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Phenotypic Plasticity of Diverse Sorghum Varieties in Response to Nitrogen Deficit Stress

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PHENOTYPIC PLASTICITY OF DIVERSE SORGHUM VARIETIES IN RESPONSE TO NITROGEN DEFICIT STRESS

by

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Nitrogen is a key component in the growth of crops and can strongly influence phenotypic variation. The movement of nutrients from old vegetative tissue to young leaves and reproductive organs is known as senescence. Senescence can be influenced by the environment of a plant, such as, drought, heat, and nutrient stress. These environmental stressors can also influence canopy architecture. Crop canopy consists of several traits including: leaf number, angle, length and width. Past research in crops like maize and rice have shown significant influences of more erect leaves, including, the ability for increased planting density, which optimizes light interception and increases yield. In a field trial of a sorghum association panel including 345 different sorghum varieties grown in a replicated design under nitrogen sufficient and nitrogen deficit conditions, we have found that sorghum leaf angle is plastic in response to nitrogen deficit stress. While the response of individual varieties varied, an overall statistically significant trend was observed towards smaller leaf angles (e.g. more erect leaves) under deficient nitrogen conditions. In parallel with field experiments, greenhouse experiments utilizing sorghum plants grown under different levels of nitrogen availability were also conducted. These confirmed the increased erectness of sorghum leaves under nitrogen deficient conditions. In addition, RGB and hyperspectral images collected from this experiment were used to train machine learning models to classify individual pixels into six classes: nonplant pixels, panicles, stems, green leaves, senescing leaves, and dead leaves. With this new method, we were able to track the progression of senescence through the life cycle of individual sorghum plants

grown under different nitrogen treatments.

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Contents

List of Figures

List of Tables

Chapter 1

Introduction

Sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is grown largely in semiarid, and underdeveloped parts of Africa and Asia. It has many uses including food for human consumption primarily in semiarid regions, and is increasingly used in the United States as animal feed and biofuels [154, 167]. Sorghum has been found to have increased nutritional value over other cereal crops. The amino acid profile for sorghum and maize is relatively similar, however, sorghum is high in alanine with 9.3 and 8.4 grams per 100 grams compared to maize with 8.6 and 6.9 grams, while maize is slightly higher in lysine with 2.4 and 3.3 grams per 100 grams of maize, with sorghum containing 2.1 and 2.9 grams [35]. Sorghum is also gluten free a good option for those with celia disease, is rich in nutrients, and is high in phenolic compounds [6, 34]. Phenolic compounds have been linked to reductions in several diseases and antioxidant power. Antioxidants are known to inhibit oxidation of oxidizable substrate that can help prevent inflammation, cardiovascular disease and aging [58, 92]. Sorghum has also increased in popularity around the world because of its productivity under undesirable environments. Sorghum has been shown to be productive under drought, and high heat; while also able to grow in high-salinity [22, 78, 140, 163]. The high root to leaf ratio, and protection from leaf wax may be linked to sorghum's ability to withstand harsh conditions such as those listed [181]. Furthermore, sorghum is efficient at maintaining high grain yields when grown under limited nitrogen condition [76, 91, 134, 179, 200].

Nitrogen in plants

Nitrogen is an important nutrient used in plant growth, and is needed for many components in cells. These include amino acids, proteins, nucleic acids, and chlorophyll [171]. Two prominent phenotypes which vary among plants and are relevant to nitrogen are: nitrogen use efficiency and nitrogen mobilization. Nitrogen use efficiency has many definitions including biomass production and harvest product per unit of nitrogen, nitrogen content within a plant during its life cycle, and several calculations that may consider: nitrogen utilization, nitrogen content, and nitrogen availability [31, 46, 61, 122, 123]. Some definitions of nitrogen efficiency can be placed into two categories, uptake and utilization efficiency [61, 122, 147]. Nitrogen uptake efficiency can be interpreted as the absorption or uptake of supplied nitrogen; while utilization efficiency can be explained as the efficiency of assimilation and remobilization within a plant. Nitrogen use efficiency includes maximizing the nitrogen content in grain yield per unit of soil nitrogen. Improvements can be obtained by a plants ability to improve nitrogen uptake capacity such as improving roots ability to take up nitrogen [62, 66]. Improvements in nitrogen mobilization and remobilization efficiency can also allow for better nitrogen use efficiency . [62]. Crop species have poor nitrogen uptake efficiency, taking up approximately 40 to 50 percent of applied nitrogen [9, 60, 132] High genetic variation within species has been found for nitrogen use efficiency for cereal crops including sorghum [109]. Even though sorghum as a crop species is efficient under limiting nitrogen levels, sorghum varieties have differing modes of nitrogen use. Tropical varieties for example, are better at nitrogen uptake at the vegetative stage than hybrids and U.S. temperate adapted varieties. Since domesticated, tropical lines have been selected under low nitrogen environments resulting in developmental differences of anatomical and physiological traits allowing for nitrogen uptake from deficient soil [171]. Although tropical varieties are able to take up nitrogen better at vegetative stage with greater biomass, hybrid and U.S. sorghum had enhanced reproductive nitrogen content [171]. However, to increase nitrogen use efficiency in sorghum as a whole, advancements in both nitrogen uptake such as those in tropical varieties, and usage of nitrogen in hybrids and U.S. varieties.

In addition to nitrogen use efficiency in crops, mobility of nitrogen in a plant is also an important advancement for crop productivity. Extensive research has been done to improve nitrogen use efficiency and identify and characterize the genes that influence nitrogen mobility and metabolism in plants [8]. Nitrogen metabolism can be separated into two stages: accumulation and remobilization. Accumulation occurs during the vegetative stage of a plant where nitrogen is taken up by roots and stored in leaves as nitrate for use when nitrate availability is a limit on growth and development. Remobilization occurs at the reproductive stage, at this point accumulation of nitrogen is slow and stored nitrogen in older leaves is moved to younger leaves and reproductive tissue such as grain [76]. In Sorghum, nitrogen is stored in cell vacuoles of leaf tissue. Nitrate and proteins are stored in the greatest amounts to be stored when nitrogen supply is high in soil and reallocate nitrogen supplies for later use in the life cycle [120,184]. To improve nitrogen use efficiency, accumulation within plant tissue and remobilization throughout the plant should both be addressed to increase yield [152].

The end stages of crop life cycle, when plants transition from nitrogen accumulation to mobilization, is known as senescence [42,112]. The rate of senescence and remobilization of leaf nitrogen is linked to nitrogen status [25,112,136]. Senescence is a process within plants that is responsible for resource allocation [72]. Senescence is used to remove photosynthetic organs where significant amounts of nutrients have been invested, it is a key component of nutrient source/sink of plants throughout its life cycle. It is controlled by several components: growth hormones, nutrient sensing, and stress response networks [15, 65, 97]. Although nitrogen remobilization is studied extensively, it is not the only nutrient remobilized in plants. Other nutrients include: Zn, Cu, Fe, S, Cr, P, Mo, and K; during senescence these elements along with nitrogen are mobilized to other portions of the plant reducing leaf content by more than 40 percent [75]. Nitrogen is the most efficient at being mobilized in plants. The main forms in which nitrogen remobilized are amino acids, small peptides, ammonium, urea, and nitrate. Remobilization of nitrate from plant storage is more common in plants grown under sufficient nitrogen supply than that of limiting nitrogen environments [12, 85].

Nitrogen fertilizers in agriculture

Before the beginning of the twentieth century, the main source of agricultural nitrogen was from nitrogen fixation by symbiotic bacteria in legumes. After the invention of the Haber Bosch process production of ammonium for fertilizer, an otherwise stagnant rate of increase crop production boomed thanks to Haber-Bosch. The Haber-Bosch process creates ammonia by inputs of unreactive nitrogen (N2), found in Earth's atmosphere, and energy [164]. This boom in fertilizer use and agricultural productivity has come at economic and environmental costs [164]. Nitrogen supply in the soil is of great importance for above ground development of plants; root development and nitrogen transport are both mechanisms responsible for nitrogen acquisition from soil [45, 59, 84, 133, 168, 186, 188]. The Green Revolution occurred between 1966 to 2000, it focused on advancements of three main cereal crops: rice, wheat, and maize [39, 74]. Advancement in technology and agronomic methods during the Green Revolution have led to increases in cereal crop yields. Since the late 1960's to now the global population has doubled, fortunately during the Green Revolution production of cereal crops tripled. These increases were achieved with only marginal increases in land use for crop production [145]. Some of the advancements during the Green Revolution include, increased use of herbicide resistant crops that reduce competition with weeds [14]. Genetic improvements in crops, including the introduction of dwarf genes in rice and wheat that resulted in shorter more sturdy varieties [146]. Since 1980 to 2010 the increase in cereal crop yield in China has increased by 65 percent, however, application of fertilizers have increased by 512 percent [20,193]. Crop production around the world is greatly impacted by application of nitrogen fertilizer. Fertilizers are linked to many environmental issues including: soil acidification, water contamination, and excess algae causing ocean dead zones due to low oxygen content [10,53,67,164]. This is due to overuse of nitrogen fertilizers and current cereal crop varieties with poor mechanisms for nitrogen uptake resulting in the loss of much of the nitrogen applied to agricultural systems [143,150,165]. The remaining applied nitrogen is lost to runoff and leaching [9, 52, 60, 132]. This has increased the importance of breeding for plants that are more adapted to decreased nitrogen content in soil, or increased nitrogen use efficiency [67, 164]. An estimate in the late 1990s, calculated that a one percent increase in nitrogen uptake by crops could save the U.S. 2.3 billion dollars annually in nitrogen fertilizer costs [150]. In fact during the 1960's, nitrogen use efficiency globally was 68 percent. In more recent years efficiency has decreased to 47 percent. Excess amounts of available nitrogen have been linked to crop deceases in nitrogen use efficiency [90]. A major challenge scientists face with improving nitrogen use efficiency is their ability to effectively phenotype a complex trait such as nitrogen use efficiency. Nitrogen use efficiency can be altered in plants several ways: root architecture, improvements in uptake has several physiological processes, metabolic genes, plant development such as vegetative growth and canopy architecture, as well as nitrogen metabolism and remobilization genes [111, 113].

Phenotyping

Phenotyping traits in crop species is one of the remaining bottlenecks, in advancing genetics of complex traits such as nitrogen use efficiency, discussed above, and crop yields. With rapid advancements in genotyping and an increase in larger data sets, a greater need has emerged for high-throughput phenotyping [4, 50].

High-throughput phenotyping can be defined as optimizing the speed at which an organism is examined, including morphological, physiological and biochemical traits. High-throughput phenotyping can be achieved by lowering cost, and automating data collection with the use of cameras and computers. In the plant community, it has been defined as analyzing morphological traits for ten to hundreds of plants per day [41, 56]. Technological advances have helped to bridge the gap in high-throughput phenotyping. In the last few years, these advancements include phenotyping staple crops in growth chambers and greenhouses. These high-throughput systems use digital imaging; such as, red green blue, near infrared, thermal, and hyperspectral [18, 21, 40, 55, 105, 130]. There can be both advantages and disadvantages to high-throughput greenhouse phenotyping. Some advantages to phenotyping crops in single pot greenhouse environments using high-throughput imaging, include limited no interference from other plants in the crop canopy and no addition changes in climate other than those important to the study [44]. High-throughput imaging in a greenhouse setting can be used to identify a range of phenotypes. For example, sorghum was imaged using red green blue and near infrared cameras for drought and fertilizer use response [130]. Red green blue and hyperspectral imaging is part of a high throughput phenotyping greenhouse (Scanalyzer3D, LemnaTec GmbH, Aachen, Germany) located at the University of Nebraska-Lincoln. Red green blue cameras use visible light to create an image that can be used in measuring plant size, biomass, and growth. Hyperspectral camera uses both visible and near infrared, and uses a spectrum of 550 nm to 1,700 nm. The camera creates a cube of 243 images bands to create a cube, and includes 320 pixels [138]. These pixels have been used in a study on sorghum as an example crop for semantic segmentation of plants in to three classes: stalk, panicle, and leaf. This was used to measure traits such plant height, panicle size, and stalk/leaf ratio [118]. Red green blue imaging has also been used to construct 3 dimensional images of crop canopies, to more accurately phenotype crop canopy architectural traits [51]. In addition to advanced phenotyping, this method is also cost effective. The cost of collecting accurate and large phenotypic traits can be costly and time consuming. High throughput phenotyping is used when either; low cost per data point, more accurate, or measure yield relevant traits. Senescence is an important agronomic trait that tells scientists both about the environment in which a plant is grown and remobilization of important nutrients such as nitrogen. However, senescence can be a challenge to phenotype, it is subject to human bias and not as easily quantified such as seed weight or plant height. As previously discussed, plants in recent studies have been able to use hyperspectral images to segment plants into three organs: leaf, stalk, and panicle in sorghum. Using red green blue images it can be challenging to differentiate between green leaf and green stalk [118]. With the use of both plant segmentation, to differentiate between leaf, stalk and panicle and the use of hyperspectral images to collect differences in tissue reluctance, we hypothesize that segmentation can also be used to track the senescence of sorghum under differing nitrogen treatments. This will allow for more uniform quantification of plant senescence.

Although these advancements in high-throughput are important, due to the controlled conditions of a greenhouse, these phenotypes can translate poorly to field experiments [131, 148]. There is still pressing need for phenotyping in a field environment. Crop canopy could be challenging to phenotype in a high-throughput phenotyping greenhouse, for example, all competition is lost in a single pot experiment. Crop canopy is greatly impacted by environment both biotic and abiotic stresses, this can be challenging to implement in a greenhouse and generally plants may exhibit different phenotypes in the field [139]. With regards to canopy, competition is important when phenotyping these traits. Crop canopy has a great impact on the productivity of crops. Several physiological attributes influence crop canopy architecture, including, leaf number, angle, length and width [86,108,119,194]. Additionally, canopy is impacted by plant field spacing. Denser planting in maize was found to result in smaller leaf length, taller plants, and more erect leaf angles [104]. Plant architecture greatly influences photosynthesis and high yields. Canopy greatly influences the interception of photosynthetically active radiation, which is obtained by the sun and is used to convert carbon in the atmosphere into energy that is used for plant growth and reproduction [200]. Leaves at the top of the canopy are generally over-saturated with light, this can cause a decrease in the ability of the leaf to convert light into energy [101, 200]. In addition to top leaves being over-saturated, lower leaves in crop canopies can be under-saturated, which can cause early senescence and loss of photosynthetic ability. This can decrease nitrogen accumulation in the plant, an important nutrient during grain fill for high yielding crops [68, 158, 174].

A crop's photosynthetic capacity is also becoming an important attribute to explore in increasing plant productivity [100, 101, 200]. Leaf angle is extensively researched for its effect on plant productivity in crops such as rice, maize, and sorghum [79, 82, 151, 172]. Leaf angle has been found to play an important role in increased interception of light and higher photosynthetic efficiency. A more erect leaf angle also decreases the stress on the plant by more even distribution of light through the crop canopy [32, 33, 106, 127, 170, 200]. In both rice and maize more erect leaf angles has been linked to an increase in yield [33, 155, 158, 190]. Increases in yield produced by more erect leaves is achieved by: maximizing light interception and increased photosynthetic ability; reduce occurrence of excess radiation at the top of the canopy; redistribute light to lower levels of the crop canopy [77, 158, 160, 174]. Plants have the natural ability to change leaf angle in response to some stimuli this can help increase productivity without increasing agricultural inputs such as fertilizer and pesticides, by being able to better capture light and increase productivity. In cereal crops such as rice a relationship was found between more erect leaves and crop yield, this trait was found during the Green Revolution, a time of great increases in agricultural productivity [155, 158, 190]. All of these agronomic advances have helped improve crop production. Leaf angle has been extensively studied in recent years, analyzing the genetic involvement of changes in leaf angle. In maize, several quantitative trait loci have been linked to leaf angle, for example: *liguleless1,2, 3 and 4, brassinosteroid deficient dwarf1.* Of these, *liguleless1* has been characterized for decreased leaf angle in maize. The *ZmLg1* mutant contained the *liguleless1* allele and was found to have 50 percent smaller leaf angle than those with the wild type [124, 151]. *Liguleless2* mutants have been found to significantly increase yield in denser planting environments than the wild type. Rice mutants with *OsDwarf4-1* allele have more erect leaves, the smaller leaf angle allows for greater grain yield under denser planting environments [155]. Finding these maize and rice mutants with more erect leaves have greater yields, suggests that leaf angle may be an important phenotype for crop improvements through breeding.

Leaf Angle in Sorghum

Sorghum (*Sorghum bicolor* (L) Moench) is grown around the world, and is the fifth most grown crop after maize, wheat rice and barley [1, 185]. Leaf angle in sorghum has been studied extensively for its impact on crop productivity and genetic control [107, 108, 172, 195]. It was found that sorghum planted under high density is more productive than that of normal density. This is largely due to the better interception of light by smaller more erect leaves [108]. Productivity of crops is dependent on the ability to effectively capture light, capture is impacted by canopy architecture. [38, 169]. In a recent study, nine canopy architectural traits were analyzed including leaf angle for 315 sorghum varieties. A genome-wide association study (GWAS) was conducted and identified several QTLs and seven significant single nucleotide polymorphisms (SNPs) associated with leaf angle. A GWAS is used to identify genetic variation associated with a particular phenotype, in this case leaf angle and eight other traits in sorghum [115, 177, 195]. The study on nine architecture traits found markers on chromosomes: 1, 3, 6, 7, and 9. In addition, QTLs found for leaf angle were found on several chromosomes: 1, 7, and 10; the QTL on chromosome 7 was found to control 45 percent of angle variation

[71]. The significance of a genetic marker on chromosome 7 is a genes known as dwarf3 (dw3), an auxin transporter, that controls plant height [172]. This gene was found to be responsible for approximately 16 percent of the phenotypic affect in leaf angle [195]. Although leaf angle has been found to influence sorghum production, the studies conducted are limited to those under optimal conditions. To date no research has been done on the effects of nitrogen on leaf angle in sorghum.

Chapter 2

Hyperspectral segmentation of senescence in sorghum over time

The use of hyperspectral imaging in a high-throughput phenotyping system has aided the ability to better quantify senescence in sorghum under differing nitrogen levels. In recent years, plant phenotyping created a bottleneck in the advancement of agricultural crops. High-throughput phenotyping helps mitigate this bottleneck, by decreasing the time it takes to phenotype a large number of plants and many different varieties. It also assists in quantifying traits that are more complex, such as, senescence. Recent studies using hyperspectral imaging have trained an algorithm to differentiate between organs in sorghum like leaves, stalk, and panicle. This same system (Scanalyzer3D, LemnaTec GmbH, Aachen, Germany) was used to difference in wavelength from a hyperspectral image to classify plant tissue into five classes: green leaf, yellow leaf, dry leaf, stalk and panicle. By using this data, we tracked and quantified senescence throughout the life cycle of a plant under differing nitrogen treatments.

Introduction

Senescence is the degradation of cells at the end of life in all cells both plant and animal. In plants, the above ground material that commonly exhibits senescence is leaf tissue. This break down includes photosynthetic organelles known as chloroplasts. The digestion of chlorophyll and macro-molecules in senescent leaves allows for transport of nutrients. In annual crops these transportable nutrients are supplied to seed, and younger leaf tissue [15, 65, 97, 183]. In addition to age, stress may also induce senescence, including: drought, nutrient stress, and extreme temperatures. Plants use senescence as a survival method in these instances, allowing plants to adjust to less than optimal conditions [96, 126]. An increased interest has begun on delayed senescence in crops. Delaying senescence can have both benefits and disadvantages. It can increase yield by maintaining photosynthetic tissue, but also decreases protein content in seed by obstructing remobilization of nitrogen [11, 30, 61, 64, 137, 173].

Programmed cell death during senescence is used to rid the plant of aging photosynthetic organs, such as older leaves. These tissues are used for nutrient storage (nitrate and proteins) and relocate nutrients to young leaves and seed production [72]. This process of mobilizing nutrients in the plant life cycle is a key component of senescence and is known as remobilization. Remobilization is used during grain fill to transport nitrogen from vegetative tissue to seed production [24, 114, 141]. Nitrogen is crucial for plant growth, especially in high-yield crop systems. Many advancements in agriculture improving high crop yield is the result of nitrogen fertilizers in addition to breeding and irrigation. However, the use of excessive amounts of nitrogen fertilizer can cause energy waste, increase production costs, and result in environmental affects such as soil acidification and water eutrophication [102]. One-hundred million tons of nitrogen fertilizer is applied to crops each year worldwide [88]. In order to decrease our nitrogen use, we can increase use of crops more suited to low nutrient soils.

Sorghum bicolor (L.) Moench has been extensively researched for its use as a cereal crop on land unsuited for major crops, such as maize and soybean. This is primarily due to sorghum's high water use efficiency, as well as tolerance to drought and short periods of flooding. Additionally sorghum has been shown to have high nitrogen use efficiency by supplying greater grain yields under limiting soil nitrogen supply. This makes it a prime candidate for growth in arid climates [54, 57, 70, 149, 153]. In addition to crops more suited to deficient climates, advancements can also be achieved through improved agricultural techniques. Another way to decrease our nitrogen use is by using crops more suited to low nutrient soils.

There is a great deal of genetic and phenotypic variation within cereal crops with respect to use of nitrogen [17, 27]. In addition to variation, crops such as maize are studied under optimal conditions, inadvertently selecting for less nitrogen efficient crops. However, it can be a challenge to improve traits related to nitrogen efficiency in crops, due to the difficulty phenotyping such traits in experiments [69]. Phenotyping senescence of leaves can be classified from several physiological indicators, for example, yellowing of leaves, changes in protein, and chlorophyll content [5]. Using hyperspectral imaging to identify changes in leaves over time, may help breeders select varieties more suited to low nitrogen environments. This method is much less labor intensive and less expensive than manual phenotyping of crops. However, there are drawbacks, such as, plants grown in individual pots have no competition like those grown in a field. Additionally, plants grown in a greenhouse setting experience no environmental variation, therefore plants selected may not perform the same in a field experiment. Recently, hyperspectral imaging was found to estimate plant nutrient status and to detect and classify disease identification [159]. It was reported that plant organs exhibit distinct spectral signatures, including a difference in reflectance pattern between leaves and stem [118]. We hypothesize that senescent and healthy leaves exhibit this same trend when analyzing hyperspectral images. Currently, there are only a few ways to analyze the process of plant senescence, but with the use of hyperspectral images and machine learning it may be possible to observe the remobilization of nitrogen during plant growth.

Methods and Materials

Plant Growth and Nitrogen Application

Sorghum plants used in this experiment include TX430. TX430 was chosen due to its published reference genome [29] and is studied for being easily transformed due to: transformation by *Agrobacterium* mediated transformation, tissue culture system, and selection [98]. However, training on data from a single genotype can lead to poor model generalization. In order to capture a broader range of the total genotypic and phenotypic diversity present within sorghum, an additional nine genetically diverse lines were chosen from the sorghum association panel (SAP). These lines include: SC85, Acme broomcorn, BTx3197, P850029, SC500, 90M, SC1439, SC301, and P898012 The results from supplemental table 3 in Casa et al, 2018, were used to define the nine sub populations for this experiment [19].

A clustering method from genotypic data was used for unlinked markers, as well as, ancestry model to determine population structure of the SAP. This population was divided into nine sub-populations; of these sub-populations, diverse varieties were chosen to represent each sub-population with nine varieties were chosen based on high kinship values from nine sub-populations. Sub-populations were determined by considering the SAP population structure, this was determined by Casa et al, 2008 [19]. SC85, a variety used in this experiment was chosen despite its low kinship value relative to other varieties, due to low seed supply for this sub-population. Imaging height restrictions (under three meters) and availability of varieties led to this choice. A total of 115 individual plants were grown in the greenhouse of the University of Nebraska-Lincoln's Greenhouse Innovation Center. Growth occurred from June 11 to September 20, 2019. Sorghum was plated in 1.5 gallon pots with a soil composition of two thirds peat moss, one third vermiculite and 2000 g/yd of

Nutrient	3 mMol N	6 mMol N	9 mMol N	12 mMol N	15 mMol N
2M KNO3	0.5 ml/L	1 ml/L	1.5 ml/L	2 ml/L	2.5 ml/L
2M Ca(NO3)2 · 4H2O	0.5 ml/L	1 ml/L	1.5 ml/L	2 ml/L	2.5 ml/L
1M KH2PO4 pH6.0	1 ml/L	1 ml/L	1 ml/L	1 ml/L	1 ml/L
2M MgSO4 · 7H2O	1 ml/L	1 ml/L	1 ml/L	1 ml/L	1 ml/L
0.05M Fe-EDTA	1 ml/L	1 ml/L	1 ml/L	1 ml/L	1 ml/L
0.5M K2SO4	3 ml/L	4 ml/L	2 ml/L	1 ml/L	-
2M CaCL · 2H2O	1.5 ml/L	2 ml/L	1 ml/L	0.5 ml/L	-
Micronutrients	1 ml/L	1 ml/L	1 ml/L	1 ml/L	1 ml/L

Table 2.1: Hoagland Solution nutrient concentrations under all levels: 3, 6, 9, 12, 15 mMol N

lime. A total of five cubic yards of media were mixed for this experiment. After pots were loaded and weighed, each pot was watered to saturation with a 100 ppm solution of calcium chelate (Miller). Each day between June 14 to June 23 a 20-10-20 NPK fertilizer as a liquid was applied to each pot to saturation. As recommended by the university greenhouse management, nutrient treatments were necessary in order to ensure survival of seedlings until the beginning of the experimental treatments. Emergence occurred on June 16, five days after planting (DAP).

Forty-five plants were given six mMol N and an additional forty-five were given 12 mMol N, each treatment contained five replicates, for each of the nine diverse SAP varieties. An additional 25 TX430 plants were included in this experiment as a control, these plants were given five fertilizer treatments including 3, 6, 9, 12, and 15 mMol N. Each individual plant was given 250 mL of solution twice a week until harvested. Solutions were made using hoagland solution (table 2.1), where nitrogen is in the form of potassium nitrate and calcium nitrate, solutions were applied to sorghum plants 27 DAP.

Imaging

All plants were placed on the conveyor belt for imaging using the University of Nebraska's Greenhouse Innovation Center's automated phenotyping greenhouse

(Lemnatec 3D High-throughput scanalyzer) on July 10th, 2019. Each plant was imaged with a hyperspectral camera, hyperspectral images were captured with the Headwall Photonics XvNir camerara [55, 118], as well as four others: Thermal Infrared, Steady State Fluorescence, and RGB (visible). Unlike previous data sets, an upgraded camera was used with higher resolution and greater dynamic range – AVT Prosilica gt6600 color with Power Over Ethernet. at a resolution of 4384 x 6576 pixels – was used to collect RGB images (figure 2.2).

Manual Pixel Annotation

Hyperspectral and RGB images from the automated phenotyping greenhouse for the same plant were paired together from the same day. A single hyperspectral image file for one plant on a single day contains 224 images each representing light intensity for a different specific light wavelength between 546 and 1700 nanometers. This is referred to as a hyperspectral cube. Each image was checked for the greatest difference in wavelength. Image 187.0.png, is the 187th image out of 224 images in the hyperspectral cube, at 1434 nanometers wavelength. Image 187 could be used to easily distinguish between classes based on greatest contrast for plant tissue and background in the gray-scale images from an individual png (image format) in the hyperspectral cube. RGB images were used to help identify classes. A class is defined as a plants specific tissue type, panicle and stalk are two different classes. The mirror images of the hyperspectral image was found by comparing different angles from the RGB camera angles, hyperspectral and RGB images were then combined and code was used to invert the RGB image to match the hyperspectral image and merge the two images (Figure 2.1). These image files were uploaded to Zooniverse crowdsourcing science platform (https://www.zooniverse.org/projects/alejandropages/sorghum-and-maize-segmentation-using-hyperspectral-imagery). A single image dataset was uploaded to the Zooniverse project for pixel collection. This data-set included 78 individual sorghum plants on the last day of imaging, September 19th, 2019 (table 2.2). An assorted number of plants from each of the ten varieties, at least one from each nutrient level, excluding BTx3197 (6mMol and 12 mMol N). TX430 variety was used for pixel collection under all five nitrogen levels (3, 6, 9, 12, 15mMol N). RGB images were used to locate the six pixel classes: green leaf, yellow leaf, dry leaf, stalk, panicle, and background on the hyperspectral images. An individual collected a total of up to ten pixels per class per image. Seventy-eight paired images were uploaded to Zooniverse for classification. A total of 8,787 pixels were collected (Background:2746; Green Leaf:1406; Panicle:796; Stalk:1305; Yellow Leaf:1162; Dry Leaf:1372). Panicle pixel numbers are low due to its small size and the absence of panicles in some later flowering sorghum varieties (figure 2.2).

Figure 2.1: Merging hyperspectral and RGB images, and correcting for mirror effect

Model Training and Model Evaluation

Four models were used as a classification method. These methods include support vector machine (SVM) [81], elastic net regularization regression (glmnet) [49], partial least squares discriminant analysis (PLS-DA) [116], and linear discriminant analysis (LDA) [175]. These methods were tested using five-fold cross validation. SVM was used for all analyses of hyperspectal images. Pixel data was unbalanced for each class, up-sampling was used to sub-sample the training data by randomly sampling from the data. Whole pixel and segmentation analysis was conducted using caret and caretEnsemble packages in Rstudio v4.0.3 [28, 87] (figure 2.2).

Whole Image Classification

Image classification was achieved using methods produced by Miao et al, 2020 with minor alterations. These alterations included the addition of two classes for segmentation: yellow leaf and dry leaf. Whole image classification was performed on a subset of images from data collected by the automated phenotyping greenhouse. This subset included all plants from the control (TX430), plants were imaged from July 13, 2019 to September 19, 2019. The plants were grown to maturity, however, those grown under low nitrogen had delayed maturity and full grain fill was not achieved in the time allotted. This subset came to a total of 1000 images used for classification. Images were cropped using an R script to remove the pot and background structure from the image. This gave a more accurate pixel count for each class. Images earlier in the study had a higher zoom level than those closer to plant maturity. All images were cropped based on the level of zoom based on the imaging date, zoom levels varied though out the experiment (figure 2.2). Images were scaled by measuring the pot circumference, 215.9 mm, and counting the number of pixels across the pot. Each pixel was then given in square millimeters.

$$mm^2 = (215.9mm/Zoomlevel)^2 * Pixelcount.$$

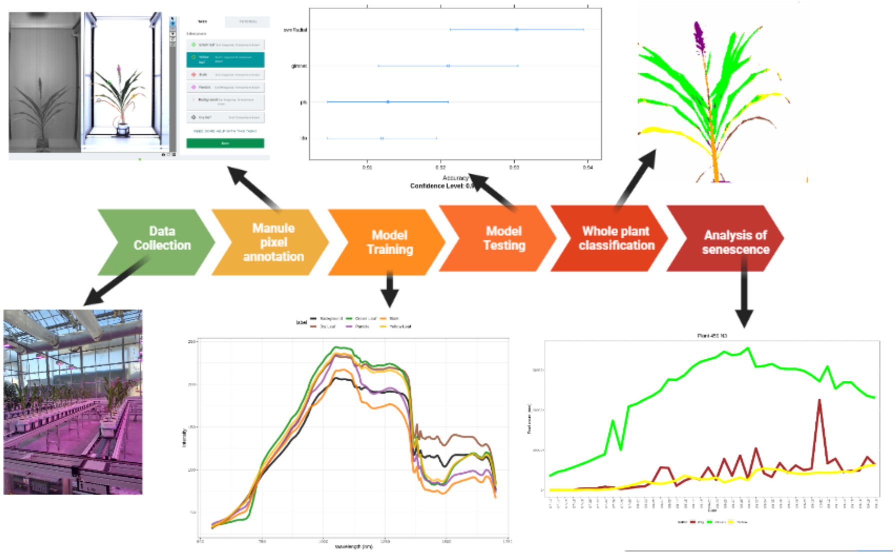


Figure 2.2: Work pipeline for data collection, model training and testing, plant classification and pixel count for senescence quantification.

Results

Hyperspectral Signatures

The average reflectance pattern for each class was computed and plotted (figure 2.3). Green leaf and yellow leaf showed a clear difference in wavelength for visible light at about 700 nm, corresponding to red in the visible light spectrum. Only a slight difference in wavelength was observed in the infrared portion of the graph. Additionally, dry leaf presented with a clear difference in infrared wavelength at about 1350 nm to 1700 nm. The first two PCs can explain 83.9 percent of classified pixel variation. Dry leaf and green leaf showed clear separation from PC1 and PC2. Yellow leaf partially overlapped both green leaf and dry leaf distribution. Stalk and panicle pixels also showed overlapping distributions (figure 2.4), these findings are similar to those found in a study to determine plant segmentation using hyperspectral images [118].

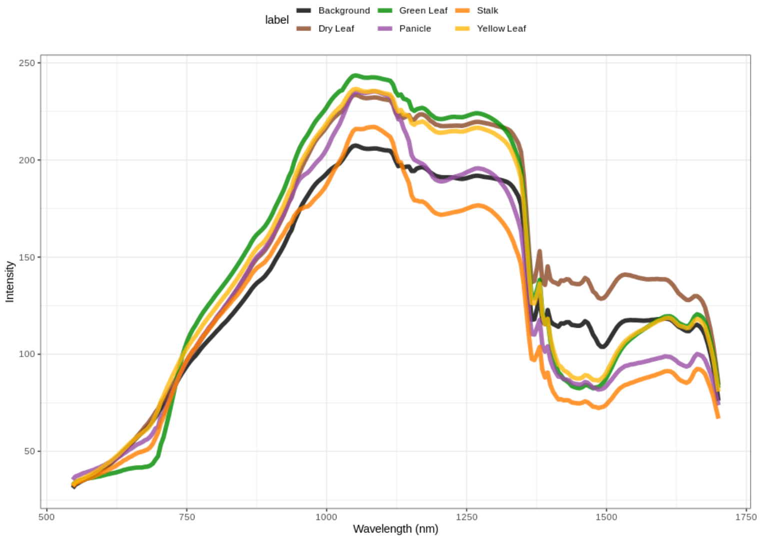


Figure 2.3: Generalized reflectance pattern of background, dry leaf, green leaf, stalk, panicle, and yellow leaf across wavelengths (nm). The blue wavelengths with in visible light (380 to 545 nm) is not captured by the camera.

Algorithm Performance

Four algorithms were initially tested for accuracy: linear discriminant analysis, partial least squares discriminant analysis, elastic net regularization regression, and support vector machine. Linear discriminant analysis algorithm was previously found to have the highest accuracy at 0.97 average for hyperspectral sorghum segmentation by [118]. When run with our data set which included two extra classes: yellow and dry leaf, as well as different images for training data; linear discriminant analysis only had an accuracy of 0.91. Partial least squares discriminant analysis and elastic net regularization regression accuracy was intermediate with a value between 0.91 and 0.92. The highest accuracy was found at 0.93 for support vector machine, and was used for all subsequent analyses.

Whole images were used for semantic segmentation of TX430 under five nitrogen levels. Support vector machine algorithm was used as it had the highest accuracy rate of the four tested algorithms, with a confidence level of 0.95 and an accuracy of 0.93. Classification of pixels was achieved at a high level; only a

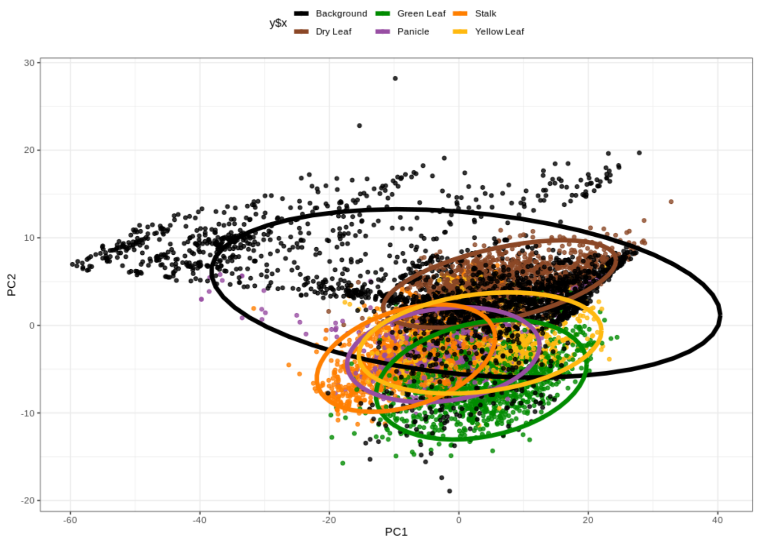


Figure 2.4: Principle components one and two for reflectance.

small portion of pixels were classified as panicle while plants were young. These pixels were concentrated along the midrib and edges of leaves. This issue is common amongst other segmentation research, where the midrib is misclassified due to its thick lignified tissue, this trait makes it similar to stalk, or panicle in our case, causing misclassification of tissue classes [118]. Segmentation of plant classes was successful and allowed for classification of leaf senescence. Under lower nitrogen levels (3mMol and 6mMol N) sorghum presented with a higher ratio of dry/yellow to green leaf tissue (figure 2.10). As expected, full nitrogen (15mMol N) showed the lowest amount of dry and yellow classified organs. Along with less senesced leaves, higher nitrogen levels exhibited greater leaf biomass. The presence of a panicle was also accelerated when compared to low nitrogen levels with 3mMol N not producing any panicle by the end of the project (segmentation photos).

Estimated senescence values

At all nitrogen levels, green leaf pixel count reached a peak vegetative growth and then began to descend when corresponding with with the beginning of sorghum reproductive stage, or boot stage. This occurs on different dates for all plants, however, decrease usually occurred approximately 70 days after planting. Over time yellow leaf and dry leaf pixel counts increased steadily with plants under low nitrogen having a ratio between 1.5 and 1.8 for green to senesed (dry and yellow) leaf pixel counts. While sorghum under high nitrogen levels has a green leaf to seneced leaf pixel count ratio ranging from 2.3 to 4.4. Additionally, plants grown under high nitrogen levels (12 and 15 mMol N) calculated higher green leaf pixel counts on average at end of life (figure 2.8 and 2.9). Pixel counts for the last day of imaging, one replicate from each treatment showed dry leaf counts: 3,656, 29,759, 18,924, 12,258, and 7,849 for treatments 3 to 15 mMol N respectively. Yellow leaf pixel counts were calculated at: 488, 12,598, 18,906, 29,336, and 20,258; green leaf returned: 7,385, 66,612, 69,336, 96,702, and 123,279. An unexpected observation was found for plants grown under nitrogen deficient conditions. An observable difference in leaf angle was found for sorghum grown under lower nitrogen conditions compared to higher nitrogen levels. Leaf angle average ranged from 3.78 degrees to 8.2 degrees under 100 percent nitrogen, although not statistically significant. Additionally sorghum exhibited delayed maturity for plants grown under nitrogen deficient conditions where after about 100 days after planting sorghum treated with 3 mMol N had not yet reached full grain fill, while sorghum at 15 mMol N was at full grain fill and able to harvest.

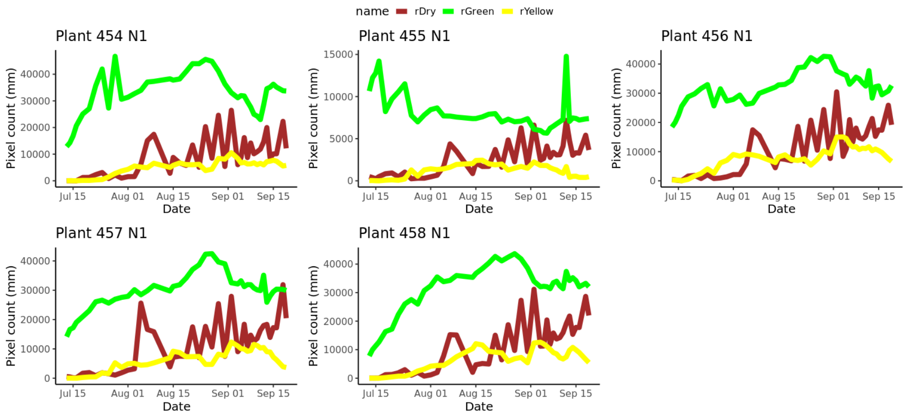


Figure 2.5: Segmented TX430 reflectance under 3 mMol N, beginning on July 10th to the end of the project on September 18th.

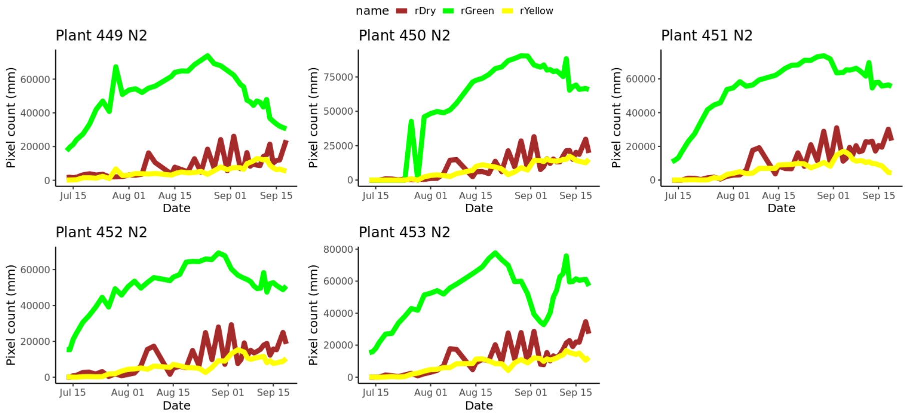


Figure 2.6: Segmented TX430 reflectance under 6 mMol N, beginning on July 10th to the end of the project on September 18th.

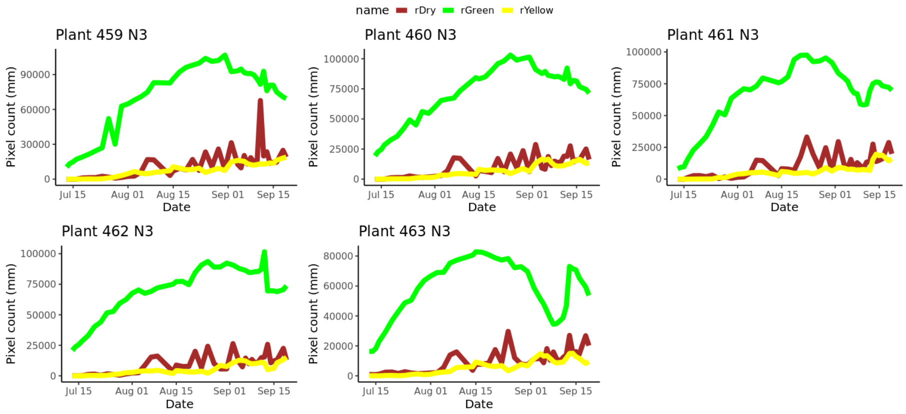


Figure 2.7: Segmented TX430 reflectance under 9 mMol N, beginning on July 10th to the end of the project on September 18th.

Discussion

In this study we collected hyperspectal data from ten diverse sorghum lines, classified pixels manually based on six classes: background, dry leaf, green leaf, yellow leaf, stalk and panicle. From these manually classified pixels we performed machine learning using four algorithms with accuracy above 0.9. Although accuracy was high for the SVM, the algorithm used for remaining plant analyses, misclassification of pixels throughout the plant did occur. Classification of dry leaf pixels was less successful than pixel classification for green leaf and yellow leaf. This was seen in pixel graphs where dry leaf count was much less smooth than that of green and yellow. On occasion some background pixels could be classified as dry leaf, leading to peaks and troughs in

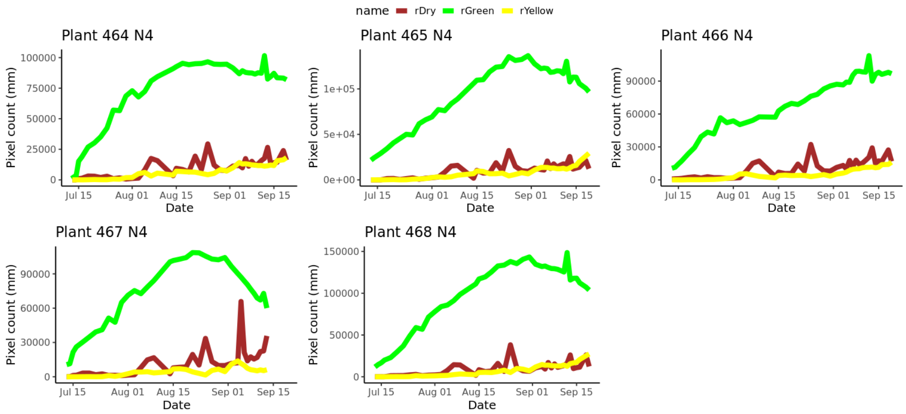


Figure 2.8: Segmented TX430 reflectance under 12 mMol N, beginning on July 10th to the end of the project on September 18th.

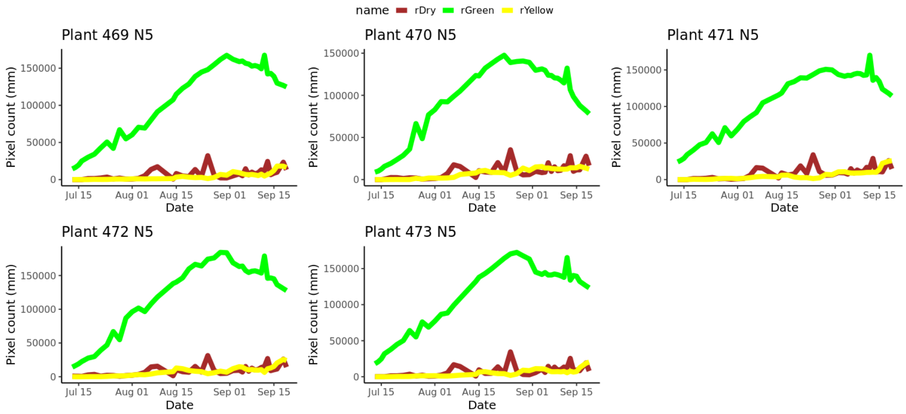


Figure 2.9: Segmented TX430 reflectance under 15 mMol N, beginning on July 10th to the end of the project on September 18th.

pixel counts. Although classification was not perfect, we were able to differentiate between leaf type, and to track senescence of sorghum over time. By tracking senescence over time, we concluded that sorghum grown under high nitrogen levels (12 and 15 mMol N) had less dry and yellow leaf material than sorghum grown under low nitrogen levels (3 and 6 mMol N). Also, Green leaf biomass, which can be calculated by pixel count, was greater for plants grown under high nitrogen treatments compared to low treatments (figure 2.5 and 2.6). Although the ratio of green leaf to senesced leaf was high, yellow and dry leaf pixel counts were higher for 12 and 15 mMol N treatments compared to sorghum under low nitrogen at 3, 6, and 9 mMol N.

Current methods used to measure phenotypes such as plant height, yield,

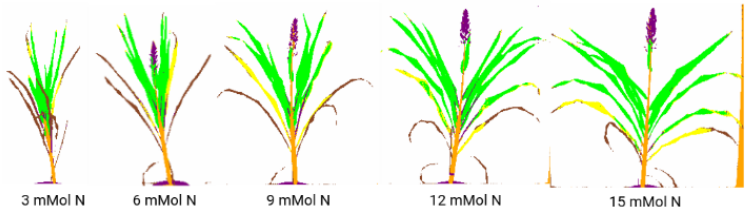


Figure 2.10: Segmented TX430 under all five nitrogen levels: 3 mMol N, 6 mMol N, 9 mMol N, 12 mMol N, and 15 mMol N. Images are end of project on September 18th.

leaf area for biomass, and even senescence can have relatively low-throughput and be costly. This can restrict the number of genotypes researchers are able to measure [118]. The ability to track senescence using hyperspectral imaging could lead to better phenotyping of this trait in plants. Plant senescence is largely determined by its surroundings, including environmental conditions [103]. As a whole, senescence is thought to be determined by many genes and involve many biological pathways throughout the plant life cycle [13, 16, 94, 95, 156]. When phenotyping senescence, a plant's whole life should be considered. Senescence is impacted by environmental factors throughout its whole life , not just a single point in time [103, 192], the ability for this project to track senescence over a most growth stages allows for better phenotyping of this trait. This allowed us to track senescence through most morphological stages and track differences in senescence under differing conditions. This method could be applied to diverse sorghum varieties and aid in future breeding and genetic methods when considering a plant's response to nitrogen stress and senescence. Understanding nitrogen's role and uses in the plant is important for understanding how we observe senescence in the leaves and what it could mean for crop production such as grain yield and flowering time [73] . A project on *Arabidopsis* tracked leaf senescence in a single leaf with the use of high throughput imaging using RGB and visible and near-infrared (VNIR) imaging. They determined this process could be used on many other phenotypic traits and reduce the bottleneck in phenotyping [103]. Our ability to image and segment the entire plant using reflectance in hyperspectal images, opens the door to many different phenotypes to measure for many plant species.

A decrease (more erect) in leaf angle was observed for some of the diverse sorghum lines grown in this greenhouse under low nitrogen levels. In addition to the differences in leaf angle for plants grown under nitrogen deficient conditions, sorghum also presented with delayed flowering time. The effects of nitrogen on leaf angle and maturity are unexpected discoveries from this study. The phenotypic variation observed in sorghum under varied nitrogen levels, led to a much larger field experiment. This experiment was conducted under nitrogen replete and nitrogen deficient conditions on 345 sorghum varieties from the sorghum association panel (SAP).

Plant number	Nitrogen level	Varietiy	Plant number	Nitrogen level	Varietiy
340	N1	ACME	414	N4	SC301
344	N1	SC500	415	N4	90M
345	N1	SC301	416	N4	P850029
347	N1	90M	417	N4	SC85
348	N1	SC1439	419	N4	P898012
350	N1	P898012	422	N4	SC85
351	N1	P850029	423	N4	90M
352	N1	SC301	424	N4	SC301
353	N1	SC500	425	N4	P898012
354	N1	ACME	426	N4	SC1439
357	N1	ACME	427	N4	SC500
358	N1	90M	429	N4	ACME
359	N1	SC1439	430	N4	SC85
360	N1	P898012	431	N4	SC500
361	N1	P850029	432	N4	P850029
362	N1	SC85	435	N4	90M
363	N1	SC500	437	N4	SC301
365	N1	SC500	438	N4	ACME
366	N1	SC85	450	N1	Tx430
368	N1	P898012	451	N1	Tx430
369	N1	SC301	452	N1	Tx430
370	N1	SC1439	453	N1	Tx430
371	N1	90M	454	N2	Tx430
372	N1	P850029	455	N2	Tx430
373	N1	ACME	456	N2	Tx430
374	N1	ACME	457	N2	Tx430
375	N1	P898012	458	N2	Tx430
376	N1	SC85	459	N3	Tx430
377	N1	90M	460	N3	Tx430
379	N1	SC301	461	N3	Tx430
380	N1	SC500	462	N3	Tx430
382	N1	SC1439	463	N3	Tx430
393	N4	SC301	464	N4	Tx430
394	N4	SC1439	465	N4	Tx430
395	N4	P850029	466	N4	Tx430
396	N4	90M	467	N4	Tx430
397	N4	SC85	468	N4	Tx430
399	N4	P898012	469	N5	Tx430
401	N4	ACME	472	N5	Tx430

Table 2.2: Sorghum plants used for pixel collection in Zooniverse, a total of 78 plants on the last day of imaging, September 19th.

Chapter 3

Nitrogen stress effects on leaf angle in sorghum

Nitrogen deficit conditions were found to decrease the leaf angle in some sorghum varieties, compared to sorghum grown under nitrogen replete conditions in an experiment conducted in a greenhouse. Several studies found specific genes in maize that influence leaf angle, causing more erect leaves. Maize mutants containing the *liguleless1* allele showed a decrease in leaf angle by about 50 percent. *Liguleless2* significantly increases yield for maize grown under denser planting. Although studies have been done on the genetic influence of leaf angle in sorghum, no studies have been done on the effects of nitrogen stress on leaf angle in sorghum. In addition to leaf angle nitrogen stress has also been found to affect the flowering time in many plant species, while also affecting the productivity or yield production of sorghum. For this study, over 300 sorghum varieties from the sorghum association panel were grown under nitrogen replete and nitrogen deficit conditions in a field. In this experiment three main phenotypes were collected: leaf angle, flowering time, and grain yield. During this study an average decrease in leaf angle was observed for sorghum grown under nitrogen stress, as well as a delay in the days to flowering, and an average decrease in yield. These findings show that the novel finding of more erect leaves in sorghum under nitrogen stress in a greenhouse was replicated in the field, as well as the decrease in yield and delayed flowering time.

Introduction

Sorghum is of great importance worldwide, however, it is especially important in semi-arid tropics of Africa and Asia due to its ability to maintain high yields under low and sporadic precipitation [3, 125]. It is the fifth most economically and widely grown grain crop, sorghum has been grown for food, animal feed, fiber, and biofuels around the world [153]. Due to its high drought tolerance and ecological adaptation, sorghum has been found to be more suitable in climates inadequate for growing maize and wheat grain crops [125, 182]. Sorghum is superior in its ability to use inputs such as nitrogen when compared to other major cereal crops [76, 91, 134, 179, 200]. Sorghum's ability to prosper in less than optimal climates makes it a candidate for future breeding programs in order to feed the world's growing population. Nitrogen is an important macronutrient for plant growth and development, and therefor a common agricultural input in the form of synthetic fertilizer [47, 93, 178]. Both light and nitrogen are key components in plant development. Up to 75 percent of nitrogen stored in the plant leaf is used during photosynthesis [36]. Crop canopy can be defined as the arrangement of leaves throughout the plant [108]. Crop canopy is strongly influenced by leaf angle, described as the inclination between leaf midrib and the stalk in maize [2]. Adjustments in leaf angle aid plants in their ability to intercept light, more erect leaves contribute to even distribution of light [101, 129, 199]. Light interception plays an important role in the productivity of a plant [172]. More erect leaves show improvements in photosynthesis and may reduce plant stress by redistributing solar radiation throughout the crop canopy in sorghum [32, 33, 106, 127, 128, 170, 200]. The effects of leaf angle on plant productivity has been extensively researched in maize, especially its impact on yield. Knowledge of the link between leaf angle and yield can be traced back to the 1970's. Leaf angle is noted as one of the top phenotypes when developing superior maize germplasm [121]. This time period, known as the Green Revolution, led to exponential increases in crop yield for staple crops such as maize, wheat and rice. This increase in yield can be explained by both new breeding techniques, the introduction of dwarfing genes, and farming techniques: fertilizer inputs, irrigation, and mechanization [37, 39]. Genetic targets of these three main crops during the Green Revolution led to reduced height, thick stalks, more erect leaves, and a large compact inflorescence [83]. The novel innovations spawned from the Green Revolution created exponential increase in yield worldwide. However, these improvements are largely one time innovations, such as irrigation, that let to substantial increases. In China research has increased for agriculture 1980 to 2000, unfortunately rate of increase in yield for maize has decreased by 64 percent, while rate of increase in rice yield are negligible [63]. As we approach the plateau of potential yield improvements in major cereal crops such as maize, rice and wheat, and sustainability of current farming tactics decrease, we must continue to improve yield through alternative methods [39, 101].

Many more genes have been linked to leaf angle in more recent years for maize, rice and sorghum [108]. Geneticists have identified genes associated with leaf angle in major crops such as maize including, *ligueless1* and *ligueless2* (*lg2*) which have helped improve crop efficiency by decreasing leaf angle and allowing for denser planting [89, 124, 142, 180]. The mutant allele of *lg2* is responsible for a decrease in leaf angle by 10 degrees [142]. The *lg2* genotype allowed for planting densities to increase from 30,000 plants per he-1 in the 1930 to 75,000 in the 2000's, these plants can be productive in densities up to 151,000 plants per ha-1 [33, 89]. Genes associated with more erect leaves have been identified in rice, *OsDwarf4-1* and *leaf inclination2*. These genes are associated with hormone response resulting in changes to leaf angle [108, 155, 196]. In rice more erect leaf angle has a positive effect on yield [155, 158, 190]. A study using RIL populations of BTx623 and IS3620 identified several QTLs associated with leaf angle and stalk height in sorghum, a well documented gene being *dwarf3*, which had a large phenotypic effect of about 30 degrees [172]. Additionally, a genome-wide association study on plant architecture in sorghum found significant single-nucleotide polymorphisms (SNPs) on five chromosomes. This study was conducted in Iowa with 315 sorghum accessions [108].

Research in cereal crops show that environmental stressors, such as drought and light intensity affect attributes of the leaf architecture such as leaf angle [43, 82, 172, 195]. However, these studies of environmental effects on leaf angle are primarily done on maize, rice, and wheat. Past research on leaf angle in sorghum has been done under optimal growing conditions [107, 172, 195]. Sorghum can be grown in resource-poor environments; the biological attributes that allow it to adapt to less optimal conditions have limited research [125]. In this study, we analyzed variation in leaf angle, flowering time, and yield in sorghum in two environments (nitrogen sufficient and nitrogen deficient), as well as the differences in leaf angles exhibited by the same genotypes between the two treatments.

Methods and Materials

Field Layout

347 diverse sorghum varieties were planted in the 2020 nitrogen study field, 85 percent of the Sorghum Association panel (SAP) is represented in the field [19]. Varieties selected were determined by SNP data and seed source availability.

The SAP field, located at 40.859, -96.597; was planted on Monday, June 8th, 2020 and sprayed with a pre-emergent herbicide within 24 hours of planting. The field was split into six sections of 390 plots, each section containing one plot each of 346 diverse genotypes, 42 check plots of BTx623, and three plots of TX430. Checks were randomized by separating each section into six subsections 13 plots long by five plots wide view figure (figure 3.2). Each subsection had seven check plots for a total of 42 check plots in each section of the field. All sections were surrounded on all four sides with border seed to reduce edge effects. Soil samples were collected and sent for nutrient testing, samples were collected from each section of the field. Sections 3000 and 8000 collected two

Figure 3.1: Sorghum boarder with optimal nitrogen (N+) on the left in this image and nitrogen deficit conditions (N-) on the right

samples, one from the east and one west ends of the field. The 4000 to 7000 section soil samples were collected from the middle of the field. All soil samples were collected before planting and after anhydrous ammonia was applied to the required standard nitrogen sections. Sections 3000, 5000, and 7000 had nitrogen fertilizer applied at 80 pounds per acre. No nitrogen was applied to sections 4000, 6000 and 8000. Nitrogen levels in sections without nitrogen application were substantially lower than those with applied nitrogen and were recorded in the ward lab report (figure 3.3).

After planting and seedlings roots were well established, weeds were managed by hand-hoeing and between row tilling. This method of weed management was maintained once a week until late August when sufficient canopy was reached.

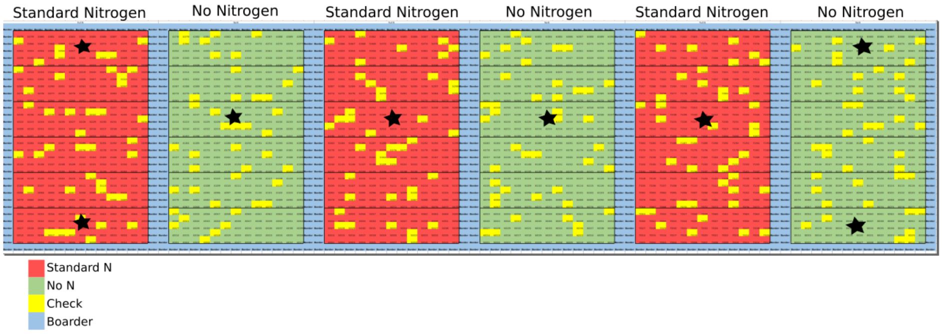
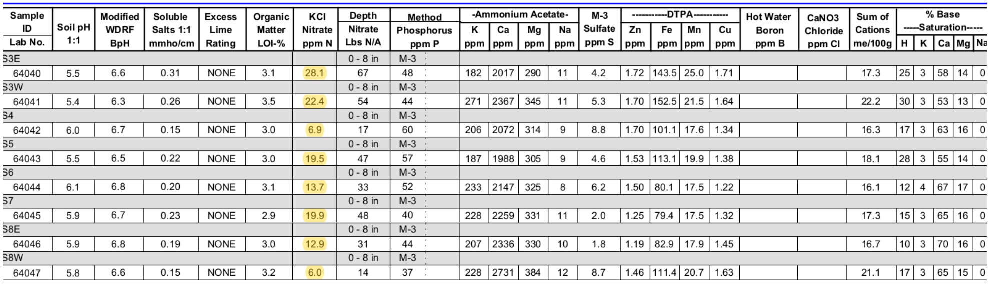


Figure 3.2: Sorghum field experiment 2020, stars indicate soil samples.



Sample ID	Soil pH 1:1	Modified WDRF BpH	Soluble Salts 1:1 mmho/cm	Excess Lime Rating	Organic Matter LOI-%	KCl Nitrate ppm N	Depth	Method	-Ammonium Acetate-				M-3 Sulfate ppm S	-----------DTPA-----------				Hot Water Boron ppm B	CaNO3 Chloride ppm Cl	Sum of Cations me/100g	% Base -----Saturation-----				
Lab No.							Nitrate Lbs N/A	Phosphorus ppm P	K ppm	Ca ppm	Mg ppm	Na ppm		Zn ppm	Fe ppm	Mn ppm	Cu ppm				H	K	Ca	Mg	Na
S3E							0 - 8 in	M-3																	
64040	5.5	6.6	0.31	NONE	3.1	28.1	67	48	182	2017	290	11	4.2	1.72	143.5	25.0	1.71			17.3	25	3	58	14	0
S3W							0 - 8 in	M-3																	
64041	5.4	6.3	0.26	NONE	3.5	22.4	54	44	271	2367	345	11	5.3	1.70	152.5	21.5	1.64			22.2	30	3	53	13	0
S4							0 - 8 in	M-3																	
64042	6.0	6.7	0.15	NONE	3.0	6.9	17	60	206	2072	314	9	8.8	1.70	101.1	17.6	1.34			16.3	17	3	63	16	0
S5							0 - 8 in	M-3																	
64043	5.5	6.5	0.22	NONE	3.0	19.5	47	57	187	1988	305	9	4.6	1.53	113.1	19.9	1.38			18.1	28	3	55	14	0
S6							0 - 8 in	M-3																	
64044	6.1	6.8	0.20	NONE	3.1	13.7	33	52	233	2147	325	8	6.2	1.50	80.1	17.5	1.22			16.1	12	4	67	17	0
S7							0 - 8 in	M-3																	
64045	5.9	6.7	0.23	NONE	2.9	19.9	48	40	228	2259	331	11	2.0	1.25	79.4	17.5	1.32			17.3	15	3	65	16	0
S8E							0 - 8 in	M-3																	
64046	5.9	6.8	0.19	NONE	3.0	12.9	31	44	207	2336	330	10	1.8	1.19	82.9	17.9	1.45			16.7	10	3	70	16	0
S8W							0 - 8 in	M-3																	
64047	5.8	6.6	0.15	NONE	3.2	6.0	14	37	228	2731	384	12	8.7	1.46	111.4	20.7	1.63			21.1	17	3	65	15	0

Figure 3.3: Soil sample resultes from the Ward lab: S3E indicating section 3000 east, S3W as section 3000 west. S4 as section 4000, S5 as section 5000, S6 as section 6000, S7 as section 7000, S8E as section 8000 east, and S8W as section 8000 west

Data Collection

Leaf angle was collected using a Husky electric protractor; the third leaf counting the flag leaf as (e.g the antepenultimate leaf) was measured on the adaxil or top portion of the leaf blade. One arm of the protractor was centered on the stock of the sorghum plant while the other pivoted to the mid rib of the plant before the leaf bend (figure 3.4). All leaf angles were collected between anthesis and grain fill for all plots, beginning July 18th 2020 and ending September 18th 2020. Up to six leaf angles were collected per plot in each section of the field. All measured plants with leaf angles collected were flagged using colored flag tape. Less than six measurements were collected from plots where low stand, damaged or diseased leaves, diseased or no panicle present before September 18th 2020, resulted in insufficient numbers of plants. When possible, plants in the middle of the plot were measured to capture the impact of competition and to avoid edge effects. Primary stalks were used for collecting leaf angle, however, a small number of tillers of less than 200 were initially misidentified. In these cases the identity of the stalk (primary vs tiller) was noted in the data and taken into account for statistical analysis by using a paired t-test to determine if differences in leaf angle was statistically significant. Leaf angles collected in the greenhouse were collected at grain fill stage with a Husky T-bevel angle finder protractor, different from the protractor used for collecting leaf angle in the field experiment. In addition to leaf angle, flowering times were also collected and determined when 50 percent of the plot is shedding pollen. Panicles for three of the six plants per plot where leaf angle measurements were made were harvested for yield calculations. The number of panicles produced in each plot were documented at the end of the planting season in sections 5000, 6000, 7000, and 8000; panicle number data was not collected from plots in the 4000 section therefore an estimated plot yield for this section was not calculated due to time limitation near harvest.

Phenotypic Data analysis

The difference in leaf angle between tillers and primary plant was calculated to ensure limited effect. This was achieved by calculating the average for all leaf angles including known tiller and plant measurements. Yield calculations included collection of one to three fully developed and dried panicles from each plot. Panicles from each plot were mechanically threshed and weighed in grams. Total weight was divided by the number of panicles collected for an average weight, this calculation gave yield per panicle. An estimated plot yield was calculated when average panicle weight was multiplied by the number of panicles present in each plot. Also, any plots marked as mixed or presented with more than one sorghum variety due to seed carry over were removed from the data set as a whole. Best linear unbiased prediction (BLUP) was performed for each of the three traits under both nitrogen conditions. Each variety was used as a random effect using the lme4 [7] package in Rstudio v4.0.3 BLUPs were used for all downstream analysis. Pearson correlation matrix was calculated between all traits under both conditions using Performance Analytics package in Rstudio.

GWAS

569,306 SNP markers were used for the sorghum association panel, SNPs with minor allele frequencies greater than 0.05 were used for analysis, bringing the reduced marker number down to 290,951 SNPs [117]. These markers were used to perform a genome wide association study (GWAS) on three phenotypes: leaf angle, yield estimate, and days to flowering. Association analysis for all traits in each condition was conducted with mixed linear model implemented in GEMMA [197]. Kinship was computed in GEMMA, principle components were computed using rMVP v1.0.0 [189]. Five principle components were used when computing MLM. GWAS was conducted under the two treatments: low nitrogen and high nitrogen; along with a genotype by environment effect (GxE) between the two treatments. GxE analysis was conducted using multivariate linear mixed model in GEMMA [198]. In addition, association analysis on leaf angle under both nitrogen conditions was conducted with FarmCPU using rMVP [99, 189]. Significance threshold was set using effective SNP number where 0.05 was divided by 82,142 effective SNPs for a threshold of 6.08 e-7. Data analysis and GWAS code can be found at: https:github.commzwiener3Sorghum$_Nitrogen_Stress$

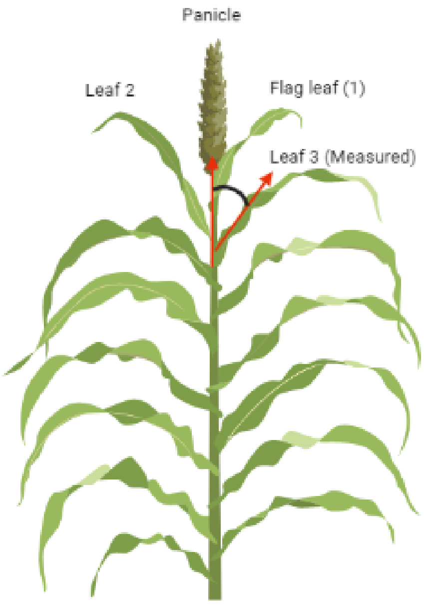


Figure 3.4: Illustration of leaf angle measured on the third leaf of each sorghum plant after flowering, counting the flag leaf as leaf one. Plants were measured after flowering.

Results

Phenotypic variation identified under varying nitrogen conditions.

Sorghum varieties were grown under standard agricultural nitrogen at 80 lbs/acre and no nitrogen application conditions.Leaf angle showed a difference in mean of 2.7 degrees and a $p = 2.47e\text{-}13$ computed from a paired t-test, where leaf angles decreased (became more erect) under no nitrogen conditions. A difference in mean plot yield between treatments was 277.16 grams less under no nitrogen conditions, with a p less than 2.2e-16; it was computed using a paired t-test in Rstudio v4.0.3. While sorghum grown under standard nitrogen had greater plot yields than sorghum grown under no nitrogen. Additionally, flowering time computed a mean difference of -3.61 days and a p less than 2.2e-16 computed from paired t-test plants under no nitrogen had a delay in flowering time compared to standard nitrogen. Standard deviation varied little for the leaf angle and days to flowering trait between standard and no nitrogen. Plot yield standard deviation was greater under no nitrogen at 282.45 grams than the 166.58 grams under standard nitrogen(table 3.1).

Sorghum varieties grown for the greenhouse experiment had leaf angles collected, these same varieties can be found in the sorghum field experiment. Acme broomcorn, BTx3197, P850029, SC500, and 90M presented with more erect leaves under no nitrogen treatment, these same lines grown in the greenhouse also exhibit more erect leaves under the low nitrogen treatment. One exception is 90M, plants grown in the greenhouse showed no difference in leaf angle under nitrogen treatments. In addition, SC1439 showed no difference in leaf angle under differing nitrogen treatments for both the greenhouse and field experiment. Some differences were observed between the greenhouse and field experiment. SC85 and P898012 had more erect leaf angles under standard nitrogen in the field, while when these varieties were grown in the greenhouse a decrease in leaf angle was observed under low nitrogen treatments. SC1439 showed no difference in leaf angle for both the greenhouse and field experiment. Finally, TX430 presented with little difference in leaf angle in the greenhouse experiment with a slight decrease in leaf angle under low nitrogen, while in the field TX430 grown under standard nitrogen leaf angles were slightly smaller compared to no nitrogen treatment (table 3.3).

Although, as a whole, sorghum presented with clear phenotypic differences between treatments, this trend does not hold true for all varieties. Some varieties showed the opposite effect with larger, less erect leaf angles under no nitrogen and more erect leaves under standard nitrogen conditions. Leaf angles ranged from 22.71 to 71.46 degrees under standard nitrogen. While leaf angle under no nitrogen ranged from 22.19 to 72.46 degrees. Although the minimum leaf angles and maximum leaf angles are not much different, the difference in mean leaf angle under these treatments is statistically significant. A paired t-test was performed showing no statistically significant differences in leaf angle between tillers and primary plants with p = 0.093.

A negative correlation between flowering time and leaf angle was observed. As leaf angle decreased (e.g. became more erect), the number of days from planting to flowering increased (figure 3.8). The connection between leaf angle and flowering time was stronger under no nitrogen conditions; R2 = 0.23 and a p = 5.96e-20. Sorghum under full nitrogen produced an R2 = 0.14 with a p = 4.13e-12. The correlation between leaf angle and plot yield was minuscule. Under standard conditions leaf angle against yield computed R2 = 0.012 with a p = 0.053, while plants in the no nitrogen environment returned R2 = 0.044 and a p = 0.0002. A similar relationship between plot yield and flowering time was found with an R2 = 0.015 and p = 0.029 for standard and R2 = 0.025 and a p = 0.006 under no nitrogen conditions. While these p-values are below 0.05 threshold, they are relatively high compared to the negative relationship between flowering and leaf angle.

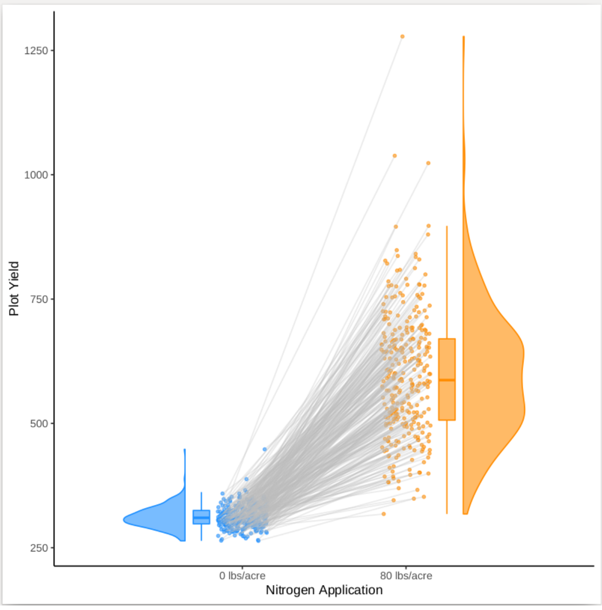


Figure 3.5: Genotypic comparison and average of plot yield in grams under no nitrogen (0 lbs/acre) and standard nitrogen (80 lbs/acre).

Identifying candidate genes associated with leaf angle, yield and

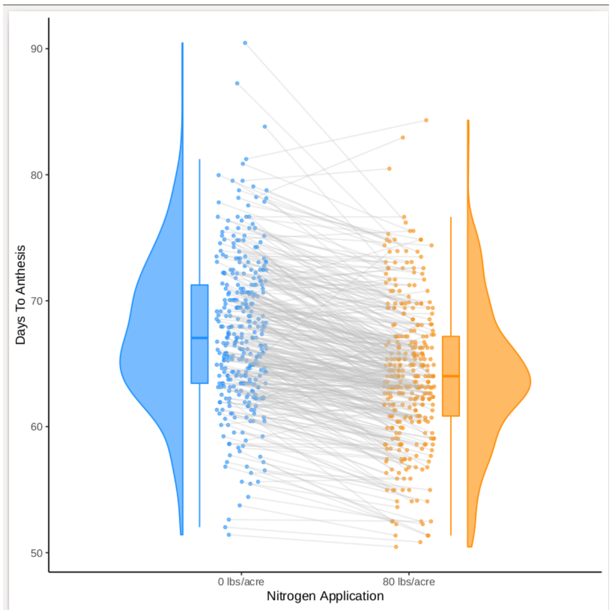


Figure 3.6: Genotypic comparison and average of flowering time (days to anthesis) under no nitrogen (0 lbs/acre) and standard nitrogen (80 lbs/acre).

Trait	Treatment	Mean	SD	Broad-Sense	Narrow-Sense
Leaf Angle	SN	46.91	9.16	0.896	0.412
Leaf Angle	NN	44.21	9.21	0.906	0.692
Flowering Time	SN	64.08	6.61	0.903	0.409
Flowering Time	NN	67.48	6.34	0.901	0.509
Plot Yield	SN	591.6	166.58	0.528	0.160
Plot Yield	NN	311.6	282.45	0.222	0.246

Table 3.1: Traits mean under nitrogen deficit and nitrogen replete conditions, leaf angle in degrees, flowering time in days, and plot yield in grams. Broad sense heritability was calculated for each trait as well as narrow-sense heritability using GEMMA

flowering time under two nitrogen environments. Narrow-sense heritability and broad sense heritability was calculated for three phenotypes: leaf angle, yield and flowering time under the two field conditions. Heritability for all phenotypes under two conditions is listed in table (table 3.1). This analysis led us to conclude our data set of measurements for both leaf angle and flowering time had the potential to enable gene discovery using genome-wide association studies. The significance threshold was determined by taking 0.05 divided by 82,142 effective SNPs. Effective SNP number is calculated by the number of markers and the amount of linkage disequilibrium in a population. GWAS results from GEMMA returned a peak on Chromosome 4:23,799,154, for the

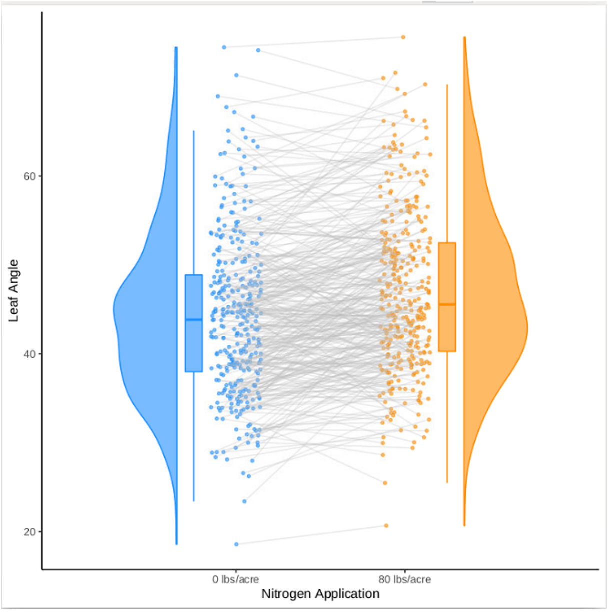


Figure 3.7: Genotypic comparison and average of leaf angle in degrees under no nitrogen (0 lbs/acre) and standard nitrogen (80 lbs/acre).

flowering time phenotype under no nitrogen conditions as well as GxE (figures 3.9 and 3.10). There was one gene identified on chromosome 4 at 23,730,325 to 23,731,465, a protein coding gene SORBI_3004G134600, and has 100 orthologs across many species. A significant peak was computed for plot yield under standard nitrogen conditions, Chromosome 1:13,111,567 this marker was located in a gene dense portion of the sorghum genome with 92 genes located 500kb up or downstream (figure 3.12). The closest genes were SORBI_3001G159032 left of the marker, and SORBI_3001G159300 located to the right. Both genes are protein coding genes, SORBI_3001G159032 is a hypothetical protein. SORBI_3001G159300 has 93 orthologs across species which include genes associated with *Zea mays* a relative of sorghum. Similar to the marker identified on Chromosome 1, the significant peak located on Chromosome 2:60,142,078 for plot yield under no nitrogen conditions was also located in a gene dense portion of the genome with 91 genes 500kb up or downstream (figure 3.11). SORBI_3002G209400 and textSORBI_3002G209600 were located close to the marker. SORBI_3002G209600 has 75 orthologs across many species including *Zea mays Zm00001d005785*. It is a protein coding gene that has not yet been

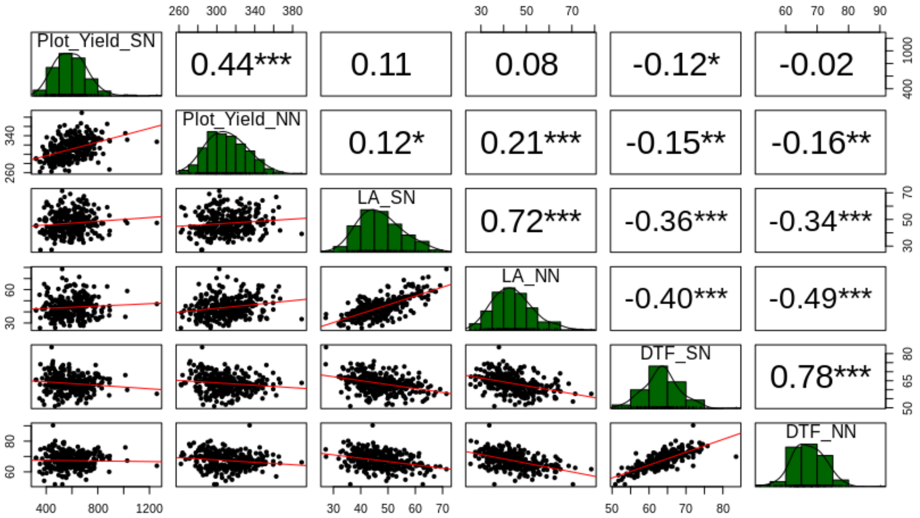


Figure 3.8: Pearson correlation (r) matrix for all traits: Leaf angle (LA), Plot yield, and Flowering time (DTF); under both nitrogen conditions: standard nitrogen (SN) and no nitrogen (NN).

characterized. Leaf angle returned no significant SNPs for MLM by GEMMA, however, FarmCPU in rMVP did identify several SNPs (table 3.2, figures 3.13 and 3.14). A peak on chromosome 7:60751873 was found, this SNP is located near *dwarf-3 (dw3)* in sorghum.

$$Narrow - senseheritability[h^2] = V_A/V_P.$$

$$Broad - senseheritability[H^2] = V_G/V_P.$$

Where V_P represents phenotypic variation, V_A represents additive genetic value, and V_G represents genetic variation.

Violin plots were created using matplotlib and seaborn in Python v3. Violin plots were used to visualize the allele effect of major and minor alleles from the three significant SNPs found for flowering time and plot yield under both conditions. An observable difference was found for flowering time for the significant SNP discovered on Chromosome 4. These varieties carrying the major

Trait	Treatment	Chromosome	SNP	Method
Flowering Time	SN	-	-	MLM
Flowering Time	NN	4	4:23,799,154	MLM
Flowering Time	GxE	4	4:23,799,154	MLM
Plot Yield	SN	1	1:13,111,567	MLM
Plot Yield	NN	2	2:60,142,078	MLM
Leaf Angle	SN	1, 6, 8, 9, 10	1:72,632,46 6:46,610,211 8:4,770,368 9:8,504,657 10:42,113,350	FarmCPU
Leaf Angle	NN	3, 4, 7	3:60,590,404 4:57,126,237 7:60,751,873 7:25,777,877	FarmCPU
Leaf Angle	SN	-	-	MLM
Leaf Angle	NN	-	-	MLM

Table 3.2: GWAS results for flowering time, plot yield, and leaf angle, including single nucleotide polymorphisms identified for each trait

allele show a decrease in days to flowering under both nitrogen conditions (figure3.15). A difference in allele effect was also observed for plot yield in no nitrogen and standard nitrogen for both significant SNPs on Chromosome 1 and 2. Plot yield had a slight increase for sorghum varieties carrying the minor allele for Chromosome 1. The difference in mean yield for no nitrogen was less than that under standard nitrogen. The SNP on Chromosome 1 was not returned in GWAS for plot yield under no nitrogen. Chromosome 2 points were more evenly split between major and minor alleles. Varieties carrying the minor allele had greater yield values. The difference in mean between minor and major allele was greater for the no nitrogen data set. The SNP located on Chromosome 2 was not significant for GWAS ran on plot yield under standard nitrogen (figure 3.16).

Discussion

Nitrogen is one of the limiting factors of plant growth and yield [80]. Nitrate affects many plant pheotypes including: root and leaf growth, flowering time, and yield [26,162,176]. The focus of this paper was to determine if leaf angle was affected by nitrogen stress, and better understand the impact of leaf angle plasticity in sorghum production. Mean difference in leaf angle, yield, and flowering time between nitrogen deficit and replete conditions were found to be statistically significant. A significant difference of 277 grams was discovered for plot yield in sorghum under two differing nitrogen levels. A majority of the sorghum varieties exhibited a reduced plot yield under no nitrogen conditions (figure 3.5). A genetic locus was identified for plot yield located at Chromosome 1:13,111,567 under standard nitrogen conditions but not no nitrogen. An observable difference in yield was determined for major and minor allele where the average yield for sorghum carrying the minor allele presented with greater yield (figure 3.15). The significant SNP found on Chromosome 1 has a clear impact on plot yield. However, undefined genes in this area make it difficult to determine the mechanism in which it impacts yield. The significant SNP located on Chromosome 2 was found for plot yield under no nitrogen, varieties that contain the minor allele exhibit higher plot yield under both standard and no nitrogen. A greater difference in mean plot yield between yield was observed under no nitrogen between major and minor alleles. This SNP could be important for sorghum response to nitrogen stress in relation to yield. The varieties containing the minor allele may be more resistant to nitrogen stress (figure 3.16).

Stresses such as lack of nutrients, drought, and high salinity have been linked to changes in flowering time in many plant species [166]. Flowering and transition from vegetative to reproductive growth stage is important to create the next generation and sustain plant species [161]. In some cropping species such as rice and wheat, stressed plants respond by flowering early to produce seed and continue on to the next generation. Flowering time affects total yields and grain quality [23]. Takeno, 2016 suggests that up to 20 days of nutrient stress is needed to induce early flowering in Japanese Morning Glory [166].

Additionally, in a study on nitrogen stress in Arabidopsis, low nitrogen was found to promote more rapid flowering. However, more extreme nitrogen stress can cause delayed flowering [110, 144, 191]. This is consistent with our findings, where plants grown under no nitrogen exhibited an increase in the number of days to flower. There was natural variation within the sorghum population for the response of flowering time to nitrogen deficit stress. Sorghum grown under stress required an average of 3.6 more days to flower. However that average value is the result of some, but not all, varieties exhibiting a delay in flowering time under nitrogen deficit conditions. In bioenergy sorghum specifically, a longer vegetative period resulting from delayed flowering time may lead to increases in biomass and nitrogen use efficiency, however this is not universally true for all sorghum [135, 153]. The significant genetic locus detected for flowering time under nitrogen stress located at Chromosome 4 at position 23,799,154 indicated an allele affect where flowering time under no nitrogen had an average increase of about 10 days for sorghum carrying the minor alleles compared the whole sorghum population with a difference of about 3 days. This SNP was also determined to be significant for flowering time with a geneotype by environment (GxE) analysis, supporting the conclusion that this genetic variation plays a role nitrogen stress response in flowering (figure 3.14).

Although no significant SNPs were found for leaf angle with MLM. FarmCPU returned several significant SNPs including a SNP chromosome located near the *dw3* gene. In sorghum this gene is responsible for plant height affecting internode to reduce lodging, as well as, having a large effect on leaf angle [172, 187]. A negative correlation was found between leaf angle and flowering time. This is a novel discovery not previously noted in literature for both flowering time and leaf angle in cropping systems. In a previous greenhouse experiment, a sub-set of sorghum varieties from the SAP grown under low nitrogen levels presented with more erect leaves and reached maturity at a later date. This phenomenon was present in some but not all sorghum varieties grown in the greenhouse. The experiment conducted in the field exhibited a similar outcome with select varieties presenting with more erect leaves under no nitrogen conditions, as well as, later maturity. This trend was observed under both standard and no nitrogen; as leaf angle decreased (became more erect), flowering time increased. The link between flowering time and leaf angle is a new discovery. Therefore, we can only hypothesize conclusions regarding this relationship. The correlation between flowering time and yield discovered was weak, although it was significant the correlation under both conditions was less than 0.05. Similarly, leaf angle showed to have little to no relationship with yield under the two environments. The lack of correlation between leaf angle and yield is unexpected. It could have been hypothesized that since the data collected from this field indicates both leaf angle and yield were significantly impacted by nitrogen stress, some relationship would be found between these two traits. Both leaf angle and yield decreased under stress. Additionally, literature has linked changes in leaf angle to yield, though primarily through the ability for higher planting densities. In rice more erect leaf angle has a positive effect on yield [155, 158, 190]. It appears from our findings that more erect leaves alone does not impact either panicle yield or plot yield; possibly other environmental factors played a role and an effect could not be detected.

Nitrogen is one of the most important macro-nutrients, is involved in many biological processes, and has significant impacts on plant growth [157]. Plants have evolved morphological features such as leaf architecture or leaf angle and adjust accordingly to optimize photosynthetic productivity increasing nitrogen content [48]. In this study we found a statistically significant increase in the erectness of sorghum leaves under under nitrogen deficit conditions relative to nitrogen replete conditions. This relationship between leaf angle and nitrogen stress was first observed in a greenhouse experiment which led to the much larger field experiment. Seven out of the ten varieties exhibiting differences in leaf angle under differing nitrogen treatments in the field experiment follow the same trend observed in the greenhouse. This indicates that the plasticity of leaf angle is transferable to a field environment and is not restricted to the climate controlled greenhouse experiment. From previous research discussed above, it is known that more erect leaves can aid the photosynthetic productivity of crops grown at high density, which can increase productivity in crops [100, 101, 200]. More erect leaves decrease over-saturation of the photosynthetic apparatus of the uppermost leaves by dispersing light throughout the canopy, increasing plants' ability to convert light into energy [101, 200]. Although the effects of leaf angle on cropping systems has been thoroughly investigated, this relationship in association with nitrogen stress in sorghum is novel. The main purpose of this study was to take findings discovered in a greenhouse experiment, and determine if similar observation for leaf angle, flowering time, and yield could be made for a sorghum population under nitrogen stress. Additional research is needed to better understanding plasticity of sorghum leaf angle, delayed flowering time, and yield when associated with nitrogen stress.

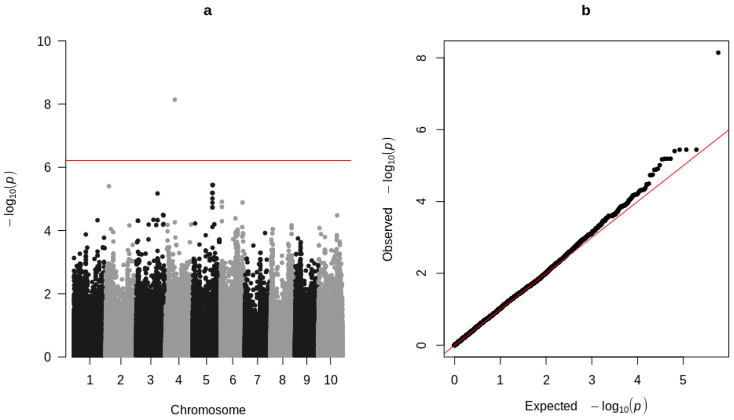


Figure 3.9: Mixed linear model results for flowering time under no nitrogen levels

Variety	Treatment	Field LA	Greenhouse LA
SC85	SN	57.2	26.1
SC85	NN	59.5	14.9
BTx3197	SN	63.6	16.4
BTx3197	NN	41.6	5.5
SC500	SN	51.9	18.8
SC500	NN	36.1	7.5
SC1439	SN	46.2	54.3
SC1439	NN	46.8	14.7
P898012	SN	50.9	25.9
P898012	NN	54.7	10.5
Acme	SN	43.1	22.6
Acme	NN	37.7	14.9
P850029	SN	38.4	21.3
P850029	NN	30.4	10.2
90M	SN	49.4	8.8
90M	NN	34.9	9.1
SC301	SN	66.2	38.7
SC301	NN	71.3	70.6
TX430	SN	40.2	5.4
TX430	NN	41.9	3.8

Table 3.3: Mean leaf angle (LA) measurements for 10 diverse lines grown in the field and in the greenhouse

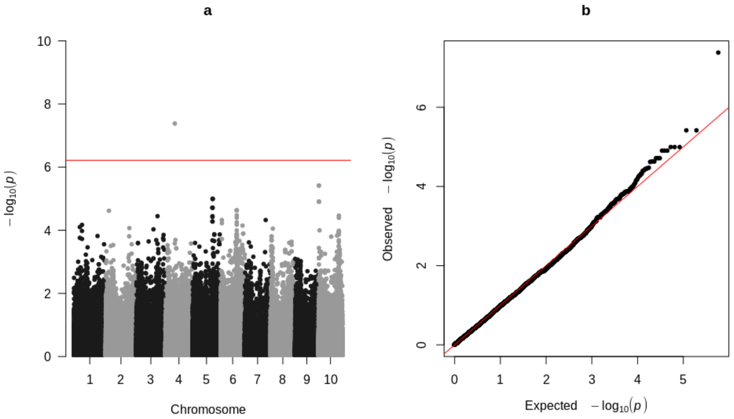


Figure 3.10: Mixed linear model results for flowering time for genotype by environment

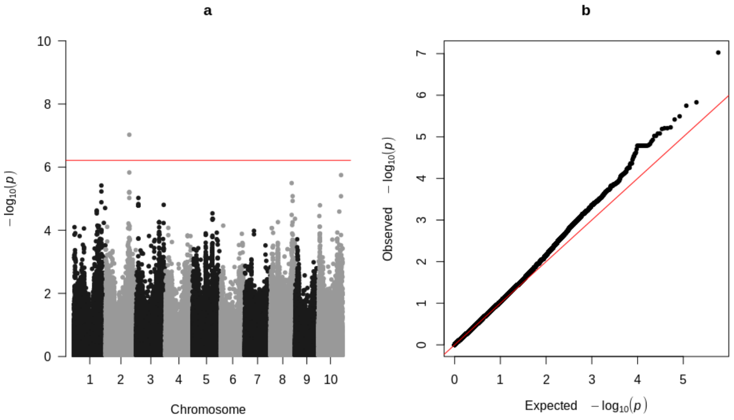


Figure 3.11: Mixed linear model results for plot yield under no nitrogen levels; a) Manhattan plot, b) Q-Q plot

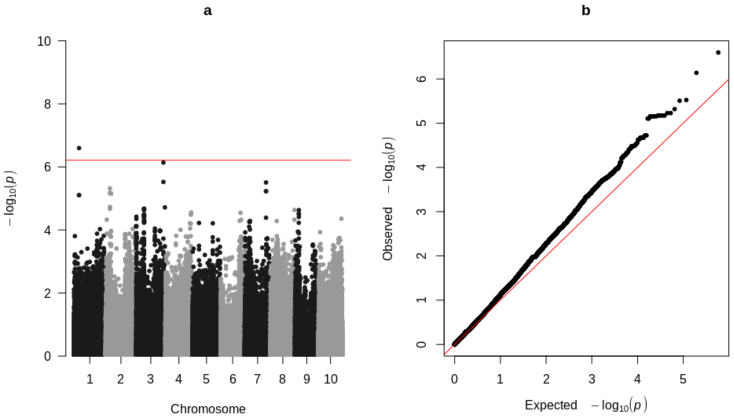


Figure 3.12: Mixed linear model results for plot yield under standard nitrogen levels; a) Manhattan plot, b) Q-Q plot

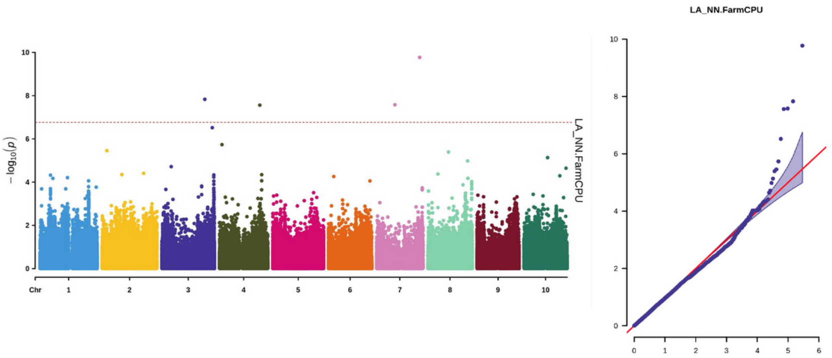


Figure 3.13: FarmCPU results for leaf angle under no nitrogen levels: Manhattan plot and Q-Q plot

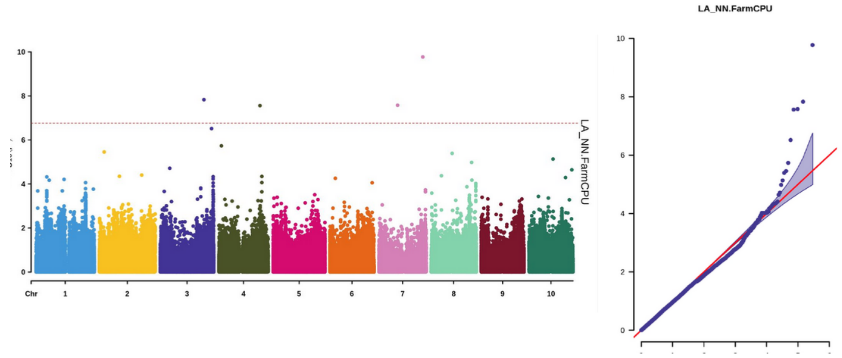


Figure 3.14: FarmCPU results for leaf angle under standard nitrogen: Manhattan plot and Q-Q plot

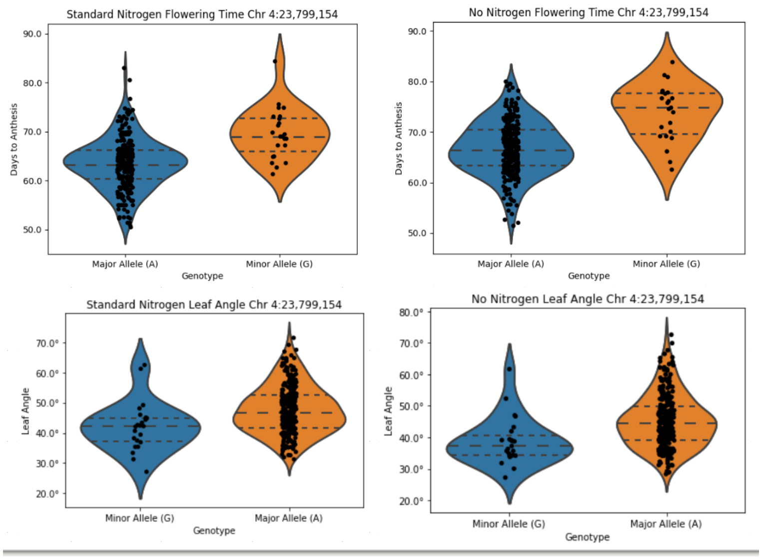


Figure 3.15: Violin plots containing major and minor allele for flowering time and leaf angle under SNP 4:23,799,154

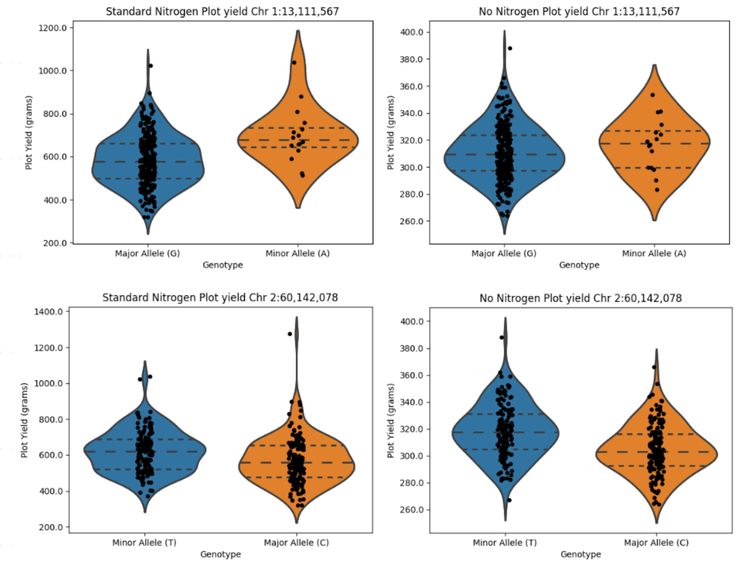


Figure 3.16: Violin plots containing major and minor allele for plot yeild for both SNP 1:13,111,567 and 2:60,142,078

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