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Estimating the relative effects of the endosperm traits of waxy and high protein digestibility on yield in grain sorghum

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ABSTRACT

Sorghum (*Sorghum bicolor* L. Moench) is an important crop that is widely grown in dry climates across the world. The grain is used for food, feed and industrial purposes and the quality of the grain influences the relative utility for each specific use. In sorghum grain, both the waxy endosperm and the high protein digestibility traits have the potential to significantly alter conversion efficiencies of sorghum in several applications including production of grain ethanol and brewing. There are concerns though, that these traits may confer a reduction in the agronomic and yield performance of lines and hybrids that possess them. The objective of this study was to assess the potential impact of both the waxy and the high digestibility traits on the yield of grain sorghum lines. From an F_2 breeding population segregating for both waxy and highly digestible endosperm, 100 $F_{2:4}$ lines were derived, with equal number in each of four categories: high digestible (HD), waxy (*WX*), HD and waxy (HD-*WX*), and normal. No selection for yield was practiced during the development of these lines. The lines and checks were evaluated in two environments in Texas. Across all environments, there was no significant difference between yields. In addition, analysis by genotype revealed that several *WX*, HD-*WX*, and HD lines were among the best yielding lines. These results imply that selection of high yielding *WX* and HD genotypes is possible, but a significant breeding emphasis on their development and selection is required to effectively identify those genotypes.

1. Introduction

Sorghum (*Sorghum bicolor* L. Moench) is an important crop grown in arid and semi-arid regions of the world. The grain is used primarily for human consumption in Asian and African countries, while in the Americas and Australia it is commonly used for animal feed and, more recently, for ethanol production. Compared to maize (*Zea mays*), sorghum grain is similar in starch content, yet has lower fat and higher protein concentrations (Dowling et al., 2002; Gualtieri and Rapaccini, 1990). Another primary difference between maize and sorghum grain is that the protein and starch in sorghum is not as readily available for enzyme degradation in many processing systems (animal, human or industrial) (Spicer et al., 1982, 1983).

The reduced protein digestibility of normal sorghum endosperm is thought to be due largely to specific endosperm storage proteins called kafirins. Sorghum kafirins form a hydrophobic matrix of protein bodies surrounding the large starch granules within the endosperm (Taylor et al., 1984; Chankrashekar and Kirlies, 1988). Kafirins are alcohol soluble prolamins that make up about 50–73% of the endosperm matrix by volume (Paulis and Wall, 1979; Hamaker et al., 1995). Kafirin storage proteins come in three types: α-, β- and γ-kafirins. The α-kafirin is highly soluble and easily digested, while the latter two are less soluble and are not easily digested because they form enzyme resistant structures. Oria et al. (2000) suggested that the lower digestibility of proteins in the sorghum endosperm is due to strong disulphide bonds formed by β- and γ-kafirins that produce an enzyme resistant structure on the periphery of the protein body. Since the highly digestible α-kafirin is located in the interior, the peripheral enzyme resistant layer of β- and γ-kafirins negatively influences protein hydrolysis. During starch hydrolysis, the disulphide bonds in the protein matrix also limits the access of amylases to the starch granules (Ezeogu et al., 2008).

Genetic variation for starch and protein digestibility exists in sorghum. The genotype P850029 has a higher protein digestibility compared to other normal grain sorghum lines (Weaver et al., 1998). The increased digestibility is due to structural rearrangement of β- and γ-kafirins in the endosperm and a reduction in the total amount of γ-kafirin in the endosperm (Tesso et al.,

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2006). Genotypes that possess these modifications produce grain that is easier to digest in any application, ranging from animal feed to ethanol production (Nyannor et al., 2007). In addition to higher protein digestibility (HD), these lines also possess 60% higher lysine content (Weaver et al., 1998; Wu et al., 2010).

In addition to the effect of sorghum endosperm proteins on bioethanol fermentation and food and feed processing; starch content and composition also influence processing characteristics. Normal sorghum genotypes produce both amylopectin and amylose in a 3:1 ratio in the endosperm. Genotypes possessing variation in the proportion of these compounds exist. For example, "waxy" endosperm sorghum types do not produce amylose, and hence, result in an endosperm in which all of the starch is amylopectin (Karper, 1933). The waxy phenotype in sorghum is conditioned by a single gene in the recessive form, designated as *wx* (Melvin and Sieglinger, 1952), which results in the absence or inactivation of granule-bound starch synthase (GBSS). Two naturally occurring waxy alleles have been identified in sorghum. The waxy GBSS− allele, designated as wx^a, has no GBSS present, while the other allele waxy GBSS+, designated as wx^b, has an inactive GBSS present (Pedersen et al., 2005). From a processing and utilization standpoint, amylopectin starch lowers gelatinization temperatures. As a result, processing and hydrolysis requires less energy and time to complete (Wu et al., 2010). Interestingly, there were no positive or negative effects observed on the growth and development of animals raised on waxy sorghums versus non-waxy sorghums (Shelton et al., 2004).

In theory, combining the high digestible and waxy traits should yield grain sorghum that has an endosperm with reduced energy gelatinization requirements and improved enzymatic hydrolysis. These traits are valued in the ethanol industry as they reduce energy requirements, yield faster conversion and turnover, and increase profit margins. The addition of the high digestibility trait, which confers 60% higher lysine, improves the animal feed value of the dried distillers grain solubles (DDGS). What is not known, is whether combining the waxy grain trait and high lysine/digestibility grain traits will confer lower grain yield potential and increase susceptibility to grain weathering, both of which would limit the potential value of these two traits in combination.

The maize high lysine trait, referred to as quality protein maize (QPM), is conferred by the recessively inherited *opaque-2* (*o2*) allele. This trait has functional similarity to the HD trait found in sorghum, where the high lysine HD trait is thought to be due to a mutation in the prolamin storage protein γ-kafirin and a redistribution of amino acids pools from low lysine kafirins to higher lysine globulin storage proteins (Tesso et al., 2006). In maize endosperm, the most abundant storage proteins are zeins and α-zeins in particular, both of which share the low lysine and tryptophan content of kafirin and other prolamins (Gibbons and Larkins, 1990). The *opaque-2* allele has been shown to encode a transcription factor regulator of zein expression (Schmidt et al., 1990). Similar to the HD trait in sorghum, the homozygous recessive form of *o2* results in a shift from a lower expression of low lysine zeins to an increased expression of naturally higher lysine globulin pools (Gibbons and Larkins, 1990). Studies have suggested that both the waxy and the QPM traits are linked to lower grain yields.

While no yield data has been reported for the HD trait of sorghum, lower yields have been reported for QPM versus wildtype maize. The dedicated efforts of the CIMMYT program to introduce high yield QPM maize provides a good example that the limiting factor in lower yield is not linkage drag or pleiotropy, but simply the need for a focused breeding effort (Krivanek et al., 2007; Vivek et al., 2008). Current results have demonstrated the grain yield competitiveness of QPM with the best normal maize cultivars (Bjarnason and Vasal, 1992; Pixley and Bjarnason, 1993).

Similarly, studies have reported reduced yield resulting from the waxy endosperm trait in several cultivated cereals. For instance, Rooney et al. (2005) reported a 17% reduction in yield between waxy and non-waxy sorghums derived from the same population. Other studies have reported similar results in sorghum (Tover et al., 1977) as well as wheat (Graybosch, 1998), maize (Fergason, 2001) and barley (Oscarsson et al., 1998). In maize, the yield of waxy hybrids averages 95% of non-waxy hybrids. As such, waxy maize is only grown for specific food and industrial applications. The lower yields in waxy maize and sorghum are likely the product of several factors such as pleiotropy, potential genetic linkage, and because breeding efforts to improve the yield of waxy inbreds and hybrids have lagged behind the extensive efforts made in traditional maize and sorghum types. As an example, Rooney et al. (2005) did find several high-yielding waxy lines among the highest yielding non-waxy lines in the population. As such, it appears that the potential to enhance and improve the yield of the waxy endosperm sorghums exists similar to that observed in waxy maize. The objective of this study is to determine the relative effect on agronomic performance of the high digestible trait, both independently and in combination with the waxy endosperm trait.

2. Materials and methods

2.1. Parental lines and population development

A set of 100 $F_{2:4}$ derived recombinant inbred lines (RIL) was developed from an F_2 population of the cross between Tx2907/P850029. Tx2907 is a waxy endosperm sorghum parental line that was released from the Texas AgriLife Research sorghum-breeding program (Miller et al., 1996). Tx2907 has protein digestibility similar to wildtype sorghum lines since it is wildtype at the HD locus described below and thus has a protein digestibility that performs equivalent to flour of wildtype grain sorghum (Yan et al., 2011). The normal or wildtype protein digestibility of the waxy trait is based on our unreported phenotypic data comparisons between the waxy, HD, HD-*wx* and wildtype sorghum lines phenotyped in this study. P850029 is a sorghum line with normal (non-waxy) starch endosperm and high digestible protein (HD), which was developed at Purdue University from a population derived from P721Q (Weaver et al., 1998; Mohan, 1975).

To develop the RILs, 200 randomly chosen F_2 panicles were self-pollinated. From these, $F_{2:3}$ progeny were self pollinated to produce the $F_{2:4}$ seed. Because the two parental lines do not segregate for major height (*Dw*) and maturity (*Ma*) genes, differences in maturity and/or height are limited to segregation in smaller effect genes. At the F_4 generation, all of the RILs were phenotyped for the high digestible and waxy traits. Lines homozygous for the high digestible trait were identified by visual observation and chemical analysis. Under visual observation, 25 seed from each RIL were halved and observed on a light box. Seed from lines with wildtype endosperm have a small oval floury center and flinty outer layer surrounding the floury center, while seed from lines with the high digestible endosperm (HD) have a completely floury endosperm (Fig. 1). For the chemical analysis verification of the HD trait, protein digestibility was measured using the in vitro pepsin digestibility assay of Mertz et al. (1984) was used as described in Yan et al. (2011).

Lines were screened for waxy endosperm using the iodine staining technique described by Pedersen et al. (2004). The seed from each entry was crushed and placed in each well of a 96 well plate. Water was added to each well, and the mixture was heated to 95 °C for 1 h. Later, after the plates were cooled, iodine stain solution was added to each well, and the wells were color scored after 60 s. The wildtype seed stained purple due to the presence of amylose, while the waxy lines stained reddish brown or magenta because of the presence of amylopectin.

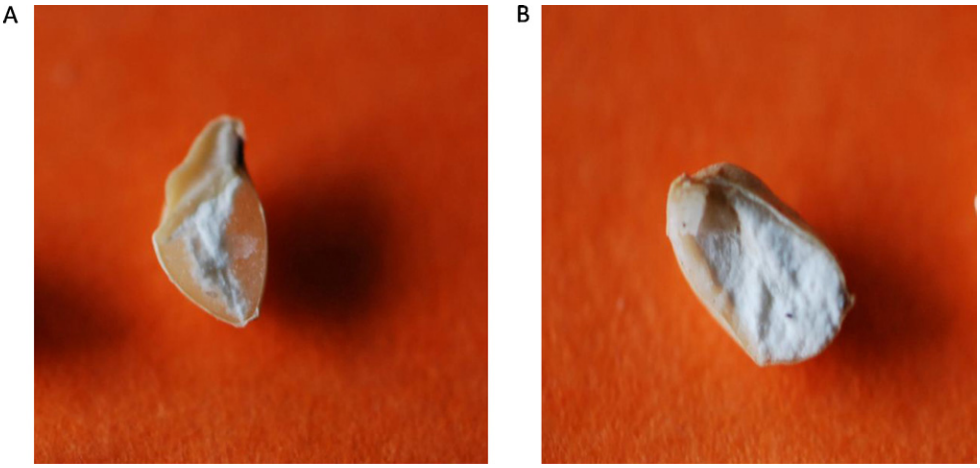

Fig. 1. Visual examination of halved seed on a light box: (A) wildtype seed and (B) HD mutant.

Any lines segregating for either trait were eliminated and all remaining RIL were categorized: (i) highly digestible protein, and wildtype endosperm, referred to herein as HD, (ii) highly digestible protein and waxy endosperm, referred to herein as HD-*wx*, (iii) normal digestibility and wildtype endosperm, referred to herein as wildtype, and (iv) normal digestibility and waxy endosperm, referred to herein as *wx*. Twenty-four lines of each phenotype were randomly chosen for evaluation.

2.2. Field study

The lines were evaluated in College Station and Halfway, Texas in 2010. In each environment, the experimental design was a randomized complete block with three replications that included the 96 experimental lines, the two parental lines, and two check lines Tx631 (Miller, 1986) and Tx2928 (Rooney, 2003) for a total of 100 entries. The soil type at College Station and Halfway were Ships Clay loam and Pullman Clay loam, respectively. The field plots were managed using agronomic practices typical for production of sorghum at each location (Rooney et al., 2005). Prior to planting, the land was prepped with shredding of stalks after grain harvest, disc harrowing twice, and bedding with a disc lister-bedder during the proceeding fall. In January/February, fertilizer was added at 168 kg ha^{-1} (10-34-0) and 4.5 kg ha^{-1} ZnEDTA. In mid-March, the seed bed was prepared with a rod-weeder, then planting occurred on March 20, prior to spraying with pre-emergent 1.42 L Atrazine and 0.6 L Brawl in 10 L ha^{-1} H_2O. After 6 weeks, plots were side-dressed with N (ammonium nitrate) at 110 kg ha^{-1} (32-0-0) then sprayed with 3.5 L Prowl in 10 L ha^{-1} H_2O. Plots were furrow irrigated as needed and aerial spraying at 9.3 L ha^{-1} of Asana was performed as needed for the control of sorghum midge and earworms. Grain was harvested on August 1. Plots were 5 m long after cutting alleyways with 75 cm row spacing. Days to 50% flowering for the grain sorghum inbreds ranged from 55 to 60 days. Days to anthesis, and plant height (cm) were measured at anthesis. At maturity, three panicles per plot were harvested, threshed using an Almaco plot thresher and the grain weighed and used to determine grain weight per panicle (g panicle^{-1}), 100 kernel weight (g), bulk test weight (kg hl^{-1}) and kernel number per panicle. The remainder of the plot was harvested using an MF8 plot combine and harvested grain weighed to determine grain yield (g). Test weight measurements were only recorded in Halfway. Test weight and 100 kernel weights were recorded twice for each field replication.

2.3. Statistical analysis

Data from each environment were analyzed separately, partitioning the sources of variation to replication, endosperm type and entries within the endosperm type. Error mean squares in the analysis of variation across environments were not heterogeneous, so the data was combined for all environments and analyzed using the PROC MIXED model with replications, environments and entries as random effects and endosperm type as a fixed effect. To identify the best performing genotypes regardless of endosperm type, the data set was analyzed using the same model except that endosperm type was removed as a source of variation.

All the data was analyzed using the general linear model procedure in SAS (2004; SAS v9.2, SAS Institute Inc., Cary, NC, USA). Means within individual and combined environments were calculated and the differences in the means were identified using least significant difference (LSD) at $P < 0.05$.

Because the lines were randomly derived from a single population, the broad sense heritability (H^2) of yield components was estimated from the variance components derived from PROC MIXED: $H^2 = \sigma^2{}_g/(\sigma^2{}_g + \sigma^2{}_{ge}/e + \sigma^2{}_{error}/re)$ where $\sigma^2{}_g$, $\sigma^2{}_{ge}$, $\sigma^2{}_{error}$, r and e represent the genotype, genotype × environment, error

Table 1
Mean squares from the combined analysis of F_4 or high digestible (HD), high digestible × waxy (HD-*WX*), wildtype and waxy (*WX*) lines from RTx2907/P850029 cross in evaluation in two environments across Texas in 2010.

Source	DF	Grain yield	Height	100 kernel weight
Env	1	280,624.2	12,411.4**	3.2*
Rep (Env)	4	2,033,367.0	1690.4	–
Endosperm type	3	1,047,599.7	7292.9**	0.15
Entry (endosperm type)	97	1,131,675.8*	1889.1**	0.27
Endosperm type × Env	3	1,131,151.1	779.4	0.14
Entry (endosperm type) × Env	97	990,613.9	560.5	0.14
Error	386	867,405.5	1167.3	0.01

* Significant at $P = 0.05$.
** Significant at $P = 0.01$.

Table 2

Means by endosperm type for grain yield, plant height, test weight, 100 kernel weight and head weight for high digestible (HD), high digestible × waxy (HD-*WX*), wildtype and waxy (*WX*) F$_4$ lines from (RTx2907/P850029) population that were evaluated in two environments across Texas in 2010.

Location	HD	HD-*wx*	Wildtype	*WX*	LSD
Grain yield (MT ha^{-1})					
Halfway	2.3	2.2	2.3	2.5	0.35
College Station	2.2	2.3	2.1	2.4	0.33
Combined	2.3	2.3	2.2	2.4	0.30
Plant height (cm)					
Halfway	146.5	137.6	151.4	145.1	4.10
College Station	159.8	140.4	160.3	152.7	6.05
Combined	153.2	139.0	155.9	148.9	2.63
Test weight (kg hl^{-1})					
Halfway	71.8	68.1	72.9	75.3	5.50
100 kernel weight (g)					
Halfway	2.9	2.8	2.9	2.9	0.23
College Station	2.7	2.7	2.7	2.6	0.20
Combined	2.8	2.7	2.8	2.8	0.13
Seed number/panicle					
Halfway	1441	1479	1394	1489	97
College Station	1425	1492	1378	1496	120
Combined	1433	1485	1386	1492	110

variances, number of replications per location and number of environments, respectively.

3. Results and discussion

3.1. Individual environment analysis

In both Halfway and College Station, variation among endosperm types was detected only for plant height at $P < 0.05$ and at $P < 0.01$, respectively. No variation due to endosperm type was detectable for grain yield, test weight, 100-kernel weight and seed number per panicle in the individual analysis at each location.

3.2. Combined analysis

As in the individual environments, endosperm type did not affect any measured variables except plant height in the combined analysis (Table 1). Variation due to entry within endosperm type was significant only for grain yield, indicating the individual lines did vary in yield potential. Environment did have an effect on 100-kernel weight only. Furthermore, no endosperm type × environment effects were detected, indicating that the response of the endosperm type was consistent across the tested environments. These results for yield per se are unique for the HD and waxy trait alone, and surprising when the two traits are combined. Initial observations from the introgression of *o2* into adapted germplasm revealed a 25% reduction in yield due to a lower density grain and an increased susceptibility to ear root and storage pests (Vasal, 2000). Similarly the waxy grain trait in sorghum has been reported to reduce sorghum yields by 17% (Rooney et al., 2005) and maize yields in hybrids by 5% (Crane and Slover, 1984; Fergason, 2001). The lower yields attributed to *o2* have now been eliminated via selection of hard flint type maize (Krivanek et al., 2007). In addition, Rooney et al. (2005) did identify waxy sorghum hybrids that yielded as high as non-waxy hybrids. In this study, no differences in the 100-kernel weight were observed, though grain hardness (not measured in this study) is known to be reduced by both the HD and the waxy endosperm traits in sorghum. This result may not be surprising given that assumed reduction of the γ-kafirin in the case of HD sorghum and the loss of GBSS and amylose may not be expected to reduce the grain's sink strength, but shift amino acids and hexose pools to other highly expressed storage protein (prolamin and globulin) and starch (amylopectin) pools. The aforementioned studies drew conclusions for reduced yields based on crosses between only a few parents. The genetic linkage resulting in reduced yields may be unique to the population and parents tested. Had a broader set of parents been used the reduced yields observed in these studies of the waxy and *o2* traits may not have been present.

The endosperm types did consistently differ for plant height with the wildtype group being the tallest while the *wx* endosperm type was the shortest (Table 2). The absence of a genotype × environment interaction indicates that these results were consistent across environments. The results have various potential causes such as pleiotropy between plant height and at least one of the endosperm traits or a genetic linkage between the traits. The reason for the differences in plant height in our study could be confirmed by using a larger population size in the future. The waxy gene is mapped to chromosome 10 of sorghum; no minor height genes have yet been identified on this chromosome. Earlier research suggests that sorghum chromosome 10 has synteny with chromosome 9 of the maize genome where a minor height gene has been identified (McIntyre et al., 2008). As such, there may be a minor

Table 3

Top 10 performing lines among the 100 lines based on average grain yield in the combined analysis in 2010 with LSD value 0.6.

Rank	Pedigree	Average yield (MT ha^{-1})	Endosperm type
1	Tx2907/P850029-WFF2-CS49	3.8	*WX*
2	Tx2907/P850029-WFF2-CS114	3.7	HD-*WX*
3	Tx2907/P850029-WFF2-CS35	3.6	Wildtype
4	Tx2907/P850029-WFF2-CS32	3.5	*WX*
5	Tx2907/P850029-WFF2-CS25	3.5	Wildtype
6	Tx2907/P850029-WFF2-CS84	3.5	*WX*
7	Tx2907/P850029-WFF2-CS89	3.4	HD
8	Tx2907/P850029-WFF2-WE51	3.3	HD-*WX*
9	Tx2907/P850029-WFF2-CS21	3.3	Wildtype
10	Tx2907/P850029-WFF2-CS9	3.3	HD

Table 4

Variance components and broad sense heritability (H^2) estimates for yield components of F_4 lines from (Tx2907/P850029) populations that were evaluated in Halfway and College Station, TX in 2010.

Traits	σ^2_{g}	σ^2_{ge}	σ^2_{error}	H^2 (%)
Grain yield	56,985.8	41,069.5	867,405.5	25.6
Plant height	1122.1	202.3	1167.3	79.1
Test weight	130.6	–	2.0	98.4
100 kernel weight	0.015	0.078	0.005	27.4
Seed number/panicle	3814.9	50,569.6	303,532.5	4.8

σ^2_{g}, σ^2_{ge}, σ^2_{error}, r and e represent the genotype, genotype × environment, error variances, number of replications per location and number of environments, respectively.

height gene close to the waxy gene that has yet to be identified in sorghum.

Rooney et al. (2005) reported that waxy sorghum lines have lower test weights when compared to wildtype sorghum. Our study did not detect significant differences in test weights among the different endosperm types such as HD, HD-*wx*, wildtype and *wx* (Table 2). The test weights were recorded only in one location, however. The 100 kernel weights for all endosperm types were lower in College Station compared to Halfway. This difference in kernel weights in the two locations may be attributed to environmental differences, as the cultural practices were similar in both locations. All the endosperm types included in the study had no significant differences in grain yield, test weight, 100-kernel weight, and seed number per panicle between Halfway and College Station. Though height differences were observed, we did not measure above ground biomass yield. Hence we do not know if there were any shoot/leaf biomass differences between the endosperm types.

3.3. Performance of individual lines

The data presented thus far clearly imply that the properties of the endosperm per se do not affect the average yield potential of a group. However, breeding for hybrid development entails a strict focus on advancing only the most elite genotypes. Therefore, it is important to evaluate all genotypes to determine if specific entries of each genotype are elite and among the highest yielding lines. Since "endosperm type" was not a significant source of variation, an analysis was completed to evaluate genotypes independent of endosperm.

As expected, significant variation for grain yield was detected in Halfway and the combined analysis. Of the 10 highest yielding lines in Halfway, four were HD endosperm type (ranked first, third, seventh and eighth), two were *wx* endosperm type (ranked fifth and sixth), and one was an HD-*wx* endosperm type (ranked ninth). None of the top 10 lines were statistically different from the top yielding line. In the combined analysis, in the top 10 high yielding genotypes, two were HD, three were *wx*, two were HD-*wx* and three were wildtype (Table 3). In the combined analysis, the top 10 yielding lines in both Halfway and College Station were not significantly different from each other. These results suggest that elite HD-*wx* lines can be identified given a large enough population size.

3.4. Heritability estimate

Depending on the trait, the estimates for heritability varied (Table 4). Test weight had the highest heritability of 98.4% where as seed number per panicle had the lowest estimated heritability of 4.8%. Heritability estimate for test weight was calculated only for Halfway, as the data was not available for College Station.

4. Conclusions

The results presented herein indicate that, in the environments sampled, there is no obvious yield penalty when the HD and waxy endosperm traits were combined in the same genetic background. This is in agreement with other recent reports that suggest that waxy grain yields similarly to normal endosperm grain in sorghum, maize, wheat and barley. For example, Pritchett et al. (2005) reported that specialty maize varieties, including waxy maize, yielded higher than normal maize in a study conducted in Indiana. Graybosch et al. (2003) also found that the highest yielding spring waxy wheat cultivar was not significantly different from that of normal spring wheat cultivars. Furthermore, it appears possible to produce specific genotypes that are both HD and waxy and are competitive to normal endosperm lines from the same cross. Finocchiaro et al. (2008) reported that waxy barley yields were lower compared to normal barley types, however the reason was attributed to the waxy barley being naked barley types. Additionally, Rooney et al. (2005) reported the yield of sorghum hybrids with different endosperm types (waxy or non-waxy) were not different, however the best genotypes were always non-waxy or normal endosperm types. It is important to test our observations in hybrid combinations to determine if the trends are consistent in sorghum hybrids. The tests in our study were conducted under favorable environmental conditions with supplemental irrigation. It would also be useful to repeat the study under stress conditions and measure the same characteristics. Replication of the results would imply the possibility to produce high yielding grain sorghums with improved processing, utilization, economic return benefits for the bioethanol and post DDGS feed industry given its high lysine characteristics.

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