# GENETIC AND ENVIRONMENTAL INFLUENCES ON THE INHERITANCE OF SORGHUM WITH A BLACK PERICARP

A Thesis

by

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## ABSTRACT

The black pericarp trait in grain sorghum [*Sorghum bicolor* (L.) Moench] is a novel trait with complex inheritance. In addition to its uniform, dark appearance, black sorghum grain contains high levels of favorable phenolic compounds such as 3-deoxyanthocyanidins (3-DOA) and condensed tannins which have applications in the specialty food industry as high-antioxidant food additives, natural food colorants, or natural food preservatives. Previous studies have indicated the trait is not fully penetrant in all environmental conditions. Additionally, black sorghum has acceptable agronomic performance, but is significantly lower yielding than other elite grain sorghum hybrids. Further improvement of black sorghum is dependent on understanding the factors—both genetic and environmental—influencing the expression of this trait.

The first of two studies investigated the effect of light shading on grain color and grain composition in black Tx3362. Increased light shading reduced, and in some cases, eliminated the black color resulting in red grain production. In addition, increased shading reduced the concentration of 3-deoxyanthocyanidins, total phenols, tannins, and fiber while increasing fat concentrations. Thus the black pericarp trait and associated high phenolic concentrations are strongly influenced by both intensity and duration of sunlight exposure.

In the second study, a generation means analysis was performed to determine the genetic factors affecting the trait. This study concluded grain color

and associated grain composition traits were influence by additive, dominance, and epistatic effects. The generation means analysis also determined the black pericarp trait is recessive, controlled by multiple genes, and is moderate to highly heritable.

Despite these challenges, there is enough variation in breeding populations between red and black parents for further improvement of the trait. Creation of high yielding hybrids with uniformly dark grain and high levels of phenolic compounds will be possible through standard plant breeding practices.

## DEDICATION

*In memory of Janet Pfeiffer, who emphasized the importance of education and instilled in me the inspiration to set high goals and the confidence to achieve them.*

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## NOMENCLATURE

| | |
|---|---|
| 3-DOA | 3-Deoxyanthocyanidins |
| CIE | *Comission Internationale de l'Eclaorage* |
| GMA | Generation means analysis |
| NIR | Near-infrared spectroscopy |
| ROS | Reactive oxygen species |
| TAES | Texas Agricultural Experiment Station |
| CS | College Station, TX |
| WE | Weslaco, TX |
| HW | Halfway, TX |

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# 1. INTRODUCTION

Anthocyanins, which are common plant pigments, are reported to have nutraceutical properties when consumed which include vasoprotective, anti-inflammatory (Lietti et al., 1976), anticancer and chemopreventative (Shih et al., 2007), antineoplastic (Kamei et al., 1995), hypoglycemic (Tsuda et al., 2003), cholesterol reduction, and cardiovascular disease reduction qualities (Awika and Rooney, 2004). In addition, a special class of anthocyanins, the 3-deoxyanthocyanidins, which are found in high levels in black sorghum [*Sorghum bicolor* (L.) Moench] (Gous, 1989) have unique processing properties (pH stability) which make them very valuable in food products (Awika et al., 2004).

Since consumers often make food choices based on health benefits and judge the nutritional value of foods based on color, the black sorghums have potential use in food products (Rooney et al., 2013b). These compounds are concentrated in the pericarp so sorghum bran could be used as an added ingredient to bring nutraceutical properties to foods that normally would not contain such benefits and as a natural food colorant. These dual properties would allow the bran to be added to foods without adversely affecting its quality (Awika, 2000; Gordon, 2001; Rooney et al., 2013b).

Despite strong demand from the food industry for new sources of natural food colorants and for ingredients high in antioxidants, the development of high yielding black sorghum hybrids has been slow because the genetic inheritance of

the trait appears to be recessive and quantitative (Rooney et al., 2013b). In sorghum, the black pericarp trait is unique because it cannot be explained by the typical digenic epistatic inheritance model of other pericarp colors (Dykes et al., 2005, 2009). Also, an extremely low number of dark transgressive segregants are recovered in crosses of red and black parents (Hayes and Rooney, 2014). The black phenotype is also not fully penetrant, perhaps due to abiotic and biotic factors (Gous, 1989; Lo et al., 1999; Dykes et al., 2005, 2009). Additionally, yield and total phenolic content appear to be in an antagonistic relationship in black sorghum (Portillo, personal communication). The average yield of black pericarp hybrids averaged just 64% of a commercial hybrid check (Hayes and Rooney, 2014). Even with all of these complexities, the trait is selectable and heritable because both pollinator and seed parents have been developed and are now used to produce a black grain sorghum hybrid (Rooney et al., 2013a; b).

Further improvements are contingent on an understanding of the genetic factors underlying the expression of this trait. Therefore, the objectives of this research were to:

- Determine the heritability of the black pericarp trait.
- Estimate the relative genetic effects (additive, dominant and epistatic), and the number of genes controlling the black pericarp trait using generation means analysis.
- Establish the timing and influence of sunlight on the penetrance of the black pericarp.

# 2. LITERATURE REVIEW

The increase in chronic health complications in the United States is typically associated with poor nutrition (National Center for Health Statistics, 2012). Healthier diets, which include the consumption of food with substantial levels of phytochemicals and dietary fiber, are correlated with tangible health benefits (Kushi et al., 1999). To increase the consumption of healthy foods, processors must integrate healthy ingredients into products that appeal to the modern food consumer. Thus, there is great demand for researchers to explore new sources of healthy dietary components (Awika et al., 2005b). Phytochemicals in cereal crops are of great interest to scientists because cereal crops are the most widely grown and consumed plants in the world (Awika et al., 2005b). Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most widely produced cereal crop in the world with grain production in 2010 of 55.6 million metric tons grown on 40.5 million hectares (FAOSTAT, 2012). Specific varieties of sorghum are known to contain high levels of beneficial phenolic compounds and fiber located in the bran layers of the grain (Awika, 2003). Therefore, sorghum has great potential to be specifically bred to produce high levels of different phenols (Dykes and Rooney, 2006).

## 2.1 Sorghum Phenols

Plants possess a wide range of phenolic compounds which can be separated into three categories: phenolic acids, tannins, and flavonoids (Gous, 1989). Phenolic acids are found in all sorghum varieties and can be further divided into free

phenolic acids which are found in the pericarp, aleurone, and testa layers and bound phenolic acids which are found in cell walls (Dykes and Rooney, 2006). Tannins are polyphenolic compounds that can be divided into condensed tannins and hydrolysable tannins. Condensed tannins are high molecular weight polyphenols and which are present only in sorghum containing the $B_1\_B_2\_$ genotype. Tannic acids, which represents hydrolysable tannins, have never been identified in sorghum (Dykes and Rooney, 2006).

The largest category of phenolic compounds is the flavonoids and the primary division of flavonoids is the anthocyanins, which encompasses most plant pigments (Dykes and Rooney, 2006). Anthocyanins are highly distributed throughout the plant kingdom. The predominant anthocyanins in sorghum are 3-deoxyanthocyanidins (3-DOA) and these compounds are unique because they lack a hydroxyl group at the C-3 position. The two most common forms of 3-DOAs found in sorghum are luteolinidin and apigeninidin (Nip and Burns, 1971; Iacobucci and Sweeny, 1983; Gous, 1989). To date, sorghum and blue maize (*Zea Mays* L.) are the only known dietary sources of 3-DOAs (Nakatani et al., 1979; Clifford, 2000).

Black sorghum has one of the highest levels of antioxidant activity of any commodity, having antioxidant potential scores similar to blueberries and over 40 times higher than white grain sorghum (Awika, 2003; Wu and Prior, 2005; Dykes and Rooney, 2006). Most other commercial sources of antioxidants are fruits and vegetables and are harvested at high moisture content. This makes storage and processing of fruits and vegetables more difficult than sorghum, which is harvested

at low moisture (Awika and Rooney, 2004; Awika et al., 2005b). This suggests a potential advantage of sorghum as a viable commercial source of anthocyanins.

## 2.2 Properties of Sorghum Phytochemicals

Many plant phenolic components, including 3-DOAs, act as antioxidants (Awika, 2000). Reactive oxygen species (ROS) in organisms can cause cell damage through oxidative stress and have been implicated in many human diseases including cancer, cardiovascular disease, rheumatoid arthritis, diabetes, Alzheimer's disease, Parkinsons disease, and are also involved with aging (Valko et al., 2007). Antioxidants can prevent damage through suppressing ROS formation, scavenging ROS, and by upregulating antioxidant defenses (Halliwell and Gutteridge, 1999). A strong correlation ($R^2$ = 0.94) was discovered between total anthocyanidin levels of sorghum lines and antioxidant activity (Awika et al., 2004). 3-DOAs are more cytotoxic to human cancer cells than other anthocyanins (Shih et al., 2007) and compared to other cereals, sorghum had the greatest antioxidant concentration per unit weight (Awika et al., 2004).

The 3-DOAs are often produced by plants in response to biotic or abiotic stresses and can be considered phytoalexins (Lo et al., 1999). The 3-DOAs are also more stable in both low and high pH solutions compared to common anthocyanins due to lack of a hydroxyl molecule. This attribute gives sorghum the potential to be used as a natural food colorant (Iacobucci and Sweeny, 1983; Mazza and Brouillard, 1987; Gous, 1989).

## 2.3 Sources of Black Pericarp Sorghum

Many different dark sorghums are known, but most are dark brown or red. Uniformly black grain sorghums are relatively rare. A true black sorghum accession from Sudan (Shawaya #2) was collected in 1979 and has since been in the Texas A&M AgriLife Research Sorghum Breeding Lab (Rooney et al., 2013b). This accession has a black pericarp color when grown under normal conditions and inheritance of this color is different than previously described digenic epistatic models of pericarp color (Rooney, 2000). After test crosses with parents of various pericarp colors, Shawaya #2 was determined to be genetically red [*R_Y_*] as all $F_1$ progeny have a red phenotype and that the black color must be caused by additional genetic factors which are not known (Rooney et al., 2013b). Shawaya #2 also showed high levels of anthocyanins and flavan-4-ols compared to other sorghum lines (Gous, 1989).

## 2.4 Genetic Effects of Phenolic Concentration

All sorghums contain phytochemicals, but the concentrations in any particular genotype can vary substantially due to genetics and the environment (Dykes et al., 2005; Taleon et al., 2012). Grain color has a major effect on phytochemical levels and since the inheritance of most grain color is sorghum is under the control of several well defined single loci, the variation can be attributed to a limited number of genes that influence the epicarp, mesocarp and testa layers. Epicarp color is determined by the *R* and *Y* genes which interact epistatically to produce red (*R_Y_)*, white (*R_yy or rryy)*, or yellow (*rrY_*) colors (Rooney, 2000).

Among these colors, the white sorghums contain just one-third the anthocyanidin concentration of red sorghum (Gous, 1989). When dominant, the intensifier gene (*I*) is expressed and creates a more intense red or yellow phenotype, or if white, an opaque phenotype (Rooney, 2000). It is not exactly clear how the presence of the intensifier gene affects total phenol content. The thickness of the mesocarp is determined by the *Z* gene (Rooney, 2000). Black and red sorghum lines with thick mesocarps (*zz*) express higher levels of total phenols than lines with thin mesocarps (*Z_*) (Dykes et al., 2005).

The presence or absence of a testa layer is determined by alleles at the $B_1$ and $B_2$ loci. These genes act in duplicate dominant epistasis; $B_1\_B_2\_$ has a pigmented testa and condensed tannins while the other types ($b_1b_1$ $B_2\_$, $B_1\_b_2b_2$, *and* $b_1b_1b_2b_2$) have neither a testa layer or condensed tannins. When a pigmented testa is present, the spreader gene in dominant form (*S_)* allows the pigmented testa layer to spread beyond the testa into the mesocarp and epicarp (Rooney, 2000). Thus, red sorghum lines with a pigmented testa express higher levels of total phenols than lines without the presence of a pigmented testa layer and in the same material the presence of a dominant allele in the *S* gene have even higher levels of total phenols. Finally, plant secondary color is controlled by the *P* and *Q* genes; *P_Q_* and *P_qq* genotypes produce plants with purple or red phenotypes, respectively, while *ppQ_ or ppqq* genotypes produce tan plants. Sorghum grains have higher levels of total phenols when produced on purple or red plants compared to tan plants, with some exceptions (Dykes et al., 2005).

## 2.5 Environmental Effects of Phenolic Concentration

The black pericarp trait is also not fully penetrant under certain environmental conditions. Dykes (2009) compared panicles of Tx3362 that were exposed to and shaded from sunlight. At maturity, uncovered panicles were black but covered panicles had a red phenotype. The uncovered panicles had three times more 3-DOA content than covered panicles, with flavone and flavanone levels remaining similar between each treatment. This indicates that the black phenotype is induced by sunlight and 3-DOA concentration is the physiological explanation of the black pericarp phenotype (Dykes et al., 2009).

## 2.6 Breeding for High 3-DOA Concentration

Six black sorghum experimental hybrids created by the Texas A&M AgriLife Sorghum Breeding Lab were used to calculate high parent heterosis for yield and total 3-DOA concentration. The mean high parent heterosis for yield among the six hybrids was 172%, indicating that hybrid performance was significantly greater than the parents. High parent heterosis averaged approximately 100% for total phenols and only 75% for 3-DOA concentration. These results indicated that total phenol and 3-DOA concentration were likely under the influence of genes with mostly additive effects and thus there is no advantage for hybridization to improve phenolic concentration and 3-DOA concentration traits (Hayes and Rooney, 2014).

The effect of the black color on grain quality is not well known. Portillo (personal communication, 2013) reported a negative correlation between 3-DOA concentration and starch concentration, lipid concentration, and yield; however,

black sorghum lines have higher protein concentrations and test weight. This may or may not be caused by the black trait but further testing and evaluation is needed to monitor the effects of 3-DOA concentration on other grain quality composition. Another obstacle of black sorghum is that mean luteolinidin and apigeninidin concentrations can vary significantly by environment (Taleon et al., 2012).

To have the highest possible antioxidant and 3-DOA content in sorghum, plants should exhibit a dark black and thick pericarp with a pigmented testa and a spreader gene. Additionally, plants should have a purple secondary plant color, be high yielding and have superior agronomic traits (Dykes et al., 2005).

## 3. THE EFFECT OF LIGHT SHADING ON PERICARP COLOR AND FLAVANOID CONCENTRATIONS IN BLACK SORGHUM Tx3362

Black sorghum genotypes have a uniformly black pericarp color when produced under typical field conditions and they also contain high levels of 3-deoxyanthocyanidins (3-DOAs) in the bran layers of the grain. Thus, these lines are potential sources of natural pigmentation that has high levels of antioxidant activity and can be used as a natural food colorant. However, prior research indicates that the black color is not fully penetrant in all environmental conditions. Specifically, black sorghum panicles shaded from sunlight between flowering and physiological maturity are dark red in color. The objective of this study was to determine if the time of light exposure affected pericarp color, 3-DOA production, and grain composition traits. Three distinct experiments were conducted that shaded developing Tx3362 black sorghum panicles for varying time intervals in two Texas environments. After harvest, samples were visually phenotyped, quantitatively measured for color via colorimeter, and scanned by near-infrared spectroscopy (NIR) to estimate grain composition values. Across both environments, increased shading reduced, and in some cases, eliminated the black color resulting in red grain production. In addition, increased shading reduced the concentration of 3-DOA, total phenols, tannins, and fiber while increasing fat concentrations. Due to the strong association between visual score and 3-DOA concentration, field selection for darkest pericarp should result in selection for highest 3-DOA content.

Additionally, maximum production of these beneficial compounds is environment specific. Therefore, environments with high sunlight intensity are conducive to the production of darker grain and higher 3-DOA, tannin and phenol content.

## 3.1 Introduction

The increase in chronic health complications in the United States is typically associated with poor nutrition (National Center for Health Statistics, 2012). Healthier diets, which include the consumption of food with substantial levels of phytochemicals and dietary fiber, are correlated with tangible health benefits (Kushi et al., 1999). This has generated interest in improving the concentrations of these compounds in food crops (Awika et al., 2005a). Specific varieties of sorghum [*Sorghum bicolor* (L.) Moench] are known to contain high levels of beneficial phenolic compounds and fiber located in the bran layers of the grain (Awika et al., 2003) and breeding is effective at developing sorghum genotypes that maximize these compounds in an agronomically acceptable hybrid (Rooney et al., 2013a; b; Hayes and Rooney, 2014).

Black grain sorghum has a uniformly dark black pericarp color when grown under normal field conditions and this grain phenotype is relatively rare within the sorghum species (Rooney et al., 2013b). Black sorghum is characterized as having high concentrations of phenolic compounds with high antioxidant activity, specifically 3-deoxyanthocyanidins (3-DOA) (Awika et al., 2004, 2005b). The 3-DOAs are unique anthocyanin compounds that are relatively rare in plants (Clifford, 2000), but are the major anthocyanin in sorghum (Awika et al., 2004). The 3-DOAs

include the compounds luteolinidin and apigeninidin and they lack a hydroxyl group at the C-3 position which increases the chemical and thermal stability of these compounds (Sweeny and Iacobucci, 1983; Mazza and Brouillard, 1987). The dark color and stability of the 3-DOAs makes them very desirable to food processors as a natural food colorant and high-antioxidant food additive (Awika et al., 2004). Several studies suggested an association between grain color and 3-DOA concentration (Dykes et al., 2005, 2009). Among black sorghum genotypes, strong associations were found between CIE *L*a*b** colorimeter values and 3-DOA concentrations (Dykes et al., 2013). Taleon et al. (2012) found no correlation between color and total flavonoid levels but that study did not investigate associations between pericarp color and specific classes within the flavonoid family. If a relationship between color and 3-DOA concentration can be established, it would provide an effective indirect selection method to increase 3-DOA concentrations with reduced reliance on laboratory tests to confirm their presence.

Grain color and phytochemical concentration can vary substantially within black pericarp genotypes due to the environment (Dykes et al., 2005; Taleon et al., 2012). Dykes et al. (2009) reported that the black pericarp trait is not fully penetrant in all environmental conditions. Specifically, shaded panicles of Tx3362 are dark red in color while sunlight exposed, whereas un-shaded panicles were black (Rooney et al., 2013b). Based on these observations, black pericarp sorghum is genetically red according to the previously described digenic epistatic model of red (*R_Y_*), white (*R_yy* or *rryy*), or yellow (*rrY_*) pericarp colors (Rooney, 2000) and

sunlight exposure is essential for development of the black color (Dykes et al., 2009).

In other crops, phytochemical concentrations are influenced by several abiotic factors including light and temperature (Christie et al., 1994; Leyva et al., 1995; Orczyk et al., 1996; Han et al., 2009; Dykes et al., 2009). In addition, biotic factors such as fungal infections can influence production of flavonoids (Hipskind et al., 1990; Snyder and Nicholson, 1990; Lafayette et al., 1996; Lo et al., 1999). It is likely that the timing of these influences affects total accumulation as well. Thus, the purpose of this experiment is to explore the effects of the timing of light exposure on the production of 3-DOAs and other compositional factors in addition to the black color itself.

### 3.2 Materials and Methods

The sorghum genotype Tx3362 was used in this study and it is an inbred line that produces a black pericarp when grown under long day production environments. The line was released in January 2012 by the Texas A&M AgriLife Research Sorghum Breeding Lab (Rooney et al., 2013b). To determine the critical time periods involved in black color induction, three distinct bagging protocols were used that affected the time and duration of sunlight exposure received by the developing grain. These tests, described subsequently, were grown in Hereford, Texas (2012) and College Station, Texas (2013). Hereford is located in the Texas High Plains and represents a temperate climate production system while College Station represents a sub-tropical production environment.

At each location, Tx3362 was planted and managed as standard for grain sorghum in each location. At mid-anthesis in each location, 600 plants were marked with a "primary tag" which randomly assigned a panicle to one of three trials (Figure 1). Thus, all panicles were of the same maturity within a location. This tag date was designated as Day 0 for all three trials.

In Trial I, 20 pollinating bags were *placed on* randomly selected and previously tagged panicles every fifth day for 35 days. In trial II, 200 tagged plants were bagged on Day 0; every fifth day 20 bags were *removed from* randomly chosen panicles for 35 days. In trial III, bags were *placed on* 20 plants every fifth day and those same bags were *removed* five days later, continuing for 35 days. Panicles from all trials were harvested on Day 40, after the grain was physiologically mature and air dried in a warehouse to minimize exposure to grain weathering. Each of the three trials had nine treatments dates including a control. For all trials, individual panicles served as the experimental unit with 20 replications per treatment.

Whole panicles were visually phenotyped for color using a subjective 1 to 5 scale where 1 was black and 5 was red. After visual ratings, panicles were threshed and cleaned using a Wintersteiger LD180 (Wintersteiger Ag; Ried, Austria). Colorimeter ratings were made on the cleaned grain using a CR-410 Colorimeter (Konica Minolta Sensing Americas, Inc.; Ramsey, NJ). Quantitative colorimeter measurements are expressed in accordance with the *Comission Internationale de l'Eclaorage* (CIE) (2004) as *L*a*b** color space values where *L** is a lightness value (0 = black, 100 = white); *a** indicates green or red (-*a** = greenness, +*a** = redness);

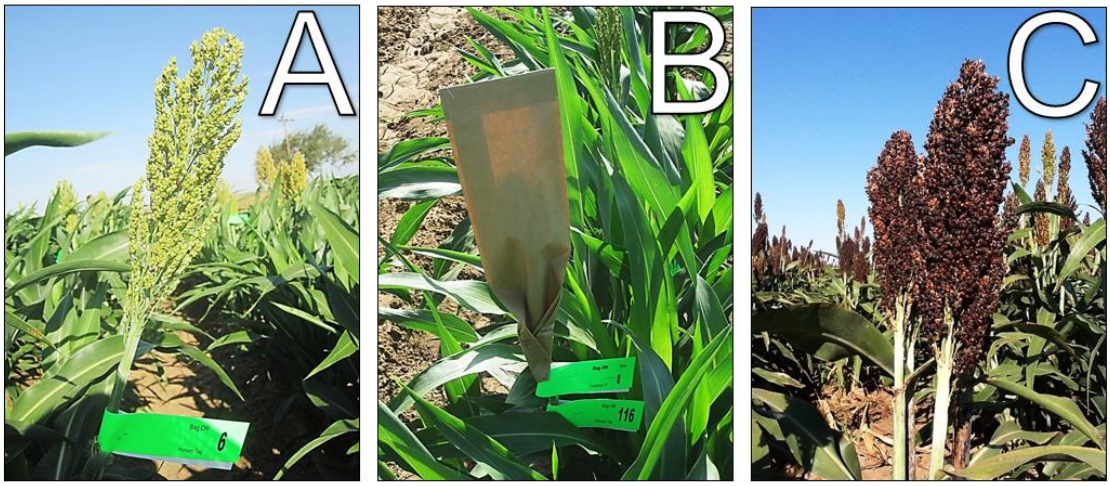


**Figure 1.** Explanation of experimental design: (a) Approximately 600 plants at peak anthesis are marked with a primary tag to ensure similar maturity; (b) Panicles are shaded with pollination bags at variable intervals; (c) Pollination bags are removed from panicles at various intervals and phenotyped at maturity.

*b** indicates blue or yellow (*-b** = blueness, *+b** = yellowness).

Compositional analysis of the grain was performed using near-infrared (NIR) spectroscopy. To meet minimum grain quantities for NIR scans, an average of four panicles were bulked to create an experimental unit. Each experimental unit was scanned using a FOSS XDS NIR spectrometer with the XDS Rapid Content modules (FOSS North America.; Eden Prairie, MN USA). Scans covered wavelengths between 400 to 2500 nm and resultant spectra were analyzed using ISIscan

v.3.10.05933 software (Infrasoft International LLC.; State College, PA). NIR predictions for ash (%), lipids (%), starch (%), fiber (%), total phenols (mg GAE/g), tannins (mg CE/g) and 3-DOA (abs/mL/g) were based on NIR calibration curves developed by the Texas A&M AgriLife Research Sorghum Breeding Lab and described by Dykes et al. (in press).

Data were analyzed by environment but general trends indicated consistency of results despite heterogeneous error terms. Consequently, environments were combined and analysis of variance was examined by JMP Pro 11.0.0 (SAS Institute, 2013) using an all fixed model of y = treatment + environment + (treatment × environment) + error. Because of environmental effects, Pearson's correlation coefficients between phenotypic variables were determined in single environments using multivariate analysis in JMP.

## 3.3 Results

### *3.3.1 Analysis of Variance*

In all trials, highly significant treatment and environment effects were detected for all compositional traits with the exception of trial III tannin and fat treatment effects (Table 1). In trial I, treatment × environment interactions were generally significant with the exception for phenols and tannins (Table 1). In trial II, treatment × environment interactions were non-significant with the exception of fat and fiber (Table 1). For trial III, treatment × environment interactions were significant for all compositional traits except tannins. (Table 1). Relative to both treatment and/or environment effects, the interaction terms were substantially

**Table 1.** Analysis of variance for grain composition traits in Tx3362 grain measured from trials I, II, and III grown in Halfway 2012 and College Station 2013.

| | | | Mean square | | | | |
|---|---|---|---|---|---|---|---|
| **Experiment** | **Source of variation** | **DF** | **Phenols** | **Tannins** | **3-DOA** | **Fat** | **Fiber** |
| **Trial I** | Treatment | 8 | 17.79** | 152.42** | 6833** | 0.52** | 0.14** |
| | Environment | 1 | 598.77** | 7514.34** | 35044** | 2.17** | 3.21** |
| | Treatment × Environment | 8 | 0.91 | 6.75 | 775** | 0.06** | 0.03** |
| | Error | 30 | 0.85 | 9.30 | 217 | 0.01 | 0.003 |
| **Trial II** | Treatment | 8 | 15.74** | 129.30** | 9197** | 0.48** | 0.12** |
| | Environment | 1 | 514.53** | 6441.67** | 7698** | 0.05* | 1.43** |
| | Treatment × Environment | 8 | 0.29 | 2.69 | 393 | 0.07** | 0.02** |
| | Error | 18 | 0.69 | 6.62 | 458 | 0.01 | 0.003 |
| **Trial III** | Treatment | 8 | 1.03* | 5.72 | 1492** | 0.01 | 0.03** |
| | Environment | 1 | 1035.54** | 14173.31** | 80260** | 3.70** | 7.01** |
| | Treatment × Environment | 8 | 0.93* | 10.67 | 719* | 0.02* | 0.02** |
| | Error | 44 | 0.42 | 5.67 | 284 | 0.01 | 0.003 |

*P <0.05

**P <0.01

**Table 2**. Analysis of variance for grain color traits in Tx3362 grain measured from trials I, II, and III grown in Halfway 2012 and College Station 2013.

| | | Mean square | | | | | |
|---|---|---|---|---|---|---|---|
| **Experiment** | **Source of variation** | **DF** | **Visual score** | **DF** | ***L**** | ***a**** | ***b**** |
| **Trial I** | Treatment | 8 | 51.85** | 8 | 185.12** | 75.94** | 259.15** |
| | Environment | 1 | 2.86* | 1 | 598.29** | 993.10** | 4212.27** |
| | Treatment × Environment | 8 | 6.56** | 8 | 45.69** | 18.68** | 41.73** |
| | Error | 304 | 0.63 | 263 | 5.31 | 2.49 | 5.55 |
| **Trial II** | Treatment | 8 | 72.55** | 8 | 211.03** | 119.01** | 288.03** |
| | Environment | 1 | 0.53 | 1 | 70.42** | 318.74** | 1243.30** |
| | Treatment × Environment | 8 | 3.60** | 8 | 36.21** | 12.31** | 44.44** |
| | Error | 245 | 0.47 | 195 | 4.55 | 2.49 | 4.33 |
| **Trial III** | Treatment | 8 | 2.91** | 8 | 6.60 | 8.18** | 8.32 |
| | Environment | 1 | 2.97** | 1 | 1985.90** | 1886.40** | 7592.63** |
| | Treatment × Environment | 8 | 1.00** | 8 | 6.06 | 7.65** | 8.93* |
| | Error | 328 | 0.35 | 313 | 3.90 | 2.96 | 4.37 |

*P <0.05
**P <0.01

smaller, implying that the main effects were of greater magnitude.

Significant treatment effects were detected for all color traits (visual score, *L**, *a**, and *b**) in trial I and trial II. In trial III, treatment effects were detected only for visual score and *a** (Table 2). Significant environment effects were present for all color traits in all three trials with the exception of visual score in trial I (Table 2). All color traits produced significant treatment × environment interactions indicating that grain color intensity differs by treatment among the environments. The relative magnitude of these effects varied, depending on which trait is considered. For example, the environment and treatment × environment interactions were small for visual score, but the opposite was observed for *L**, *a** and *b** values (Table 2). This was one indication that subjective visual scores interpret colors from a substantially different perspective than the quantitative colorimeter scores.

### *3.3.2 Color and Composition Trends*

In trial I, the longer the panicle was shaded, the higher the visual score rating (more red color) and *L*a*b** values (Figures 2 and 3, Appendix 1). Alternatively, the longer the panicle was exposed to sunlight prior to shading, the blacker the pericarp. The effects appeared to be additive with consistent reductions in values with each treatment date through approximately 20-25 days when the effects were relatively reduced or not present (Figure 3). In trial I, the most pronounced changes to colors occurred in treatment Day 0 (anthesis) through Day 20 (Figure 3). This 20 day interval is responsible for approximately 80% of the total

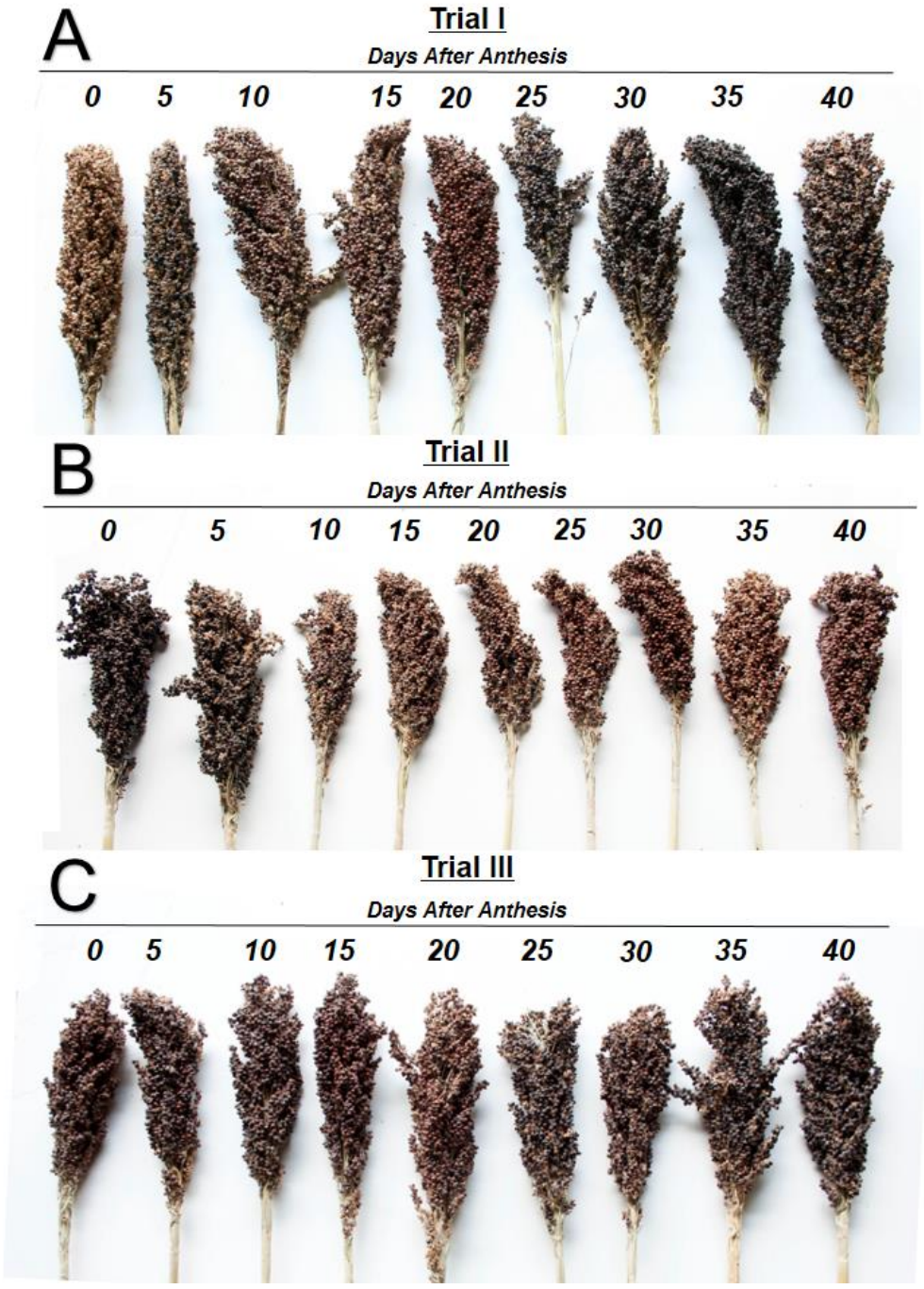


**Figure 2.** A representative panicle from each treatment in each trial grown in the Hereford, Texas 2012 environment. (A) Trial I, (B) trial II, and (C) trial III.

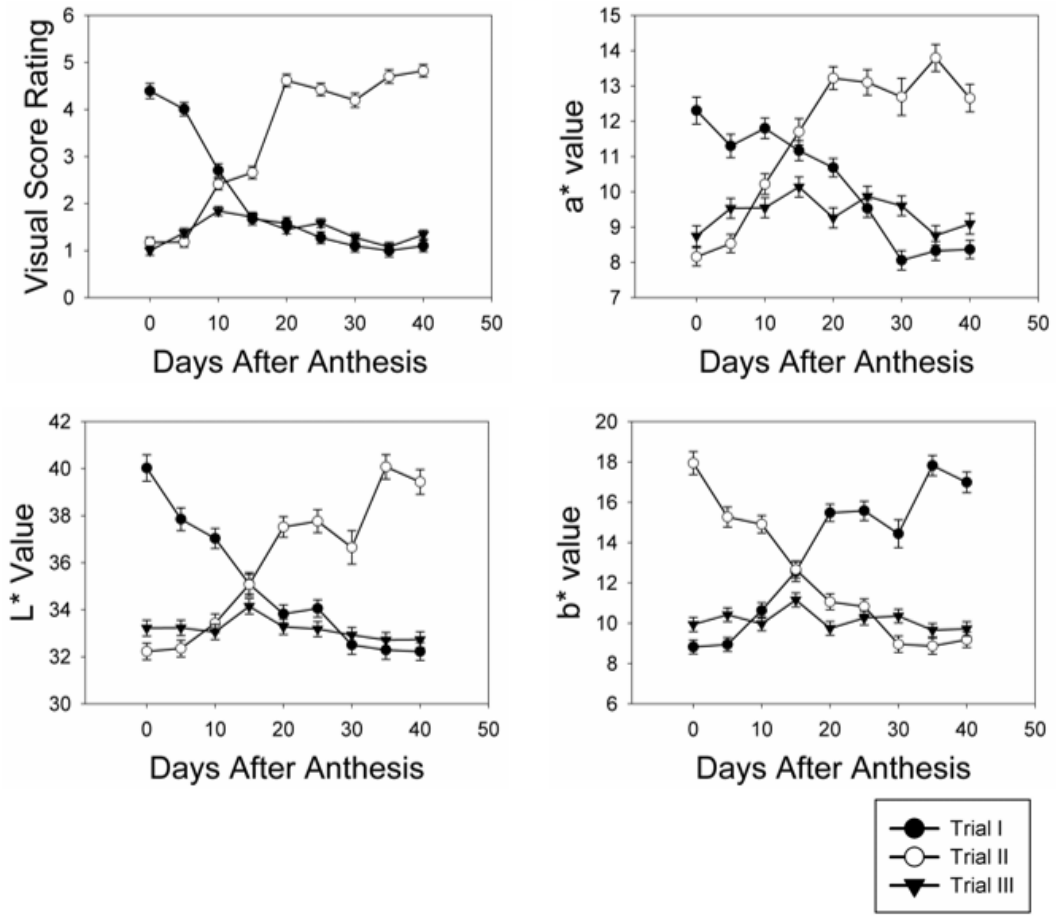


**Figure 3.** Scatter plot results of visual score ratings and *L*a*b** colorimeter results from trials I, II and III from combined analysis of both Hereford 2012 and College Station 2013 environments. Visual score ratings reflect 1 (black) to 5 (red).

change in visual score and $L^*$ value. Post Day 20 treatments showed additional darkening, but with much smaller effects.

Results in trial II where similar to those observed in trial I; the longer the panicle was shaded, the lighter the color of the panicle (Figure 2). Since this trial removed bags placed on the panicle at Day 0, the exposure to sunlight was later in development and visual scores decreased as panicles were unshaded (Figure 3). The unshaded control was darkest by visual score (1.18) and the completely shaded treatment the lightest in color (4.82). These trends were similar in colorimeter results (Figure 3). Also similar to trial I, the largest treatment effects occurred between the Day 5 and Day 20 treatments (Figure 3) and this interval was responsible for two-thirds of the total range for $L^*$value and over 80% of the total range in visual score. Only very small changes in color were detected in post-Day 20 treatments.

The effects of trial III treatments were small relative to those observed in trials I and II. This was reflected in both the analysis of variance (Table 1) as well as color ratings (Figures 2 and 3). In visual and colorimeter scores, the greatest treatment effects in this trial were Day 10 and 15 wherein visual scores increased to a maximum of 1.84 relative to untreated controls of 1. These represent a relatively small change in color which is difficult to detect visually (Figure 2). Five day shading periods between Day 10 and Day 20 did have the greatest effect, but overall, five days of shading did not result in major changes in color.

In addition to altering pericarp color, shading also influenced 3-DOA, tannin and total phenol concentrations. Shading in trial I and II reduced the concentrations of all these compounds, following trends very similar to the visual scores (Figure 4, Appendix 2). In trial III, shading in five day intervals affected 3-DOA concentrations but had little to no effect on the total phenol and tannin concentrations. In this trial, the 3-DOA concentrations dropped to the lowest levels in the Day 10 and Day 15 treatments which coincides with the lightest colors in visual scores as well. Because the black color has been associated with 3-DOA concentration (Dykes et al., 2009, 2013) reductions were expected. The results also imply that tannin and phenol concentrations are affected by shading. Variation in tannin and phenol concentrations are known to be influenced by both genotype and environment (Maxson et al., 1972; Dykes et al., 2013), but the effect of shading had not previously been reported in sorghum. It has been reported in wine grapes where higher levels of total tannins were detected in sun-exposed fruit compared to shaded fruit (Downey et al., 2004; Cortell and Kennedy, 2006).

### *3.3.3 Correlation Analysis*

Several compositional traits were significantly correlated with grain color measurements (Table 3). As expected, 3-DOA and *L*a*b** values were highly associated, but the strongest association was with *a** which is a measure of relative red to green (2012 r= -0.88; 2013 r = -0.96). Visual score was significantly strongly and negatively associated with 3-DOA (2012 r = -0.82; 2013 r = -0.89) indicating that, among black sorghum genotypes, field selection for darkest pericarp results in

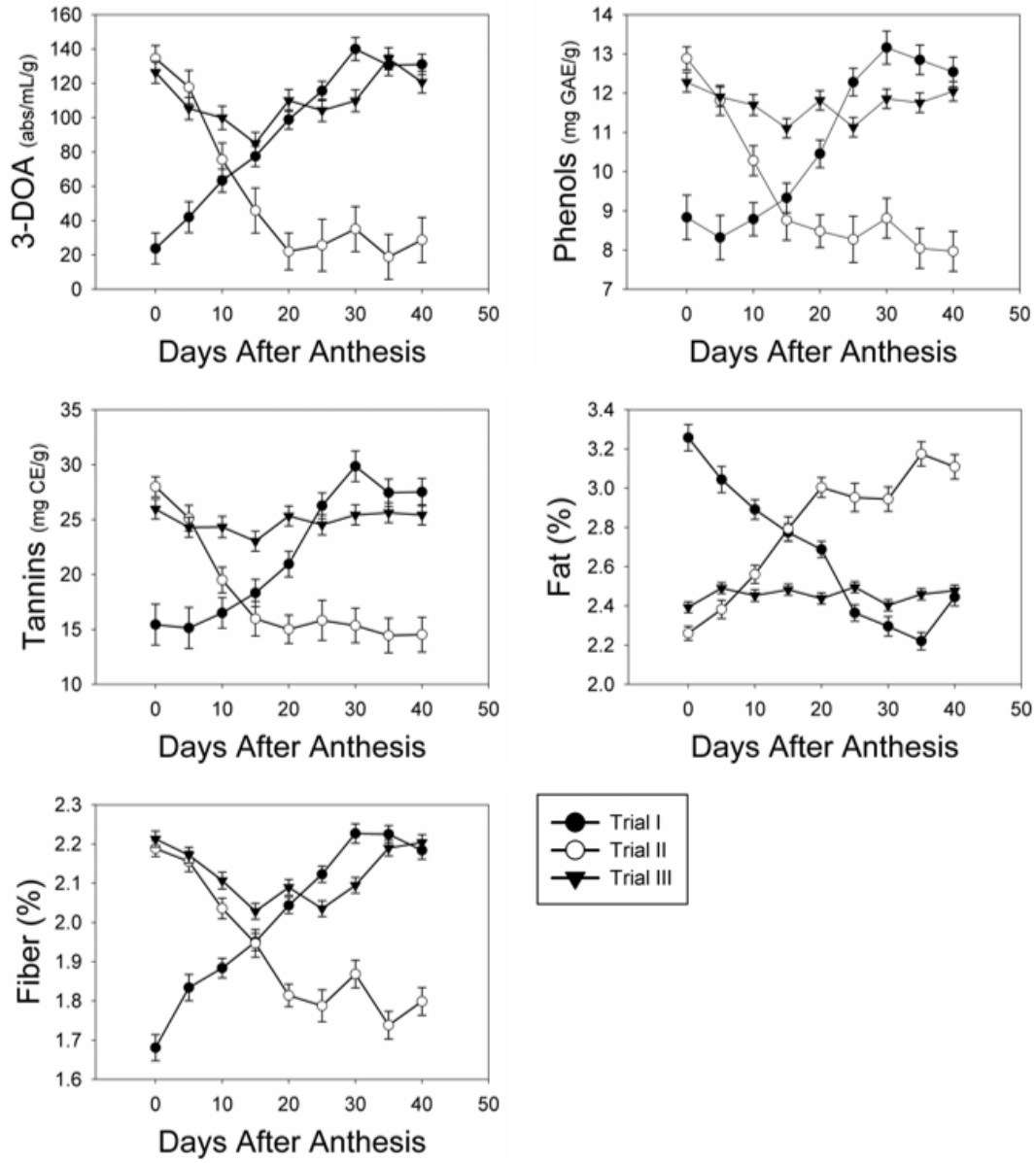


**Figure 4.** Scatter plot results of selected NIR estimated grain composition traits from trials I, II, and III from both Hereford 2012 and College Station 2013 environments.

**Table 3.** Pearson's coefficients of correlation between visual scores, *L**, *a**, *b** and total phenols (mg GAE/g), tannins (mg CE/g), 3-DOA (abs/mL/g), fat (%) and fiber (%) in Tx3362 grain from trials I, II, and III grown in Halfway 2012 (top diagonal) and College Station 2013 (bottom diagonal).

| | Mean visual score | Mean *L** | Mean *a** | Mean *b** | Phenols | Tannins | 3-DOA | Fat | Fiber |
|---|---|---|---|---|---|---|---|---|---|
| **Mean visual score** | | 0.69** | 0.78** | 0.68** | -0.75 | -0.79** | -0.82** | 0.66** | -0.78** |
| **Mean *L**** | 0.90** | | 0.87** | 0.97** | -0.73 | -0.75** | -0.79** | 0.83** | -0.79** |
| **Mean *a**** | 0.84** | 0.86** | | 0.90** | -0.68** | -0.73** | -0.88** | 0.67** | -0.72** |
| **Mean *b**** | 0.93** | 0.98** | 0.92** | | -0.74** | -0.76** | -0.81** | 0.82** | -0.75** |
| **Phenols** | -0.79** | -0.81** | -0.92** | -0.86** | | 0.97** | 0.75** | -0.79** | 0.75** |
| **Tannins** | -0.76** | -0.76** | -0.87** | -0.81** | 0.95** | | 0.79** | -0.82** | 0.76** |
| **3-DOA** | -0.89** | -0.88** | -0.96** | -0.93** | 0.93** | 0.89** | | -0.69** | 0.80** |
| **Fat** | 0.92** | 0.89** | 0.91** | 0.94** | -0.90** | -0.85** | -0.92** | | -0.76** |
| **Fiber** | -0.86** | -0.90** | -0.94** | -0.94 | 0.91** | 0.85** | 0.97** | -0.90** | |

*P <0.05

**P <0.01

the highest 3-DOA content. Protein, ash and starch content had weak relationships with color data. Total phenols, tannins and fiber content were negatively correlated with all color scores in both environments while fat content was positively correlated with them.

### 3.4 Discussion

The significant treatment effects in trial I and II combined with the relatively minimal effects observed in trial III indicated that the increase in black pericarp color, and 3-DOA, phenol and tannin contents results from a sustained period of exposure to sunlight in the early phases (Day 0 – Day 20) of caryopses development. While exact duration and intensity of light required to fully induce the black pericarp phenotype cannot be determined from this study, results from trial III indicate that five days of shading is not sufficient to completely change the color of the grain.

The negative relationship of fiber content with phenols, tannins and 3-DOA is likely based on the fact that fiber and all of these compounds are located within the pericarp (Waniska and Rooney, 2000). The reduction of tannins and phenols in response to shading has not been reported in sorghum and merits further evaluation to determine if this is a consistent phenomenon.

The relationship between fat content and 3-DOA, phenols, and tannins is intriguing as there is no obvious relationship between these traits. Most of the fat or lipid content in grain sorghum is in the embryo which has essentially none of these flavonoids. Fatty acids and luteolinidin (one of the 3-DOA compounds) do

share a common precursor, malonyl-CoA, in their respective biosynthetic pathways (Stafford, 1994; Thelen and Ohlrogge, 2002). This relationship may provide a link between production of both fat content and 3-DOA concentration, but an additional study is needed to further elucidate that relationship.

Based on these limited number of environments and treatments, it appears that the black color intensity, and to some extent both tannin and phenol concentrations in grain sorghum are strongly influenced by duration of sunlight exposure. This reaction is probably the result of photo-induced gene promoter(s) which either regulate or are involved in the biosynthetic pathways of these compounds. Given the relatively large effect on several different traits, it implies that this is a regulatory factor much like what is observed in sorghum for other complex pathways, such as maturity (Murphy et al., 2011). Further research into the genetic inheritance and the loci underlying this trait will be needed to characterize this reaction.

In the meantime, this research confirms that maximum production of these compounds is environment specific and that high sunlight intensity appears to be conducive to the production of darker grain and higher 3-DOA, tannin and phenol content. As more specialty grain sorghums are grown, producers should be aware of the role of sunlight in the production of these compounds and elucidation of the biosynthetic pathways may result in useful induction systems for not only these compounds but others as well.

# 4. INHERITANCE OF PERICARP COLOR, NUTRITIONAL QUALITY, AND GRAIN COMPOSITION TRAITS IN BLACK SORGHUM

Black pericarp grain sorghum [*Sorghum bicolor* (L.) Moench] has high levels of phenolic compounds, especially 3-deoxyanthocyanidins (3-DOAs), which have application in food science and human nutrition as a high-antioxidant food additive, natural food colorant, and natural food preservative. The inheritance of this trait is complex and has not been studied, thus creating a barrier for further genetic improvement. In order to determine the genetic mechanisms governing this novel trait, a generation means analysis was performed using Tx378 (red), Tx3362 (black), and $F_1$, $F_2$, and backcross generations derived from these parents. These six generations were evaluated in 2013 in three diverse Texas growing environments. Significant additive, dominance, and epistatic effects were detected for grain color and associated grain composition traits. Segregating distributions confirmed black sorghum is recessive to red. Estimates of broad-sense heritability ranged from 55% to 100%. 3-deoxyanthocyanidn (3-DOA) content was moderate to highly heritable (0.77). Between two and ten genes are estimated to control the black pericarp trait. Despite the complicated mode of inheritance, enough variation exists for future improvement of black sorghum. Creation of high yielding hybrids with uniformly dark grain and high levels of phenolic compounds will be possible through standard plant breeding practices.

## 4.1 Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] with a uniformly black pericarp is a novel trait within elite breeding germplasm (Rooney et al., 2013b; Hayes and Rooney, 2014). Sources of very black sorghum are rare: the source of black sorghum used in the Texas A&M AgriLife Research Sorghum Breeding Program was an accession from Sudan (Rooney et al., 2013b). From this source, both pollinator (Rooney et al., 2013b) and seed parents (Rooney et al., 2013a) have since been developed and are used to generate a temperately adapted black grain sorghum hybrid that is grown commercially for the specialty food market. Because black sorghums combine high-antioxidant and phenolic compounds in an easily processed and stable source (bran) (Kushi et al., 1999; Anderson, 2003), the demand for black sorghum is expected to increase as a nutraceutical additive (Awika et al., 2005b; Dykes and Rooney, 2006; Dykes et al., 2013), natural food colorant (Awika et al., 2004; Awika, 2005), and natural preservative (Awika and Rooney, 2004).

One compound particularly rich in black sorghum is the 3-deoxyanthocyanidins (3-DOAs) (Gous, 1989; Awika et al., 2004; Dykes et al., 2009, 2013; Hayes and Rooney, 2014). Anthocyanins are pigments common to nearly all plant species, but the 3-DOAs are a unique subset (Clifford, 2000) that are the predominant anthocyanin type in sorghum (Awika et al., 2004). The 3-DOAs lack a hydroxyl group at the C-3 position which increases the molecules' stability and desirability to food processors (Sweeny and Iacobucci, 1983; Mazza and Brouillard,

1987). Not all sorghum have 3-DOA content; black sorghum contains substantially higher levels of 3-DOAs compared to red, yellow and white pericarp types (Awika et al., 2004).

The 3-DOAs and other phenolic compounds found in sorghum, such as tannins, are known to demonstrate high levels of antioxidant activity (Awika et al., 2005b; Dykes and Rooney, 2006). Black sorghum inbreds have antioxidant values equal or higher than blueberries on a per weight basis (Awika et al., 2005b; Dykes and Rooney, 2006). Therefore, consuming substantial amounts of these naturally occurring phenolic compounds in sorghum can be beneficial to human health as they have the potential to diminish the effects of the most widespread chronic health conditions including cardiovascular disease (Kushi et al., 1999; Awika and Rooney, 2004), high blood pressure (Tsuda et al., 2003), and even cancer (Chen et al., 1993; Shih et al., 2007).

Because black sorghum inbred lines have recently been released (Rooney et al., 2013b; a), the trait is heritable. However, selection for the black pericarp trait and associated high phenolic compounds is challenging due to the complex inheritance of these traits. While much is known regarding grain color in sorghum, the black color cannot be fully explained by the two gene model for epicarp color in which the *R* and *Y* genes interact epistatically to produce red *(R_Y_*), white (*R_yy or rryy*), or yellow (*rrY_*) colors (Rooney, 2000). Black sorghum is genetically red (*R_Y_*), but additional, unknown genetic factors promote the expression of the black pericarp trait in the presence of sunlight (Dykes et al., 2009).

Additional known factors may affect color as well. For example, the intensifier gene (*I*), when dominant, creates a more intense pericarp color in red and yellow material, but it is not clear how the intensifier effects the appearance of black pericarps or the composition of phenolic compounds (Rooney, 2000). Mesocarp thickness is controlled by the *Z* gene (Rooney, 2000) and a thicker layer (zz) enhances total phenols in red and black sorghums (Dykes et al., 2005). The presence of condensed tannins is controlled by the $B_1$ and $B_2$ genes which operates in an epistatic manner, in which *B1_B2_* has a pigmented testa layer and condensed tannins while the other allele combinations have no tannin production (Rooney, 2000). When a pigmented testa is present, the spreader gene (*S*) allows the tannins in the testa layer to "spread" into the mesocarp and epicarp (Rooney, 2000). Therefore, red sorghum lines with a pigmented testa express higher levels of total phenols than lines without the presence of the testa layer (Dykes et al., 2005). In the same material, the presence of a dominant allele in the spreader gene gives the grain even higher levels of total phenols (Dykes et al., 2005).

Breeding efforts suggest these known genes explain only a small fraction of the inheritance of the black pericarp trait, as $F_2$ populations have an extremely low frequency of plants with pericarp color as dark as the black parental line (Rooney et al., 2013b). However, once a transgressive segregant is observed, it is easily selectable and highly heritable. This suggests that multiple recessive genes control the trait (Rooney et al., 2013b).

Although genetic factors are the primary control for this trait, environment also affects specific concentrations of 3-DOAs, tannins, and total phenols as well as the physical appearance of the grains (Dykes et al., 2009; Hayes and Rooney, 2014). As the developing grain receives increased exposure to sunlight, there is increased production of these compounds as well as a darker grain appearance. Additionally, flavanoids are commonly expressed as a plant defense mechanism in response to plant pathogens and accumulate rapidly in vegetative tissue at the site of infection (Lo et al., 1999).

Although black sorghum lines have been created, hybrid yields are low relative to the best modern hybrids. For example, the best black sorghum hybrid yielded only 78% of the elite commercial check (white pericarp) in a recent study (Hayes and Rooney, 2014). Further improvement of specialty sorghums that contain high levels of phenolic compounds and are also high yielding is contingent upon understanding the genetic factors underlying the expression of this trait. Therefore, the purpose of this research is to determine the inheritance of the black pericarp trait and associated specialty compounds by estimating these traits' relative genetic effects using generation means analysis (GMA). Concurrently, the heritability and number of genes contributing to these traits will also be determined.

## 4.2 Materials and Methods

### *4.2.1 Generation Means Analysis*

A generation means analysis (GMA) (Mather and Jinks, 1971) was conducted to understand the various genetic effects involved with black pericarp inheritance. GMA is a popular tool that has been employed by breeders to understand the inheritance of a wide-array of quantitative traits (Piepho and Möhring, 2010). Two divergent parents, $P_1$ and $P_2$, are crossed and selfed to create $F_1$ and $F_2$ generations. The $F_1$ generation is backcrossed to each parental line to create $BC_1P_1$ and $BC_1P_2$ generations. The six generations ($P_1$, $P_2$, $F_1$, $F_2$, $BC_1P_1$, $BC_1P_2$) are subsequently evaluated in field trials in multiple environments (Piepho and Möhring, 2010). Individuals in $P_1$, $P_2$, and $F_1$ families are genetically identical within their respective populations and thus are referred to as non-segregating generations. Variation observed in non-segregating generations can be attributed to environmental variation within families, rather than genetic variation. The $F_2$, $BC_1P_1$, and $BC_1P_2$ generations contain many different genotypes resulting from segregation and are thus referred to as segregating generations (Kearsey and Pooni, 1996).

### *4.2.2 Plant Material*

The GMA was conducted using familial generations derived from the parental cross of B.Tx378 and Tx3362. B.Tx378 is a red pericarp inbred line released in 1965 by the Texas Agricultural Experiment Station (TAES) (Stephens and Karper, 1965). Tx3362 is an inbred line with a black pericarp that was released in 2013 by the Texas A&M AgriLife Research Sorghum Breeding Program (Rooney

et al., 2013b). It was developed from a cross between Shawaya #2 and RTx430, a common restorer line with good combining abilities (Miller, 1984).

To create the populations, three distinct versions of Tx378 were used as seed parents (B.Tx378, B.Tx378ms3 and A3.Tx378). B.Tx378 was the standard male fertile version, B.Tx378ms3 is a version of the line that segregates for genetic male sterility, and A3.Tx378 is a cytoplasmic male sterile in A3 cytoplasm. These three versions of the hybrid were necessary to have $F_1$ male sterile panicles essential in the production of the $F_1$, $BC_1P_1$, and $BC_1P_2$ generations. All three of these version are isogenic or isocytoplasmic and were developed in the Texas A&M AgriLife Research Sorghum Breeding Program.

In June 2012, Tx378 was crossed to Tx3362 in College Station, TX using B.Tx378 and A3.Tx378 as seed parents and Tx3362 as the pollinator parent (Table 4). In October 2012 in Weslaco, TX, the $F_1$, $F_2$ and backcross generation seed was produced. The $F_1$ hybrid was created again using B.Tx378ms3 as the seed parent and crossing male sterile plants with Tx3362 as the pollinator parent. The $F_2$ generation was produced by self-pollinating $F_1$ hybrids created from the cross B.Tx378/Tx3362. The backcrosses were created using male sterile A3.Tx378/Tx3362 $F_1$ plants as the seed parent and B.Tx378 and Tx3362 as the pollinator parents to produce the $BC_1P_1$ and $BC_1P_2$ generations, respectively (Table 4, Appendix 3). The original parental lines (B.Tx378 and Tx3362) were also replanted in Weslaco for self-pollination to recreate seed for parental generations and cross- pollinated to recreate $F_1$ seed. This allowed for all seed planted in the

**Table 4.** Summary of plant material used in the generation means analysis.

| Generation | Pedigree | Male Fertility | Individuals Evaluated |
|---|---|---|---|
| $P_1$ | B.Tx378 | Fertile | ≥ 25 |
| $P_2$ | Tx3362 | Fertile | ≥ 25 |
| $F_1$ | B.Tx378ms3 × Tx3362 | Fertile | ≥ 25 |
| $BC_1P_1$ | (A3.Tx378 × Tx3362) × Tx378 | Sterile | ≥ 75 |
| $BC_1P_2$ | (A3.Tx378 × Tx3362) × Tx3362 | Sterile | ≥ 75 |
| $F_2$ | B.Tx378 × Tx3362 | Fertile | ≥ 150 |

GMA to be produced from parents grown in the same environment—Weslaco, TX in Fall 2012— which we expected to limit variance in seed quality due to different production environments (Kearsey and Pooni, 1996).

The use of male sterility to facilitate hybridization results in several generations that differ for the presence and absence of male fertility (Table 4). Measured plants were open pollinated in the field so for generations that were male sterile, pollination in the field trial was primarily by pollen from the fertile generations in the trial. Since 3-DOA, phenols and tannins are located in the pericarp and testa which is maternal tissue derived from the ovary wall (Evers and Millar, 2002), they generally are unaffected by cross-pollination. It is possible that traits influenced by the germ and endosperm are subject to xenia effects. This would include grain composition parameters such as oil, protein and starch composition which in turn can impact the proportion of 3-DOA, phenol, tannins in the grain.

### *4.2.3 Experimental and Field Layout*

The six generations from each parental cross were planted in three diverse locations in Texas in 2013—Weslaco, College Station, and Halfway. All locations were irrigated to maximize growth and minimize potential drought stress. The agronomic practices used were standard (eg., fertilization, tillage, pest control) for grain sorghum production in each area. In College Station and Halfway the plots were 5.5 meters long with row spacing of 0.76 meters; in Weslaco, each plot was 5.18 meters in length with rows spaced 1.02 meters apart. Each generation was randomized with a block and experimental units for this study were individual panicles within a generation. To minimize the effect of grain weathering on grain color, panicles were harvested at or just past physiological maturity and allowed to dry in a warehouse.

The number of panicles randomly harvested varied by generation; more panicles were harvested from segregating generations than non-segregating generations in order to increase precision (Table 4). For each non-segregating generation ($P_1$, $P_2$, and $F_1$), at least 25 plants were harvested and phenotyped. For each backcross generation, at least 75 plants were harvested for evaluation, and in the $F_2$ generation, at least 150 panicles were harvested for phenotyping.

### *4.2.4 Harvest and Phenotyping*

Whole panicles from individual plants were visually phenotyped on a one to five color scale by Brian Pfeiffer; one representing red and five representing black. Panicles were then threshed and cleaned using a Wintersteiger LD180

(Wintersteiger Ag; Ried, Austria). The color of the cleaned grain (free of glumes) was quantitatively measured using a CR-410 Colorimeter (Konica Minolta Sensing Americas, Inc.; Ramsey, NJ). Each data output from the colorimeter was actually an average of three successive measurements. Additionally, each sample was measured three times from three different views and averaged to create a single data point. Therefore, samples were measured nine times in total to increase the precision of the data. Measurements are expressed in accordance with the *Comission Internationale de l'Eclaorage* (CIE) (Comission Internationale de l'Eclaorage, 2004) as *L*a*b** color space values. *L** is a lightness value (0 = black, 100 = white); *a** indicates green or red *(-a** = greenness, +*a** = redness); *b** indicates blue or yellow (-*b** = blueness, +*b** = yellowness).

Compositional analysis of whole seeds was performed using a FOSS XDS MasterLab with the XDS Rapid Content modules (FOSS North America; Eden Prairie, MN, USA). The samples were scanned with wavelengths between 400 to 2500 nm, using ISIscan v.3.10.05933 software (Infrasoft International LLC.; State College, PA). NIR predictions for protein (%), moisture (%), fat (%), fiber (%), ash (%), total phenols (mg GAE/g), tannins (mg CE/g), and 3-DOA (abs/mL/g) were based on NIR calibration curves described by Dykes et al. (in press) and calibration curves developed by the Texas A&M AgriLife Research Sorghum Breeding Program (unpublished results). NIR analysis required a greater minimum volume than colorimeter examination. Single panicle samples that did not meet minimum

requirements were not included in the NIR study, leading to a smaller number of observations reported for composition traits.

### *4.2.5 Statistical Analysis*

#### 4.2.5.1 Analysis of Variance and Descriptive Statistics

General trends indicated consistency of results in individual environments and therefore environments were combined. Significant environment and generation × environment interactions were detected, but the magnitude of these effects was small compared to the generation effect. Analysis of variance was examined using JMP Pro 11.0.0 (SAS Institute, 2013) standard least squares report using a mixed model of y = generation + environment + (generation × environment) + error with environment as a random effect. Mean, standard error, and variance of each generation was calculated using the tabulate platform in JMP.

#### 4.2.5.2 Gene Effects

A joint scaling test (Singh and Chaudhary, 1985) using a three-parameter model was applied to all traits but did not sufficiently explain the inheritance pattern of any color or composition traits (Appendix 18). Lack of fit to this model indicates the existence of epistatic effects (Kearsey and Pooni, 1996). Thus, a six-parameter model was used to estimate *m*, a, and *d*, as well as epistatic effects, including additive × additive gene interaction [$aa$], dominance × dominance interaction [$dd$], and additive × dominance interaction [$ad$], written as $\mu_i = m + [a]x_{i1} + [d]x_{i2} + [aa]x_{i1}^2 + [dd]x_{i2}^2 + [ad]x_{i1}x_{i2}$ (Kearsey and Pooni, 1996). Calculations were performed using JNTSCALE software (Ng, 1990).

#### 4.2.5.3 Heritability

Broad sense heritability ($H^2$) is the proportion of phenotypic variation that is due to genetic variation. In this study, $H^2$ was estimated on a single plant basis as $H^2 = \frac{F_2 - \frac{P_1+P_2+F_1}{3}}{F_2}$ (Allard, 1960) where $P_1$, $P_2$, $F_1$, and $F_2$ denote the means of B.Tx378, Tx3362, and the $F_1$ and $F_2$ generations, respectively. Special consideration was required in the measurement of broad-sense heritability for 3-DOA. Due to the limitations in the ability of the calibration curve to estimate high concentrations of 3-DOA, there is more error variation in estimates of high 3-DOA concentrations than in estimates of low 3-DOA concentrations (Dykes et al., in press). Since this abnormally high variance in the 3-DOA parent (Tx3362) could result in incorrect heritability estimates, the broad sense heritability equation was modified for the 3-DOA trait. In this case, the Tx3362 ($P_2$) was removed from the model, written as $H^2 = \frac{F_2 - \frac{P_1+F_1}{2}}{F_2}$. Heritability estimates were also performed through JNTSCALE software.

#### 4.2.5.4 Number of Genes

The minimum number of genes controlling the black pericarp trait was estimated for grain color by $N = \left(\frac{(P_1-P_2)^2}{8}\right)\left(\hat{\sigma}^2_{F_2} - \hat{\sigma}^2_{P_1,P_2,F_1,\text{pooled}}\right)$ (Wright, 1934; Lande, 1981). In this equation, $P_1$ and $P_2$ refer to the means of B.Tx378 and Tx3362, respectively, while $\hat{\sigma}^2_{F_2}$ is the variance of the $F_2$ generation, and $\hat{\sigma}^2_{P_1,P_2,F_1,\text{pooled}}$ is the pooled variance of B.Tx378, Tx3362 and the $F_1$ generation. This formula assumes

that there is no dominance, linkage, or epistasis (Wright, 1934). Estimates were also calculated via JNTSCALE.

## 4.3 Results

### *4.3.1 Analysis of Variance*

In the combined environment analysis, generation and environment were significant sources of variation for all traits measured ($p < 0.01$) (Table 5 and 6). Additionally, all traits with the exception of tannin and oil had significant generation × environment interactions. The significant interaction term indicates generations reacted differentially in each environment. However, generation means demonstrate that these shifts did not affect relative performance of the generation, i.e. Tx378 was always red and Tx3362 was consistently black (Table 7 and 8, Appendix 4-17). Thus, the significant G × E term is due to shifts in the absolute values of each trait rather than a cross-over effect. Furthermore, the environment and generation × environment effects are substantially smaller in comparison to the effect of the generation, implying that genetics influences this trait to a greater extent than environment.

Black Tx3362 (5.00) is much darker than red B.Tx378 (1.00) in visual score rating (Table 7). B.Tx378 is lighter ($L^*$), redder ($a^*$), and yellower ($b^*$) than the black Tx3362 and all other generations (Table 7, Appendix 4). Tx3362 has higher values than B.Tx378 for phenols, tannins, 3-DOA, protein, and fiber, but B.Tx378 actually has a higher concentration for starch (67.25 ± 0.05 %) than Tx3362 (62.51% ± 0.09 %) (Table 8, Appendix 5). Minimal differences were identified

**Table 5.** Mean squares for color traits of the B.Tx378 × Tx3362 GMA family grown in three locations across Texas in 2013.

| Source | df | Visual Score | *L** | *a** | *b** |
|---|---|---|---|---|---|
| Generation | 5 | 222.34** | 4609.79** | 1181.05** | 5340.02** |
| Environment | 2 | 5.67** | 3366.03** | 51.63** | 2537.53** |
| Generation × Environment | 10 | 2.48** | 90.27** | 44.78** | 81.60** |
| Error | 1479 | 0.83 | 10.72 | 2764.21 | 50505.73 |

**Table 6.** Mean squares for NIR estimated composition traits of the B.Tx378 × Tx3362 GMA family grown in three locations across Texas in 2013.

| Source | df | Phenols | Tannins | 3-DOA | Protein | Moisture | Fat | Fiber | Ash | Starch |
|---|---|---|---|---|---|---|---|---|---|---|
| Generation | 5 | 368.36** | 2788.07** | 319626** | 197.23** | 10.29** | 3.11** | 4.00** | 0.24** | 141.99** |
| Environment | 2 | 249.10** | 1327.35** | 55162** | 22.99** | 112.53** | 29.88** | 0.11** | 0.04** | 6.63** |
| Generation×Environment | 10 | 36.79** | 140.45 | 4343** | 6.51** | 1.36** | 0.33** | 0.10** | 0.02** | 5.34** |
| Error | 896 | 13.76 | 100.09 | 463 | 1.67 | 0.48 | 0.28 | 0.01 | 0.002 | 0.79 |

**Table 7.** Means and standard errors for color traits in parents, $F_1$, $F_2$, and backcross generations evaluated in three Texas environments in 2013.

| Generation | N† | Visual score‡ | *L** | *a** | *b** |
|---|---|---|---|---|---|
| B.Tx378 | 100 | 1.00 ± 0.00 | 49.59 ± 0.39 | 15.15 ± 0.11 | 26.42 ± 0.22 |
| $BC_1$B.Tx378 | 300 | 1.31 ± 0.03 | 42.92 ± 0.28 | 15.78 ± 0.08 | 22.84 ± 0.22 |
| $F_1$ | 99 | 2.00 ± 0.00 | 39.16 ± 0.33 | 14.75 ± 0.10 | 19.57 ± 0.31 |
| $F_2$ | 599 | 1.85 ± 0.04 | 41.12 ± 0.20 | 14.28 ± 0.06 | 20.15 ± 0.17 |
| $BC_1$Tx3362 | 300 | 2.56 ± 0.08 | 37.48 ± 0.24 | 13.05 ± 0.11 | 15.76 ± 0.25 |
| Tx3362 | 100 | 5.00 ± 0.00 | 30.39 ± 0.21 | 6.76 ± 0.13 | 6.81 ± 0.28 |

† N, number of samples
‡ 1 = red, 5 = black

**Table 8.** Means and standard errors for NIR estimated composition traits in parents, $F_1$, $F_2$, and backcross generations evaluated in three Texas environments in 2013.

| Generation | N† | Phenols‡ | Tannins§ | 3-DOA¶ | %Protein | %Moisture | %Fat | %Fiber | %Ash | %Starch |
|---|---|---|---|---|---|---|---|---|---|---|
| B.Tx378 | 57 | 5.48 ± 0.09 | 8.85 ± 0.33 | 7.72 ± 1.07 | 9.31 ± 0.05 | 10.33 ± 0.10 | 2.52 ± 0.05 | 1.74 ± 0.01 | 1.28 ± 0.00 | 67.25 ± 0.05 |
| $BC_1$B.Tx378 | 179 | 8.65 ± 0.29 | 17.02 ± 0.81 | 11.40 ± 0.91 | 11.33 ± 0.11 | 10.32 ± 0.07 | 2.89 ± 0.06 | 1.74 ± 0.01 | 1.28 ± 0.00 | 66.18 ± 0.07 |
| $F_1$ | 69 | 14.14 ± 0.21 | 30.18 ± 0.63 | 21.72 ± 1.87 | 12.23 ± 0.10 | 10.50 ± 0.09 | 2.95 ± 0.05 | 1.81 ± 0.01 | 1.27 ± 0.00 | 65.33 ± 0.07 |
| $F_2$ | 377 | 9.04 ± 0.22 | 15.82 ± 0.57 | 22.06 ± 1.34 | 11.96 ± 0.08 | 10.16 ± 0.05 | 2.86 ± 0.04 | 1.83 ± 0.01 | 1.26 ± 0.00 | 65.80 ± 0.05 |
| $BC_1$Tx3362 | 167 | 8.96 ± 0.34 | 14.75 ± 0.88 | 43.44 ± 2.49 | 13.31 ± 0.10 | 10.70 ± 0.06 | 2.91 ± 0.04 | 1.96 ± 0.01 | 1.27 ± 0.00 | 64.80 ± 0.07 |
| Tx3362 | 65 | 8.77 ± 0.31 | 12.03 ± 0.78 | 197.86 ± 5.25 | 15.51 ± 0.09 | 9.48 ± 0.11 | 2.43 ± 0.05 | 2.38 ± 0.02 | 1.11 ± 0.00 | 62.51 ± 0.09 |

† N, number of samples
‡ Total phenols (mg GAE/g)
§ Condensed tannins (mg CE/g)
¶ 3-deoxyanthocyanidins (abs/mL/g)

among the generations for moisture, ash, and oil concentrations even though significant generation effects were detected for these traits. Grain from the $F_1$ generation had the highest concentration of phenols (14.14 ± 0.21 mg GAE/g), tannins (30.18 ± 0.63 mg CE/g), and oil (2.95 ± 0.05 %), indicating that these traits are more heterosis than other measured traits (Table 8).

### *4.3.2 Gene Effects*

The six-parameter model indicates that visual score is under the control of significant additive (-2.00 ± 0.00), dominance (-2.57 ± 0.60), additive × dominance (*a*×*d*) (1.50 ± 0.16), and dominance × dominance (*d*×*d*) (1.91 ± 0.37) effects (Table 9). Smaller visual score means indicate a redder appearance to the grain; thus, negative values for additive and dominance effects indicate that these effects promote expression of the red pericarp color, where as positive values for *a*×*d* and *d*×*d* epistatic interactions promoted expression of the black phenotype.

On the contrary, lower values for CIE *L*a*b** variables indicate darker (to black) pericarp colors. Therefore, effects with negative values promote black pericarp expression. The lightness (*L**) trait demonstrates a significantly positive additive effect (9.60 ± 0.22) and significantly negative dominance (-5.71 ± 2.79), additive × additive (*a*×*a*) (-3.69 ± 1.07), and *a*×*d* (-8.30 ± 0.86) effect. The redness (*a**) variable establishes a significant positive additive (4.19 ± 0.09) and dominance (11.15 ± 0.97) effect and a negative *a*×*d* (-2.93 ± 0.32) and *d*×*d* (-6.80 ± 0.64) effect. The *a** variable has a much greater dominance effect compared to other grain color appearance traits (visual score, *L** or *b**). The *b** variable reveals a positive additive

**Table 9.** Six parameter model estimates of midparent [*m*], additive [*a*], dominance [*d*], and additive × additive [*a*×*a*], additive × dominant [*a*×*d*], and dominant × dominant [*d*×*d]* epistatic effects (and their standard errors) from a joint scaling test for grain color and composition traits in parents (B.Tx378 and Tx3362), their $F_1$, $F_2$, and backcrossed generations grown in three Texas environments in 2013.

| Trait | *m* | *a* | *d* | *a* × *a* | *a* × *d* | *d* × *d* |
|---|---|---|---|---|---|---|
| Visual Score | 2.66 ± 0.24** | -2.00 ± 0.00** | -2.57 ± 0.60** | 0.34 ± 0.24 | 1.50 ± 0.16** | 1.91 ± 0.37** |
| *L** | 43.68 ± 1.09** | 9.60 ± 0.22** | -5.71 ± 2.79** | -3.69 ± 1.07** | -8.30 ± 0.86** | 1.19 ± 1.84 |
| *a** | 10.40 ± 0.37** | 4.19 ± 0.09** | 11.15 ± 0.97** | 0.55 ± 0.36 | -2.93 ± 0.32** | -6.80 ± 0.64** |
| *b** | 20.03 ± 0.96** | 9.80 ± 0.18** | 0.95 ± 2.47 | -3.42 ± 0.94** | -5.46 ± 0.76** | -1.41 ± 1.65 |
| Phenols† | 8.09 ± 1.26** | -1.64 ± 0.16** | -2.23 ± 3.25 | -0.96 ± 1.25 | 2.68 ± 0.96** | 8.28 ± 2.07** |
| Tannins‡ | 10.19 ± 3.35** | -1.59 ± 0.42** | 2.52 ± 8.66 | 0.25 ± 3.32 | 7.72 ± 2.55** | 17.47 ± 5.54** |
| 3-DOA§ | 81.36 ± 7.99** | -95.07 ± 2.68** | -177.56 ± 20.87** | 21.43 ± 7.53** | 126.05 ± 7.54** | 117.92 ± 13.56** |
| Protein | 10.98 ± 0.43** | -3.10 ± 0.05** | 2.69 ± 1.11** | 1.43 ± 0.43** | 2.24 ± 0.32** | -1.44 ± 0.71** |
| Moisture | 8.50 ± 0.29** | 0.42 ± 0.07** | 4.63 ± 0.73** | 1.41 ± 0.28** | -1.60 ± 0.24** | -2.64 ± 0.49** |
| Fat | 2.32 ± 0.20** | 0.04 ± 0.04 | 1.55 ± 0.52** | 0.15 ± 0.20 | -0.13 ± 0.16 | -0.93 ± 0.33** |
| Fiber | 1.98 ± 0.03** | -0.32 ± 0.01** | -0.44 ± 0.09** | 0.08 ± 0.03** | 0.20 ± 0.03** | 0.28 ± 0.06** |
| Ash | 1.12 ± 0.02** | 0.08 ± 0.00** | 0.40 ± 0.04** | 0.07 ± 0.02** | -0.15 ± 0.01** | -0.25 ± 0.03** |
| Starch | 66.11 ± 0.30** | 2.37 ± 0.05** | -0.48 ± 0.77 | -1.23 ± 0.30** | -1.97 ± 0.23** | -0.30 ± 0.50 |

† Total phenols (mg GAE/g)
‡ Condensed tannins (mg CE/g)
§ 3-deoxyanthocyanidins (abs/mL/g)

(9.80 ± 0.18) effect and negative *a*×*a* (-3.42 ± 0.94) and *a*×*d* (-5.46 ± 0.76) effects.

For composition traits, which included total phenols, tannins, 3-DOA, protein, moisture, fat, fiber, ash, and starch, higher means indicated greater levels (or concentrations) of these compounds. Therefore, positive gene effects indicated increases in these compounds (Table 9). Total phenols and tannins had very similar effects. For both traits, significantly positive additive, *a*×*d*, and *d*×*d* effects are observed. Both dominance and *a*×*a* effects were non-significant. However, the *a*×*d* (7.72 ± 2.55) and *d*×*d* (17.47 ± 5.54) effects calculated for tannins were much greater in magnitude than *a*×*d* (2.68 ± 0.96) and *d*×*d* (8.28 ± 2.07) effects calculated for phenols. 3-DOA, protein, and moisture content were the only traits where all six-parameters were significant. 3-DOA had a highly negative additive (-95.07 ± 2.68) and dominance component (-177.56 ± 20.87), but positive *a*×*a* (21.43 ± 7.53), *a*×*d* (126.05 ± 7.54), and *d*×*d* (117.92 ± 13.56) effects. Protein showed negative additive (-3.10 ± 0.05) and *d*×*d* (-1.44 ± 0.71) effects, but positive dominance (2.69 ± 1.11), *a*×*a* (1.43 ± 0.43) and *a*×*d* (2.24 ± 0.32) effects. Finally, starch revealed a positive additive effect (2.37 ± 0.05) as well as negative *a*×*a* (-1.23 ± 0.30) and *a*×*d* (-1.97 ± 0.23) effects.

All traits observed, besides oil, had significant additive gene action. Dominance gene action also influenced about two-thirds of observed traits. Epistatic interactions had more variable significance, but most traits were affected by significant additive × dominance and dominance × dominance interactions.

**Table 10**. Broad sense heritability ($H^2$) estimates on a single plant basis for color and composition traits. The estimates were made using B.Tx378 × Tx3362 GMA generations evaluated in Weslaco (WE), College Station (CS), and Halfway (HW), Texas in 2013.

| | Environment | | | |
|---|---|---|---|---|
| **Trait** | **WE** | **CS** | **HW** | **Combined** |
| Visual score | 1.00 | 1.00 | 1.00 | 1.00 |
| *L** | 0.82 | 0.78 | 0.80 | 0.55 |
| *a** | 0.60 | 0.70 | 0.66 | 0.42 |
| *b** | 0.76 | 0.85 | 0.61 | 0.54 |
| Phenols | 0.92 | 0.83 | 0.95 | 0.82 |
| Tannins | 0.88 | 0.73 | 0.90 | 0.80 |
| 3-DOA | 0.83 | 0.85 | 0.97 | 0.77 |
| Protein | 0.90 | 0.81 | 0.85 | 0.80 |
| Moisture | 0.54 | 0.91 | 0.85 | 0.37 |
| Fat | 0.91 | 0.89 | 0.90 | 0.68 |
| Fiber | 0.42 | 0.68 | 0.29 | 0.34 |
| Ash | 0.60 | 0.73 | 0.88 | 0.65 |
| Starch | 0.86 | 0.62 | 0.78 | 0.68 |

### *4.3.3 Heritability*

In general, grain color and specialty compounds are moderate to highly heritable (Table 10). All traits had higher heritability estimates when calculated in individual environments as opposed to combined environments, presumably due to genotype × environment interactions and estimation on a per plant basis because

individuals in segregating generations cannot be replicated in multiple environments. Visual score rating has a broad sense heritability of 1.0. Since this trait was measured on a subjective rating scale, it is subject to human bias and it interprets grain color from a substantially different perspective than a quantitative colorimeter measurement that has the ability to detect small differences between samples. However, it clearly confirms why selection was successful once segregants were identified. *L*a*b** CIE color space variables also have high heritability estimates, indicating selection should be effective. The specialty compounds of interest in this study, like their associated grain color traits, also have moderately high heritability. In combined environment analysis, total phenol concentration appears to be the most heritable (0.82), followed by tannins (0.80), and then 3-DOA (0.77). Grain moisture and fiber concentration are more influenced by the environment resulting in low to moderate heritability.

### *4.3.4 Number of Genes*

Combined environment analysis estimated that between approximately two genetic loci were affecting the visual score rating while up to 10 genes influenced the redness (*a**) of the grain (Appendix 19). Values for *L** and *b** were intermediate with approximately four loci affecting *L** and five loci affecting *b**.

## 4.4 Discussion

Results from this study indicate that multiple genes with epistatic interactions control the black pericarp trait and associated prominent levels of beneficial health compounds. The results confirm that black grain color is a

recessive trait. This is confirmed by the color of the $F_1$ hybrid grain as well as the very limited number of individuals in the $F_2$ and $BC_1P_1$ or $BC_2P_2$ generations with a black pericarp. Given the number of panicles sampled, the presence of several panicles that were equally as dark as Tx3362 implies the black pericarp trait is not likely to be highly quantitative with somewhere between two to ten genes affecting this trait. Similarly, distribution of 3-DOA concentration in all advanced breeding generations ($F_1$, $F_2$, and backcrossed generations), is greatly skewed to the B.Tx378 parent as well. Therefore, control of the black pericarp color and 3-DOA compounds are likely under the same genetic control because these two traits were directly correlated with each other as was confirmed in previous studies.

In this population, much of the genetic effects for tannins could be explained using the $B_1$ and $B_2$ loci. Neither B.Tx378 nor Tx3362 had a pigmented testa layer and thus had low or residual levels of condensed tannins (values less than 10 mg CE/g are considered to have minimal to no tannins). However, the $F_1$ generation contained high levels of tannins ($\bar{x}$ = 30.18 ± 0.63 mg CE/g) compared to the parents (B.Tx378 $\bar{x}$ = 8.85 ± 0.33 mg CE/g; Tx3362 $\bar{x}$ = 12.03 ± 0.78 mg CE/g). This observation is due to epistatic complementation at the $B_1$ and $B_2$ loci. From previous reports, (unpublished results) Tx378 is genetically $b_1\_B_2\_$ and does not contain a pigmented testa layer or condensed tannins. The elevated levels of these compounds in the $F_1$ generation meant that the $F_1$ individuals were genetically $B_1\_B_2\_$ and contained both tannins and a pigmented testa. Thus, Tx3362, which also had low to no tannins and did not have an obvious testa layer, is genetically $B_1\_b_2\_$.

This interaction explains why the epistatic terms for this trait were high. Since tannins and phenol concentrations are related, they also reflected similar patterns.

Moisture, fat and ash were measured in this study, but generation means and effects, although often significant, appeared to account for only trivial fluctuations. Hence there are only minor genetic factors segregating within material generated from this biparental cross. Divergence between the two parents was observed for protein, fiber, and starch; however, the estimated genetic effects were of small magnitude for fiber. For these traits, the xenia effect present in the male sterile generations (both backcrosses) could affect the results obtained.

The number of genes reported for the black pericarp trait varied substantially between different methods of color measurement. A number of genetic factors, especially linkage, could greatly affect our ability to mathematically estimate the number of genetic components affecting this trait (Wright, 1934; Rodriguez-Herrera et al., 2000; Wilson et al., 2013). Our high throughput estimation of composition traits by NIR spectrometry has the limitation of higher measurement error in blacker samples and therefore, Wright's (1934) equation may not be representative of the true values. Because of this, the minimum number of genes was not reported for composition traits.

In conclusion, additive, dominance, and epistatic effects control the black pericarp trait and related phenolic compounds. Specifically, the black pericarp is a recessive trait under the control of multiple genes and is moderate to highly heritable. The creation of high yielding hybrids with elite agronomic traits and high

levels of phenolic compounds should be possible through recurrent selection, backcross breeding, or conventional pedigree breeding methods. Furthermore, once the visual black color is fixed in a breeding line, it may be possible to further enhance color via selection for *L*a*b** values that are not detectable visually. Given the strong relationship between 3-DOA concentration and black color this could be an effective means of further increasing 3-DOA concentrations in these specialty sorghums.

# 5. CONCLUSIONS

From this research, many of the factors influencing the inheritance of the black pericarp trait have been elucidated. The generation means analysis experiment determined that the trait is recessive, controlled by multiple genes, but is highly heritable. A combination of additive, dominance, and epistatic effects controlled most color and composition traits measured. All color traits and phenolic compounds had moderate to high heritability and approximately two to ten genes were estimated to be associated with the black pericarp trait.

Additionally, the light shading experiment confirmed the black pericarp appearance, 3-DOA levels, and to some extent, tannins and total phenols were affected by sunlight. The effects of sunlight shading was additive—the longer the panicles were shaded, the redder their appearance and the lower the concentration of 3-DOA, tannins, and total phenols. Therefore, the maximum expression of the black pericarp is environment dependent. Despite the complexities of this trait, the information generated from this research has increased the body of scientific knowledge which will allow for further improvement of black pericarp sorghum.

High throughput estimation of phenolic compounds and other grain composition traits was possible through NIR spectroscopy. Without the availability of this technology, there would be a need to perform wet chemistry analysis for all the compounds of interest in both light shading and generation means analysis studies, greatly increasing the time and resources required to perform this

research. Additionally, the Konica Minolta colorimeter allowed for a quantitative measurement of color. Although visual score rating was highly effective within environments, this manual method did not detect large differences in color between environments, an observation that was clearly obvious from colorimeter analysis.

One major limitation of the generation means analysis experiment is the existence of sterile backcross generations. In the experimental development phase, the male sterile lines were not considered an issue as the presence of phenolic compounds are generally located in maternal tissue of the developing grain. However, the pollination of backcross generations by neighboring plants potentially resulted in uncontrolled phenotypes due to xenia effects. This confounds our ability to make inferences on non-phenolic grain composition traits.

Research from these projects will lay the foundation for future work on the subject. Research should expand upon the statistically inferred number of genes associated with the black pericarp trait (Section 4.2.5.4) by identifying the physical location of chromosomal regions, and eventually individual genes, that are involved with the trait. Additionally, further generation means analysis work should be conducted in different material, including white × black and yellow × black populations. As for the physiology of the trait, future research should attempt to characterize the biosynthetic pathway of the 3-DOA molecule.

The information gathered here regarding the genetic and environmental influences on the inheritance of the black pericarp trait explains why more sources

of the black sorghum have not been identified and used in breeding programs until recently. Although the inheritance is complicated, further improvement is possible through normal breeding practices. If collaboration is sustained between food scientists, human nutritionists, and plant breeders, and these compounds can be demonstrated to have health benefits, black sorghum will be a popular commodity among food processors and farmers in the future providing health benefits to consumers everywhere.

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# APPENDIX

## A1. Tables and Figures

**Appendix 1.** Means and ranges of each treatment for pericarp color traits.

| | | | | Visual score | | *L** | | *a** | | *b** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Environment** | **Trial** | **DAA†** | **N‡** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** | **Mean** | **Range** |
| 2012 | I | 0 | 14 | 3.79 | 4.00 | 39.52 | 9.33 | 12.97 | 4.33 | 19.55 | 7.92 |
| 2012 | I | 5 | 20 | 3.40 | 4.00 | 37.59 | 7.81 | 12.67 | 6.69 | 17.80 | 8.88 |
| 2012 | I | 10 | 19 | 3.05 | 4.00 | 38.08 | 7.28 | 12.96 | 3.38 | 18.32 | 6.39 |
| 2012 | I | 15 | 20 | 2.35 | 4.00 | 37.74 | 8.06 | 12.80 | 5.16 | 17.66 | 9.49 |
| 2012 | I | 20 | 19 | 2.16 | 4.00 | 36.55 | 10.13 | 12.52 | 7.15 | 16.13 | 10.52 |
| 2012 | I | 25 | 20 | 1.55 | 3.00 | 36.50 | 11.45 | 12.55 | 5.86 | 16.07 | 12.45 |
| 2012 | I | 30 | 20 | 1.20 | 2.00 | 33.59 | 7.21 | 10.49 | 5.13 | 12.88 | 7.40 |
| 2012 | I | 35 | 18 | 1.00 | 0.00 | 33.57 | 8.32 | 10.78 | 6.27 | 12.86 | 9.16 |
| 2012 | I | 40 | 20 | 1.20 | 1.00 | 35.39 | 5.02 | 11.32 | 4.50 | 14.52 | 7.39 |
| 2012 | II | 0 | 20 | 1.10 | 1.00 | 33.59 | 5.84 | 10.23 | 5.01 | 12.57 | 6.55 |
| 2012 | II | 5 | 19 | 1.37 | 1.00 | 33.84 | 6.72 | 10.65 | 5.71 | 12.86 | 6.74 |
| 2012 | II | 10 | 17 | 2.94 | 4.00 | 35.96 | 6.89 | 12.77 | 4.59 | 15.42 | 5.36 |
| 2012 | II | 15 | 15 | 3.07 | 3.00 | 36.26 | 4.84 | 12.57 | 4.11 | 15.69 | 2.88 |
| 2012 | II | 20 | 13 | 4.23 | 2.00 | 37.66 | 3.37 | 14.09 | 1.66 | 17.36 | 4.57 |
| 2012 | II | 25 | 14 | 3.93 | 2.00 | 37.84 | 5.57 | 13.87 | 2.46 | 17.43 | 4.66 |
| 2012 | II | 30 | 11 | 4.09 | 3.00 | 38.11 | 5.22 | 13.94 | 3.80 | 17.43 | 5.21 |
| 2012 | II | 35 | 10 | 4.40 | 3.00 | 39.33 | 7.11 | 14.57 | 3.28 | 19.37 | 7.49 |
| 2012 | II | 40 | 14 | 4.64 | 1.00 | 37.68 | 4.50 | 13.62 | 5.26 | 17.28 | 6.21 |
| 2012 | III | 0 | 20 | 1.00 | 0.00 | 35.54 | 7.32 | 11.70 | 6.38 | 15.31 | 8.89 |
| 2012 | III | 5 | 18 | 1.33 | 2.00 | 35.78 | 10.04 | 11.74 | 5.98 | 15.32 | 11.48 |
| 2012 | III | 10 | 19 | 1.68 | 2.00 | 35.24 | 7.34 | 11.37 | 5.65 | 14.14 | 7.99 |
| 2012 | III | 15 | 20 | 1.80 | 2.00 | 36.17 | 8.06 | 12.24 | 6.58 | 15.68 | 10.03 |
| 2012 | III | 20 | 20 | 1.80 | 2.00 | 35.25 | 6.86 | 11.42 | 6.10 | 13.97 | 9.40 |
| 2012 | III | 25 | 20 | 1.75 | 2.00 | 35.50 | 9.37 | 11.94 | 5.68 | 14.60 | 9.17 |
| 2012 | III | 30 | 20 | 1.40 | 2.00 | 35.69 | 10.17 | 12.06 | 6.81 | 15.47 | 12.43 |
| 2012 | III | 35 | 19 | 1.11 | 1.00 | 35.40 | 4.42 | 11.26 | 4.29 | 14.71 | 5.82 |
| 2012 | III | 40 | 19 | 1.63 | 2.00 | 36.01 | 9.05 | 12.37 | 5.64 | 15.22 | 8.48 |
| 2013 | I | 0 | 20 | 1.25 | 1.00 | 30.87 | 8.60 | 6.09 | 6.32 | 5.07 | 8.67 |
| 2013 | I | 5 | 11 | 5.00 | 0.00 | 40.53 | 9.88 | 11.63 | 5.14 | 16.33 | 10.31 |
| 2013 | I | 10 | 17 | 1.00 | 0.00 | 30.90 | 5.34 | 5.79 | 4.65 | 4.56 | 4.66 |
| 2013 | I | 15 | 20 | 1.00 | 0.00 | 30.87 | 7.62 | 6.41 | 6.38 | 5.02 | 6.40 |
| 2013 | I | 20 | 13 | 4.62 | 1.00 | 38.11 | 9.50 | 9.93 | 11.93 | 12.73 | 17.71 |
| 2013 | I | 25 | 19 | 1.42 | 2.00 | 30.68 | 7.84 | 7.33 | 8.85 | 5.51 | 9.23 |
| 2013 | I | 30 | 17 | 1.88 | 3.00 | 30.89 | 6.79 | 7.67 | 8.45 | 5.84 | 7.42 |
| 2013 | I | 35 | 14 | 2.36 | 2.00 | 35.98 | 9.69 | 10.64 | 5.11 | 11.49 | 7.31 |
| 2013 | I | 40 | 19 | 2.00 | 2.00 | 30.87 | 6.56 | 7.73 | 4.18 | 5.79 | 4.93 |
| 2013 | II | 0 | 12 | 2.25 | 4.00 | 33.88 | 9.16 | 10.84 | 6.32 | 9.44 | 9.16 |
| 2013 | II | 5 | 18 | 1.00 | 0.00 | 32.44 | 5.10 | 9.53 | 4.11 | 7.66 | 3.26 |
| 2013 | II | 10 | 19 | 1.63 | 2.00 | 32.12 | 5.16 | 8.03 | 7.44 | 6.63 | 6.67 |
| 2013 | II | 15 | 14 | 5.00 | 0.00 | 37.37 | 6.57 | 12.35 | 3.70 | 13.59 | 6.51 |
| 2013 | II | 20 | 19 | 1.00 | 0.00 | 31.10 | 8.01 | 8.85 | 7.62 | 5.99 | 7.73 |
| 2013 | II | 25 | 20 | 1.10 | 1.00 | 31.30 | 6.01 | 7.11 | 5.30 | 5.51 | 4.66 |
| 2013 | II | 30 | 12 | 4.92 | 1.00 | 37.69 | 9.50 | 12.33 | 7.22 | 13.72 | 9.57 |
| 2013 | II | 35 | 20 | 1.00 | 0.00 | 31.61 | 7.55 | 6.51 | 5.87 | 5.59 | 6.16 |
| 2013 | II | 40 | 19 | 1.42 | 2.00 | 30.86 | 4.65 | 7.80 | 8.07 | 5.92 | 6.93 |
| 2013 | III | 0 | 10 | 4.30 | 2.00 | 35.18 | 3.74 | 11.44 | 4.19 | 11.44 | 5.31 |
| 2013 | III | 5 | 18 | 1.00 | 0.00 | 31.42 | 7.07 | 5.63 | 4.85 | 5.04 | 5.82 |
| 2013 | III | 10 | 19 | 1.16 | 1.00 | 30.17 | 8.63 | 7.15 | 6.83 | 5.25 | 6.74 |
| 2013 | III | 15 | 12 | 5.00 | 0.00 | 40.81 | 12.03 | 13.02 | 6.05 | 16.26 | 9.90 |
| 2013 | III | 20 | 19 | 1.00 | 0.00 | 31.01 | 5.81 | 5.87 | 3.30 | 4.87 | 4.76 |
| 2013 | III | 25 | 19 | 1.05 | 1.00 | 30.04 | 5.13 | 6.26 | 3.48 | 4.60 | 4.71 |
| 2013 | III | 30 | 13 | 5.00 | 0.00 | 41.19 | 9.38 | 11.69 | 2.96 | 16.69 | 6.34 |
| 2013 | III | 35 | 20 | 1.00 | 0.00 | 29.06 | 7.03 | 5.41 | 4.22 | 3.84 | 3.82 |
| 2013 | III | 40 | 20 | 1.05 | 1.00 | 29.45 | 5.82 | 5.82 | 5.00 | 4.22 | 4.84 |

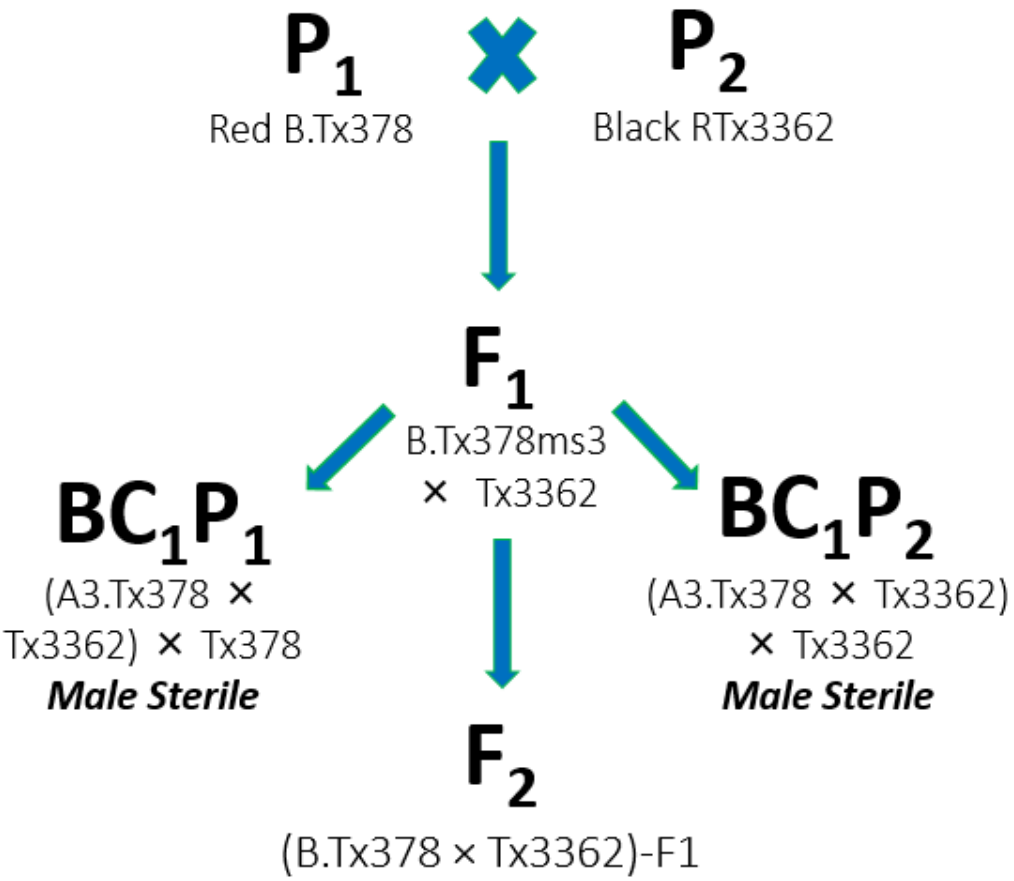
† DAA, days after anthesis

‡ N, number of samples

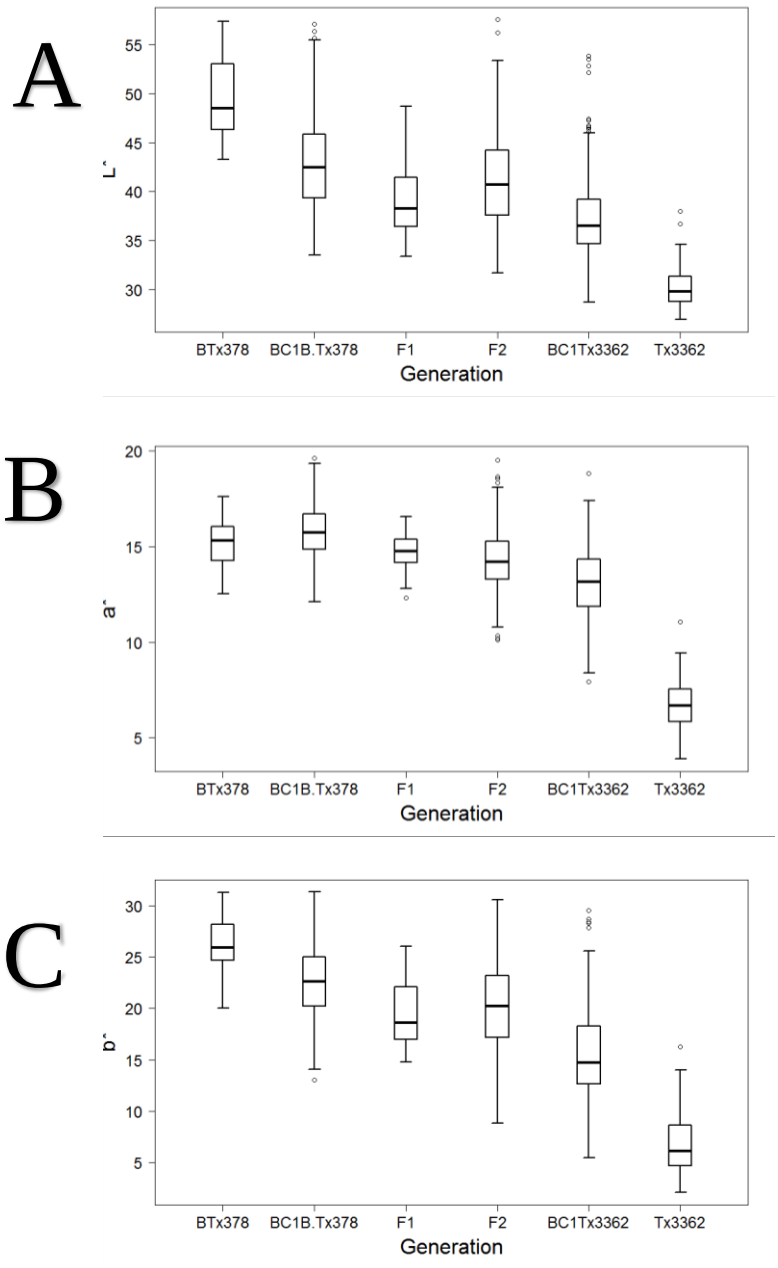
**Appendix 2.** Means and ranges of each treatment for grain composition traits.

| | | | | Phenols§ | | Tannins¶ | | 3-DOA# | | Fat†† | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Env. | Trial | DAA† | N‡ | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| 2012 | I | 0 | 1 | 13.78 | 0.00 | 30.87 | 0.00 | 13.31 | 0.00 | 3.29 | 0.00 |
| 2012 | I | 5 | 2 | 12.43 | 1.34 | 30.24 | 3.53 | 35.94 | 1.87 | 3.04 | 0.03 |
| 2012 | I | 10 | 3 | 12.33 | 2.30 | 28.72 | 4.60 | 46.67 | 16.10 | 3.08 | 0.11 |
| 2012 | I | 15 | 3 | 13.14 | 1.47 | 31.44 | 4.72 | 46.35 | 27.74 | 3.11 | 0.23 |
| 2012 | I | 20 | 4 | 14.23 | 3.68 | 33.74 | 12.32 | 64.96 | 50.55 | 3.01 | 0.49 |
| 2012 | I | 25 | 4 | 15.61 | 1.71 | 38.83 | 5.65 | 76.38 | 32.70 | 2.64 | 0.18 |
| 2012 | I | 30 | 2 | 16.32 | 1.55 | 41.59 | 3.49 | 97.96 | 11.95 | 2.62 | 0.11 |
| 2012 | I | 35 | 3 | 16.64 | 2.43 | 41.96 | 9.91 | 92.48 | 34.26 | 2.47 | 0.25 |
| 2012 | I | 40 | 3 | 16.29 | 2.24 | 41.32 | 4.76 | 86.37 | 64.36 | 2.78 | 0.13 |
| 2012 | II | 0 | 4 | 17.16 | 1.95 | 44.03 | 4.70 | 107.97 | 93.42 | 2.48 | 0.17 |
| 2012 | II | 5 | 2 | 15.66 | 1.76 | 39.40 | 5.43 | 92.42 | 4.51 | 2.51 | 0.36 |
| 2012 | II | 10 | 2 | 14.17 | 2.03 | 33.41 | 5.22 | 43.57 | 10.47 | 2.72 | 0.15 |
| 2012 | II | 15 | 1 | 13.56 | 0.00 | 31.94 | 0.00 | 32.79 | 0.00 | 2.88 | 0.00 |
| 2012 | II | 20 | 2 | 12.88 | 0.57 | 29.99 | 2.88 | 16.87 | 1.38 | 2.92 | 0.02 |
| 2012 | II | 25 | 1 | 12.28 | 0.00 | 30.41 | 0.00 | 16.88 | 0.00 | 2.99 | 0.00 |
| 2012 | II | 30 | 2 | 13.02 | 1.15 | 30.70 | 3.94 | 17.43 | 16.96 | 3.03 | 0.29 |
| 2012 | II | 35 | 1 | 12.38 | 0.00 | 28.90 | 0.00 | 14.04 | 0.00 | 3.08 | 0.00 |
| 2012 | II | 40 | 1 | 12.11 | 0.00 | 29.05 | 0.00 | 15.39 | 0.00 | 2.95 | 0.00 |
| 2012 | III | 0 | 4 | 16.36 | 0.25 | 42.28 | 4.84 | 80.08 | 32.65 | 2.72 | 0.14 |
| 2012 | III | 5 | 3 | 16.01 | 0.84 | 39.85 | 3.23 | 66.22 | 2.95 | 2.79 | 0.03 |
| 2012 | III | 10 | 3 | 16.51 | 0.21 | 41.58 | 0.48 | 76.23 | 25.56 | 2.65 | 0.08 |
| 2012 | III | 15 | 4 | 15.19 | 1.65 | 38.00 | 5.29 | 59.48 | 34.67 | 2.74 | 0.16 |
| 2012 | III | 20 | 4 | 16.24 | 1.77 | 41.37 | 5.58 | 75.92 | 34.77 | 2.68 | 0.05 |
| 2012 | III | 25 | 4 | 15.43 | 2.16 | 38.82 | 6.78 | 79.50 | 75.03 | 2.67 | 0.18 |
| 2012 | III | 30 | 4 | 15.81 | 1.75 | 40.69 | 5.29 | 70.53 | 53.05 | 2.62 | 0.16 |
| 2012 | III | 35 | 4 | 15.75 | 1.15 | 40.58 | 4.18 | 93.70 | 24.57 | 2.71 | 0.15 |
| 2012 | III | 40 | 4 | 15.45 | 1.78 | 38.23 | 4.39 | 66.84 | 23.57 | 2.74 | 0.05 |
| 2013 | I | 0 | 2 | 3.88 | 0.00 | 0.00 | 0.00 | 34.13 | 10.48 | 3.23 | 0.12 |
| 2013 | I | 5 | 1 | 4.20 | 0.00 | 0.02 | 0.00 | 48.07 | 0.00 | 3.05 | 0.00 |
| 2013 | I | 10 | 2 | 5.24 | 0.37 | 4.27 | 3.33 | 79.95 | 0.39 | 2.70 | 0.03 |
| 2013 | I | 15 | 3 | 5.52 | 1.66 | 5.20 | 5.88 | 108.59 | 13.71 | 2.43 | 0.31 |
| 2013 | I | 20 | 3 | 6.67 | 1.29 | 8.15 | 6.82 | 132.79 | 16.55 | 2.36 | 0.23 |
| 2013 | I | 25 | 3 | 8.95 | 1.06 | 13.70 | 2.68 | 154.76 | 25.74 | 2.09 | 0.09 |
| 2013 | I | 30 | 3 | 10.00 | 0.33 | 18.10 | 4.84 | 181.92 | 18.03 | 1.97 | 0.07 |
| 2013 | I | 35 | 3 | 9.06 | 1.23 | 12.94 | 4.48 | 168.50 | 12.35 | 1.97 | 0.22 |
| 2013 | I | 40 | 3 | 8.79 | 0.23 | 13.70 | 0.97 | 175.64 | 6.83 | 2.11 | 0.12 |
| 2013 | II | 0 | 4 | 8.61 | 1.56 | 11.94 | 9.11 | 160.96 | 60.07 | 2.04 | 0.16 |
| 2013 | II | 5 | 3 | 7.96 | 1.65 | 10.85 | 2.90 | 143.09 | 22.56 | 2.25 | 0.17 |
| 2013 | II | 10 | 3 | 6.39 | 1.40 | 5.58 | 2.78 | 107.49 | 14.11 | 2.40 | 0.14 |
| 2013 | II | 15 | 2 | 3.96 | 0.17 | 0.00 | 0.00 | 58.87 | 22.42 | 2.70 | 0.15 |
| 2013 | II | 20 | 2 | 4.08 | 0.13 | 0.03 | 0.05 | 27.25 | 5.14 | 3.09 | 0.04 |
| 2013 | II | 25 | 1 | 4.26 | 0.00 | 1.23 | 0.00 | 34.44 | 0.00 | 2.91 | 0.00 |
| 2013 | II | 30 | 1 | 4.60 | 0.00 | 0.00 | 0.00 | 52.76 | 0.00 | 2.86 | 0.00 |
| 2013 | II | 35 | 2 | 3.71 | 0.24 | 0.00 | 0.00 | 23.62 | 6.73 | 3.27 | 0.07 |
| 2013 | II | 40 | 2 | 3.83 | 1.79 | 0.00 | 0.00 | 42.06 | 7.32 | 3.27 | 0.02 |
| 2013 | III | 0 | 3 | 8.20 | 1.72 | 9.63 | 6.76 | 172.74 | 20.16 | 2.07 | 0.17 |
| 2013 | III | 5 | 4 | 7.80 | 0.89 | 8.74 | 3.45 | 144.40 | 43.54 | 2.19 | 0.33 |
| 2013 | III | 10 | 3 | 6.89 | 1.09 | 7.07 | 2.08 | 123.55 | 18.92 | 2.25 | 0.19 |
| 2013 | III | 15 | 3 | 7.02 | 0.43 | 8.06 | 3.70 | 110.51 | 20.81 | 2.22 | 0.13 |
| 2013 | III | 20 | 3 | 7.39 | 0.15 | 9.29 | 1.40 | 144.10 | 9.19 | 2.20 | 0.15 |
| 2013 | III | 25 | 3 | 6.84 | 1.43 | 10.23 | 4.99 | 128.88 | 40.28 | 2.33 | 0.10 |
| 2013 | III | 30 | 3 | 7.90 | 0.94 | 10.16 | 4.43 | 149.09 | 22.55 | 2.18 | 0.15 |
| 2013 | III | 35 | 3 | 7.76 | 1.54 | 10.67 | 8.29 | 174.96 | 26.64 | 2.21 | 0.15 |
| 2013 | III | 40 | 3 | 8.64 | 1.06 | 12.62 | 4.84 | 174.52 | 24.30 | 2.22 | 0.07 |

† DAA, days after anthesis
‡ N, number of samples
§ Total phenols (mg GAE/g)
¶ Condensed tannins (mg CE/g)
# 3-deoxyanthocyanidins (abs/mL/g)
†† % Fat

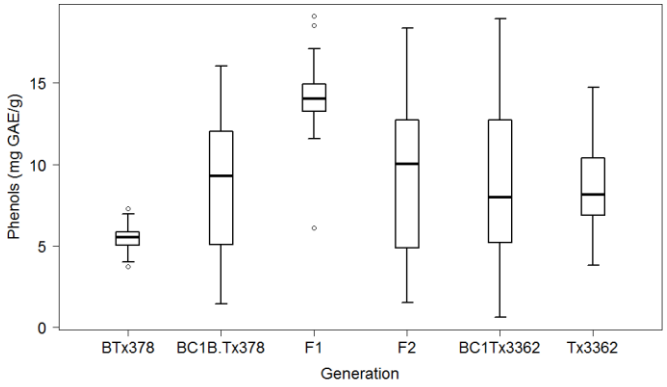



**Appendix 3.** Graphical summary of plant material used in the generation means analysis.

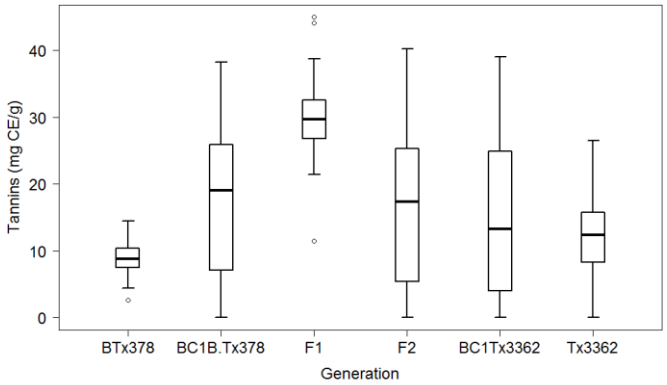



**Appendix 4.** Boxplots representing CIE (A) *L**, (B) *a**, and (C) *b** values in parents, $F_1$, $F_2$, and backcross generations evaluated in three Texas environments in 2013.

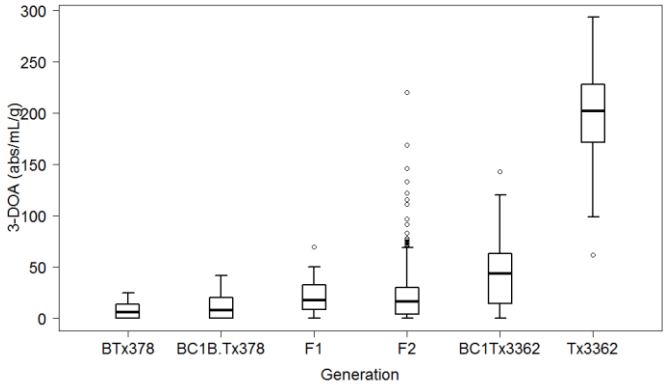
A




B




C




**Appendix 5.** Boxplots representing (A) total phenols (mg GAE/g), (B) tannins (mg CE/g), and (C) 3-DOA (abs/mL/h) levels in parents, $F_1$, $F_2$, and backcross generations evaluated in three Texas environments in 2013.

**Appendix 6.** Basic data of visual score rating by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 25 | 1.00 | 0.02 | 0.10 |
| | $BC_1$B.Tx378 | 75 | 1.24 | 0.43 | 1.00 |
| | $F_1$ | 25 | 2.00 | 0.02 | 0.10 |
| | $F_2$ | 150 | 1.75 | 0.63 | 3.00 |
| | $BC_1$Tx3362 | 75 | 2.25 | 0.59 | 3.00 |
| | Tx3362 | 25 | 5.00 | 0.02 | 0.10 |
| HW | B.Tx378 | 30 | 1.00 | 0.02 | 0.10 |
| | $BC_1$B.Tx378 | 80 | 1.23 | 0.45 | 2.00 |
| | $F_1$ | 30 | 2.00 | 0.02 | 0.10 |
| | $F_2$ | 160 | 1.55 | 1.43 | 13.00 |
| | $BC_1$Tx3362 | 80 | 2.13 | 1.77 | 13.00 |
| | Tx3362 | 30 | 5.00 | 0.02 | 0.10 |
| WE | B.Tx378 | 50 | 1.00 | 0.01 | 0.10 |
| | $BC_1$B.Tx378 | 150 | 1.39 | 0.58 | 2.00 |
| | $F_1$ | 50 | 2.00 | 0.01 | 0.10 |
| | $F_2$ | 299 | 2.05 | 1.03 | 4.00 |
| | $BC_1$Tx3362 | 150 | 2.93 | 1.19 | 4.00 |
| | Tx3362 | 50 | 5.00 | 0.01 | 0.10 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 7.** Basic data of CIE *L** value (lightness) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 25 | 50.45 | 2.19 | 8.48 |
| | $BC_1$B.Tx378 | 75 | 42.99 | 3.08 | 15.17 |
| | $F_1$ | 25 | 40.23 | 2.40 | 14.12 |
| | $F_2$ | 150 | 42.17 | 4.15 | 18.48 |
| | $BC_1$Tx3362 | 75 | 36.11 | 2.92 | 16.93 |
| | Tx3362 | 25 | 28.95 | 0.95 | 3.56 |
| HW | B.Tx378 | 30 | 54.91 | 1.45 | 5.26 |
| | $BC_1$B.Tx378 | 80 | 47.83 | 4.47 | 18.30 |
| | $F_1$ | 30 | 43.42 | 1.45 | 4.91 |
| | $F_2$ | 160 | 45.51 | 3.93 | 19.79 |
| | $BC_1$Tx3362 | 80 | 42.53 | 4.00 | 20.35 |
| | Tx3362 | 30 | 32.56 | 2.22 | 9.32 |
| WE | B.Tx378 | 50 | 46.43 | 1.59 | 6.92 |
| | $BC_1$B.Tx378 | 150 | 40.43 | 3.70 | 17.69 |
| | $F_1$ | 50 | 36.44 | 1.05 | 5.51 |
| | $F_2$ | 299 | 38.39 | 3.44 | 16.96 |
| | $BC_1$Tx3362 | 150 | 35.63 | 2.07 | 10.38 |
| | Tx3362 | 50 | 30.03 | 1.61 | 6.75 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 8.** Basic data of CIE *a** value (-*a** = greeness, +*a** = redness) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 25 | 13.88 | 0.74 | 3.14 |
| | $BC_1$B.Tx378 | 75 | 16.25 | 1.26 | 6.59 |
| | $F_1$ | 25 | 14.57 | 0.86 | 3.35 |
| | $F_2$ | 150 | 14.02 | 1.51 | 7.99 |
| | $BC_1$Tx3362 | 75 | 13.11 | 1.52 | 8.42 |
| | Tx3362 | 25 | 5.46 | 0.89 | 2.90 |
| HW | B.Tx378 | 30 | 14.98 | 0.82 | 3.21 |
| | $BC_1$B.Tx378 | 80 | 15.34 | 1.27 | 5.73 |
| | $F_1$ | 30 | 15.43 | 0.77 | 3.26 |
| | $F_2$ | 160 | 14.45 | 1.55 | 7.82 |
| | $BC_1$Tx3362 | 80 | 14.77 | 1.41 | 7.83 |
| | Tx3362 | 30 | 7.89 | 1.08 | 5.52 |
| WE | B.Tx378 | 50 | 15.88 | 0.79 | 4.39 |
| | $BC_1$B.Tx378 | 150 | 15.76 | 1.34 | 7.25 |
| | $F_1$ | 50 | 14.49 | 0.92 | 4.25 |
| | $F_2$ | 299 | 14.32 | 1.48 | 9.40 |
| | $BC_1$Tx3362 | 150 | 12.16 | 1.53 | 6.67 |
| | Tx3362 | 50 | 6.85 | 1.08 | 4.36 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 9.** Basic data of CIE *b** value (-*b** = blueness, +*a** = yellowness) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 25 | 25.53 | 1.06 | 4.02 |
| | $BC_1$B.Tx378 | 75 | 22.42 | 2.39 | 11.60 |
| | $F_1$ | 25 | 19.89 | 1.76 | 9.41 |
| | $F_2$ | 150 | 20.50 | 3.48 | 17.59 |
| | $BC_1$Tx3362 | 75 | 14.17 | 3.11 | 17.64 |
| | Tx3362 | 25 | 4.16 | 1.18 | 4.75 |
| HW | B.Tx378 | 30 | 29.59 | 0.81 | 3.03 |
| | $BC_1$B.Tx378 | 80 | 26.83 | 2.75 | 10.98 |
| | $F_1$ | 30 | 23.96 | 1.16 | 4.41 |
| | $F_2$ | 160 | 23.99 | 2.68 | 13.85 |
| | $BC_1$Tx3362 | 80 | 21.46 | 3.47 | 17.15 |
| | Tx3362 | 30 | 9.65 | 2.51 | 11.20 |
| WE | B.Tx378 | 50 | 25.25 | 1.48 | 8.06 |
| | $BC_1$B.Tx378 | 150 | 21.05 | 3.21 | 15.21 |
| | $F_1$ | 50 | 17.17 | 1.05 | 5.08 |
| | $F_2$ | 299 | 18.06 | 3.38 | 18.89 |
| | $BC_1$Tx3362 | 150 | 13.71 | 2.38 | 11.50 |
| | Tx3362 | 50 | 6.71 | 2.20 | 9.53 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 10.** Basic data of total phenols (mg GAE/g) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 5.84 | 0.19 | 0.26 |
| | $BC_1$B.Tx378 | 22 | 7.21 | 3.16 | 9.47 |
| | $F_1$ | 14 | 12.99 | 2.10 | 8.52 |
| | $F_2$ | 91 | 8.75 | 3.78 | 13.58 |
| | $BC_1$Tx3362 | 13 | 5.98 | 4.54 | 12.98 |
| | Tx3362 | 10 | 8.65 | 1.66 | 6.00 |
| HW | B.Tx378 | 30 | 5.11 | 0.53 | 2.23 |
| | $BC_1$B.Tx378 | 80 | 8.66 | 4.07 | 13.69 |
| | $F_1$ | 29 | 13.51 | 0.87 | 3.39 |
| | $F_2$ | 151 | 8.69 | 4.11 | 13.86 |
| | $BC_1$Tx3362 | 78 | 7.83 | 4.03 | 14.29 |
| | Tx3362 | 30 | 6.76 | 1.21 | 5.24 |
| WE | B.Tx378 | 25 | 5.91 | 0.61 | 2.78 |
| | $BC_1$B.Tx378 | 77 | 9.05 | 3.85 | 12.49 |
| | $F_1$ | 26 | 15.45 | 1.38 | 5.84 |
| | $F_2$ | 135 | 9.63 | 4.53 | 16.84 |
| | $BC_1$Tx3362 | 76 | 10.62 | 4.23 | 15.09 |
| | Tx3362 | 25 | 11.24 | 1.63 | 6.01 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 11.** Basic data of condensed tannins (mg CE/g) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 11.76 | 3.79 | 5.36 |
| | $BC_1$B.Tx378 | 22 | 13.96 | 7.92 | 27.19 |
| | $F_1$ | 14 | 27.65 | 5.39 | 22.11 |
| | $F_2$ | 91 | 15.54 | 9.69 | 32.96 |
| | $BC_1$Tx3362 | 13 | 8.39 | 10.85 | 29.61 |
| | Tx3362 | 10 | 13.10 | 5.66 | 19.14 |
| HW | B.Tx378 | 30 | 7.65 | 1.95 | 8.76 |
| | $BC_1$B.Tx378 | 80 | 16.75 | 11.15 | 34.21 |
| | $F_1$ | 29 | 27.57 | 2.66 | 10.09 |
| | $F_2$ | 151 | 14.56 | 10.75 | 33.46 |
| | $BC_1$Tx3362 | 78 | 12.60 | 10.88 | 35.59 |
| | Tx3362 | 30 | 8.23 | 4.94 | 17.54 |
| WE | B.Tx378 | 25 | 10.06 | 2.24 | 8.99 |
| | $BC_1$B.Tx378 | 77 | 18.17 | 11.29 | 35.99 |
| | $F_1$ | 26 | 34.47 | 4.49 | 19.03 |
| | $F_2$ | 135 | 17.42 | 12.26 | 40.22 |
| | $BC_1$Tx3362 | 76 | 18.04 | 11.20 | 39.00 |
| | Tx3362 | 25 | 16.17 | 5.26 | 18.95 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 12.** Basic data of 3-deoxyanthocyanidins (3-DOA) (abs/mL/h) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 20.95 | 5.62 | 7.95 |
| | $BC_1$B.Tx378 | 22 | 15.00 | 10.67 | 34.95 |
| | $F_1$ | 14 | 20.53 | 10.37 | 38.40 |
| | $F_2$ | 91 | 30.58 | 21.51 | 115.79 |
| | $BC_1$Tx3362 | 13 | 49.67 | 23.25 | 75.47 |
| | Tx3362 | 10 | 212.29 | 24.82 | 83.56 |
| HW | B.Tx378 | 30 | 1.30 | 3.49 | 18.00 |
| | $BC_1$B.Tx378 | 80 | 0.83 | 2.93 | 21.85 |
| | $F_1$ | 29 | 8.52 | 6.07 | 21.58 |
| | $F_2$ | 151 | 13.41 | 30.29 | 220.09 |
| | $BC_1$Tx3362 | 78 | 21.25 | 23.53 | 98.23 |
| | Tx3362 | 30 | 169.70 | 39.96 | 177.28 |
| WE | B.Tx378 | 25 | 14.36 | 5.06 | 16.36 |
| | $BC_1$B.Tx378 | 77 | 21.34 | 9.41 | 39.18 |
| | $F_1$ | 26 | 37.10 | 10.62 | 50.04 |
| | $F_2$ | 135 | 25.99 | 20.02 | 122.07 |
| | $BC_1$Tx3362 | 76 | 65.16 | 25.35 | 125.25 |
| | Tx3362 | 25 | 225.89 | 26.69 | 108.97 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 13.** Basic data of percent protein by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 10.38 | 0.04 | 0.06 |
| | $BC_1$B.Tx378 | 22 | 12.62 | 1.25 | 4.08 |
| | $F_1$ | 14 | 12.95 | 1.25 | 5.18 |
| | $F_2$ | 91 | 12.04 | 1.74 | 7.57 |
| | $BC_1$Tx3362 | 13 | 14.98 | 1.07 | 3.87 |
| | Tx3362 | 10 | 16.33 | 0.36 | 1.07 |
| HW | B.Tx378 | 30 | 9.43 | 0.31 | 1.07 |
| | $BC_1$B.Tx378 | 80 | 11.30 | 1.39 | 5.80 |
| | $F_1$ | 29 | 11.75 | 0.46 | 1.90 |
| | $F_2$ | 151 | 12.00 | 1.31 | 5.88 |
| | $BC_1$Tx3362 | 78 | 13.32 | 1.42 | 7.48 |
| | Tx3362 | 30 | 15.23 | 0.69 | 2.73 |
| WE | B.Tx378 | 25 | 9.08 | 0.29 | 1.10 |
| | $BC_1$B.Tx378 | 77 | 11.00 | 1.57 | 6.04 |
| | $F_1$ | 26 | 12.38 | 0.56 | 2.06 |
| | $F_2$ | 135 | 11.87 | 1.55 | 7.76 |
| | $BC_1$Tx3362 | 76 | 13.01 | 0.76 | 4.48 |
| | Tx3362 | 25 | 15.51 | 0.55 | 2.04 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 14.** Basic data of percent moisture by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 10.91 | 0.04 | 0.06 |
| | $BC_1$B.Tx378 | 22 | 11.01 | 0.46 | 1.57 |
| | $F_1$ | 14 | 11.13 | 0.24 | 0.72 |
| | $F_2$ | 91 | 10.46 | 0.78 | 4.15 |
| | $BC_1$Tx3362 | 13 | 11.04 | 0.51 | 1.57 |
| | Tx3362 | 10 | 10.52 | 0.31 | 1.04 |
| HW | B.Tx378 | 30 | 9.69 | 0.31 | 1.29 |
| | $BC_1$B.Tx378 | 80 | 9.49 | 0.62 | 2.83 |
| | $F_1$ | 29 | 9.66 | 0.25 | 1.00 |
| | $F_2$ | 151 | 9.52 | 1.08 | 7.61 |
| | $BC_1$Tx3362 | 78 | 10.17 | 0.49 | 2.82 |
| | Tx3362 | 30 | 8.75 | 0.60 | 2.32 |
| WE | B.Tx378 | 25 | 11.06 | 0.20 | 0.89 |
| | $BC_1$B.Tx378 | 77 | 10.99 | 0.40 | 1.65 |
| | $F_1$ | 26 | 11.09 | 0.34 | 1.31 |
| | $F_2$ | 135 | 10.66 | 0.64 | 3.66 |
| | $BC_1$Tx3362 | 76 | 11.18 | 0.77 | 3.03 |
| | Tx3362 | 25 | 9.95 | 0.64 | 2.29 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 15.** Basic data of percent fat (oil) by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 2.05 | 0.07 | 0.10 |
| | $BC_1$B.Tx378 | 22 | 2.38 | 0.64 | 2.06 |
| | $F_1$ | 14 | 2.65 | 0.31 | 1.26 |
| | $F_2$ | 91 | 2.44 | 0.59 | 2.54 |
| | $BC_1$Tx3362 | 13 | 2.77 | 0.22 | 0.68 |
| | Tx3362 | 10 | 1.98 | 0.13 | 0.40 |
| HW | B.Tx378 | 30 | 2.85 | 0.20 | 0.79 |
| | $BC_1$B.Tx378 | 80 | 3.26 | 0.68 | 2.23 |
| | $F_1$ | 29 | 3.32 | 0.23 | 0.79 |
| | $F_2$ | 151 | 3.25 | 0.61 | 2.67 |
| | $BC_1$Tx3362 | 78 | 3.30 | 0.46 | 2.51 |
| | Tx3362 | 30 | 2.79 | 0.15 | 0.59 |
| WE | B.Tx378 | 25 | 2.16 | 0.15 | 0.54 |
| | $BC_1$B.Tx378 | 77 | 2.65 | 0.64 | 2.53 |
| | $F_1$ | 26 | 2.69 | 0.20 | 0.89 |
| | $F_2$ | 135 | 2.72 | 0.63 | 2.67 |
| | $BC_1$Tx3362 | 76 | 2.54 | 0.35 | 1.62 |
| | Tx3362 | 25 | 2.19 | 0.21 | 0.80 |

† N, Number of samples
‡ St Dev, Standard deviation

**Appendix 16.** Basic data of percent fiber by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 1.62 | 0.05 | 0.06 |
| | $BC_1$B.Tx378 | 22 | 1.73 | 0.08 | 0.33 |
| | $F_1$ | 14 | 1.77 | 0.04 | 0.17 |
| | $F_2$ | 91 | 1.78 | 0.13 | 0.69 |
| | $BC_1$Tx3362 | 13 | 1.96 | 0.14 | 0.50 |
| | Tx3362 | 10 | 2.55 | 0.10 | 0.27 |
| HW | B.Tx378 | 30 | 1.76 | 0.03 | 0.09 |
| | $BC_1$B.Tx378 | 80 | 1.73 | 0.08 | 0.36 |
| | $F_1$ | 29 | 1.82 | 0.03 | 0.13 |
| | $F_2$ | 151 | 1.80 | 0.09 | 0.54 |
| | $BC_1$Tx3362 | 78 | 1.90 | 0.12 | 0.50 |
| | Tx3362 | 30 | 2.31 | 0.12 | 0.47 |
| WE | B.Tx378 | 25 | 1.72 | 0.02 | 0.08 |
| | $BC_1$B.Tx378 | 77 | 1.74 | 0.06 | 0.26 |
| | $F_1$ | 26 | 1.83 | 0.04 | 0.15 |
| | $F_2$ | 135 | 1.88 | 0.10 | 0.62 |
| | $BC_1$Tx3362 | 76 | 2.01 | 0.14 | 0.59 |
| | Tx3362 | 25 | 2.39 | 0.13 | 0.50 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 17.** Basic data of percent ash by environment and genotype from a generation means analysis grown in College Station (CS), Halfway (HW), and Weslaco (WE), Texas in 2013.

| Environment | Generation | N† | Mean | St Dev‡ | Range |
|---|---|---|---|---|---|
| CS | B.Tx378 | 2 | 1.30 | 0.01 | 0.01 |
| | $BC_1$B.Tx378 | 22 | 1.30 | 0.05 | 0.19 |
| | $F_1$ | 14 | 1.29 | 0.02 | 0.06 |
| | $F_2$ | 91 | 1.25 | 0.05 | 0.22 |
| | $BC_1$Tx3362 | 13 | 1.26 | 0.04 | 0.12 |
| | Tx3362 | 10 | 1.12 | 0.04 | 0.13 |
| HW | B.Tx378 | 30 | 1.25 | 0.02 | 0.10 |
| | $BC_1$B.Tx378 | 80 | 1.25 | 0.05 | 0.23 |
| | $F_1$ | 29 | 1.24 | 0.02 | 0.09 |
| | $F_2$ | 151 | 1.25 | 0.07 | 0.47 |
| | $BC_1$Tx3362 | 78 | 1.28 | 0.04 | 0.23 |
| | Tx3362 | 30 | 1.12 | 0.03 | 0.12 |
| WE | B.Tx378 | 25 | 1.30 | 0.02 | 0.07 |
| | $BC_1$B.Tx378 | 77 | 1.31 | 0.03 | 0.15 |
| | $F_1$ | 26 | 1.29 | 0.03 | 0.14 |
| | $F_2$ | 135 | 1.27 | 0.04 | 0.20 |
| | $BC_1$Tx3362 | 76 | 1.26 | 0.06 | 0.26 |
| | Tx3362 | 25 | 1.10 | 0.03 | 0.14 |

† N, Number of samples

‡ St Dev, Standard deviation

**Appendix 18.** Three parameter model estimates of midparent [*m*], additive [*a*], and dominance [*d*] effects (and their standard errors) from a joint scaling test for grain color and composition traits, in addition to the chi-squared goodness of fit values, in parents (BTx378 and Tx3362), their $F_1$, $F_2$, and backcrossed generations grown in three Texas environments in 2013.

| Trait | *m* | *a* | *d* | χ2 |
|---|---|---|---|---|
| Visual score | 3.00 ± 0.00** | -2.00 ± 0.00** | -1.00 ± 0.00** | 403.99** |
| *L** | 39.96 ± 0.19** | 8.72 ± 0.19** | 0.68 ± 0.37** | 139.65** |
| *a** | 11.88 ± 0.07** | 3.73 ± 0.07** | 3.90 ± 0.13** | 398.30** |
| *b** | 17.28 ± 0.16** | 9.08 ± 0.16** | 3.84 ± 0.32** | 118.84** |
| Phenols | 6.55 ± 0.14** | -1.14 ± 0.15** | 6.74 ± 0.25** | 71.65** |
| Tannins | 9.01 ± 0.38** | -0.44 ± 0.39** | 18.47 ± 0.72** | 67.46** |
| 3-DOA | 59.35 ± 1.72** | -51.85 ± 1.66** | -49.34 ± 2.62** | 457.19** |
| Protein | 12.32 ± 0.05** | -2.97 ± 0.05** | -0.25 ± 0.10** | 62.44** |
| Moisture | 10.04 ± 0.06** | 0.09 ± 0.06** | 0.58 ± 0.12** | 78.58** |
| Fat | 2.55 ± 0.03** | 0.02 ± 0.03 | 0.54 ± 0.06** | 23.31** |
| Fiber | 1.97 ± 0.01** | -0.24 ± 0.01** | -0.19 ± 0.01** | 220.43** |
| Ash | 1.21 ± 0.00** | 0.07 ± 0.00** | 0.09 ± 0.00** | 291.56** |
| Starch | 65.19 ± 0.04** | 2.08 ± 0.04** | 0.48 ± 0.08** | 161.53** |

**Appendix 19.** Estimates for the minimum number of genes associated with the black pericarp trait. Estimates were made using GMA generations from the cross of BTx378 × Tx3362 and evaluated in three Texas environments in 2013.

| Trait | Genes |
|---|---|
| Visual score | 1.69 |
| *L** | 3.67 |
| *a** | 9.16 |
| *b** | 5.39 |

## A2. Vita


| | |
|---|---|
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