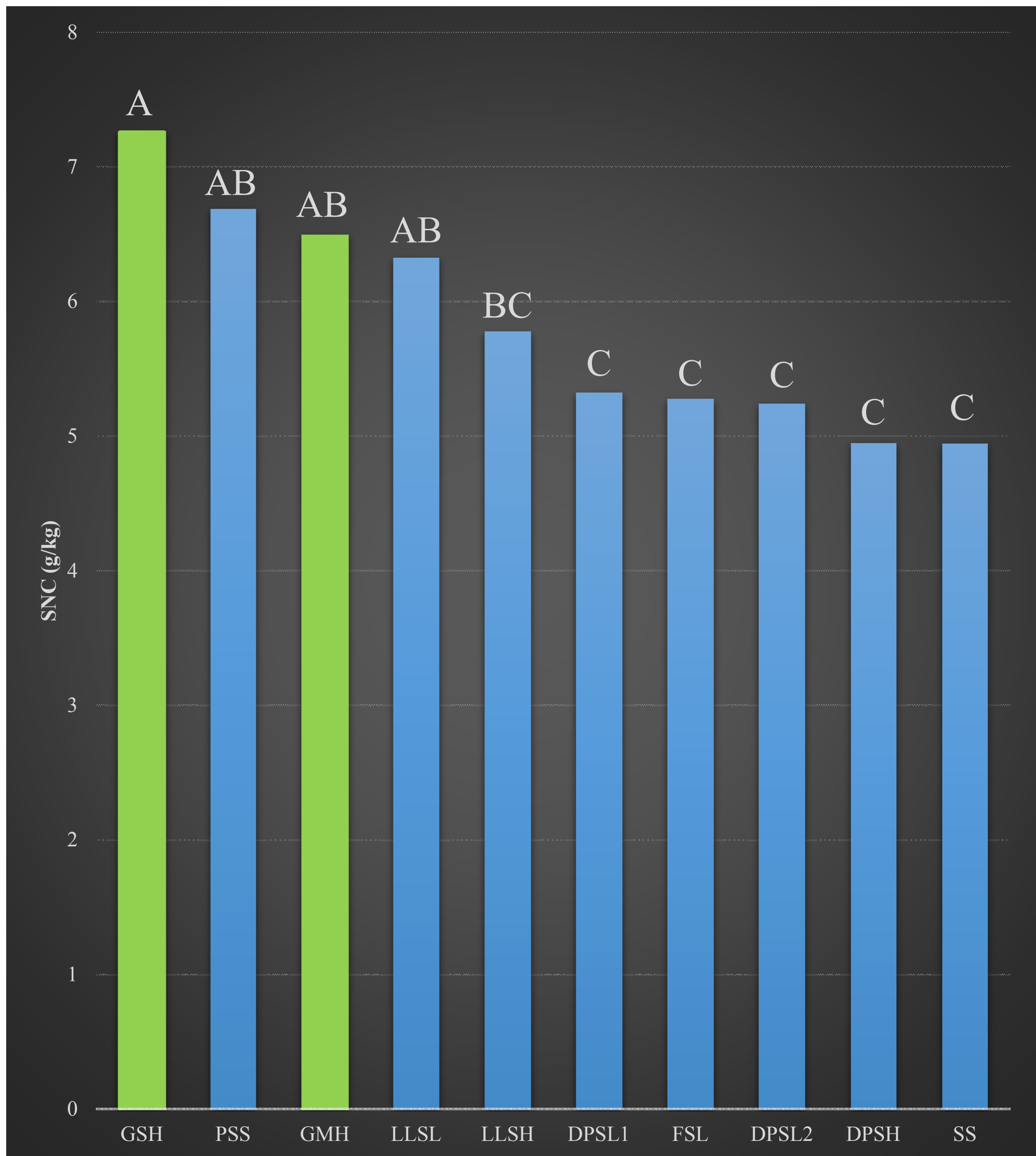
		N concentration			N uptake		
		Mean Square			Mean Square		
Source of variation	df	SNC	TBNC	GNC^{+}	SNU	TBNU	GNU^{+}
NRate	3	133 *	140 *	144 *	61117 **	234800 ***	64704 ***
Genotype	9	21 **	29 *	55	36114 *	7610	7914 **
NRate×Genotype	27	1	1	2	1372	771	704 *
Year	1	23	3	102	35401	567	30356 *
Year×Nrate	3	5	7 *	12 *	782	297	197
Year×Genotype	9	3	8 **	32 **	7034 ***	3786 **	665
Year×NRate×Genotype	27	2	2 **	3	790	1135	282 *
Residual	215	1	1	2	930	1100	162

GNC= grain nitrogen concentration (g/kg), SNC= stover nitrogen concentration (g/kg), TBNC= total biomass nitrogen concentration (g/kg), GNU= grain nitrogen uptake (kg/ha), SNU= stover nitrogen uptake (kg/ha), TBNU= total biomass nitrogen uptake (kg/ha). * P-value is less than 0.05, **P-value is less than 0.01 and *** P-value is less than 0.001. $^{+}$Only 9 genotypes were evaluated for grain yield as the photoperiod sensitive sorghum did not produce grain.



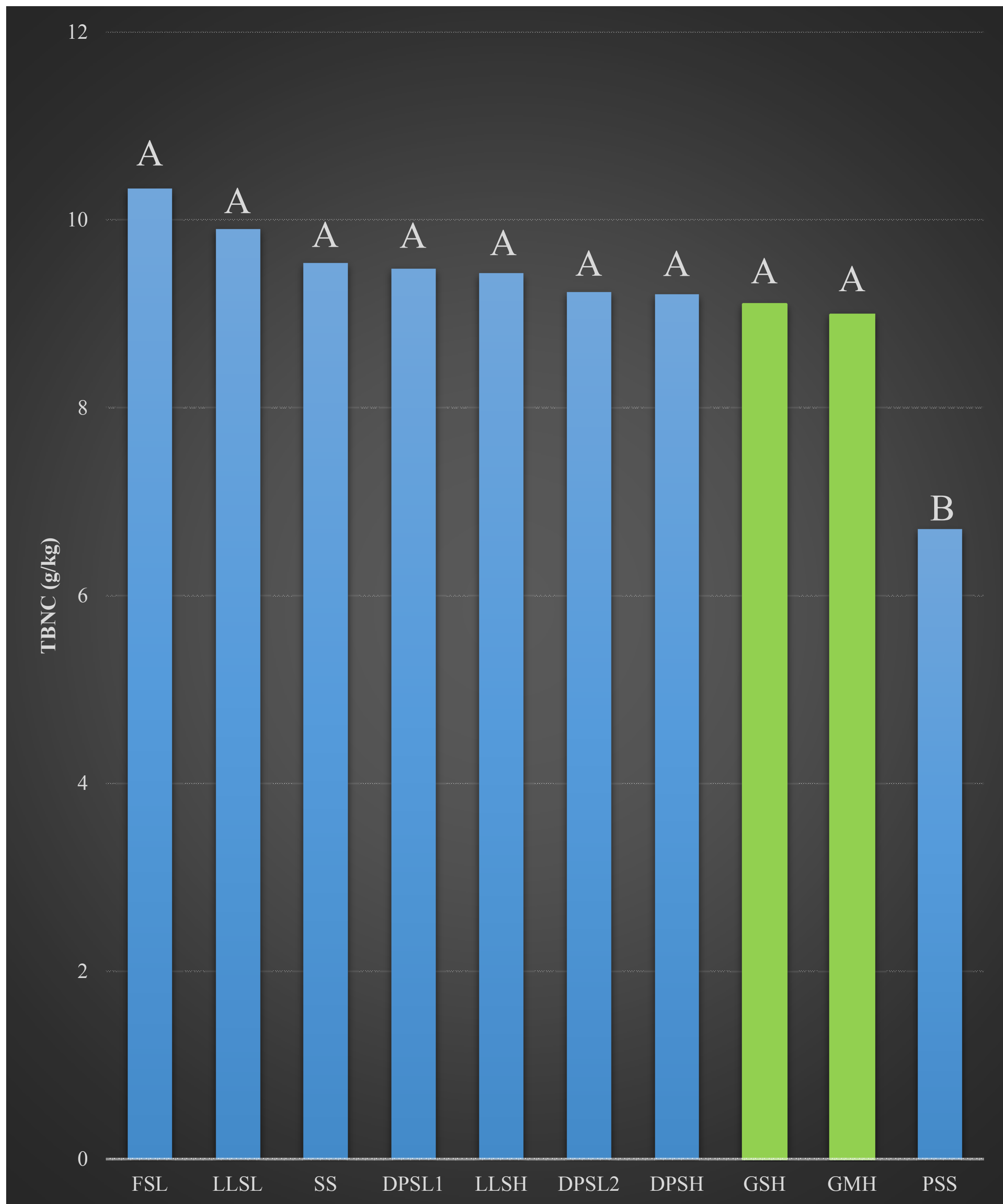
DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum. Bars represent SE.

Figure 4.5 Nitrogen by genotypes interaction of grain nitrogen uptake (GNU) in eight sorghums and maize genotypes.



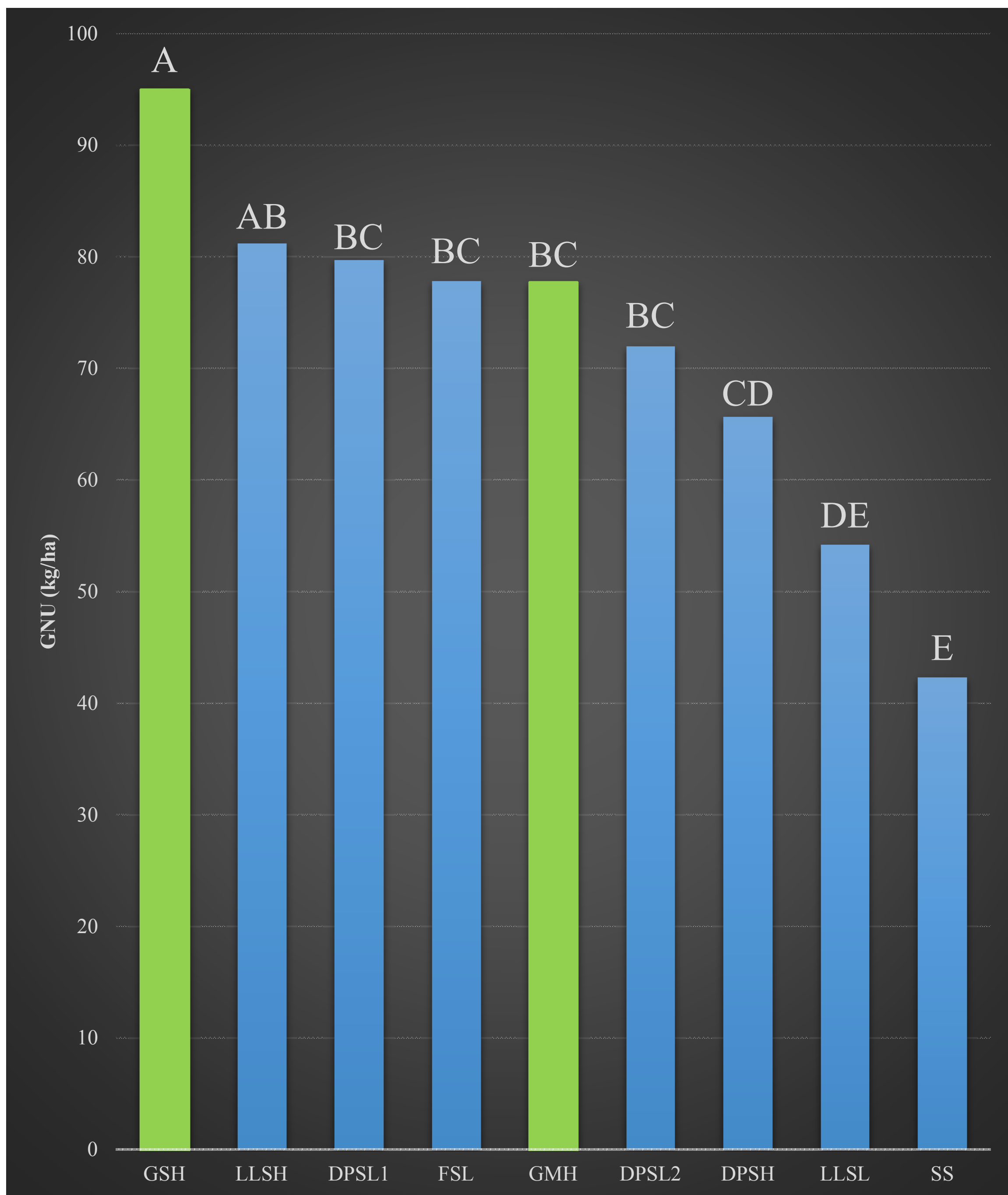
Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.6 Means of stover nitrogen concentration (SNC) of nine sorghums and maize genotypes.



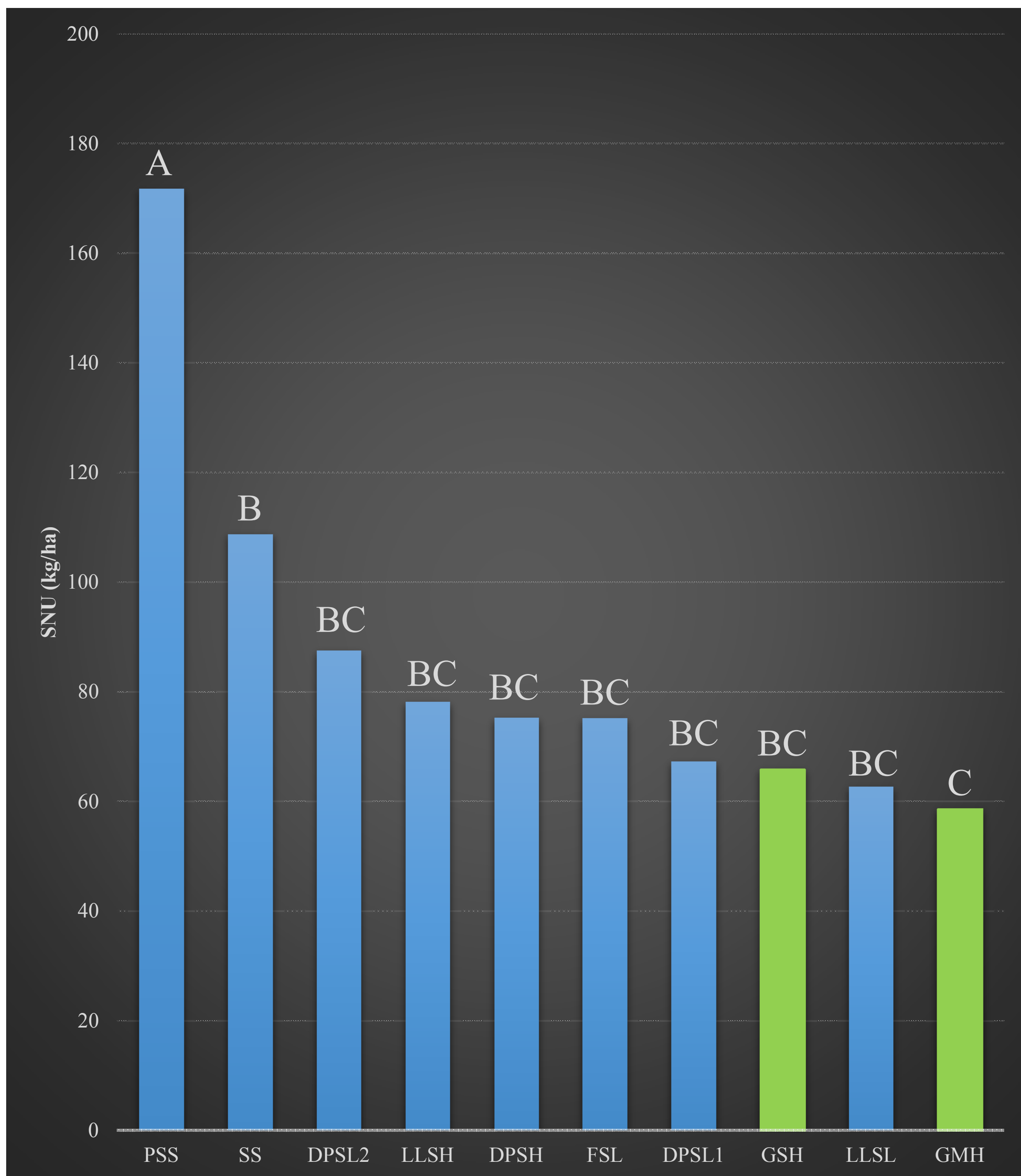
Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.7 Means of plant total biomass nitrogen concentration (TBNC) of nine sorghums and maize genotypes.



Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.8 Means of grain nitrogen uptake (GNU) of eight sorghums and maize genotypes.



Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.9 Means of stover nitrogen uptake (SNU) of nine sorghums and maize genotypes.

4.4.4 Biomass carbon concentration and biomass carbon uptake

Estimates of grain carbon concentration (GCC) and stover carbon concentration (SCC) in grams per kilogram of grain+stover were obtained and used to determine differences in means (Table 4.7). Similarly, estimates of grain carbon uptake (GCU), stover carbon uptake (SCU) and total biomass carbon uptake (TBCU) in kilograms per hectare of grain+stover were used to determine differences in means (Table 4.7). Significant differences were observed with Nrates for SCC, GCU, SCU and TBCU. Genotype source of variation showed significant differences for GCU and SCU. Year source of variation showed significant differences for GCC and GCU. The interaction NRate×Genotype showed significant difference only for GCU, while the interaction Year×Genotype showed significant differences for GCU, SCU and TBCU. Figure 4.10 shows NRate×Genotype interaction of grain carbon uptake estimates (GCU). Within each Nrate, significant effects among the grain bearing sorghums and maize were observed in grain carbon uptake estimates (kg/ha) at all different Nrates (0, 67, 135 and 202 kilograms of nitrogen per hectare; Appendix C.4). Within each genotype, significant effects among four Nrates were observed in grain carbon uptake estimates for all genotypes (Appendix C.5). Overall, all genotypes uptake more carbon in grain when nitrogen is applied. This positive trend was clearly observed in GMH and GSH.

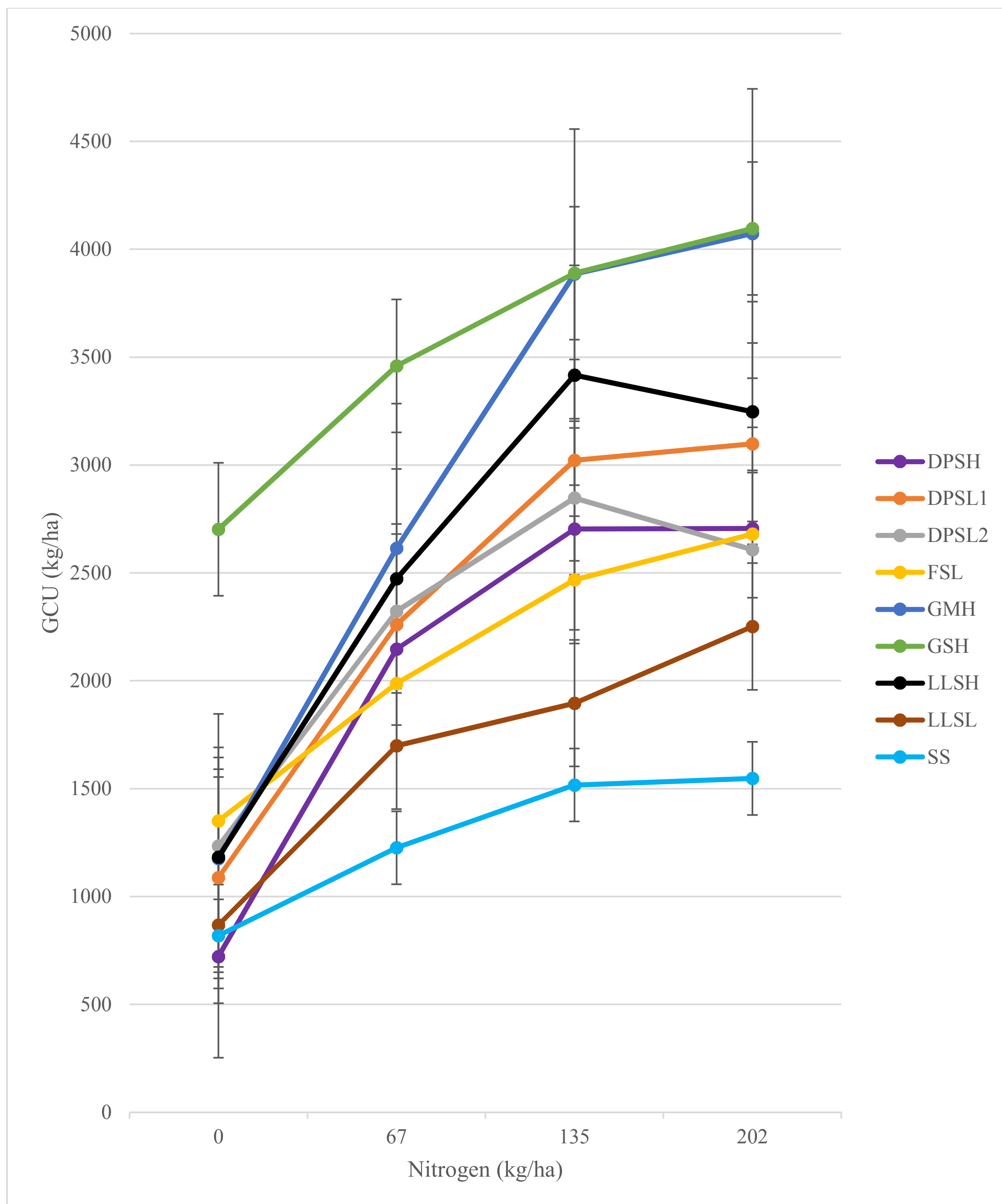
Figure 4.11 shows mean comparisons of grain carbon uptake (GCU) in the grain bearing sorghums and maize genotypes. A maximum of 3537 kg/ha and a minimum of 1277 kg/ha of grain carbon uptake estimates were obtained by GSH and SS respectively. Significant differences were observed between GSH and the other genotypes, except with GMH.

Similarly, no significant differences were observed among GMH, LLSH, DPSL1 and DPSL2. Also no significant differences were observed among DPSL1, DPSL2, FSL and DPSH. Finally, no significant differences were observed between DPSH, LLSL and SS. Overall, three of the four hybrid genotypes (GSH, GMH and LLSH) showed the highest estimates of grain carbon uptake in comparison to the other genotypes. Figure 4.12 shows means of stover carbon uptake (kg/ha) in nine sorghums and maize genotypes. A maximum of 11319kg/ha and a minimum of 3819kg/ha of stover carbon uptake estimates were obtained by PSS and GSH, respectively. Significant differences were observed between PSS with the other genotypes, except with SS. These two sorghum genotypes, PSS and SS, showed no significant differences. This two sorghum obtained the best estimates of stover carbon uptake in comparison to the other genotypes (11319 and 9292 kg/ha). Also, no significant differences were observed among SS, DPSL2, DPSH, FSL and LLSH. Finally, no significant differences were observed among DPSL2, DPSH, FSL, LLSH, DPSL1, LLSL, GMH and GSH.

Table 4.7 Combined analysis of variance of biomass carbon concentration (g/kg) and biomass carbon uptake (kg/ha) in nine sorghums and maize genotypes

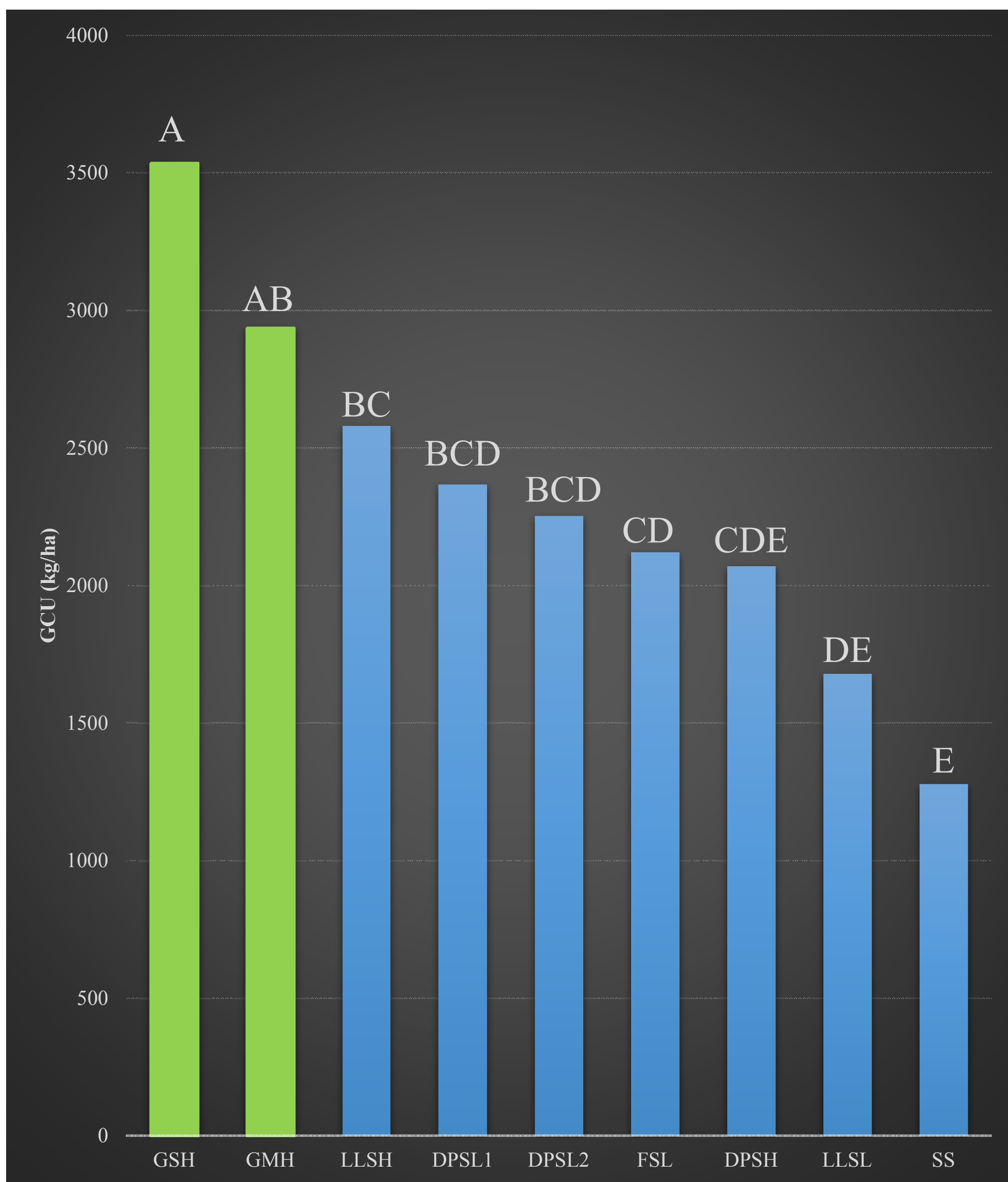
		C concentration				C uptake					
		Mean Square				Mean Square					
Source of variation	df	SCC		GCC^{+}		SCU		TBCU		GCU^{+}	
NRate	3	10667	*	998		74458728	*	216185311	**	43555758	***
Genotype	9	5778		435		182699337	*	84973607		14071571	**
NRate*Genotype	27	2409		283		3968461		2868738		804464	*
Year	1	582		35680	**	92883265		24668373		24496238	**
Year*Nrate	3	744		579		2874619		3901525		178475	
Year*Genotype	9	2693		489		45526861	***	42939766	***	1919075	***
Year*NRate*Genotype	27	3182		370		2295284		2741028		363029	***
Residual	215	3067		350		3164279		3298138		114877	

GCC= grain carbon concentration (g/kg), SCC= stover carbon concentration. (g/kg), TBCC= total biomass carbon concentration (g/kg), GCU= grain carbon uptake (kg/ha), SCU= stover carbon uptake (kg/ha), TBCU= total biomass carbon uptake (kg/ha). * P-value is less than 0.05, **P-value is less than 0.01 and *** P-value is less than 0.001.
$^{+}$Only 9 genotypes were evaluated for grain yield as the photoperiod sensitive sorghum did not produce grain.



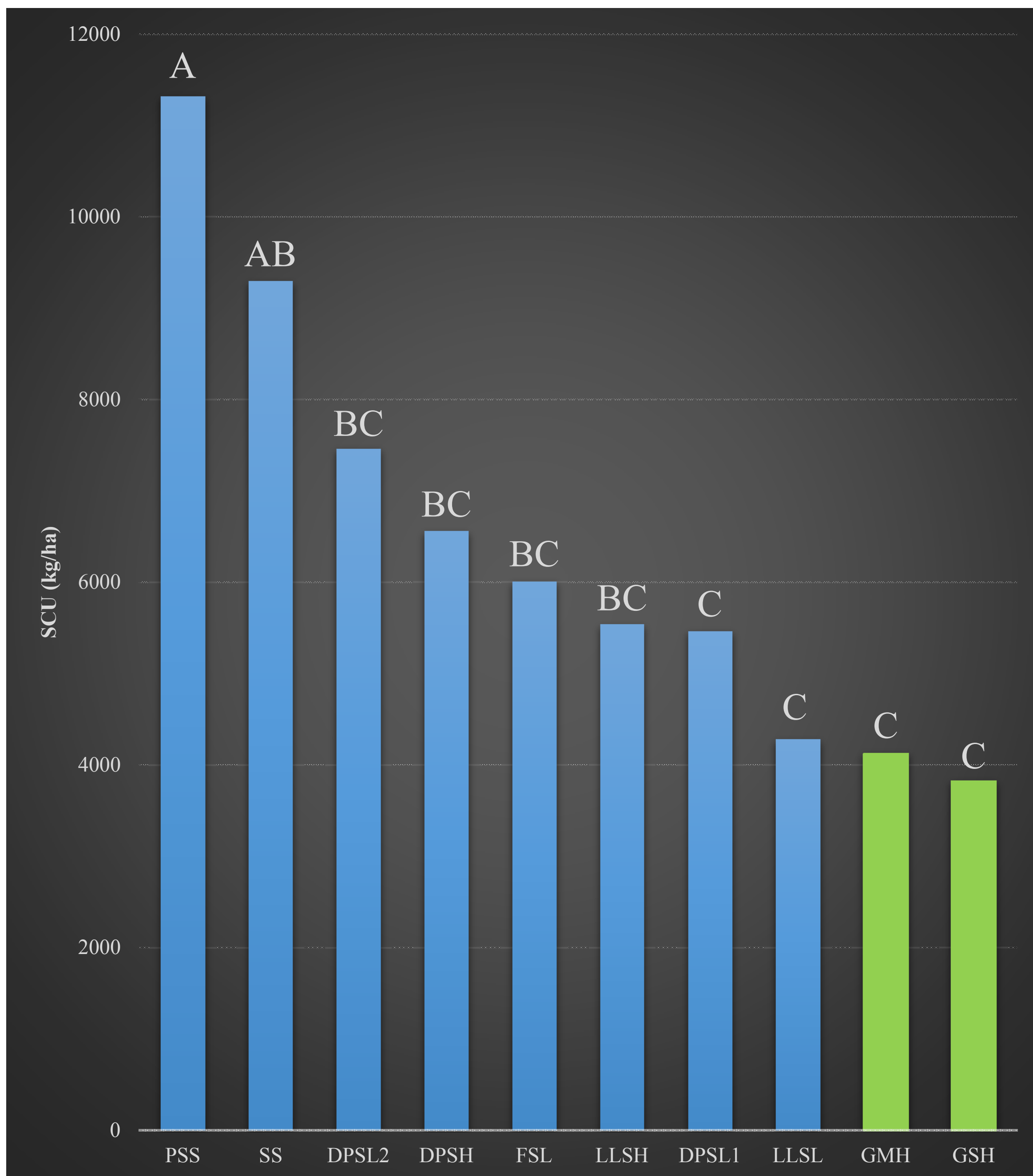
DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum.

Figure 4.10 Nitrogen by genotypes interaction of grain carbon uptake (GCU) in eight sorghums and maize genotypes.



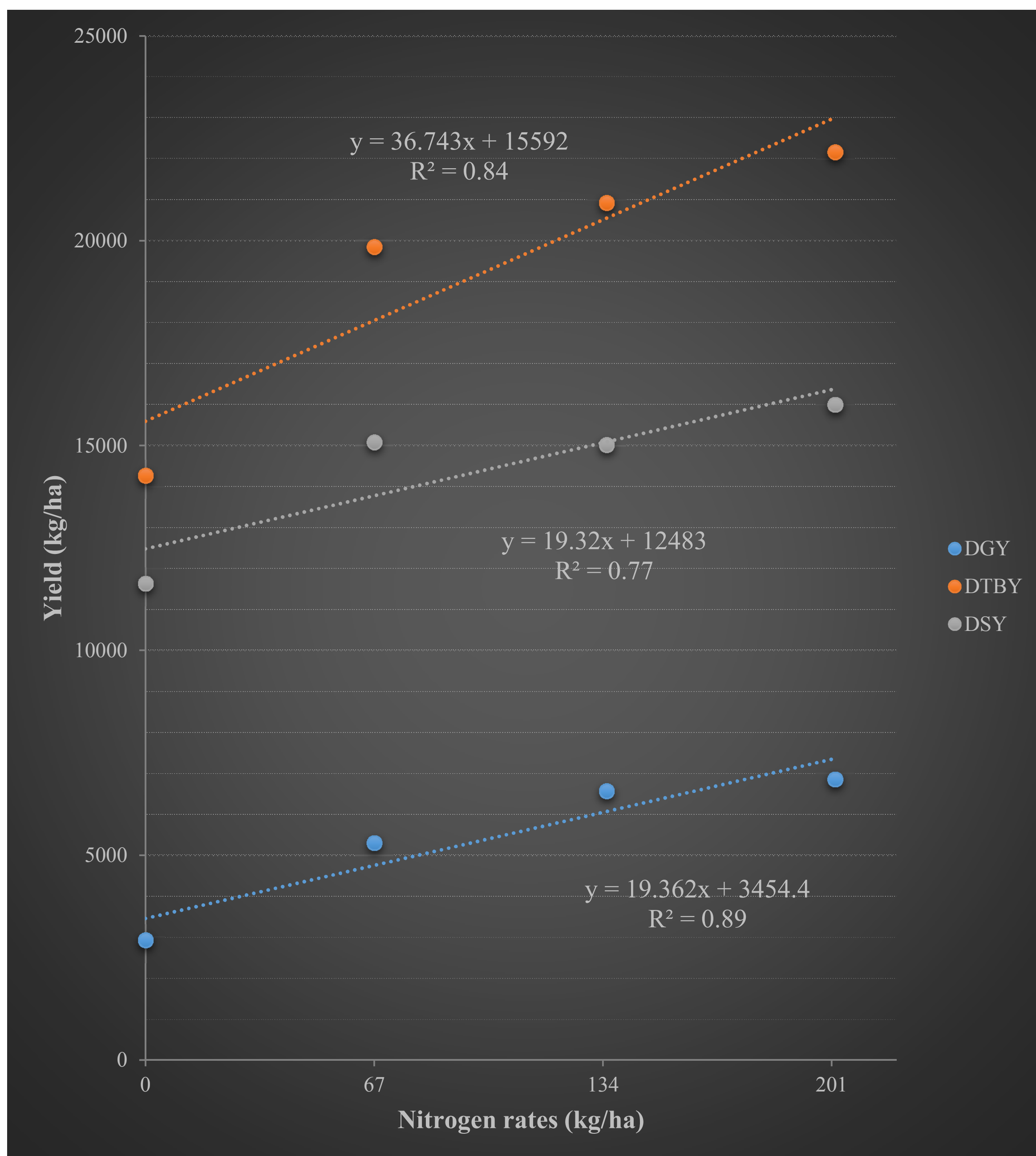
Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.11 Means of grain carbon uptake (GCU) in eight sorghum and maize genotypes.



Blue bar= sorghums genotypes, Green bar=commercial grain hybrids, DPSH=dual-purpose sorghum hybrid, GMH=grain maize hybrid, GSH=grain sorghum hybrid, LLSH=low lignin sorghum hybrid, DPSL1= dual-purpose sorghum line (PU216B), DPSL2= dual-purpose sorghum line (P90344), FSL=forage sorghum line, LLSL=low lignin sorghum line, SS=sweet sorghum, (LSD 0.05). Bars represent SE.

Figure 4.12 Means of stover carbon uptake (SCU) in nine sorghums and maize genotypes.



DGY=dry grain yield (kg/ha), DSY=dry stover yield (kg/ha) and DTBY=dry total biomass yield (kg/ha).

Figure 4.13 Effect of four applied nitrogen rates on biomass performance of nine sorghums and maize genotypes.

4.5 Discussion

Sorghum harbors important genetic variability for commercial production of grain and/or stover in both optimal and marginal lands. With the high cost of nitrogen fertilizer, it is important to understand the effect of nitrogen in plant biomass performance. A sound strategy of using nitrogen more efficiently is choosing appropriate crop among available species by comparing the effect of nitrogen fertilizer in a diverse group of genotypes. Crop variety selection based on the AONR and NUE and combined with complementary fertilizer application can not only cut production costs but can also prevent adverse environmental consequences to fertilizer use.

Our results showed that application of nitrogen fertilizer had significant effects on biomass components (dry grain yield and dry stover yield). This indicates that at least one of the nitrogen rates increased dry grain yield, dry stover yield and total biomass yield (Table 4.2). Indeed, across all materials, linear relationships of 89%, 77% and 84% between nitrogen rates and dry grain yield, dry stover yield and dry total biomass yield were obtained (Figure 4.13). These results suggest a higher nitrogen dependency of plants to produce grain, rich in non-structural carbohydrates (starch) than stover consisting mainly of structural carbohydrates (Muchow 1988, Messman et al., 1991; Brink and Fairbrother, 1992, Blumenthal et al., 2008, Wiley 2008, Wortmann et al., 2013). Evidence of significant differences in genotype effects were observed in the analysis of variance for the biomass components, dry grain yield and dry stover yield (Table 4.2). Overall, most hybrid genotypes, except DPSH, performed better in terms of dry grain yield than the inbred lines and SS (Figure 4.3), perhaps a reflection of the high yield potential of the inbred parental

lines as the increase due to heterosis was minimal in these hybrids. Although no significant differences were observed between GSH and GMH, the grain sorghum hybrid, ranked first in grain yield across nitrogen rates. It is not surprising that these grain hybrids out-performed the other varieties in this regard since these hybrid genotypes were bred to produce high grain yields. Indeed, these specialized genotypes provide good sources of non-structural carbohydrates for animal consumption and perhaps for ethanol production (Vogel et al., 2010). Different results in genotype performance were observed for dry stover yield (Figure 4.4). PSS and SS did not show significant differences for dry stover yield; however, these two sorghum genotypes ranked first in comparison to the other genotypes. This is evidence that these genotypes carry important traits to produce high amounts of lignocellulosic biomass (Wiedenfeld, 1984, Murray et al., 2009, Wang et al., 2009, Chen et al., 2014; Mankanda et al., 2009). The photoperiod sensitive trait allowed the PSS to obtained high lignocellulosic biomass due to its lacks of adaptability to long days (Mccollun et al., 2004). Indeed, the lack of adaptability to long days was translated as zero grain production by PSS, but higher stover yield (Figure 4.4). This physiological adjustment increases plant height, number of stems, leaves and tillers, as a positive response associated with high dry stover yield (Corredor et al., 2009, Shoemaker and Bransby, 2010). During the last three decades, sweet sorghums (SS) have been the most attractive crops for the ethanol industry (Han et al., 2013). Indeed, several studies about the ability of producing soluble sugars in theirs stems was a main priority by the scientific community (ICRISAT, 2006; Nghiem et al., 2013). Importantly, these sorghums are also capable to produce considerable amounts of lignocellulosic biomass. The reason is because

sweet sorghums are tall plants with high multiple tillers and great adaptability to abiotic stress conditions (Reddy et al., 2004; Reddy et al., 2010). Evidence in this study suggests that sweet sorghum is an attractive biomass crop, perhaps for animal consumption or lignocellulosic ethanol production.

Overall, results obtained in this study suggest that dry grain yield is more responsive to applied nitrogen fertilizer during the growing season than dry stover yield. However, depending of the genotype, DGY and DSY can be simultaneously responsive to applied nitrogen as it was observed in the dual-purpose sorghum hybrid genotype. Significant evidence of Nrate×Genotype interaction was observed for DGY and DSY (Table 4.2). For DGY, hybrids generally performed better across Nrates than inbred lines, SS and the PSS (Figure 4.1). On average, grain yield of hybrids were at least 30% more than the lines. The GSH showed more stable DGY across Nrates than the other genotypes. On average, GSH yielded 62% more grain than the other genotypes at zero Nrate. This is evidence of good adaptability of this hybrid under nutrient deficiencies, as occurs on marginal lands. For DSY, PSS and SS performed better across Nrates than the other genotypes (Figure 4.2). On average, these two genotypes yielded 48% more stover than lines and hybrids. The genetic architecture of PSS and SS played were major contributors to these results. The lack of adaptability to long days of PSS promotes vegetative over reproductive growth. This vegetative growth, at least its above ground components, all captured in stover yield components is very responsive to nitrogen fertilizer. Generally sweet sorghums are tall plants with multiple tillers. Similarly to photoperiod sensitive sorghum, sweet sorghums positively interact with applied nitrogen fertilizer. Unlike the PSS, after 135 kg ha^{-1} of

nitrogen fertilizer is supplied no more significant difference in DSY yield were reached in SS (Figure 4.2).

Maximization of biomass yield is key knowledge for crop production. AONR is the amount of applied nitrogen fertilizer that maximizes grain yield, stover or total biomass yield. Assuring that applied nitrogen fertilizer is efficiently used by plants is a major goal in agriculture. Estimates of AONR and NUE for grain yield are reported in Table 4.3. Grain AONR varied from 67 to 135 kg ha^{-1} of nitrogen fertilizer. This is evidence that applications above 135 kg ha^{-1} of nitrogen fertilizer do not increase grain yield. Generally, hybrid genotypes required more nitrogen fertilizer to produce grain, except for DPSH (AONR=135 kg/ha). At this Nrate, the GSH and GMH showed higher grain yield than the other genotypes. Inbred lines and the sweet sorghum cultivar maximized grain yield with only 67 kg ha^{-1} of nitrogen fertilizer. At this AONR, DPSL2 and DPSH, showed maximum grain yields of 5486 and 5077 kg/ha, respectively, while the SS showed the lowest grain yield (2867 kg/ha) at this AONR.

Estimates of NUE and its components (NRE and NIE) have been reported by many studies at high and low N inputs (Sinebo et al., 2004). In our study we reported grain NUE and its components at AONR of 10 genotypes (Table 4.3). DPSH, GMH, DPSL2, LLSH and DPSL1 showed higher estimates of grain NUE than LLSL, GSH, FSL and SS. These NUE estimates are above typical values of cereal crops (Novoa and Loomis 1981; Casmann et al., 2002; Dobermann, 2007). From this group of diverse genotypes, DPSH showed the highest NUE estimate (50 kg grain kg^{-1} N applied). These results indicate a gain of 67%

for grain NUE by this sorghum hybrid (Dobermann 2005; 2007). GMH showed NUE estimates of 44 kg grain kg^{-1} N applied. This represents a gain of 47% for grain NUE by corn. LLSH and DPSL2 obtained a gain of 28% for grain NUE. Finally the sweet sorghum cultivar did not show gains in NUE. The results indicate that hybrids had better estimates of grain NUE than inbred lines and the sweet sorghum cultivar. For a better understanding of these results, NRE and NIE for grain were estimated (Table 4.3). Similarly to grain NUE estimates, most hybrids obtained also high estimates of NRE, except the GSH. These estimates of NRE were above typical values reported in the literature (Dobermann, 2005; 2007). Consistently, most of the hybrid genotypes also showed high estimates of grain NIE (Table 4.3). These estimates were also above typical values reported in the literature (Dobermann, 2005; 2007). Interestingly, LLSL and DPSL2 also showed good estimates of NIE. Perhaps these lines are not the best to uptake nitrogen but they are efficient at utilizing N which contributes to the N protein deposition in the grain. Based on the contrasting genetic background of the plant material used in our study, we observed hybrids were the best grain NUE genotypes. The possible explanation of this results are related to genetic improvement of these selected group of genotypes. Grain hybrids are developed by crossing two inbred lines with desirable traits for grain production. Perhaps an association of high grain yield and NUE in grain hybrids results from heterosis (Ciampitti and Vyn 2013).

Stover AONR differed widely among genotypes (from 0 to 202 kg ha^{-1} of nitrogen fertilizer, Table 4.4). This is evidence that genotypes respond differently to nitrogen fertilizer for DSY (Pandey et al., 2001). The DPSL1, DPSHL2, DPSH and the GSH

obtained AONR at 0 kg ha^{-1} of applied nitrogen. The DSY of these genotypes were unresponsive to N fertilizer. Amazingly, the dual purpose sorghums produced on average above 12 tons of dry stover per hectare without N fertilizer application. The FSL, LLSL, LLSH, GMH and SS had an AONR of 67 kg/ha of nitrogen fertilizer. Remarkably, SS yielded above 24 tons/ha of dry stover at the minimum input of N fertilizer (67 kg/ha), showing that this sorghum genotype is extremely responsive to minimum N fertilization. The PSS maximized DSY at the maximum input of nitrogen fertilizer (202 /ha). Over 30 tons per hectare of non-grain biomass were produce by PSS, showing its extreme responsiveness to maximum N fertilization. The best estimates of stover NUE were obtained by SS and PSS (Table 4.4). Although PSS produced more DSY than SS, PSS was less NUE than SS. Indeed, SS was twice as efficient at uptaking and utilizing nitrogen fertilizer. These results were supported by high estimates of NRE and NIE obtained by SS in comparison to the other genotypes (Table 4.4). This is strong evidence that sweet sorghums could be economically more profitable to produce lignocellulosic biomass at a commercial scale than other types of sorghum and hybrid maize.

Estimates of dry total biomass AONR suggest that SS can maximize dry total biomass at minimal inputs of N fertilizer (Table 4.5). In comparison to GMH, SS yielded twice the dry total biomass with higher NUE. In comparison to PSS, SS yielded around the same amount of dry total biomass but with minimum inputs of N fertilizer, with twice the NUE. Within the hybrid genotypes, the DPSH and LLSH were more consistent at producing total biomass yield at the minimum input of nitrogen fertilizer in comparison to GMH. Although DPSH and GMH had similar NUEs, the DPSH yielded around five more tons of dry total

biomass per ha than GMH. Supporting this result, high estimates of NUE were observed for SS, DPSH and LLSH. These sorghums genotypes were able to uptake above 75% of applied nitrogen at their corresponding AONR and at least 112kg of total biomass was produce for one kilogram of uptaken nitrogen.

Variation for biomass nitrogen concentration and uptake in plant tissue has been observed in sorghum (Muchow 1990). This variation was associated to climatic, soil and genotypic factors across years and locations (Chardon et al., 2010). Our results indicate that there were genotypic differences in SNC, GNU and SNU (Table 4.6). Among genotypes, the GSH and LLSH tended to take up more N than inbred lines and the other genotypes. This agrees with findings of Nakamura et al. (2002) that N absorption was regulated by root anatomy and morphology, and it was higher in hybrids than in local cultivars or inbred lines in low–N conditions among sorghum genotypes (Pandey et. al., 2001). As expected, greater grain N uptake was associated with higher grain yields and NUE (Figure 4.4 and 4.6, Table 4.4). Similarly, greater estimates of stover N uptake were associated with dry stover yield (Figure 4.4 and 4.9). However, N concentration in stover was not associated with higher stover yields and NUE (Figure 4.4 and 4.6, Table 4.4). The only genotype with greater SNC and SNU associated with DSY was PSS, but its NUE was lower in comparison to the other genotypes. This result can be explained by the photosensitivity of this genotype. PSS does not produce grain so the plant prioritize the accumulation of N to stems and leaves. Additionally, nutrient uptake by sorghum is also influenced by other factors including nutrient availability, soil water availability, soil organic matter, soil chemical and physical properties, type of previous crop, plant population and the genotype (Wortmann,

2007). All of these factors could influence the genotypic difference in the stover and grain N uptake that has been observed in this current study.

Evidence of variation for GCU and SCU are presented in Table 4.7. Genotype had a significant effect in GCU and SCU. This results suggests that breeding for these traits would be feasible. As expected, greater estimates of grain carbon uptake and stover carbon uptake (kg/ha) were associated with DGY and DSY, respectively (Figure 4.3, 4.4, 4.11 and 4.12). This is an indication of a positive response of carbon uptake associated to increments in applied N fertilizer, and also a positive response of structural sugar yield associated increments in N fertilizer.

4.6 Conclusion

Sorghum and maize genotypes vary in their response to nitrogen fertilizer for grain and stover yield. Hybrid genotypes had the best response to nitrogen fertilizer for dry grain yield (5486 to 9160 kg /ha). Sweet stalked and photoperiod sensitive (SS and PSS) genotypes were the most responsive to nitrogen fertilizer for dry stover yield (24167 and 30505 kg /ha). Dual purpose sorghum hybrid (DPSH) was the most consistent to produce grain and stover with minimum input of nitrogen fertilizer, but above typical grain NUE values. Interestingly, the GSH showed significantly higher grain yield than the other varieties even at the zero nitrogen rate. This sorghum genotype showed consistent grain and stover yield responses across nitrogen rates. Over all, grain yields showed AONRs of 135 kg/ha, while dry stover yields had AONRs of 67 kg/ha for most sorghum genotypes. Additionally, most sorghum genotypes had better NUE than maize. Of all the genotypes, hybrids generally had better NUE, NRE and NIE for grain yield. However, SS had remarkably high NUE for stover yield, suggesting that perhaps this genotype could be selected as a dedicated lignocellulosic biomass crop. Our results show opportunities to breed for higher NUE in grain sorghum. There were differences in N uptake and C uptake among the genotypes used in this study. Indeed, GNU, GCU, SNU and SCU were associated to high grain and stover yield, giving evidence for the high performance of N uptake by sorghum genotypes.

Sources of genetic variation to improve agronomic and NUE traits could be found in the dual-purpose sorghum hybrid, low lignin sorghum hybrid, sweet sorghum and photoperiod sensitive sorghum. An ideal biomass crop must meet high standards of agronomic

performance and NUE. Sorghum shows great promise as a lignocellulosic biomass crop, able to maximize grain and stover yields at low N inputs with an efficient nitrogen utilization (high yield + low AONR + high NUE).

4.7 References

Acquaah G (2009) Principles of plant genetics and breeding. John Wiley & Sons. 569 pages.

Ben-Israel, Imri, et al. (2012) "Heterotic trait locus (HTL) mapping identifies intra-locus interactions that underlie reproductive hybrid vigor in Sorghum bicolor." PloS one 7(6):e38993.

Bernardo R (2010) Breeding for quantitative traits in plants. Second edition, 400 pages.

Blum A (1970) Nature of heterosis in grain production by the sorghum panicle. Crop Science 10(1): 28-31.

Brink GB, Fairbrother TE (1992) Bermuda grass-subterranean clover response to nitrogen application. J. Prod. Agric. 5:591–595.

Buah S.S.J, Mwinkaara S (2009) Response of sorghum to nitrogen fertilizer and plant density in the guinea savanna zone. Journal of Agronomy, 8:124-130.

Burow G, Franks CD, Xin Z, Burke JJ (2012) Genetic diversity in a collection of chinese sorghum landraces assessed by microsatellites. 2012:1722–1729.

Canilha L, Kumar Chandel A, dos Santos Milessi TS, et al. (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. J Biomed Biotechnology 2012:989572.

Cassman KG, Dobermann A, Walters DT (2002) Agroecosystems, nitrogen-use efficiency, and nitrogen management. AMBIO: A Journal of the Human Environment, 31(2):132-140.

Cassman KG, Dobermann A, Walters DT, Yang H (2003) Meeting cereal demand while protecting natural resources and improving environmental quality. Annual Rev. Environ. Resource 28:315–358.

Chen S-F, Danao M-GC, Singh V, Brown PJ (2014) Determining sucrose and glucose levels in dual-purpose sorghum stalks by Fourier transform near infrared (FT-NIR) spectroscopy. J Sci. Food Agric. 2014:1-8.

Ciampitti IA, Vyn TJ (2011). A comprehensive study of plant density consequences on nitrogen uptake dynamics of maize plants from vegetative to reproductive stages. Field Crops Res. 121:2–18.

Ciampitti IA, Vyn TJ (2012) Physiological perspectives of changes over time in maize yield dependency on nitrogen uptake and associated nitrogen efficiencies: A review. Field Crop Res. 133:48–67.

Ciampitti, IA, Vyn, TJ (2013). Grain nitrogen source changes over time in maize: A review. Crop Science 53(2):366-377.

Corredor DY, Salazar JM, Hohn KL, et al. (2009) Evaluation and characterization of forage Sorghum as feedstock for fermentable sugar production. Appl. Biochem. Biotechnol. 158:164–79.

Danalatos NG, Archontoulis SV TK (2009) Comparative analysis of sorghum vs corn growing under optimum and under water/nitrogen limited conditions in central Greece. 17th Eur. Biomass Conf. Exhib. 2:538–544.

Dawson JC, Huggins DR, Jones SS (2008) Characterizing nitrogen use efficiency in natural and agricultural ecosystems to improve the performance of cereal crops in low-input and organic agricultural systems. Field Crops Research 107:89-101.

Dien BS, Sarath G, Pedersen JF, et al. (2009) Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* L. Moench) lines with reduced lignin contents. BioEnergy Res. 2:153–164.

Erickson JE, Woodard KR, Sollenberger LE (2011) Optimizing sweet sorghum production for biofuel in the southeastern USA through nitrogen fertilization and top removal. BioEnergy Res 5:86–94.

Espinoza L, Kelley J, Ballantyne P (2005) Yield response of grain sorghum to varying nitrogen rates under irrigated and non-irrigated conditions. Wayne E Sabbe Arkansas Soil Fertility Stud Soil 25–29.

Field C (1983) Allocating leaf nitrogen for the maximization of carbon gain: Leaf age as a control on the allocation program. Oecologia 56:341-347.

Fridman E. (2014) Investigating the role of allelic and inter-locus Interactions that drive heterosis for grain quantity and quality of sorghum bicolor. In plant and animal genome XXII conference. Plant and animal genome. San Diego, CA.

Garner JC, Maranville JW, Paparozzi ET (1994) Nitrogen use efficiency among diverse sorghum cultivars. Crop Sci. 34:728-733.

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S B-ŁR (2010) Hemicelluloses for fuel ethanol: A review. Bioresource Technology 101:4775–800.

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends in Plant Science 9:597–605.

Hamelinck CN, Hooijdonk G Van, Faaij AP (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28:384–410.

Han K J, Pitman W D, Kim M, Day D F, Alison W, McCormick ME, Aita G (2013) Ethanol production potential of sweet sorghum assessed using forage fiber analysis procedures. GCB Bioenergy 5:358–366.

Hayes CM, Rooney WL (2014) Agronomic performance and heterosis of specialty grain sorghum hybrids with a black pericarp. 459–466.

Henry F, C. Raper C (1989) Cyclic variations in nitrogen uptake rate of soybean plants. Plant Physiol. 91:1345-1350.

Hirel B, Chardon F, Durand J (2005) The Contribution of Molecular Physiology to the Improvement of Nitrogen Use Efficiency in Crops. Biotech, J Crop Science 10:123–132.

ICRISAT Report (2006) Food, Fuel, Fodder and Fuel Crop. Report presentation 627.

Jacobsen SE, Wyman CE (2000) Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Applied Biochemical Biotechnology 84(86):81–96.

Johnson GV, Raun WR (2003) Nitrogen response index as a guide to fertilizer management, Journal of Plant Nutrition, 26(2):249-262,

Kimber CT, Dahlberg JA, Kresovich S (2013) The gene pool of *Sorghum bicolor* and its improvement. A.H. Paterson (ed.), Genomics of the *Saccharinae*, Plant Genetics and Genomics: Crops and Models 11. Springer, 23-41.

Kirby JS, Atkins RE (1968) Heterotic response for vegetative and mature plant characters in grain sorghum, *Sorghum bicolor* (L.) Moench. 20–24.

Lea PJ, Azevedo RA (2007) Nitrogen use efficiency 2: Amino acid metabolism. Ann Appl. Biol. 151:269-275.

Makanda I, Tongoona P, Derera J (2009) Quantification of genotypic variability for stem sugar accumulation and associated traits in new sweet sorghum varieties. African Crop Science Conference Proceedings 9:391–398.

Maranville JW, Madhavan S (2002) Physiological adaptations for nitrogen use in sorghum. In: Food security in nutrient-stressed environments: Exploiting plants genetic capabilities developments in plant and soil sciences volume 95:81-90.

Messman MA, Weiss WP, and Erickson DO (1991) Effects of nitrogen fertilization and maturity of bromegrass on in situ ruminal digestion kinetics of fiber. J. Anim. Sci. 69:1151–1161.

McAllister CH, Beatty PH, Good AG (2012) Engineering NUE crop plants: the current status. Plant Biotechnology Journal 10:1011–1025.

McCollum FT III, McCuistion K, Bean B (2005) Brown midrib and photoperiod-sensitive forage sorghums. In: Proceedings of the 2005 Plains Nutrition Council Spring Conference. Publication No. AREC 05-20. Texas A&M University Agricultural Research and Extension Center, Amarillo, 36-46.

McKenzie BA, Richey CB (1914) Harvesting, drying and storing grain sorghum. Cooperative Extension work in Agriculture and Home Economics, state of Indiana, Purdue University, and U.S. Department of Agriculture cooperating, West Lafayette, IN, AE-82W.

Moose SP, Below FE (2009) Biotechnology approaches to improving maize nitrogen use efficiency. A.L. Kriz, B.A. Larkins (eds.), molecular genetic approaches to maize improvement biotechnology in agriculture and forestry 63:65-77.

Muchow RC and Sinclair TR (1994) Nitrogen response of leaf photosynthesis and canopy radiation use efficiency in field grown maize and sorghum Crop Sci. 34: 721–727.

Murray SC, Rooney WL, Hamblin MT, et al. (2009) Sweet sorghum genetic diversity and association mapping for brix and height. Plant Genome J. 2(1):48-62.

Navas GE, Clark RB (2001) Sorghum nitrogen efficiency in Colombia. Plant Nutrition Developments in Plant and Soil Sciences 92:66-67.

Nielsen RL (2006) Nitrogen loss mechanism and nitrogen use efficiency, Purdue Nitrogen Management Workshops. 1-5 pp.

Nielsen RL, Johnson W, and Nice G (2008) Grain Sorghum Considerations for Late Planting in Southern Indiana. Purdue University Extension Specialists. URL:http://www.kingcorn.org/news/timeless/LatePlantGrainSorghum.html

Novoa R, Loomis RS (1981) Nitrogen and plant production. Plant Soil 58:177-204.

Osborne S (2007) Energy in 2020 : Assessing the economic effects of commercialization cellulosic ethanol. US Department Commercial Int. Trade A:1–20.

Pandey RK, Maranville JW, Bako Y (2001) Nitrogen fertilizer response and use efficiency for three cereal crops in Nige. Commun. Soil Sci. Plant Anal., 32(9):1465-1482.

Pavlik BM (1993) Nutrient and productivity relations of the dune grasses *Ammophilia arenaria* and *Elymus mollis I. Blasé* photosynthesis and nitrogen use efficiency in the laboratory and fiel. Oecoligia 57:227-234.

Pradhan S, Chopra UK, Bandyopadhyay KK (2013) Effect of water and nitrogen management on water productivity and nitrogen use efficiency of wheat in a semi-arid environment. 4:727–732.

Ragauskas AJ, Williams CK, Davison BH, et al. (2006) The path forward for biofuels and biomaterials. Science 311:484–9.

Raun WR, Johnson GV (1995) Soil-plant buffering of inorganic nitrogen in continuous winter wheat. Agron. J. 87:827–834.

Raun WR, Johnson GV (1999) Improving nitrogen use efficiency for cereal production. Agron. J. 91:357–363.

Raun WR, Solie JB, Johnson GV, Stone ML, Mullen RW, Freeman, KW, Thomason, W E, Lukina, Erna V (2002) Improving nitrogen use efficiency in cereal grain production with optical sensing and variable rate application. Agron. J. 94:815–820.

Reddy, Belum VS, et al. (2005) Sweet sorghum-a potential alternate raw material for bio-ethanol and bio-energy. ICRISAT: International Sorghum and Millets Newsletter 46: 79-86.

Rooney WL, Blumenthal J, Bean B, Mullet JE (2007) Designing sorghum as a dedicated bioenergy feedstock. Biofuels, Bioproducts and Biorefining, 1(2):147-157.

Shoemaker CA, Bransby DI (2010) The role of sorghum as a bioenergy feedstock, sustainable alternative fuel feedstock opportunities, challenges and roadmaps for six US regions. Soil and Water Conserv. Soc., Ankeny, IA, 149-159.

Shroyer J (1991) Agronomy research briefs: Optimal nitrogen and phosphorus fertilizer rates for irrigated corn and grain sorghum Part 2. Kansas Fertilizer Research. K-State Res & Ext Report of Progress 647:30-36.

Sindelar AJ, Lamb JA, Sheaffer CC, et al. (2012) Response of corn grain, cellulosic biomass, and ethanol yields to nitrogen fertilization. Agronomy Journal 104:363.

Snyder C, Bruulsema T. (2007) Nutrient use efficiency and effectiveness in North America: Indices of agronomic and environmental benefit. Int. Plant Nutrition Inst., Norcross, GA. Reference 07076.

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83:1–11.

Sweeney DW, Moyer JL (2007) Nitrogen management affects sorghum grown for grain and forage. Crop management 6(1):1-14.

Tamang PL, Brinsin KF, Malapati A, Schwartz R, Johnson J, Moore-Kucera J (2011) Nitrogen requirements for ethanol production from sweet and photoperiod sensitive sorghums in southern high plains 103(2):431-440.

United Nations, Department of Economic and Social Affairs, Population Division (2011) World population prospects: the 2010 revision.

U.S. Department of Energy (2007) Energy Information Administration. Quarterly Coal Report, Tables 24 and 37.

Vermerris W, Saballos A, Felderhoff T, et al. (2011) Genetic dissection of bioenergy traits in sorghum. Breakout Session A: Plant Feedstocks Genomics for Bioenergy. Joint Meeting 2011: U.S. Department of Energy Genomic Science Awardee Meeting IX and U.S. Department of Agriculture–DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting.

Vermerris W, Saballos A, Ejeta G, et al. (2007) Molecular breeding to enhance ethanol production from corn and sorghum stover. Crop Sci. 47(S3):142–153.

Vogel KP, Dien BS, Jung HG, et al. (2010) Quantifying actual and theoretical ethanol yields for switchgrass strains using NIRS analyses. BioEnergy Res. 4:96–110.

Wang ML, Zhu C, Barkley N a, et al. (2009) Genetic diversity and population structure analysis of accessions in the US historic sweet sorghum collection. Theor. Appl. Genet. 120:13–23.

Wang Z, Gao J, Ma BL (2014) Concurrent improvement in maize yield and nitrogen use efficiency with integrated agronomic management strategies. Agronomy Journal 1243–1250.

Wiedenfeld RP (1984) Nutrient requirements and use efficiency by sweet sorghum. Energ. Agr. 3:49-59.

Wortmann CS, Regassa T (2011) Sweet sorghum as a bioenergy crop for the US great plains. Econ Eff. Biofuel Prod InTech, Rijeka, Croat 225–240.

Wortmann CS, Ferguson RB, Hergert GW, et al. (2013) Nutrient management suggestions for grain sorghum. University of Nebraska-Lincoln Extension, Institute of Agriculture and Natural Resources. G1669.

Wylie P (2008) Managing sorghum for high yields. Grain Research and Development Corporation. Core text ISBN 978-1-875477-57-9.

Yang B, Gray MC, Liu C, et al. (2004) Unconventional relationships for hemicellulose hydrolysis and subsequent cellulose digestion. In ACS symposium series. Washington, DC; American Chemical Society 19:100-125.

APPENDICES

Appendix A

(Chapter 2)

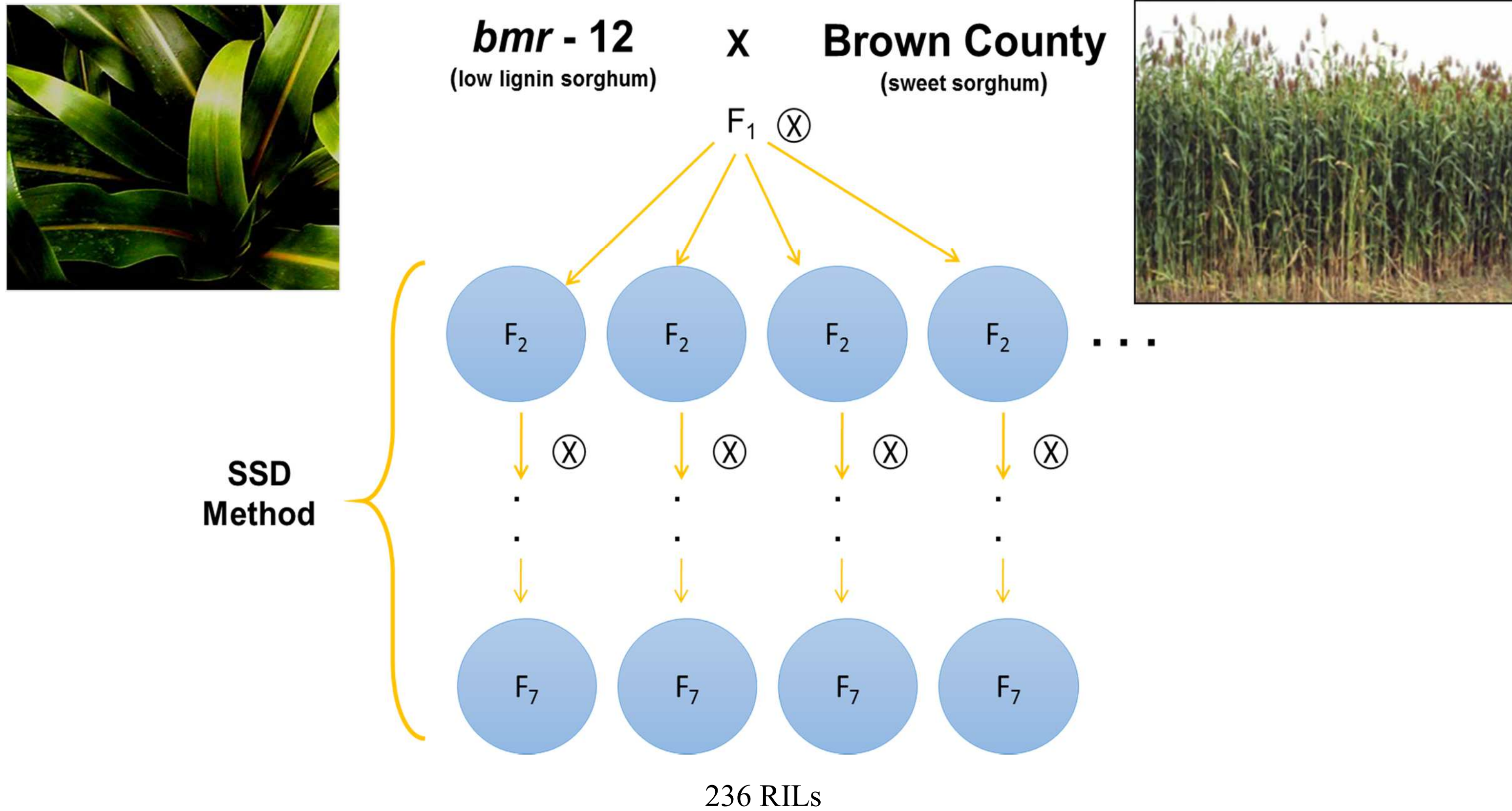


Figure A.1 Development of a brown midrib sweet sorghum RILs population by Single Seed Method.

High Throughput DNA Extraction and PCR

Adapted from Xin et al., 2003 (Biotechniques 34:820-24)

Stock solutions:
1M NaOH (Fw=40) (kept on shelf by Nutrient stocks)
20% Tween 20 (v/v) (kept on shelf by Nutrient stocks)
20% PVP (PolyVinylPyrrolidone 40kDa) (w/v) (kept in glass vial in refridgerator)
20% BSA fraction V (Sigma B-4287) (w/v) (kept in microcentrifuge tube in refrigerator)

Table A.3 Buffer A (100mM NaOH, 2% Tween 20)

***Make fresh from stock solutions just before using.**

	For 10ml (ml) (96-well plate)	For 25 ml (ml)	For 100ml (ml)
1M NaOH	1	2.5	10
20% Tween 20	1	2.5	10
ddH_20	8	20	80

Buffer B (0.1M Tris-HCl, 2mM EDTA)

3.15 g Tris-HCl
0. 15g EDTA
200ml ddH_2O

1. Put leaf punch tissue (89mm^2, standard paper punch) or seedling stem tissue (1cm) into 0.5ml microtubes or 96-well plate.
2. Add 50µl Buffer A to each sample (Same tips, don't touch sample)
3. Incubate for 10min in thermocycler at 95°C (thermocycler program "95").
4. Add 50µl Buffer B and mix immediately with tips (Use new tips for each).
5. The extracted DNA is stable now. Place extracted DNA plate in 4°C refrigerator until ready for PCR setup.

PCR Following High Throughput DNA Extraction

1. **Make 5 µM Primer Mix.** Using 100 µM primer stocks, in a 2 ml tube, combine 100 µl of F primer and 100 µl of R primer. Add 1.8 ml sterile ddH_2O. Total volume will be 2 ml with a concentration of 5 µM of each primer.

2. **Make PCR Master Mix** (for established polymorphic marker).

Table A.4 PCR Master Mix (20µl reaction)

	Single PCR Rxn (µl)	96-well plate (µl) (x110 single rxn)	Final Conc.
My Taq Red 2x Mix	**10***	**1100***	**50%***
20 % BSA	0.1	11	0.5%
20 % PVP	1	110	5 %
Primer Mix (5 µM)	2	220	10%
ddH_2O	5.9	649	29.5%
Totals:	**19**	**2090**	**95%**

***ALWAYS MUST BE 50% OF TOTAL PCR RXN VOLUME INCLUDING DNA- NO MORE, NO LESS!**

3. Aliquot **19 µl** PCR mixture to new PCR tube or 96-well plate (You can use same tips for this step)
4. Transfer **1.0 µl** DNA to PCR tube. (Make sure to use new tips for each sample). You may need to add more DNA, in that case, be sure to adjust the volume of Master Mix accordingly (generally by adding less water).
5. Cover tubes/plate with plastic caps or a sticker. Your samples are now ready for PCR.

Example PCR Conditions- Program: 10. HTPCR.CYC (MWG thermocycler)

***Your conditions may change based on primer melting temperature and expected product length**

Step 1	94°C	2 min
Step 2	94°C	20 sec
Step 3	59°C	30 sec
Step 4	72°C	1.5 min
Step 5	Go to step 2 for 35 more times	
Step 6	72°C	5 min
Step 7	4°C	Forever
Step 8	End	

SAS MACRO to estimate genetic and phenotypic correlations for Chapter 2

```
Title2 "Mean for genetic Correlations for YEARS 2008-2009 - COMBINATED";
Data RILs_08_09;
Length RIL$ 20;
infile "RILs_08_09.csv" dsd firstobs=2 missover;
input Year Block Key $ RIL Code $ FSY FHY FTBY DSY DHY DTBY DGY Hcm Brix
Diam Mat;
run;
*USE DATA FROM ONLY ONE ENVIRONMENT FOR THIS EXAMPLE!;
data one;
set RILs_08_09;
*/if env = 98; */proc print;
*first, estimate variance components for each trait separately to compare to multivariate
analysis below;
%macro varcomp(trait);
proc mixed data = RILs_08_09;
class Year Block RIL;
model &trait = ;
random Year RIL Block(Year) Year*RIL;
*also check effect of setting reps fixed on other variance components;
proc mixed data = RILs_08_09;
class Year Block RIL;
model &trait = Block;
random Year RIL Year*RIL;
run;
%mend;
%varcomp(FSY);
%varcomp(FHY);
%varcomp(FTBY);
%varcomp(DSY);
%varcomp(DHY);
%varcomp(DTBY);
%varcomp(DGY);
%varcomp(Hcm);
%varcomp(Brix);
%varcomp(Diam);
%varcomp(Mat);
*restructure data set for multivariate reml analysis;
data two; length trait $ 5; set one;
trait = "FSY"; y = fsy; output;
```

```
trait = "FHY"; y = fhy; output;
trait = "FTBY"; y = ftby; output;
trait = "DSY"; y = dsy; output;
trait = "DHY"; y = dhy; output;
trait = "DTBY"; y = dtby; output;
trait = "DGY"; y = dgy; output;
trait = "Hcm"; y = hcm; output;
trait = "Brix"; y = brix; output;
trait = "Diam"; y = diam; output;
trait = "Mat"; y = mat; output;
drop fsy fhy ftby dsy dhy dtby dgy hcm brix diam mat;

* analyze variables pair-wise;
%macro corr(trait1, trait2);
data traits;
set two;
if trait = "&trait1" or trait = "&trait2";
proc mixed asycov data = traits;
class trait Year Block RIL;
model y = Year(trait) Block(Year*trait);
random trait /subject = RIL type = un;
random trait /subject = RIL*Year type=un;
repeated trait/ sub = Block*RIL(Year) type = un;
ods output covparms = estmat;
ods output asycov = covmat;
run;
proc iml;
use estmat; read all into e;
use covmat; read all into cov;
* Note that SAS introduces an extra first column into the covariance matrix which must
be removed;
C = cov(|1:nrow(cov), 2:ncol(cov)|);
* Obtain genotypic and phenotypic covariance and variance components;
CovG = e(|2,1|);
VG1 = e(|1,1|);
VG2 = e(|3,1|);
CovP = CovG + e(|5,1|) + e(|8,1|);
VP1 = VG1 + e(|4,1|) + e(|7,1|);
VP2 = VG2 + e(|6,1|) + e(|9,1|);
* Create a module called "correl" that will estimate genotypic and phenotypic
correlations
and their standard errors;
```

```
start correl(C, CovG, VG1, VG2, CovP, VP1, VP2, RG, RP, SERG, SERP);
RG = CovG/sqrt(VG1*VG2);
*Make the derivative vector for rg, note that the order of the rows and columns of the
variance
covariance matrix is VG1, CovG, VG2, VError1, CovError, VError2;
dg = (-1/(2*VG1))//(1/CovG)//(-1/(2*VG2))//0//0//0//0//0//0;
varrg = (RG**2)*dg`*C*dg; serg = sqrt(varrg);
RP = CovP/sqrt(VP1*VP2);
*Make the derivate vector for rp;
d1p = -1/(2*VP1);
d2p = 1/CovP;
d3p = -1/(2*VP2);
dp= d1p//d2p//d3p//d1p//d2p//d3p//d1p//d2p//d3p;
varrp = (RP**2)*dp`*C*dp;
serp = sqrt(varrp);
finish correl;
call correl(C, CovG, VG1, VG2, CovP, VP1, VP2, RG, RP, SERG, SERP);
print "Genotypic Correlation Between &trait1 and &trait2";
print RG serg;
print "Phenotypic Correlation Between &trait1 and &trait2";
print RP serp;
quit;
run;
```

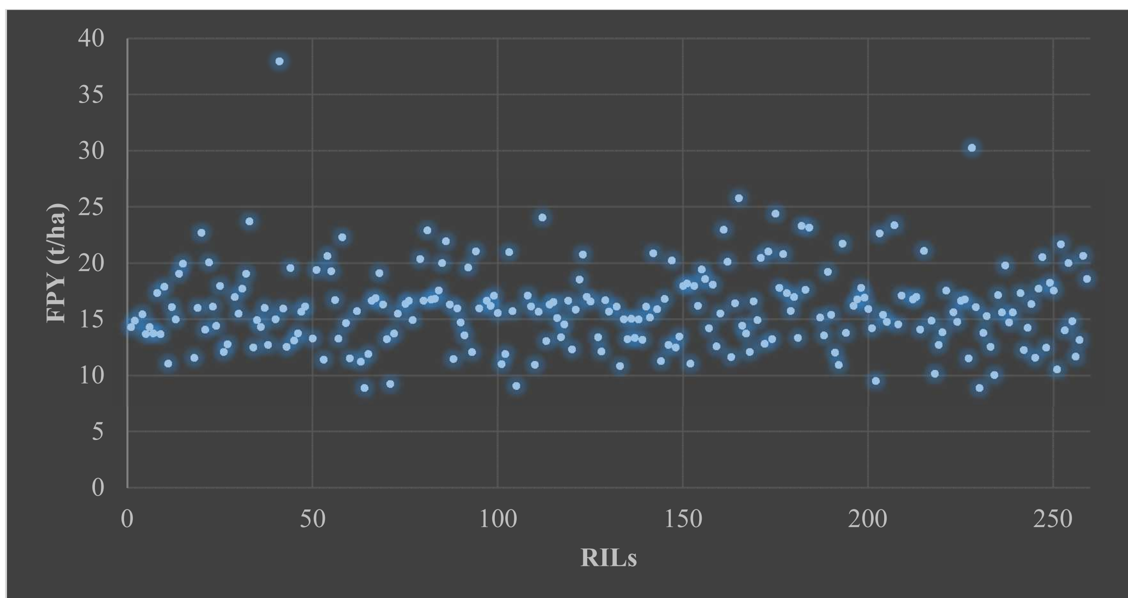


Figure A.2. RILs fresh panicle yield (FPY) scatter plot (Ȳ= 15.84 t/ha).

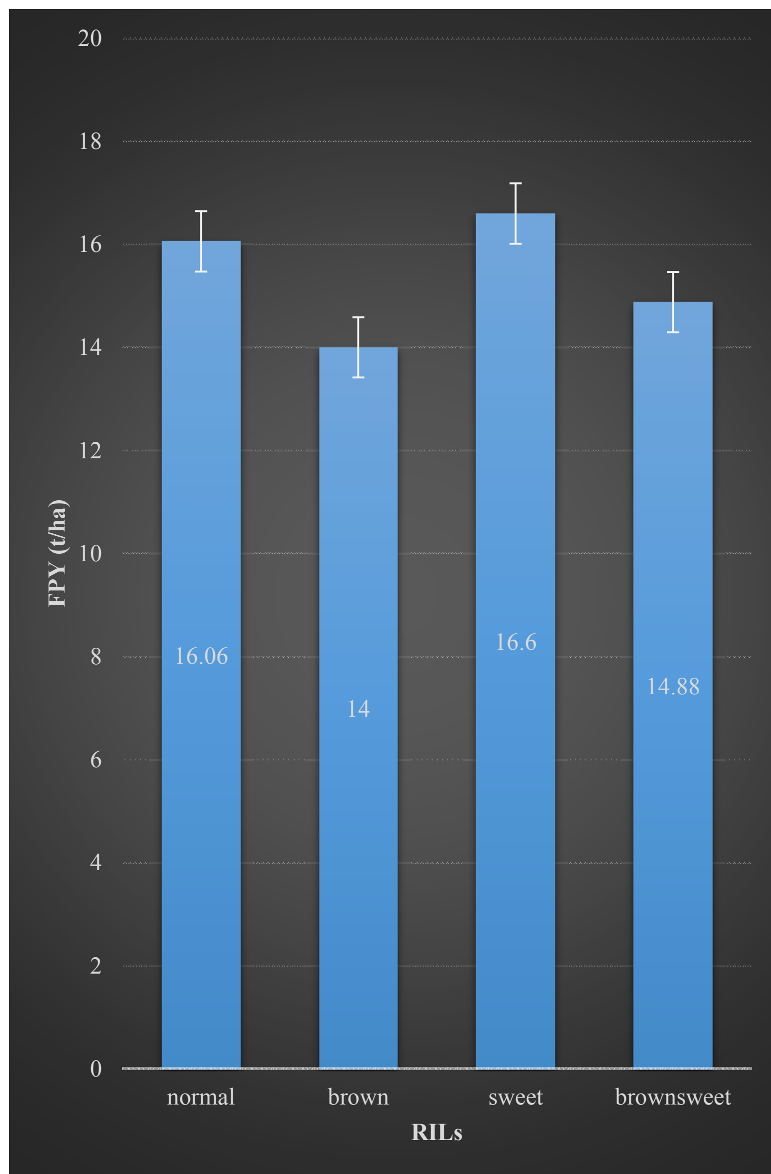


Figure A.3. Mean fresh panicle yield (FPY) among four different RILs phenotypic classes. Bars represent standard errors.

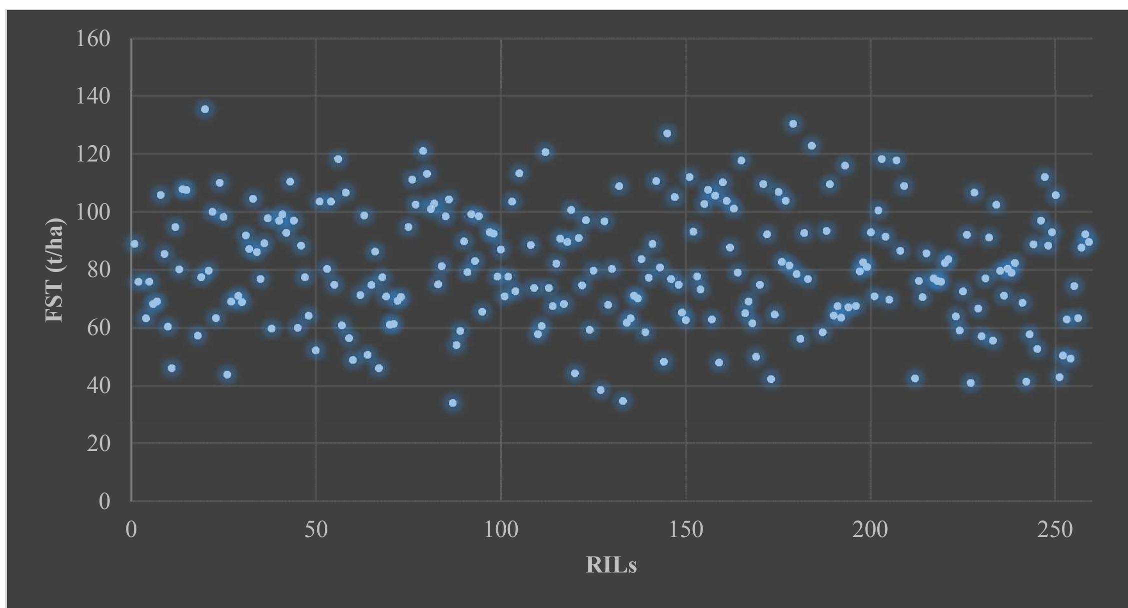


Figure A.4. RILs fresh stover yield (FSY) scatter plot ($\bar{Y}$= 79.86 t/ha).

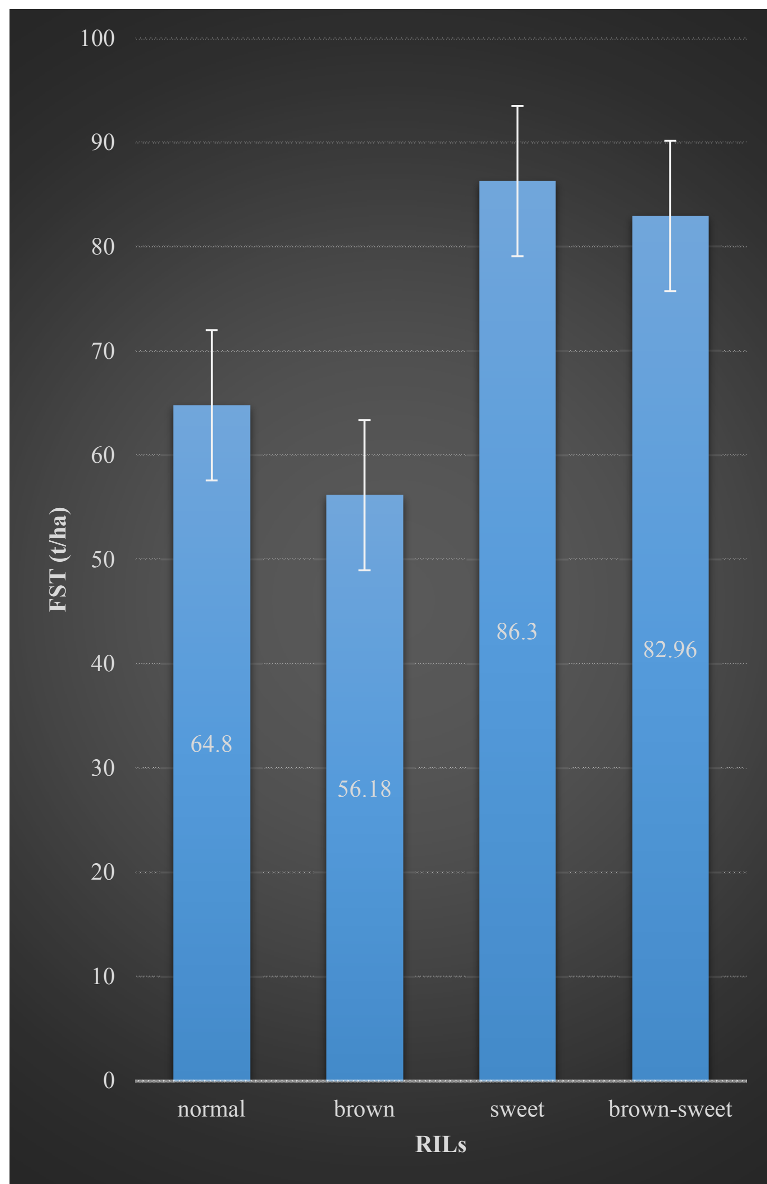


Figure A.5. Mean fresh stover yield (FSY) among four different RILs phenotypic classes. Bars represent standard errors.

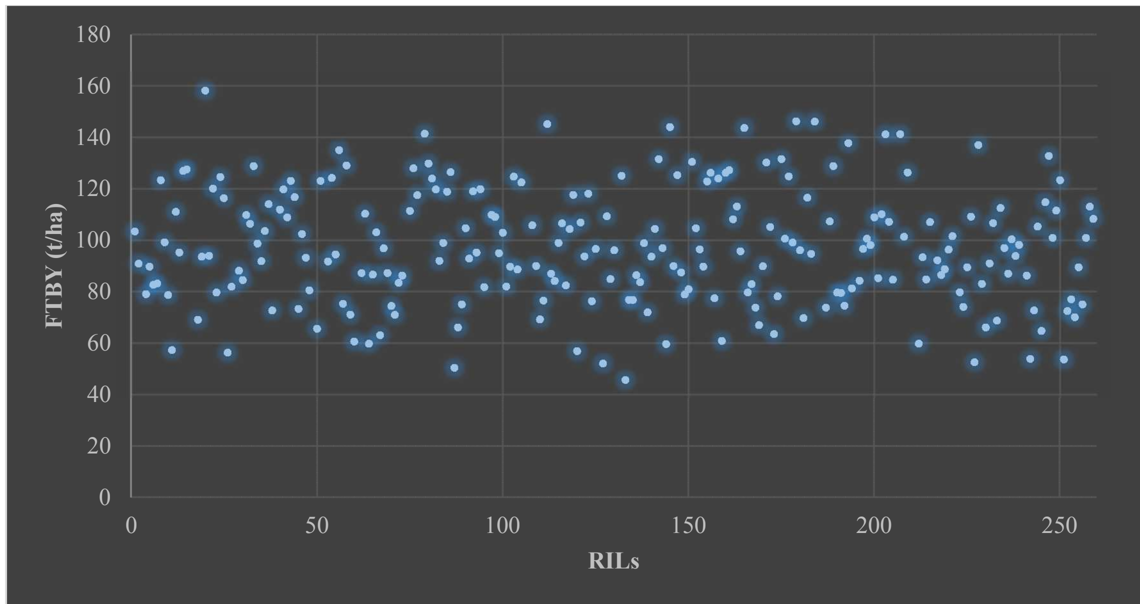


Figure A.6 RILs fresh total biomass yield (FTBY) scatter plot ($\bar{Y}$= 96.03 t/ha).

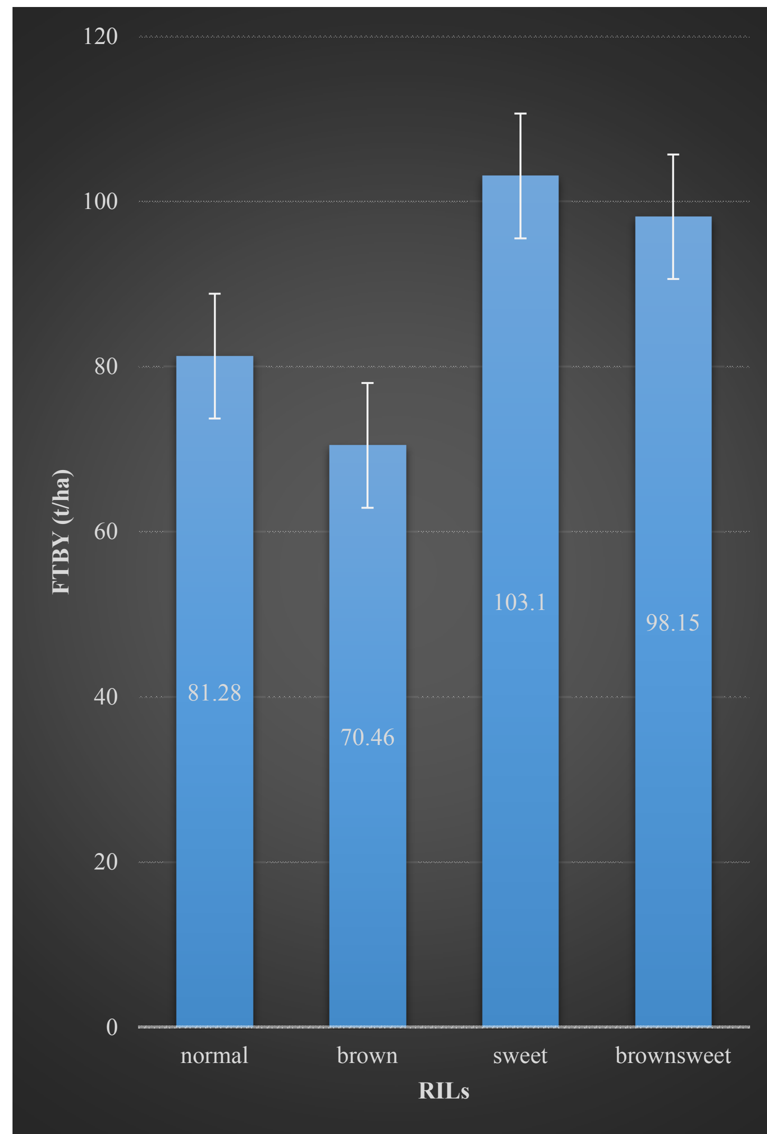


Figure A.7 Mean fresh total biomass yield (FTBY) among four different RILs phenotypic classes. Bars represent standard errors.

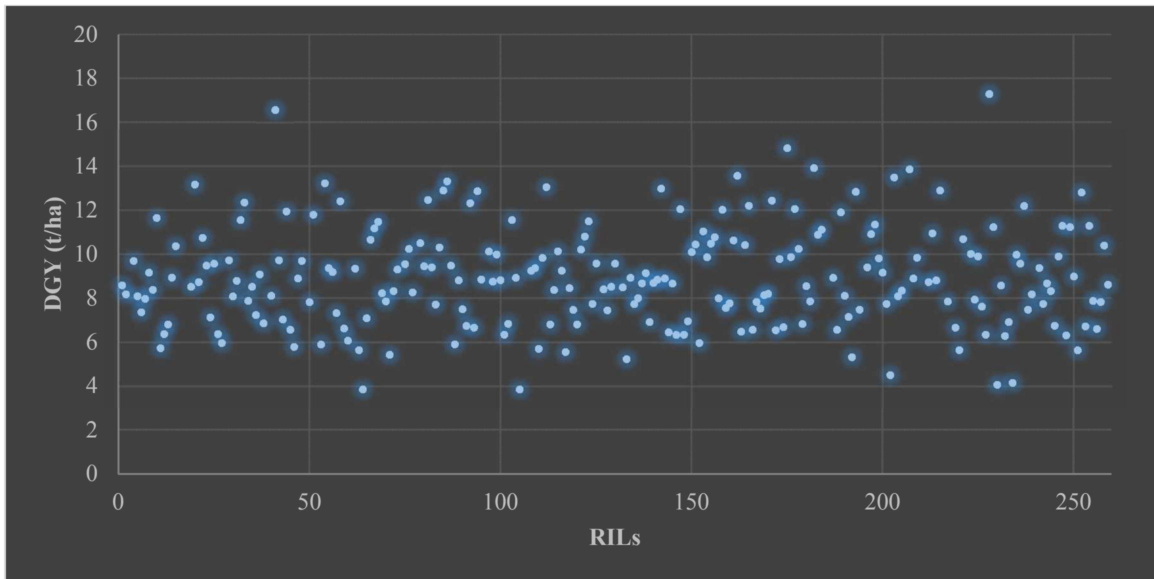


Figure A.8. RILs dry grain yield (DGY) scatter plot ($\bar{Y}$= 8.96 t/ha).

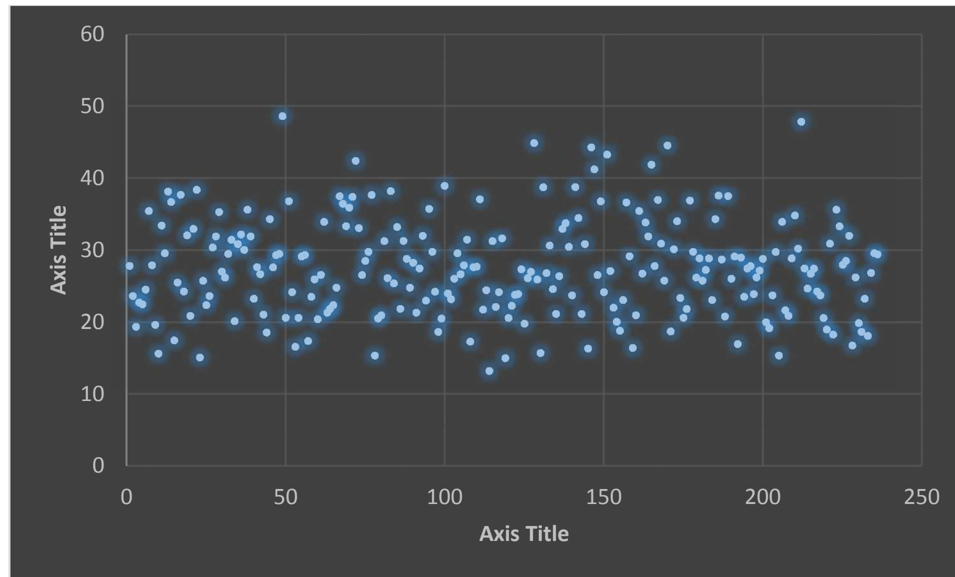


Figure A.9. RILs dry stover yield (DSY) scatter plot ($\bar{Y}$ = 22.52 t/ha).

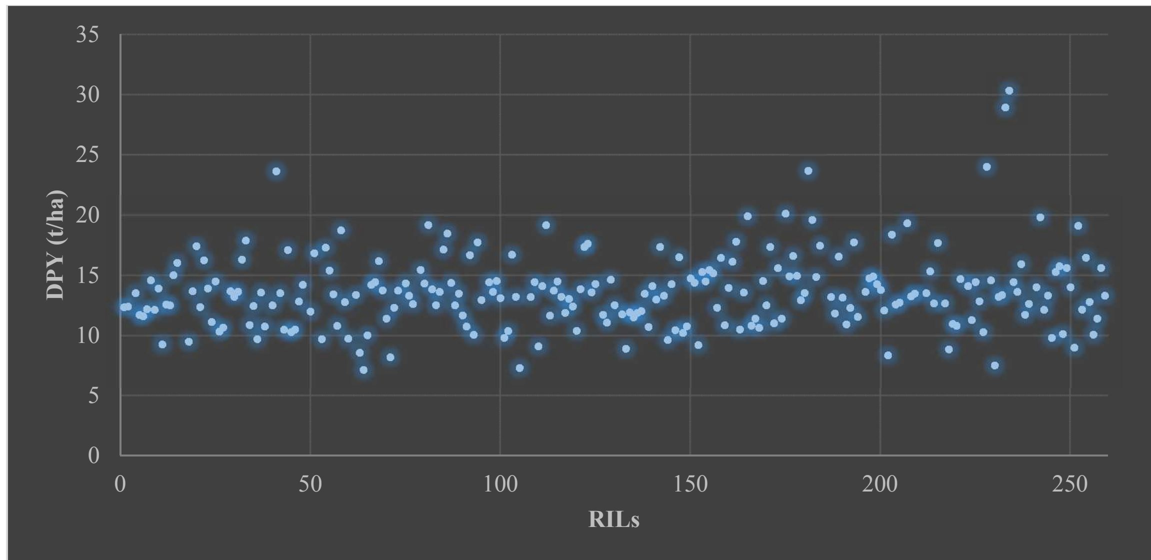


Figure A.10. RILs dry panicle yield (DPY) scatter plot ($\bar{Y}$ = 13.64 t/ha).

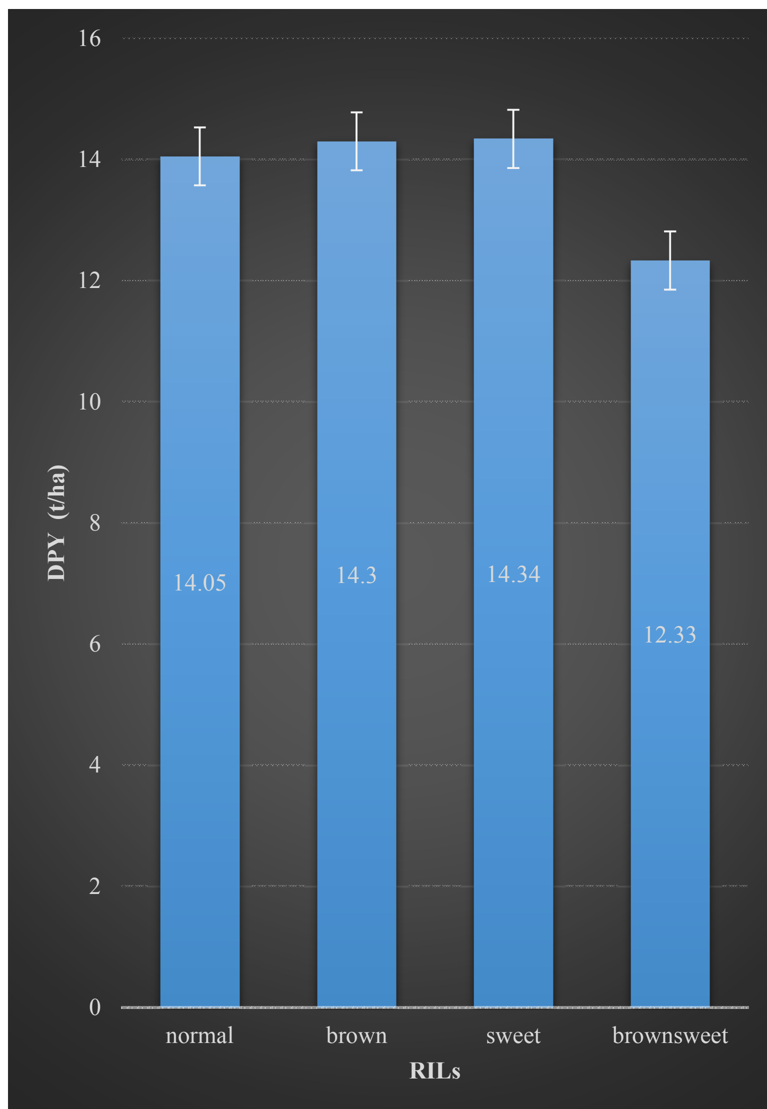


Figure A.11. Mean dry panicle yield (DPY) among four different RILs phenotypic classes. Bars represent standard errors.

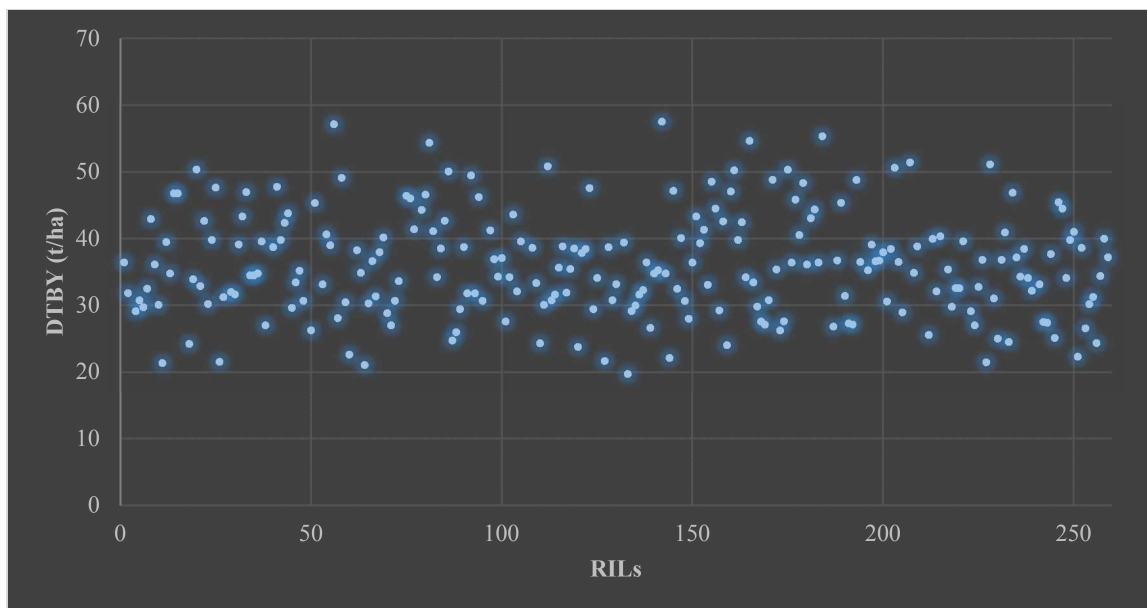


Figure A.12. RILs dry total biomass yield (DTBY) scatter plot ($\bar{Y}$= 35.22 t/ha).

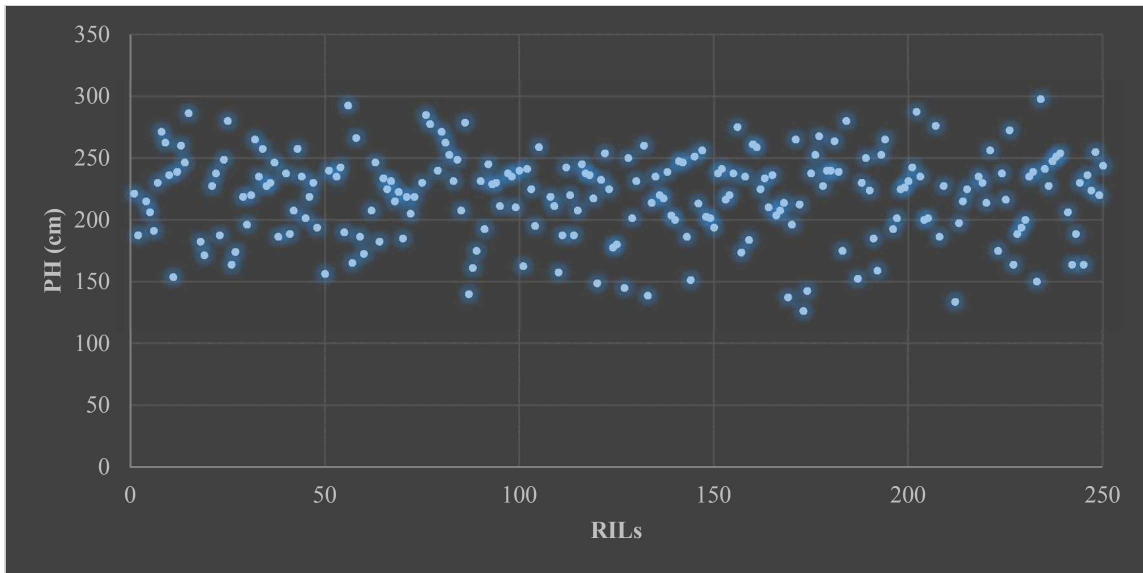


Figure A.13. RILs plant height (PH) scatter plot ($\bar{Y}$ = 218.3 cm).

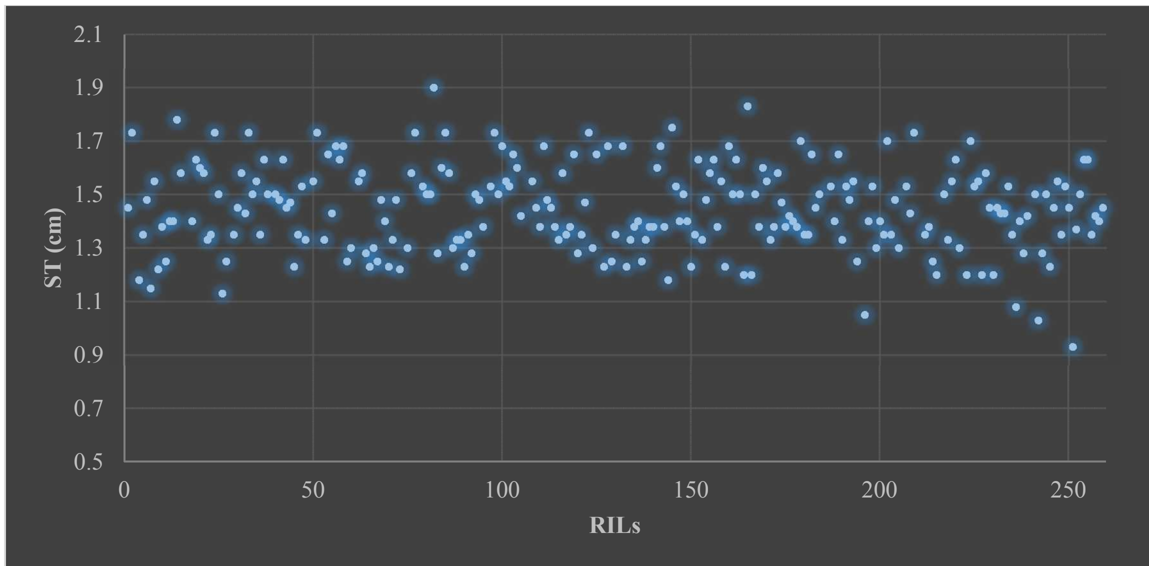


Figure A.14. RILs stem thickness (ST) scatter plot ($\bar{Y}$ = 1.44 cm).

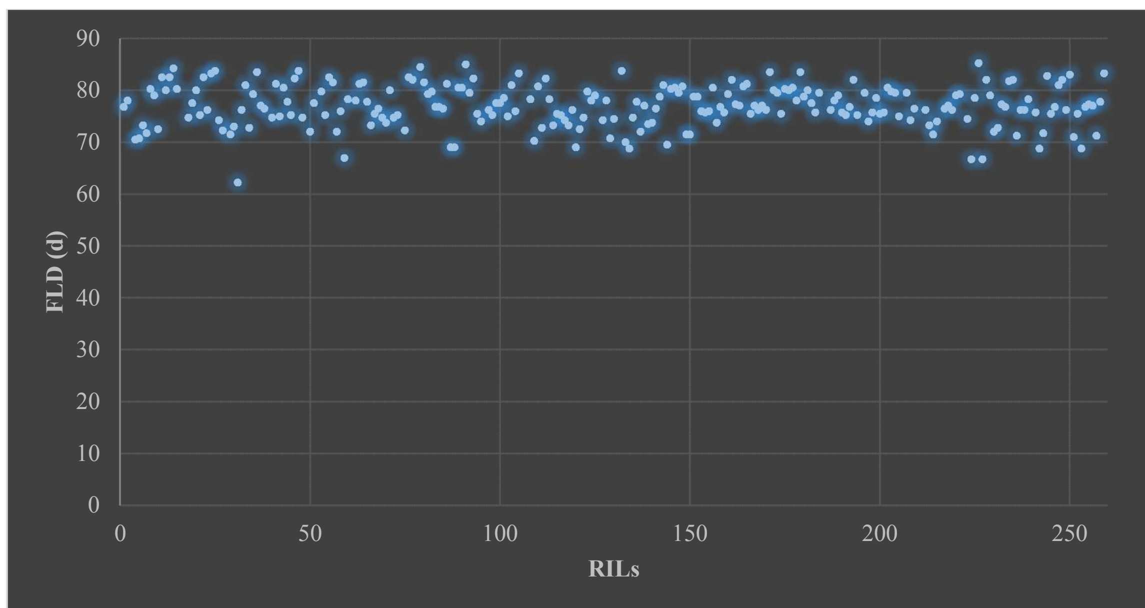


Figure A.15. RILs plant maturity (d) scatter plot ($\bar{Y}$= 77 days).

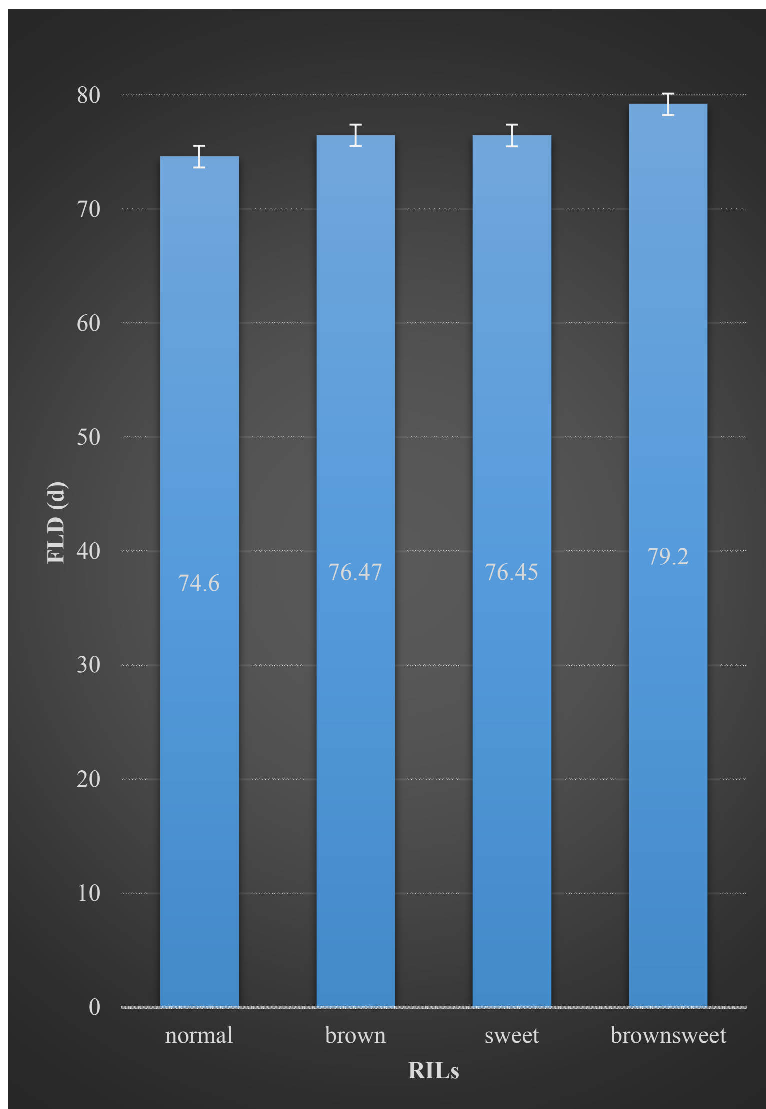


Figure A.16. Mean plant maturity (PM) among four different RILs phenotypic classes. Bars represent standard errors.

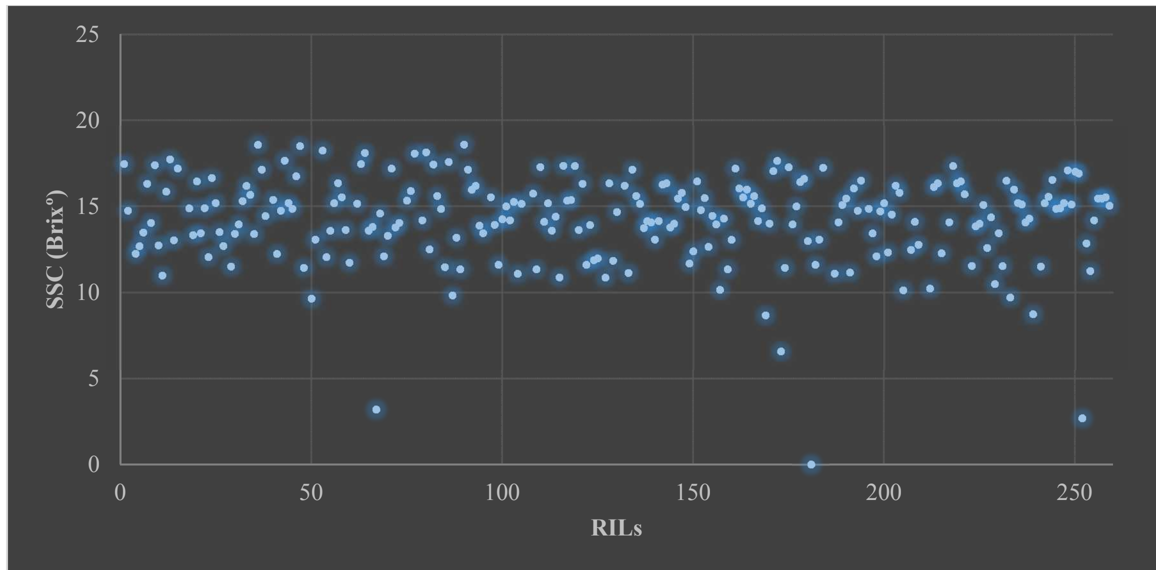


Figure A.17. RILs stem sugar concentration (SSC) Scatter Plot ($\bar{Y}$ = 14.25 ºBrix).

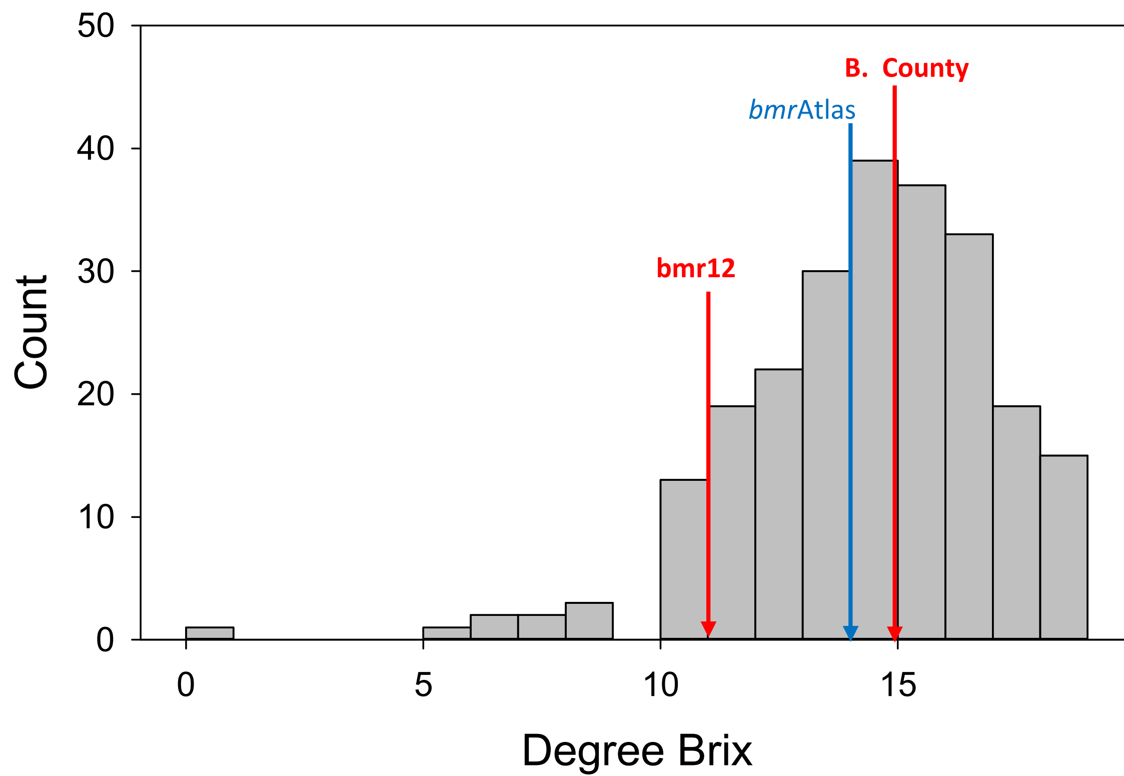


Figure A.18. Brix measurements distribution. B County (Stem sweet parent), bmr12 (Non-stem sweet parent) and bmrAtlas (control)

Table A.1. ANOVA with years (2008 and 2009)

Source of variation	df	DGY		DSY		DTBY		PH		ST		Brix		PM	
		Mean square		Mean square		Mean square		Mean square	Mean square	Mean square		Mean square		Mean square	
RIL	235	14	**	1.1	**	0.02	**	3060	**	0.09	**	15	**	69.2	**
brown-sweet vs normal	1	15		23.9	**	0.16	**	45228	**	0.00		1669	**	2157.4	**
brown-sweet vs sweet	1	85	**	1.2	**	0.12	**	1070		0.36	**	179	**	1101.0	**
brown-sweet vs brown	1	25	*	19.2	**	0.37	**	65198	**	0.16	*	291	**	253.4	**
Error	235	5		0.2		0.01		280		0.04		4		19.2	
RIL	235	15	**	1.1	**	0.02	**	2324	**	0.07	**	18	**	13.0	**
brown-sweet vs normal	1	126	**	8.5	**	0.03		32124	**	0.16		1019	**	460.2	**
brown-sweet vs sweet	1	174	**	4.9	**	0.22	**	252		0.19	*	4		341.4	**
brown-sweet vs brown	1	19		10.0	**	0.11	**	34261	**	0.06		527	**	35.1	**
Error	235	9		0.5		0.01		250		0.04		5		2.2	

*P-value is less than 0.05, **P-value is less than 0.01

Appendix B

(Chapter 2)

Table B.1. Year analysis of variance of Glucose Recovery

	SOV	DF	MS	
1	RIL	235	660	**
	brown-sweet vs normal	1	27053	**
	brown-sweet vs sweet	1	75814	**
	brown-sweet vs brown	1	168	
	Error	235	205	
2	RIL	235	1555	**
	brown-sweet vs normal	1	118594	**
	brown-sweet vs sweet	1	154610	**
	brown-sweet vs brown	1	996	
	Error	235	324	

*P-value is less than 0.05, **P-value is less than 0.01

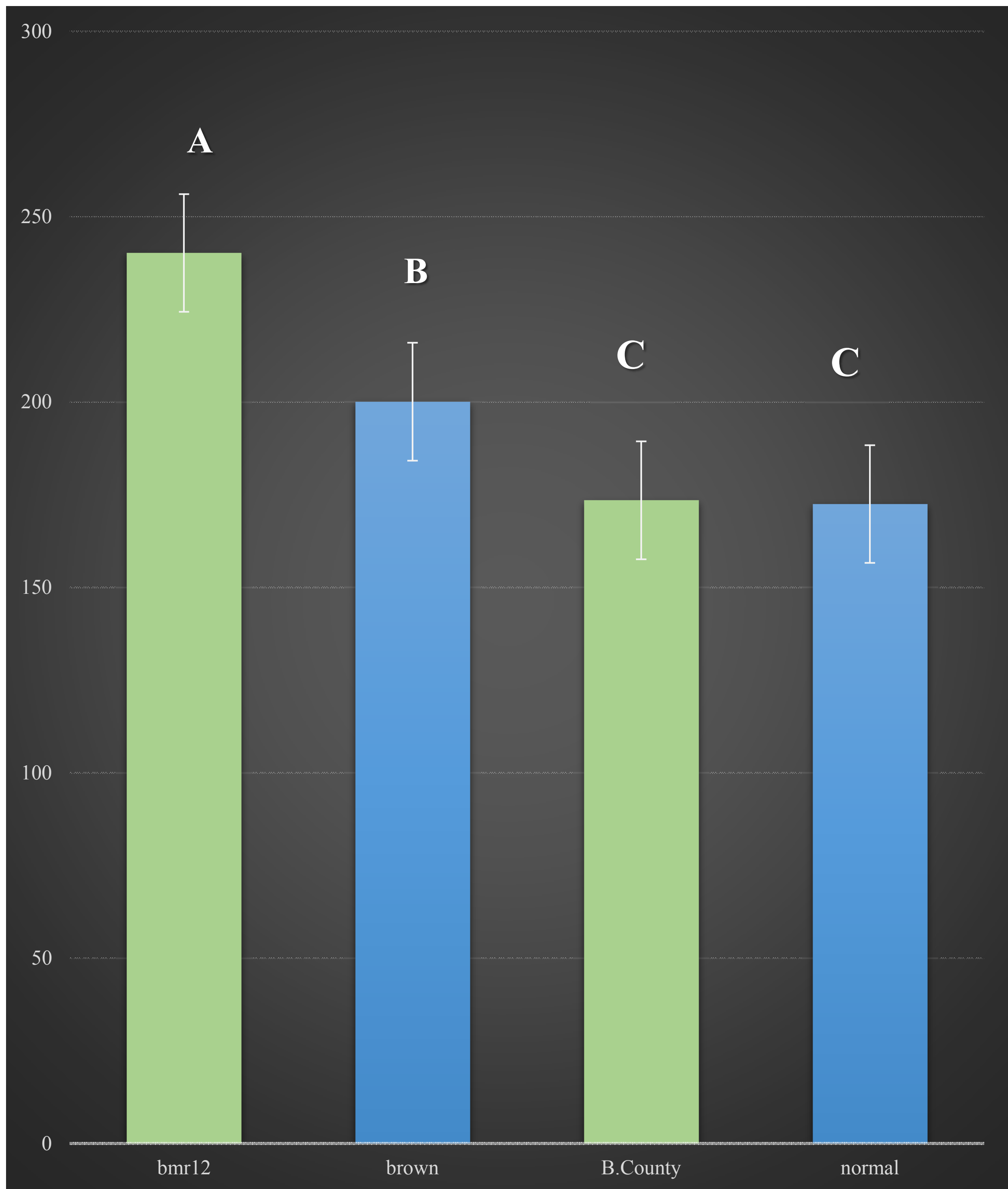


Figure B.1. Mean glucose recovery 2008 (LSD P<.05)

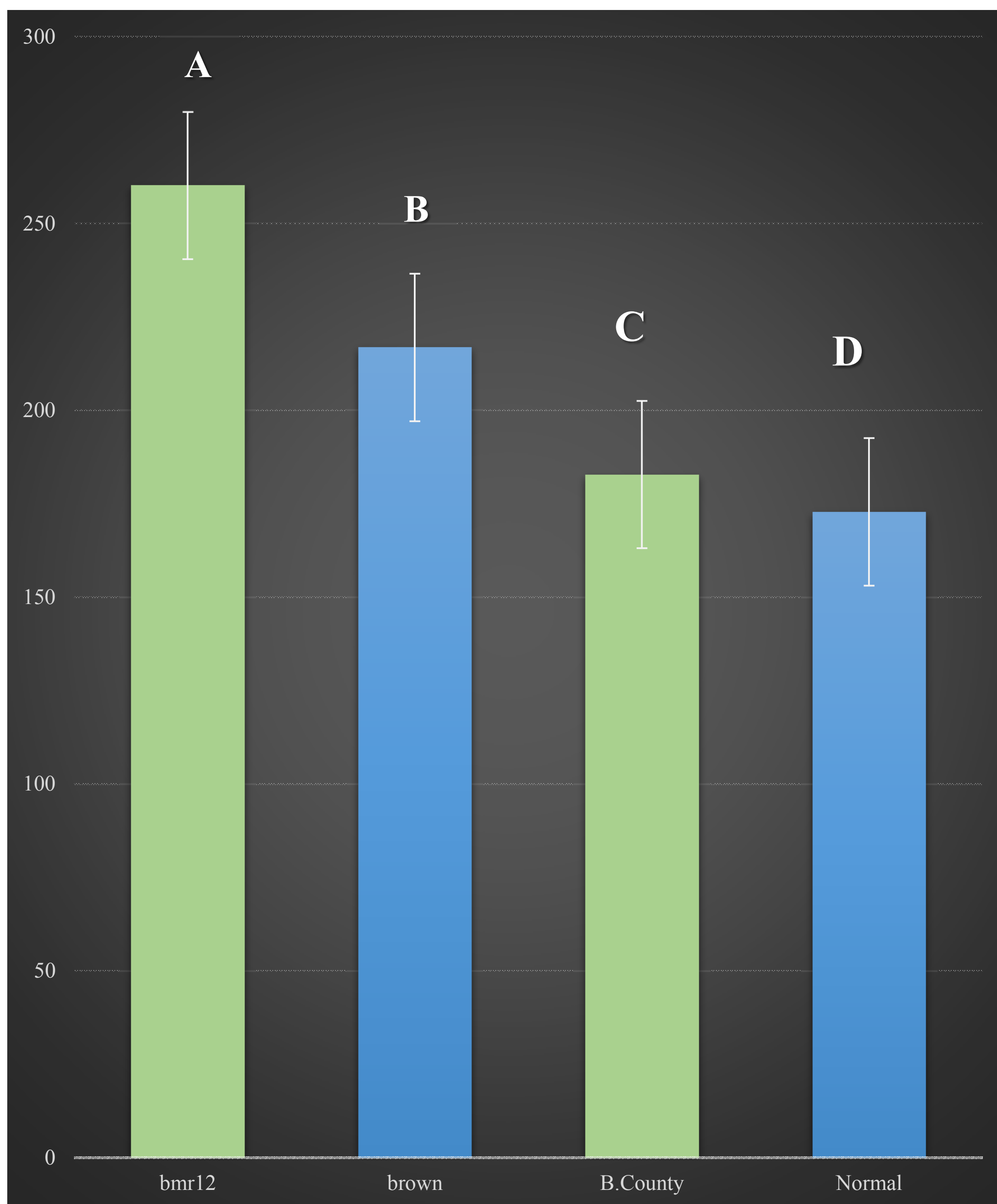


Figure B.2. Mena glucose recovery 2009 (LSD P<.05).

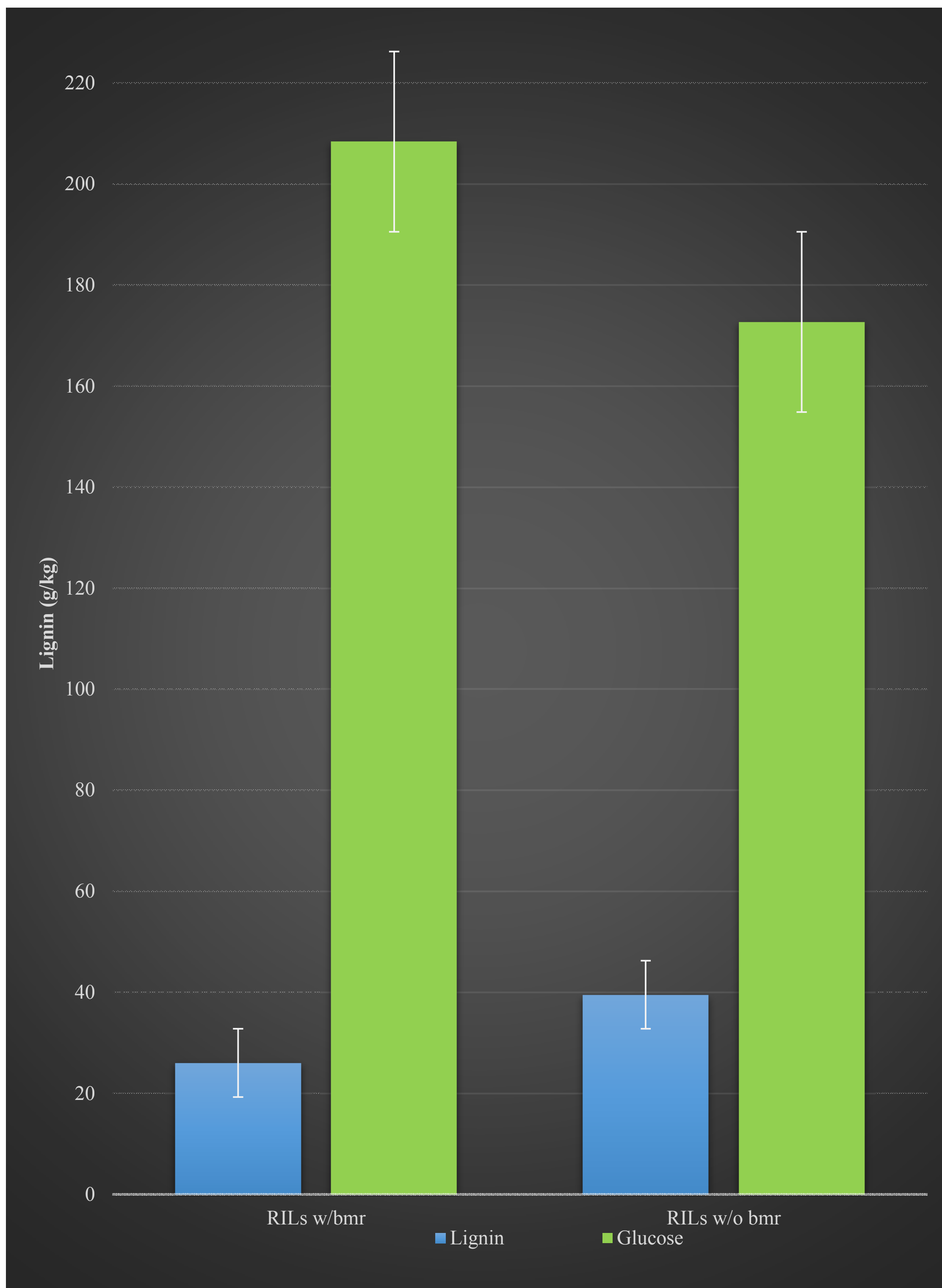


Figure B.3. Lignin/Glucose ratio (g/kg)

Table B.2. Year analysis of variance of theoretical ethanol production

	Source of variation	df	Mean square	
1	RIL	235	0.0441	**
	brown-sweet vs normal	1	2.4926	**
	brown-sweet vs sweet	1	0.0401	**
	brown-sweet vs brown	1	1.1036	**
	Error	235	0.0049	
2	RIL	235	0.0484	**
	brown-sweet vs normal	1	1.8723	**
	brown-sweet vs sweet	1	0.0016	
	brown-sweet vs brown	1	0.9359	**
	Error	235	0.0132	

*P-value is less than 0.05, **P-value is less than 0.01

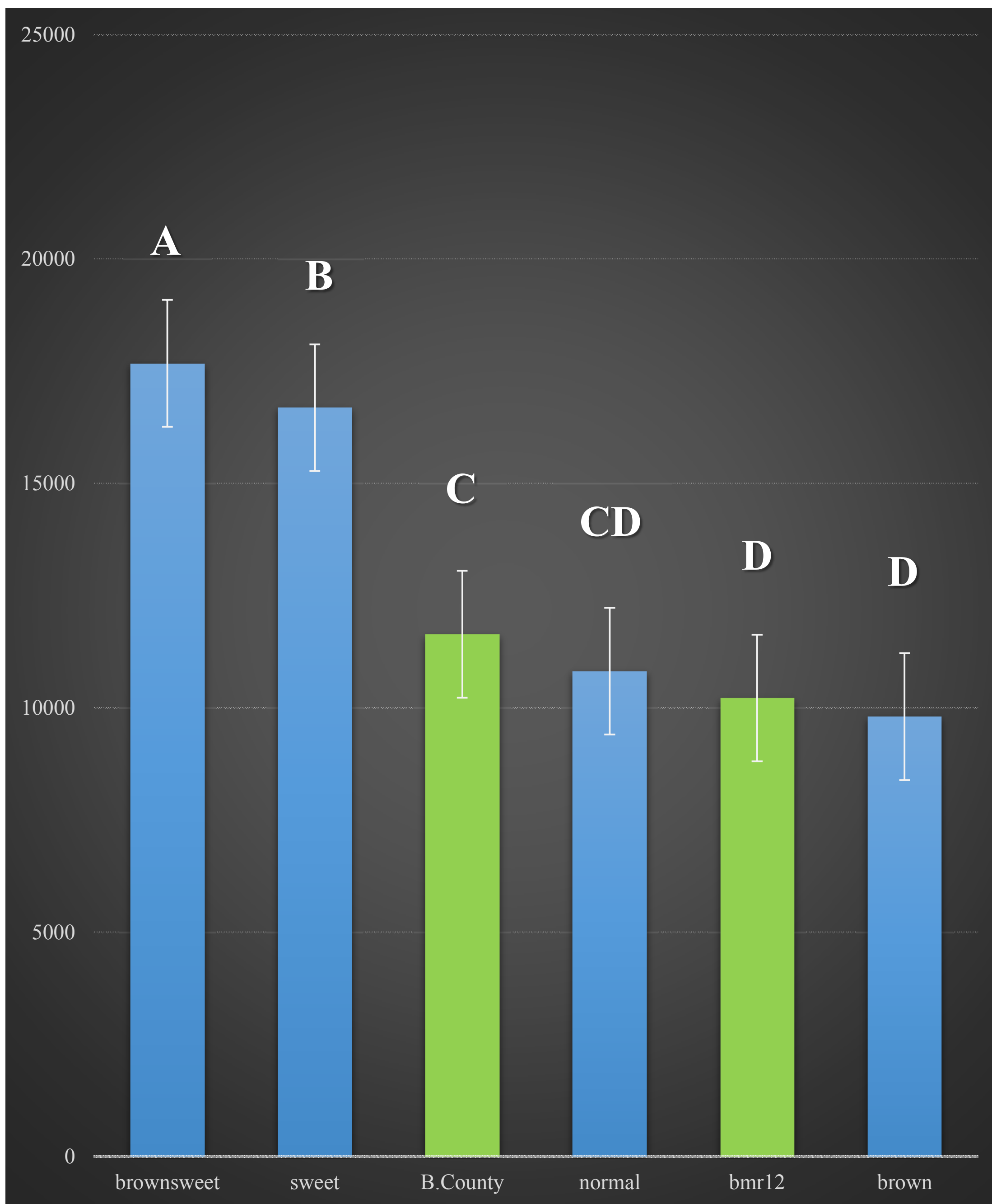


Figure B.4. Mean theoretical ethanol production 2008 (LSD P<.05).

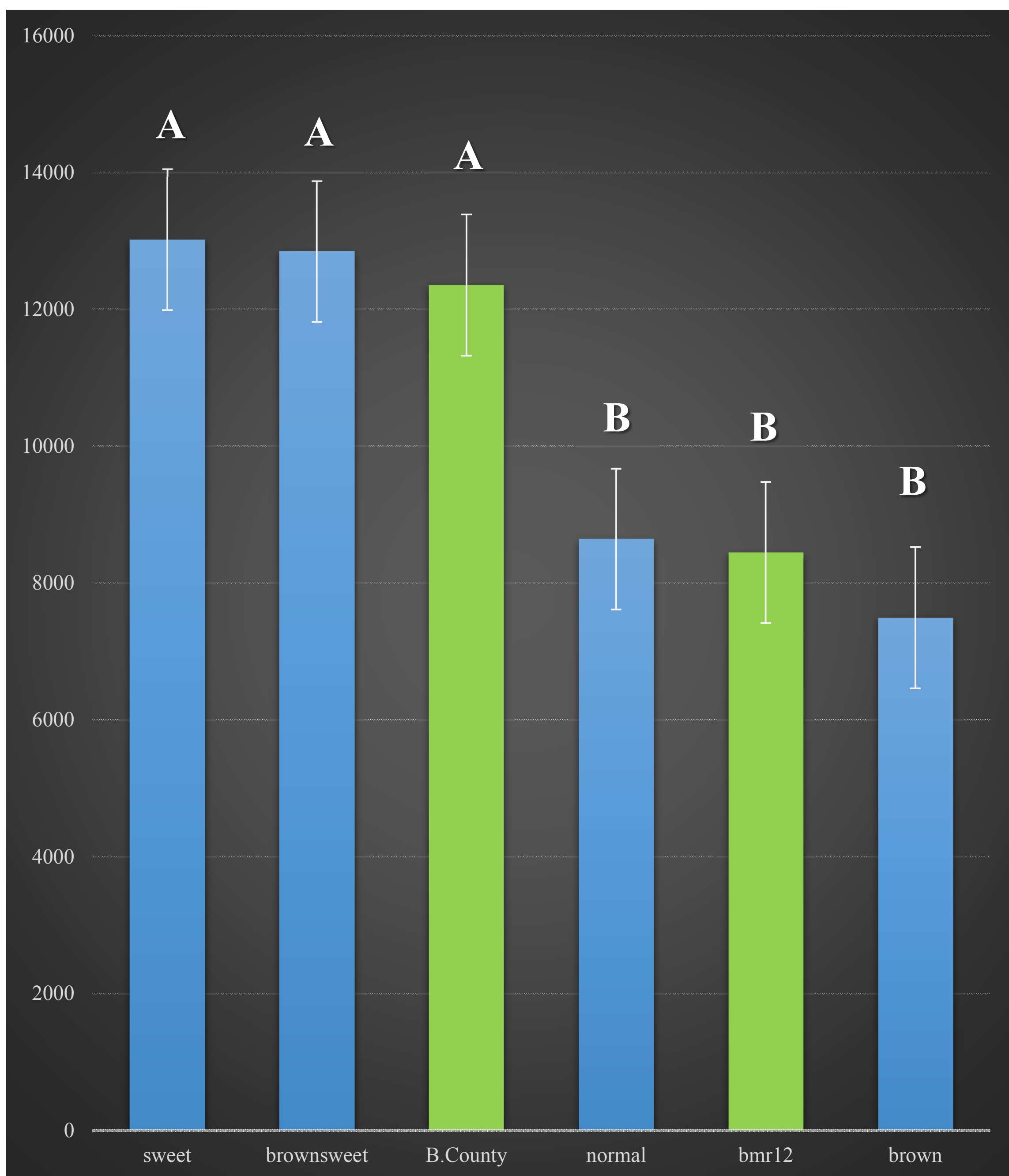


Figure B.5 Mean theoretical ethanol production 2009 (LSD P<.05)

Appendix C

(Chapter 3)

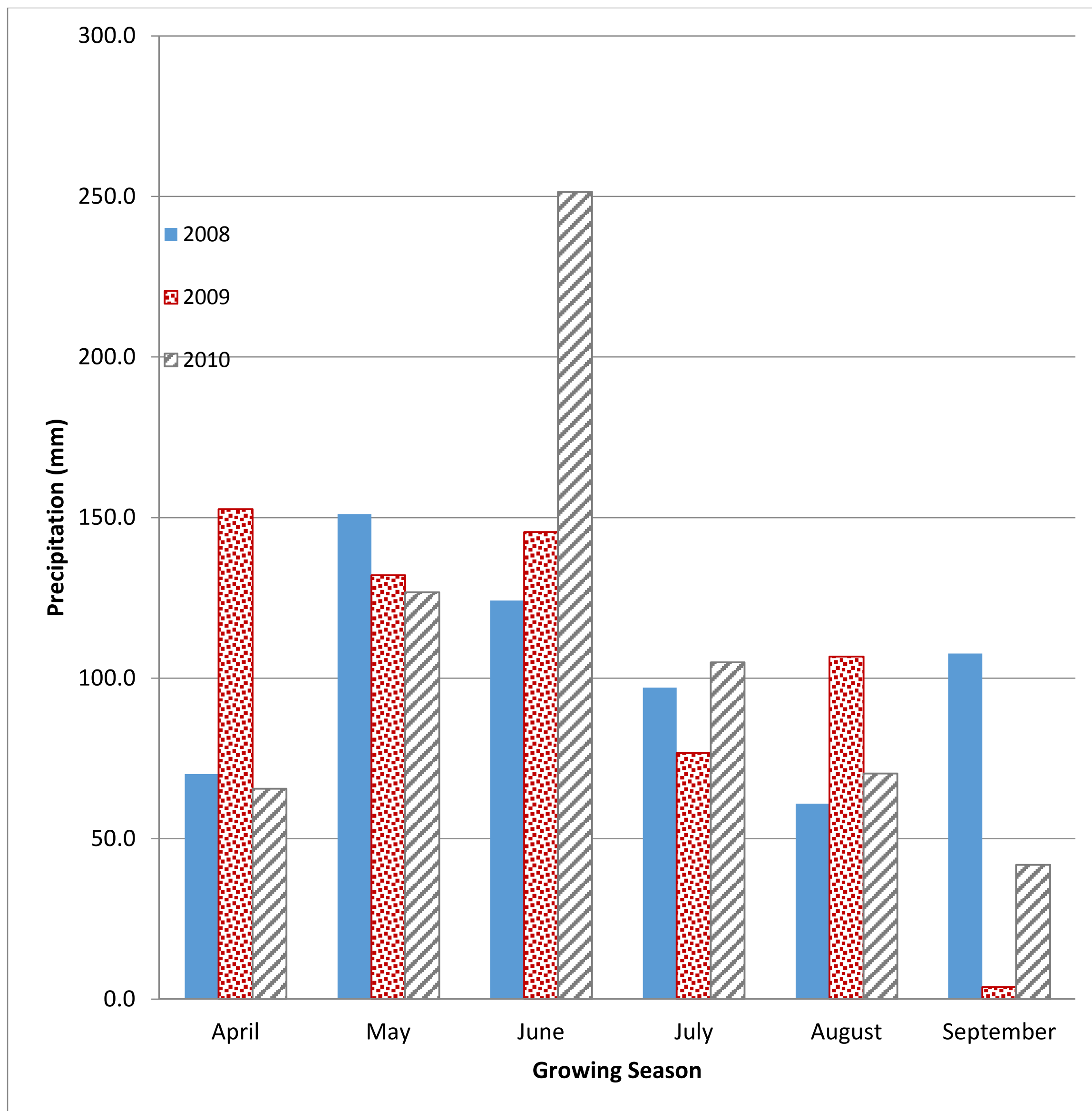


Figure C.1. – Monthly rain fall during growing season at ACRE – Purdue University, West Lafayette, Indiana for 2008, 2009 and 2010. Source: ACRE Meteorology Climate Center.

Table C.1 Rain precipitation and temperatures for 2008, 2009 and 2010 at ACRE.

Date	Precipitation		Avg. Mean Temp		Avg. Min Temp		Avg. Max Temp	
	(in)	mm	(°F)	(ºC)	(°F)	(ºC)	(°F)	(ºC)
May-08	6.0	151	57	14	46	8	68	20
Jun-08	4.9	124	72	22	62	17	83	28
Jul-08	3.8	97	73	23	62	17	83	29
Aug-08	2.4	61	69	21	57	14	82	28
Sep-08	4.2	108	66	19	53	12	80	27
Oct-08	1.8	45	52	11	38	3	66	19
Average	**4**	**98**	**65**	**18**	**53**	**12**	**77**	**25**
May-09	5.2	132	62	17	50	10	74	23
Jun-09	5.7	146	72	22	62	16	82	28
Jul-09	3.0	77	69	20	59	15	79	26
Aug-09	4.2	107	70	21	59	15	81	27
Sep-09	0.6	14	64	18	52	11	76	25
Oct-09	6.1	155	50	10	40	4	59	15
Average	**4**	**105**	**64**	**18**	**53**	**12**	**75**	**24**
May-10	5.0	127	64	18	53	12	75	24
Jun-10	9.9	251	74	23	63	17	84	29
Jul-10	4.1	105	76	25	66	19	87	31
Aug-10	2.8	70	75	24	63	17	87	30
Sep-10	2.1	54	66	19	52	11	80	27
Oct-10	0.9	23	54	12	37	3	71	21
Average	**4**	**105**	**68**	**20**	**56**	**13**	**80**	**27**

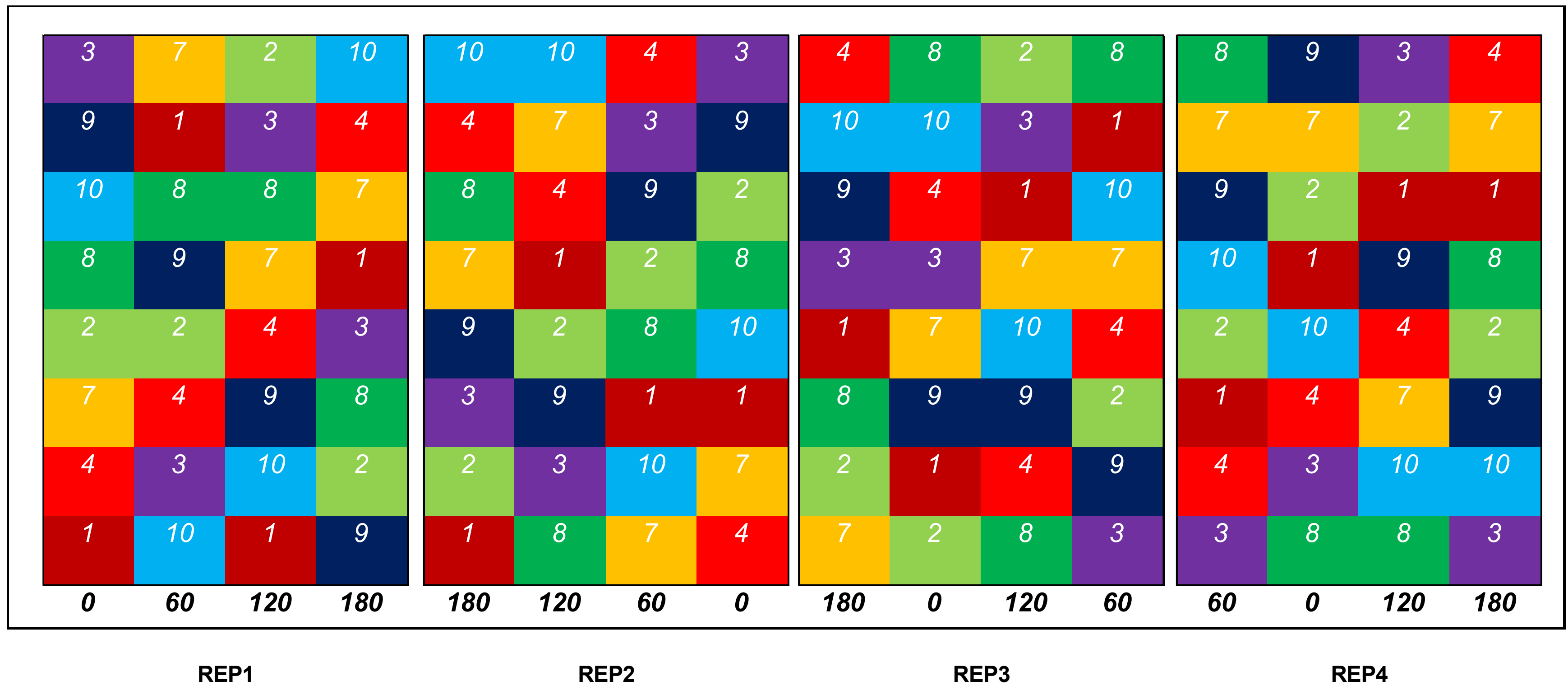


Figure C.2 Experimental design and randomization for 2008, ACRE. 1 = PR915AxBMR27, 2 = BMR27, 3 = PR915B, 4 = PU216A x P90344, 7 = AgriGoldAG585RR, 8 = Crosbyton A747 X R50, 9 = Sugar Drip, 10 = IS7777.

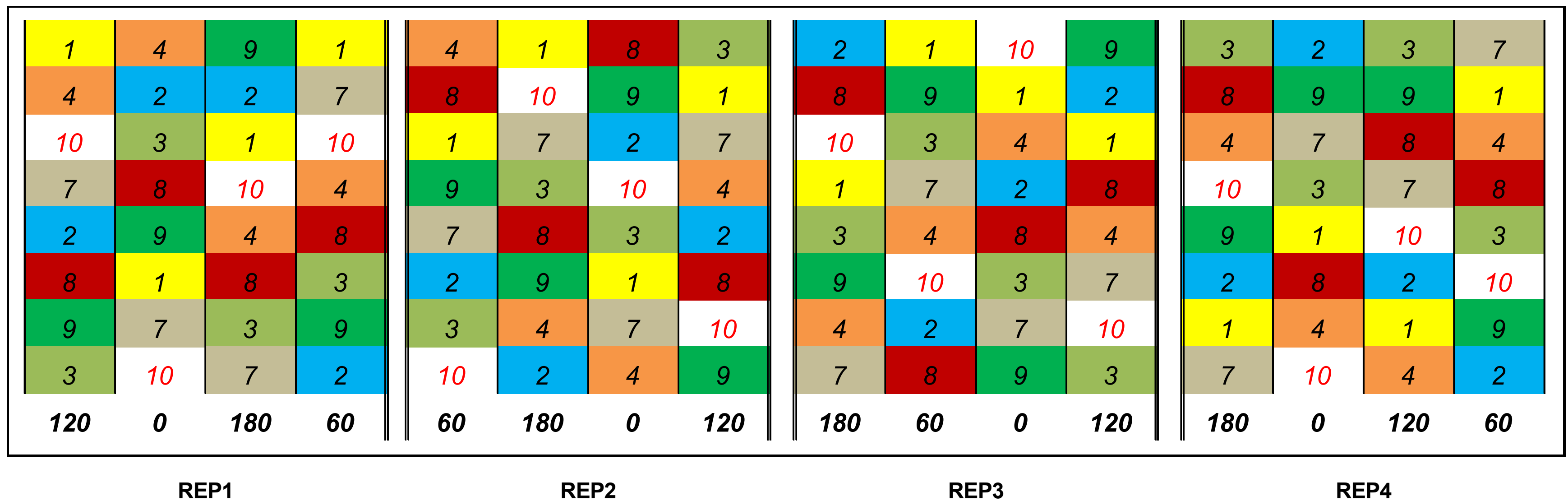


Figure C.3 Experimental design and randomization for 20010, ACRE. 1 = PR915AxBMR27, 2 = BMR27, 3 = PR915B, 4 = PU216A x P90344, 7 = AgriGoldAG585RR, 8 = Crosbyton A747 X R50, 9 = Sugar Drip, 10 = IS7777

Table C.2. Means dry grain yield and mean dry stover yield of nine sorghums and maize genotypes within each nitrogen rate.

	Dry grain yield (kg/ha)								Dry stover yield (kg/ha)							
Phenotype	0		60		120		180		0		60		120		180	
LLSH	2774	b	5808	b	7868	abc	7644	abc	10240	abc	13558	c	12966	c	14660	bc
FSL	3200	b	4667	bc	5803	cd	6315	cd	11919	abc	14670	c	14757	bc	15075	bc
LLSL	2066	b	4000	bc	4350	de	5299	de	7288	bc	9350	c	9972	c	10900	c
DPSH	1704	b	5077	bc	6353	cd	6391	cd	13190	abc	16248	bc	14359	bc	14826	bc
DPSL1	2595	b	5577	b	7107	abc	7231	bcd	11256	abc	11070	c	13315	c	13713	c
DPSL2	2875	b	5486	b	6568	bcd	6177	cd	14307	abc	16939	bc	16534	bc	17342	bc
SS	1906	b	2867	c	3225	e	3543	e	15816	ab	24167	ab	22626	ab	23096	ab
PSS	0		0		0		0		18023	a	26389	a	26715	a	30505	a
GSH	6483	a	8177	a	9160	a	9664	a	8020	bc	8627	c	9172	c	9956	c
GMH	2717	b	6017	ab	8665	ab	9399	ab	6323	c	9748	c	9810	c	9933	c

Table C3. Mean dry grain yield and mean dry stover yield of four nitrogen rates within each genotypes.

Phenotype	N rate	Biomass Components (kg/ha)			
	kg/ha	Grain		Stover	
LLSH	0	2774	c	10240	b
	60	5808	b	13558	a
	120	7868	a	12966	ab
	180	7644	a	14660	a
FSL	0	3200	c	11919	b
	60	4667	b	14670	ab
	120	5803	ab	14757	ab
	180	6315	a	15075	a
LLSL	0	2066	b	7288	b
	60	4000	a	9350	ab
	120	4350	a	9972	ab
	180	5299	a	10900	a
DPSH	0	1704	b	13190	a
	60	5077	a	16248	a
	120	6353	a	14359	a
	180	6391	a	14826	a
DPSL1	0	2595	c	11256	a
	60	5577	b	11070	a
	120	7107	a	13315	a
	180	7231	a	13713	a
DPSL2	0	2875	b	14307	a
	60	5486	a	16939	a
	120	6568	a	16534	a
	180	6177	a	17342	a

Continued. Table C.3. Mean of dry grain yield and mean dry stover yield of four nitrogen rates within each genotypes.

Phenotype	N rate	Biomass Components (kg/ha)			
	kg/ha	Grain		Stover	
SS	0	1906	b	15816	b
	60	2867	ab	24167	a
	120	3225	ab	22626	a
	180	3543	a	23096	a
PSS	0	0		18023	c
	60	0		26389	b
	120	0		26715	b
	180	0		30505	a
GSH	0	6483	c	8020	a
	60	8177	b	8627	a
	120	9160	ab	9172	a
	180	9664	a	9956	a
GMH	0	2717	c	6323	b
	60	6017	b	9748	a
	120	8665	a	9810	a
	180	9399	a	9933	a

Table C4. Means grain nitrogen uptake (g/kg) and mean grain carbon uptake (kg/ha) of nine sorghums and maize genotypes within each nitrogen rate.

	GNU (kg/ha)								GCU (kg/ha)							
Phenotype	0		60		120		180		0		60		120		180	
LLSH	34	b	68	ab	109	a	115	a	1183	b	2473	bc	3416	ab	3247	ab
FSL	42	ab	67	ab	92	ab	110	ab	1350	b	1987	bcd	2467	cd	2680	bc
LLSL	25	b	50	bc	61	c	81	c	868	b	1698	cd	1896	de	2252	cd
DPSH	22	b	62	b	85	b	93	bc	722	b	2146	bc	2704	bcd	2706	bc
DPSL1	33	b	68	ab	101	ab	116	a	1088	b	2261	bc	3022	abc	3099	bc
DPSL2	36	b	66	ab	97	ab	89	c	1234	b	2323	bc	2847	bc	2608	bc
SS	24	b	38	c	51	c	56	d	818	b	1226	d	1517	e	1547	d
GSH	59	a	85	a	108	a	128	a	2702	a	3460	a	3889	a	4096	a
GMH	28	b	61	b	103	ab	120	a	1176	b	2614	ab	3886	a	4073	a

Table C.5. Mean grain nitrogen uptake (g/kg) and mean grain carbon uptake (kg/ha) of four nitrogen rates within each genotypes.

Phenotype	N rate	Biomass Components (kg/ha)			
	kg/ha	GNU		GCU	
LLSH	0	34	c	1183	c
	60	68	b	2473	b
	120	109	a	3416	a
	180	115	a	3247	a
FSL	0	42	d	1350	c
	60	67	c	1987	b
	120	92	b	2467	ab
	180	110	a	2680	a
LLSL	0	24.8	c	868	b
	60	50.0	b	1698	a
	120	60.6	b	1896	a
	180	81.4	a	2252	a
DPSH	0	22.4	c	722	b
	60	61.8	b	2146	a
	120	85.0	a	2704	a
	180	93.4	a	2706	a
DPSL1	0	33.4	c	1088	c
	60	68.4	b	2261	b
	120	100.9	a	3022	a
	180	116.3	a	3099	a
DPSL2	0	35.9	c	1234	b
	60	65.6	b	2323	a
	120	97.3	a	2847	a
	180	89.1	a	2608	a

Continued. Table C.5. Mean grain nitrogen uptake (g/kg) and main grain carbon uptake (kg/ha) of four nitrogen rates within each genotypes.

Phenotype	N rate	Biomass Components (kg/ha)			
	kg/ha	GNU		GCU	
SS	0	24	c	818	b
	60	38	bc	1226	ab
	120	51	ab	1517	a
	180	56	a	1547	a
GSH	0	59	d	2702	c
	60	85	c	3460	b
	120	108	b	3889	ab
	180	128	a	4096	a
GMH	0	28	c	1176	c
	60	61	b	2614	b
	120	103	a	3886	a
	180	120	a	4073	a

Table C.6. Estimation of AONR for Grain Yield eight sorghum and maize genotype.

	FSL (PR915B)			LLSL (bmr27)			LLSH (PR915Axbmr27)			DPSL1 (PU216B)		
	N-Rate	**Yield**		**N-Rate**	**Yield**		**N-Rate**	**Yield**		**N-Rate**	**Yield**	
	202	5721	A	202	5299	A	135	**8552**	A	202	7977	A
	135	5300	A	135	4435	A	202	8267	AB	135	7806	A
	67	**4300**	AB	67	**4169**	A	67	6911	B	67	**6628**	A
	0	2917	B	0	2079	B	0	3411	C	0	3051	B
AONR		**67**			**67**			**135**			**67**	

LSD(P>.05)

Continued Table C.6. Estimation of AONR for Grain Yield eight sorghum and maize genotype

DPSL2			DPSH			SS		
(P90344)			(PU216AxP90344)			(Sugar Drip)		
N-Rate	**Yield**		**N-Rate**	**Yield**		**N-Rate**	**Yield**	
135	**8502**	A	202	13313	A	202	3216	A
202	7859	AB	135	**12124**	A	135	2992	A
67	6848	B	67	9947	B	67	2620	A
0	3299	C	0	3573	C	0	1793	A
135			**135**			**0**		

LSD (P>.05)

Continued Table C.6. Estimation of AONR for dry grain yield of nine sorghum and maize genotype.

	Grain Maize Hybrid			Grain Sorghum Hybrid		
	(AgriGoldAG585RR)			(CrosbytonA747xR50)		
	N-Rate	**Yield**		**N-Rate**	**Yield**	
	202	12977	A	202	8968	A
	135	**11857**	A	135	8949	A
	67	7788	B	67	**7602**	A
	0	3782	C	0	5804	B
AONR	**135**			**67**		

LSD (P>.05)

Table C.7. Estimation of AONR for dry stover yield of nine sorghum and maize genotype.

	FSL			LLSL			LLSH			DPSL1		
	(PR915B)			(*bmr*27)			(PR915AxBMR27)			(PU216B)		
	N-Rate	**Yield**		**N-Rate**	**Yield**		**N-Rate**	**Yield**		**N-Rate**	**Yield**	
	202	15014	A	202	11744	A	202	17212	A	202	17154	A
	135	14709	AB	135	10731	AB	67	**16805**	A	135	16369	A
	67	**14521**	AB	67	**10285**	AB	135	15368	A	67	**14469**	AB
	0	11680	B	0	7894	B	0	12061	B	0	13009	B
AONR	**67**			**67**			**67**			**67**		

LSD (P>.05)

Continued. Table C. 7. Estimation of AONR for dry stover yield of nine sorghum and maize genotype.

DPSL2				DPSH				SS				PSS			
(P90344)				(PU216AxP90344)				Sugar Drip				(IS7777)			
N-Rate	**Yield**			**N-Rate**	**Yield**			**N-Rate**	**Yield**			**N-Rate**	**Yield**		
202	22260	A		67	**32387**	A		67	**22233**	A		202	**32624**	A		
135	21871	A		202	31160	A		202	20787	A		135	28242	B	
67	**20894**	A		0	28088		B	135	19376	A		67	27931	B	
0	15285		B	135	28011		B	0	15199		B	0	18952		C
67				**67**				**67**				**202**			

LSD (P>.05)

Continued. Table C. 7. Estimation of AONR for dry stover yield of nine sorghum and maize genotype.

	GMH			GSH		
	(AgriGoldAG585RR)			(CrosbytonA747xR50)		
	N-Rate	**Yield**		**N-Rate**	**Yield**	
	202	22026	A	202	11278	A
	135	20869	A	135	11015	A
	67	**19799**	A	67	9946.82	A
	0	12628	B	0	**8455**	A
AONR	**67**			**0**		
LSD (P>.05)						

VITA

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