A MYCOLOGICAL ASSESSMENT OF HIGHLY DIGESTIBLE PROTEIN SORGHUM LINES

A Thesis

by

OSTILIO ROLANDO PORTILLO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Plant Breeding

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Approved by:

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ABSTRACT

A Mycological Assessment of Highly Digestible Protein Sorghum Lines.

(December 2007)

Ostilio Rolando Portillo, B.S., Universidad Nacional Autonoma de Honduras, Honduras

Chair of Advisory Committee: Dr. Dirk B. Hays

The improved protein digestibility of the highly digestible protein (HD) sorghum lines is attributed to the invaginated shape of the endosperm protein bodies that provides better proteolytic access to the kafirins containing protein bodies. Recent evidence suggests that by virtue of their modified endosperm matrix the HD sorghum lines are more susceptible to the grain mold disease complex (GMDC). This study tests the hypothesis that the HD sorghum endosperm matrix confers a greater susceptibility to grain molds compared to the wild type endosperm matrix in sorghum (i.e. spherical non-invaginated protein bodies).

The parental lines and progeny generated by crosses of two HD lines (P850029 and P851171) with three wild type (WT) sorghum lines (B.Tx635, R.Tx436 and 96GCPOB124) were used in this study. The progeny was advanced through seven generations of self-pollination to develop recombinant inbred lines (RILs). The RILs were grown in five locations in Texas during 2005 (College Station, Weslaco, Beeville, Corpus Christi and Halfway) and two in 2006 (College Station and Weslaco). Finally, grain samples were analyzed using a protease turbidity assay to estimate their level of protein digestibility and classify the RILs into digestible groups (DGs).

Several caryopsis characteristics associated with the grain mold disease (seed hardness, endosperm texture, thousand kernel weight, starch content and germination) were also analyzed to estimate the resistance of the HD lines to grain molds. The HD lines susceptibility to grain molds was measured using a threshed grade score and a mycoflora analysis. These studies revealed that the HD sorghum lines have caryopsis characteristics associated with a higher susceptibility to grain molds and significant differences were found between the HD lines and the WT sorghums lines when compared on the basis of fungal incidence providing statistical evidence in support of the tested hypothesis. However, the germination analyses provided evidence that a higher susceptibility to grain mold infection did not render the HD RILs more vulnerable to grain mold damage.

The analysis of molds pathogenicity revealed that although several pathogens were isolated from the grain's internal mycoflora not all of them were found significantly and meaningfully associated with the GMDC.

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I would like to thank God for his blessings which made this cultural and academic experience at Texas A&M University a reality. I would like to express my gratitude to Dr. Dirk B. Hays, chair of my advisory committee, for his support during research and thesis preparation. Individuals deserving gratification are Dr. Lloyd W. Rooney for providing valuable information and logistical support during the research stage, Dr. William L. Rooney for his suggestions during statistical evaluations and results interpretation, Dr. Louis K. Prom, Dr. Brian D. Shaw, and Dr. Thomas Isakeit for their help with the mycoflora analysis, which represents a major part of this thesis.

Finally, I want to express my deepest gratitude to Wendy W. Dye for her support and dedication during the long hours required to prepare this thesis.

NOMENCLATURE

ANOVA-	Analysis of Variance
DG-	Digestible Group
GMDC-	Grain Mold Disease Complex
hr-	Hour
HD-	Highly Digestible Protein
Fisher's LSD-	Least Significant Difference Test
min-	Minute
M-	Molar
MD-	Medium Digestible Protein
NIR-	Near Infrared Reflectance Spectrophotometer
ND-	Normally Digestible Protein
PDA-	Potato Dextrose Agar
RIL-	Recombinant Inbred Line
RCBD-	Randomized Complete Block Design
$r \cdot min^{-1}$-	Revolutions Per Minute
SKHT-	Single Kernel Hardness Test
TCA-	Trichloro Acetic Acid
Tukey's HSD-	Tukey's Honestly Significant Differences Test
WT-	Wild Type
µL-	Microliter

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CHAPTER I

INTRODUCTION

The highly digestible protein (HD) sorghum lines have a high *in vitro* and *in vivo* digestibility of their endosperm protein fraction. This high digestibility is attributed to the invaginated conformation of endosperm protein bodies which is thought to provide the proteases easy access to the kafirin containing protein bodies. The HD lines are digested more efficiently (catabolism) and their amino acids are more readily available. In addition to this phenomenon, the highly digestible sorghum lines have a higher lysine content. The combination of these factors makes the HD lines a suitable option for the nutrition of monogastrics and polygastrics.

The grain mold disease complex (GMDC) could be a major factor that limits the practical use of HD sorghums because the soft grain deteriorates more readily. The GMDC is ubiquitously distributed and negatively affects sorghum yield and overall grain quality particularly in warm and humid environments. The grain mold disease is considered a complex problem due to the diversity of genera of pathogens that cause the disease, as well as our incapacity to control the environmental factors (temperature and humidity) that promote their incidence and severity.

Several strategies have been devised to decrease the level of grain mold damage while still in the field. One of the most practical and economically feasible strategies is the development of tolerant cultivars. That said, little has been accomplished in developing HD sorghum lines with an acceptable level of grain mold tolerance. As a

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This thesis follows the style of Cereal Chemistry.

result, there is no information regarding the level of mold resistance in HD lines, the adaptability of HD lines to environments that favor grain mold or the combinability of the HD trait with grain mold resistance.

Our hypothesis is that as a result of the modified endosperm matrix the HD lines are more susceptible to the GMDC than wild type sorghum lines. This assumption is based on the argument that the HD endosperm protein matrix due to its invaginated protein bodies provides greater access of fungal proteolytic and glycolytic enzymes to the endosperm nutritional resources resulting in a higher susceptibility to grain mold infection and damage.

The specific objectives of this project are:

1. Determine if the HD sorghum lines are preferentially more susceptible to the grain mold disease versus WT sorghum RILs.
    a. Identify the HD and the WT sorghum lines in the RILs by determining the level of protein digestibility through a protease digestion assay as described by Aboubacar et al., (2003).
    b. Determine differences between the HD and the WT sorghum lines based on caryopsis' characteristics associated with the grain mold disease.
    c. Determine differences between the HD and the WT sorghum lines based on grain damage and susceptibility to fungal infection.
2. Identify the pathogens (genera and species) responsible for the grain mold disease, their importance to the grain mold disease and preferential pathogenicity to HD versus WT sorghum RILs based on caryopsis quality and germination.

CHAPTER II

SUSCEPTIBILITY OF THE HD SORGHUM LINES TO THE GRAIN MOLD DISEASE COMPLEX (GMDC)

1. Introduction.

1.1. The highly digestible protein (HD) sorghum lines. Among cereals, sorghum is one of the most important grains due to its capacity to adapt to a wide range of environments including those with semi-arid conditions (Klein et al., 2001). It also constitutes a main source of protein to millions of people in Africa, Asia and Latin America where it is grown (Aboubacar et al., 2003).

Sorghum is a versatile cereal used in a variety of applications such as animal feed, alcoholic and non-alcoholic beverages and foods (MacLean et al., 1981; Beta et al., 1999). Sorghums nutritional value, however, is negatively affected by its low content of essential amino acids, specifically lysine (Shull et al., 1992), and by the documented low digestibility of its protein. According to the *in vivo* studies conducted by MacLean et al., (1981) on pre-school children whose ages ranged between 6 months and 2.5 years, cooked sorghum had the lowest rates of protein absorption and retention compared to other protein sources such as maize, potatoes, wheat and rice. While the study showed the low availability of the sorghum polypeptides; it provided no clarification on the reasons for such phenomenon.

In the sorghum grain, the endosperm accounts for approximately 82 percent of the seed structure (Hoseney, 1994). It is comprised of cells containing protein bodies enmeshed in a protein matrix that surrounds the larger starch granules (Kulp K. & Ponte

J.G., 2000). More information about physical structures of the sorghum kernel is presented by Rooney & Miller (1982). The protein bodies consist of prolamins storage proteins, termed kafirins in sorghum (Hoseney, 1986). Kafirins constitute between 77 to 82 percent of the total protein content in the kernel endosperm (Belton et al., 2006).

Similar to the classification of prolamins (zeins) in maize, kafirins are classified based on their configuration, molecular weight and alcohol solubility properties. α-Kafirins (*M*r 25,000 and 20,000) are soluble in 95 percent ethanol, while β-kafirins (*M*r 20,000, 18,000, and 16,000) are soluble in lower concentrations of alcohol (10-60 percent). Lastly, γ-kafirins (*M*r 28,000) are soluble in the presence of a reducing agent such as 2-mercaptoethanol (Shull et al., 1991).

Several plausible explanations have been proposed for the low protein digestibility of sorghum. Some explanations propose that the interaction of the prolamins with polyphenolic tannins, inositol hexaphosphate (phytic acid), cell wall constituents and starch render them less digestible. Others take into account the inherent properties of the proteins and the interactions among themselves in response to pH or temperature changes in the surrounding environment (Duodu et al., 2003). The protein properties and interactions may include protein hydrophobicity, crosslinkings and changes in the polypeptides structure (Duodu, et al., 2003). The presence of tannins and protein cross-linking will be discussed further in the next section (1.2 and 1.3).

1.2. Tannins reduce protein digestibility. Sorghum cultivars are classified as type II based on the extractability of tannins in acidified methanol and localization in the pigmented testa. Others are classified as type III because the tannins are extractable in

both acidified and non-acidified methanol and are localized in both the pigmented testa and the pericarp (Earp et al., 2004b; Waniska et al., 2001).

Tannins are secondary plant metabolites localized in the inner integument generally referred as the seed coat or testa which is found between the pericarp and the aleurone layer (Earp et al., 2004a; Earp et al., 2004b). Tannins are bitter-tasting plant polyphenolic compounds rich in hydroxyls and carboxyl groups. Although tannins confer desirable agronomic characteristics such as grain mold disease resistance/tolerance and protection against birds and insects; they are undesirable due to ability to bind and precipitate proteins during digestion (Duodu et al., 2003). Tannins form tannin-protein complexes via hydrogen bonding, hydrophobic association or covalent bonding (Nyachoti et al., 1996). Protein precipitation *per se* via insoluble tannin-protein complexes could be a desirable outcome depending on the intended application. In brewing, the formation of tannin-protein complexes precipitates the soluble proteins and tannins which otherwise produce reddish and brown pigments in the beer (Hoseney, 1986). On the other hand, the same phenomenon leads to a reduction of the nutritional value of sorghum proteins in feeds and foods since the insoluble tannin-protein complexes render the polypeptides resistant to proteases. However, recent evidence of low protein digestibility among low and tannin free cultivars (Elkin et al., 1996) suggests the existence of other mechanisms responsible for the reduced protein digestibility.

1.3. Protein cross-linking reduces protein digestibility. α-Kafirins are localized in the lightly stained zones inside the protein bodies; whereas, the β-kafirins are found in

both the light and dark stained peripheral zones. Similar to the β-kafirins, the γ-kafirins are also localized in the peripheral zone as well as in the dark stained inclusions inside of the protein bodies (Shull et al., 1992). Since both β and γ-kafirins are rich in the sulfur containing amino acid cysteine (5 & 7 mol % respectively) and β-kafirins have a significant methionine content (5.7 mol%) (Shull et al., 1992), they may form polypeptide polymers via disulphide bonds at the periphery of the protein body. This may act as a protease resistant barrier blocking access to the α-kafirins located inside the protein bodies during extreme environmental conditions such as high temperatures (Oria et al., 1995). This hypothesis is supported by the findings of Hamaker et al., (1986) who used an *in vitro* approach of raw and cooked whole grain sorghum flour samples to show that the protein digestibility of the raw samples plummeted around 15 percent after cooking (Hamaker et al., 1986). Likewise, reducing agents which block disulphide bonds increases the digestibility of the sorghum proteins by 11 and 25 % respectively (Hamaker et al., 1987).

The recently discovered high digestible (HD) protein sorghum mutant which has a higher lysine content (60 percent) (Weaver et al., 1998) and high protein digestibility in cooked and raw flour samples (Oria et al., 2000), provides one possible solution to the low protein bioavailability of sorghum.

The improved protein availability of HD lines has been related to invaginations in the protein bodies compared to normal spherical WT protein bodies. Additionally, the γ-kafirins are mostly present in the inclusions at the base of the folds, and less at the periphery of the protein bodies compared to WT sorghum (Oria et al., 2000).

The higher protein availability of the HD sorghum mutant is hypothesized to be a consequence of the γ-kafirin modified localization. Reduced in content and at the base of the invaginations, the number of protein polymers formed via disulphide bonds is thought to be reduced at the enzyme-resistant barrier. As such, the catabolism of α-kafirins during enzyme digestion is increased (Oria et al., 2000).

Recent results (data not published) based on viscosity analysis showed that HD sorghum lines also have a higher level of starch bioavailability providing evidence of a possible association between protein and starch digestibilities. In other words, these outcomes support the hypothesis that as the protein digestibility increases the starch digestibility also increases. The synergic action of a higher lysine content, improved protein digestibility, and higher starch bioavailability not only showed the nutritional advantages of the HD lines, but a higher availability of fermentable carbohydrates also suggests their potential for industrial applications such as ethanol production. In contrast, a higher starch bioavailability may also lead to a higher level of grain mold deterioration in the field; however this hypothesis has not yet been tested. Unfortunately, due to the lack of breeding efforts it is not known if the development of HD sorghum lines with an acceptable level of grain mold resistance could be achieved.

1.4. The grain mold disease complex (GMDC). The GMDC of sorghum develops from fungal colonization during fertilization during early caryopsis development or at maturity. The latter is referred as grain weathering and both are correlated with warm and humid conditions. Grain mold is a disease complex since it is caused by multiple saprophytic and/or facultative parasitic fungal genera and species (Esele et al., 1993). As

a result of the growth and development of the GMDC, kernel milling, yield and quality of the grain for feed or food is negatively affected (Bejosano et al., 2003). The GMDC can reduce 1,000-kernel weight, kernel size, seed viability, nutritional quality and alter grain appearance (grain surface discoloration). All of which reduces market value (Menkir et al., 1996).

Many physical and chemical caryopsis traits have been associated with resistance to grain mold. These traits include a dominantly inherited red and brown pericarp, (Menkir et al., 1996), a thin mesocarp regulated by the presence and number of starch granules, the presence of a pigmented inner integument (Esele et al., 1993), grain hardness (Jambunathan et al., 1982), polyphenolic tannins and flavan-4-ols (Menkir, 1996), panicle shape (Rao, & Rana, 1989), greater plant height and dark glumes (Audilakshmi et al., 1999).

Resistance in dark pericarps is associated with high amounts of flavan-4-ols (Menkir et al., 1996). Consistent with this hypothesis sorghum genotypes which maintain high levels of flavan-4-ols during grain maturity were associated with mold resistance. Additionally, genotypes in which a significant decline in the flavan-4-ols concentration was observed were susceptible to the grain mold disease (Melake-Berhan et al., 1996). Unfortunately, colored sorghum cultivars have a low consumer acceptance especially in parts of the world where it is used as a food (Bandyopadhyay et al., 1988).

The pigmented testa is a kernel micro structure located between the pericarp and the aleurone cell layer (Earp et al., 2004a; Earp et al., 2004b; Bandyopadhyay et al., 1988). It acts as a physical barrier due to the high tannin content found in the structure

which restrains disease development by inhibiting spore germination and fungal growth (Esele et al., 1993). Tannins, however, also precipitate proteins and consequentially reduce the nutritional value of the sorghum grain. As such, sorghum cultivars with pigmented testa (type II and III) have not been pursued (Rodriguez-Herrera et al., 2000).

The association of the grain hardness and resistance to grain mold has been well documented. Jambunathan et al., (1982) determined the grain hardness of several sorghum accessions along with grain mold damage and labeled them as *colored resistant with pigmented testa, colored susceptible without pigmented testa, colored resistant without pigmented testa*, *white susceptible with pigmented testa*, and *white resistant without pigmented testa*. They found that resistant genotypes have harder grains. The opposite was confirmed for the susceptible genotypes. Finally, Jambunathan et al., (1982) hypothesized that the resistance displayed by the *white resistant without pigmented testa* accessions is attributed to their grain hardness based on their lack of flavan-4-ols and tannins. This outcome could be of great importance given the general interest of developing white sorghum cultivar without tannins.

It has been hypothesized that the HD sorghum lines are more susceptible to grain mold infection and deterioration as a result of their soft and floury endosperms; however, due to the lack of information this assumption has not yet been clarified. In this chapter, this hypothesis will be tested to confirm or deny such assumption.

In sorghum, several approaches have been developed for measuring the response to the grain mold disease such as field grade score, threshed grade score, ergosterol

content, percent germination (Audilakshmi et al., 1999) 1,000 kernel weight, and myco-flora analysis.

The field grade score consists of a visual examination and evaluation of the panicles from each genotype under study. The panicles after harvest are visually scored using a 1 to 5 scale to describe the grain mold severity on the panicle surface, where 1 corresponds to no mold damage is observed and 5 corresponds to cases in which more than 50 percent of the kernels surface in the panicle is covered by molds (Audilakshmi et al., 1999). The threshed grade score uses the same score on threshed grain (Audilakshmi et al., 1999). Ergosterol content is used to estimate the biomass of the microorganisms colonizing the grain. Ergosterol is a sterol functional group on fungal cell membranes. Its concentration provides direct assessment of the fungal content in sorghum flour (Jambunathan et al., 1982). Seed mycoflora analysis on the other hand is used to identify the grain mold disease fungal genera and species associated with the infected samples (Prom, 2004).

2. Methodology.

2.1. Plant material. Three families with 4 distinct RILs lines each were developed by crossing two HD lines (P850029 and P851171) with three WT lines (B.Tx635, R.Tx436 and 96GCPOB124). The HD RILs were selected as parental lines based on their high protein digestibility whereas the WT RILs were selected because of their high and moderate grain mold disease resistance. The RILs were grown in 5 different locations in Texas (Beeville, College Station, Weslaco, Corpus Christi and Halfway) in 2005 and

two locations (College Station and Weslaco) in 2006. As a result, grain samples were collected from seven different environments (location*year) for subsequent analysis.

2.2. Identification of highly digestible protein (HD) sorghum lines. The RILs (parental lines and offspring) were first phenotyped using the protease turbidity assay developed by Aboubacar et al., (2003) (described below). Seed harvested from Weslaco, Texas in 2005 was used based on its low disease incidence. The results were used to distribute the RILs into digestible groups (DGs) across environments. From a statistical view point, a digestible group was defined as one or more RILs which absorbances (after 60 min of dilution in 72% TCA) are not significantly different according to Tukey's HSD at a 0.05 level of significance.

Protease Turbidity Assay. Grain samples were ground to 1 mm flour particles using a UDY cyclone lab sample mill. 50 mg. of sorghum flour from each RIL was weighed into 2 ml Eppendorf tubes and diluted in 1 ml of pepsin solution (20 mg. of pepsin/ml of 0.1 *M* KH_2PO_4, pH 2.0). The samples were agitated and digested for 1 hr in a water bath calibrated at 37^{o}C. After stopping the reaction by adding 100 µL of 2N NaOH, the solution was centrifuged for 10 min at 14,000 $r \cdot min^{-1}$ and the digested protein containing supernatant was discarded. The recovered pellets were resuspended in 1 ml of 0.1 *M* KH_2PO_4 (pH 7.0), vortexed, centrifuged for 10 min at 14,000 $r \cdot min^{-1}$, and the supernatant was again discarded. A final wash was performed using double distilled autoclaved water and the pellet was stored at -20^{o}C for further protein extraction.

Proteins extraction. To extract the remaining proteins, 50 mg of digested flour sample from each RIL was weighed into 2 ml Eppendorf tubes and diluted in a protein

extraction buffer made of 0.5 ml of 0.0125M sodium tetraborate buffer (pH 10.0) containing 1% sodium dodecyl sulfate (w/v) and 0.5% 2-mercaptoethanol (v/v) for 1 hr at room temperature with shaking. Finally, the mixtures were centrifuged for 10 min at 14,000 $r \cdot min^{-1}$ to recover the protein containing supernatants which were stored at -20^{o}C for further analysis.

Turbidity Measurements. A 25 µL sample of protein extract from each RIL was transferred into 2 ml Eppendorf tubes where they were diluted and vortexed in 1 ml of purified water and 200 µL of 72% of TCA. The A 562 of each sample was recorded at 5, 30, 45 and 60 min using a Spectrophotometer. Prior measuring absorbances, the Spectrophotometer was recalibrated every time using a protein-free sample prepared with 25 µL of protein extraction buffer, 200 µL of 72% TCA, and 1 ml of purified water.

2.3. Determination of physical RIL grains' characteristics. Three replicates of 300 sorghum kernels were each used to estimate the average seed hardness and 1,000 kernel weight of each RIL across environments using the single kernel hardness test (SKHT) (Perten Single Kernel Characterization System SKCS 4100, Perten Instruments, Springfield IL).

Near-infrared reflectance spectrophotometry (NIR) was used to determine the average grain starch content of the RILs (parental lines and offspring) in three separate replicates across environments (Perten PDA 7000 Dual Array with Grams Software, Perten Instruments, Springfield IL).

An endosperm texture index was used to phenotype the RILs using grain harvested from Weslaco, Texas in 2005 based on the low disease incidence. The

endosperm texture was described using a 1 to 5 scale visual examination of longitudinal half kernels as described by Rooney & Miller (1982) where 1 and 2 corresponds to a flinty endosperm and 5 to a chalky endosperm. The results were extrapolated across environments.

2.4. Evaluation of the level of susceptibility to GMDC. The RILs and DGs susceptibility to the GMDC was measured using three different approaches: a threshed grade score, a mycoflora analysis and germination test.

The Threshed Grade Score. The RILs and DGs level of susceptibility to the GMDC was determined through visual evaluations of clean grain samples across 5 environments in Texas using the seed collected in 2005. The test was conducted using a 1 to 9 scale where 1 corresponds to no observable damaged and 9 corresponds to 100 percent of the grain surface covered by molds.

The Mycoflora Analysis. A modified mycoflora analysis (Prom, 2004) was used to measure the RILs and DGs susceptibility to the GMDC. Briefly, seed from each RIL was surface disinfected first by rinse in running deionized water for 30 min, then by immersion with shaking in a 10% Clorox solution for 3 min, in 95% ethanol for 5 min, and three times in double distilled water for 15 min each. After dried overnight in a laminar air flow cabinet, the seeds were incubated for 7 to 10 days at 25^{o}C in Petri dishes containing half-strength potato dextrose agar (PDA) medium. The identification of the fungal genera and species was based on conidia, conidiosphores, colony morphology and color. Finally, a randomized complete block design (RCBD) with 15 replications per RIL was used and the combined analysis performed on the data collected

from 5 environments (Beeville 2005, College Station 2006, Corpus Christi 2005, Halfway 2005 and Weslaco 2005) based on consistency of results.

The Germination Test. The RILs and DGs level of susceptibility to the GMDC was measured by analyzing their germination potential across 7 environments in Texas using the seed collected in 2005 and 2006. The experiment was conducted in three separate replicates in soil containing trays to emulate field conditions.

2.5. Statistical analysis and interpretation. SPSS and SAS were used to estimate correlations (Pearson product moment correlations), compare the means of the DGs (Contrast), and detect significant differences between RILs, and DGs via analysis of variance (ANOVA) and test for significance by Tukey's HSD test.

Identification of HD Sorghum Lines. The results of the protease turbidity assay were recorded and used to phenotype the RILs based on turbidity absorbance using a one-way ANOVA under the following hypothesis:

Hypothesis: H_o= the absorbance mean of all RILs are equal.

H_a= at least one RIL is different.

Model: $Y= \mu + \alpha_i + error$

Where Y = absorbance (nm).

μ = intercept.

α_i = RIL effect.

A Tukey's HSD test was used to identify the significant differences and group the RILs into DGs.

Determination of RIL Grain Physical Characteristics

a. The data collected form the SKHT, NIR, endosperm texture index, and germination test was analyzed using Pearson correlations to estimate associations between traits and absorbance across environments under the following hypothesis:

   $\rho_{xy} = 0$

   $\rho_{xy} \neq 0$

   Where X= absorbance (nm).

   Y= seed hardness index, germination rate (%); 1,000 kernel weight (g), starch content (%), and endosperm texture index.

b. An ANOVA factorial design was used to detect significant differences between the RILs, and DGs across environments on the basis of seed hardness, thousand kernel weight, starch content and endosperm texture where the main and interaction effects were estimated by an all fixed model under the following hypothesis:

   H_o= the mean of all RILs/DGs are equal.

   H_a= at least one RIL/DG is different.

   Model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha_i*\beta_j + error_{ij}$

   Where Y = seed hardness index, endosperm texture index, thousand kernel weight (g), starch content (%).

   μ = intercept.

   α = RIL/DG effect.

   β = environment effect.

   $\alpha_i*\beta_j$ = RIL*environment interaction effect.

DG*environment interaction effect.

A Tukey's HSD test was used to identify the significantly different RILs and DGs.

c. Significant differences between DGs based on grain' physical characteristics (seed hardness index, endosperm texture index, thousand kernel weight and starch content) were identified via contrast analyses. The selected contrasts were tested under the following hypotheses:

HD vs. MD

H_o: $l = \mu_1 - \mu_2 + 0\mu_3 = 0$

H_a: $l = \mu_1 - \mu_2 + 0\mu_3 \neq 0$

MD vs. ND

H_o: $l = 0\mu_1 + \mu_2 - \mu_3 = 0$

H_a: $l = 0\mu_1 + \mu_2 - \mu_3 \neq 0$

HD vs. ND

H_o: $l = \mu_1 + 0\mu_2 - \mu_3 = 0$

H_a: $l = \mu_1 + 0\mu_2 - \mu_3 \neq 0$

Where: μ_1= HDμ

μ_2= MDμ

μ_3= NDμ

Evaluation of the Level of Susceptibility to GMDC

a. The data collected from the threshed grade score, the mycoflora analysis and the germination test, was examined individually to detect significant differences between the RILs and DGs across environments using an ANOVA factorial design where the main and interaction effects were estimated by an all fixed model under the following hypothesis:

H_o= the mean of all RILs/DGs are equal.

H_a= at least one RIL/DG is different.

Model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha_i*\beta_j + error_{ij}$

Where Y = threshed grade score index, fungal incidence (number of fungal colonies), germination (%).

μ = intercept.

α = RIL/DG effect.

β = environment effect.

$\alpha_i * \beta_j$ = RIL*environment interaction effect.

DG*environment interaction effect.

A Tukey's HSD test was used to identify the significantly different RILs and DGs.

b. Significant differences between DGs based on germination rate, threshed grade score and fungal incidence were identified via contrast analyses. The selected contrasts were tested under the following hypotheses:

HD vs. MD

H_o: $l = \mu_1 - \mu_2 + 0\mu_3 = 0$

H_a: $l = \mu_1 - \mu_2 + 0\mu_3 \neq 0$

HD vs. ND

H_o: $l = \mu_1 + 0\mu_2 - \mu_3 = 0$

H_a: $l = \mu_1 + 0\mu_2 - \mu_3 \neq 0$

MD vs. ND

H_o: $l = 0\mu_1 + \mu_2 - \mu_3 = 0$

H_a: $l = 0\mu_1 + \mu_2 - \mu_3 \neq 0$

Where: μ_1= HDμ

μ_2= MDμ

μ_3= NDμ

3. Results.

3.1. Identification of highly digestible protein (HD) sorghum lines.

Protease Turbidity Assay. The statistical analysis of recorded absorbances after 60 min of dilution in 72% TCA detected the presence of highly significant differences among

the RILs (Table I). As a result the RILs were classified into DGs identified as highly (HD), medium (MD) and normally digestible protein (ND) (Figure 1).

Table I: Mean squares from ANOVA of recombinant inbred lines based on turbidity at 60 min

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
RILs	1.246	17	0.073	68.589	0.000

Level of significance= 0.05
R Squared = 0.968 (Adjusted R Squared = 0.954)

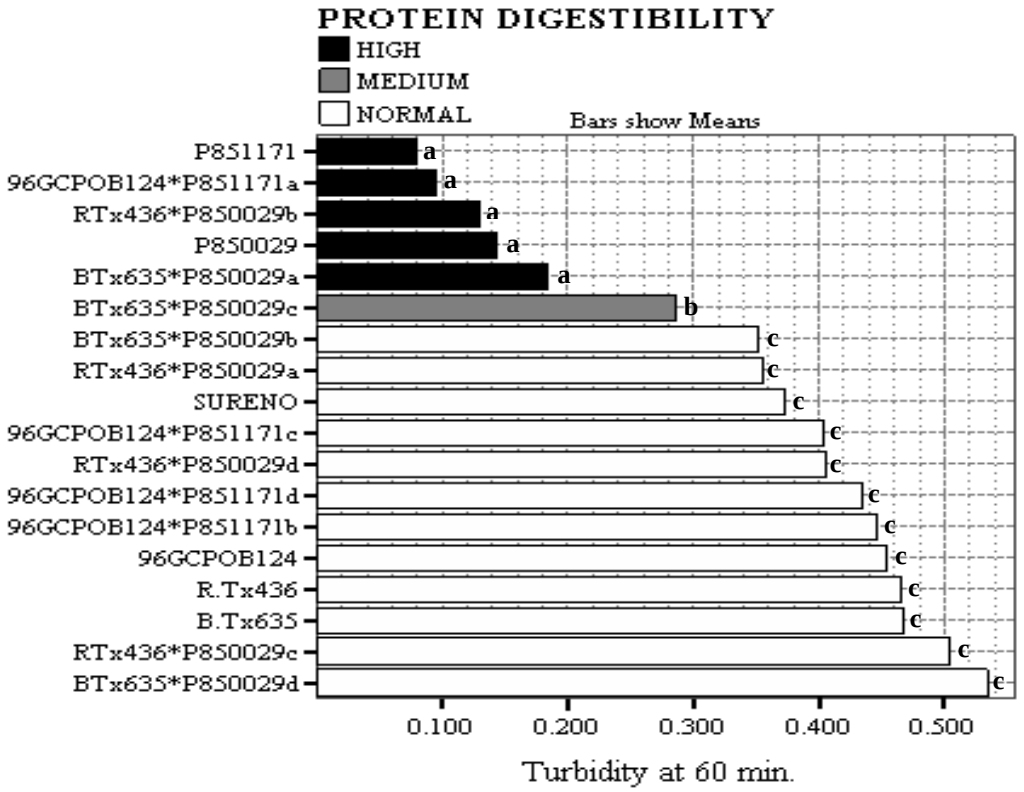

Figure 1: Digestible groups based on the turbidity assay.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

3.2. Determination of physical RIL grains' characteristics.

Endosperm Texture Index. The endosperm texture was evaluated using clean grain samples of RILs grown in Weslaco 2005 based on its low disease incidence. The results were extrapolated to the remaining 6 environments for subsequent correlation to other caryopsis traits. The index was assigned using a 1 to 5 scale visual examination of

longitudinal half kernels as described by Rooney & Miller (1982) where 1 and 2 corresponds to a flinty endosperm, 3 and 4 correspond to an intermediate texture and 5 corresponds to a chalky endosperm texture (Figure 2). The DGs analysis revealed highly significant differences between them (Table II). Additionally, Tukey's HSD grouped them as follow: HD > MD, ND (Figure 3). This outcome was corroborated via contrast among the DGs (Table III). These results propose that as the protein digestibility increases the endosperm texture becomes flourier. This hypothesis seems to be supported by the highly significant correlation (-0.914**) between the level of turbidity and the endosperm texture index of the RILs under study (Table IV).

Table II: Mean squares from ANOVA of digestible groups based on endosperm texture index.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
DG	105.250	2	52.625	1033.364	0.000

Level of significance= 0.05
R Squared = 0.975 (Adjusted R Squared = 0.974)

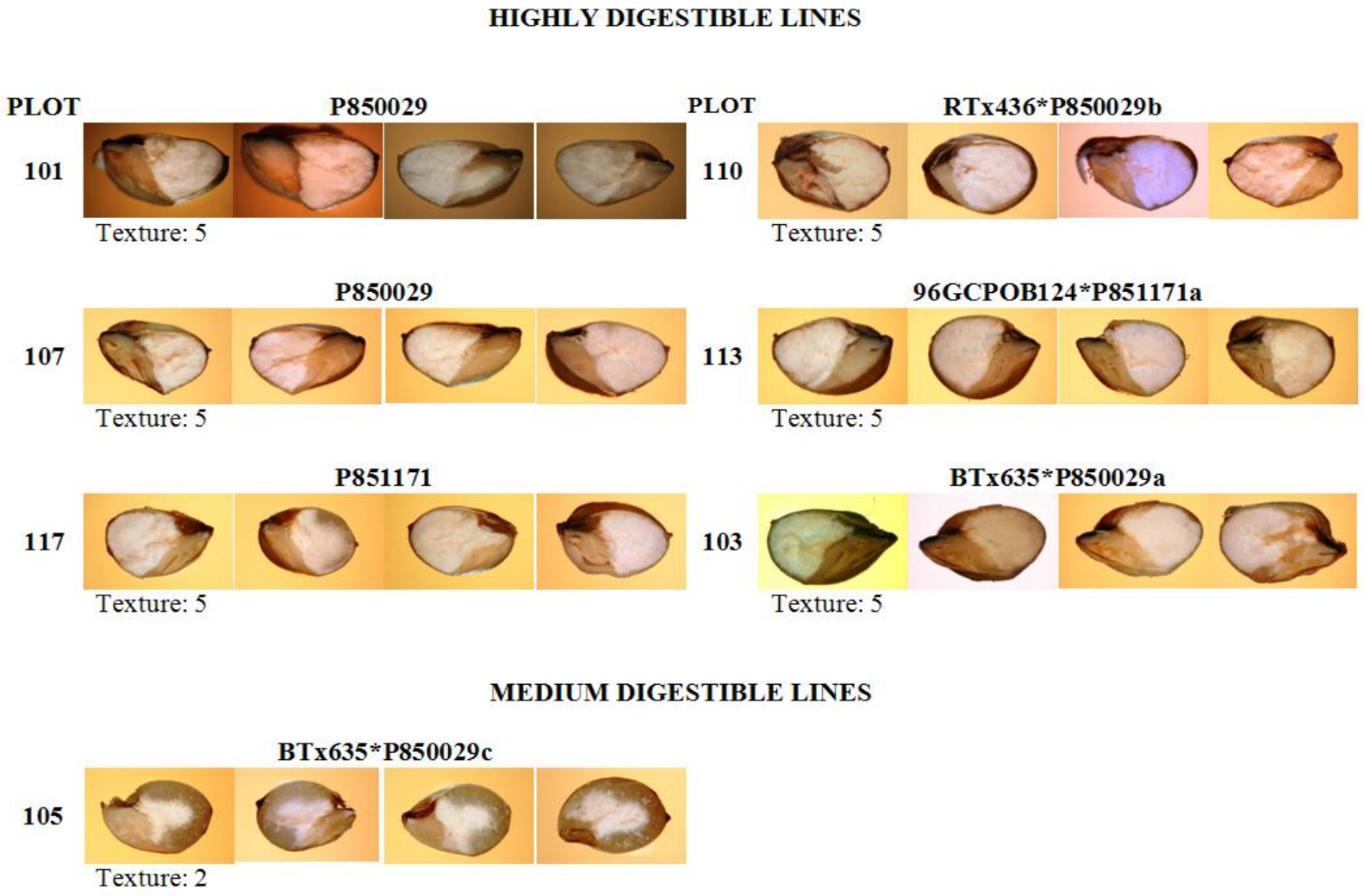

Figure 2: Endosperm texture index.
1 and 2: flinty endosperm, 3 and 4: intermediate texture, and 5: chalky endosperm texture.

Figure 2: Continued.

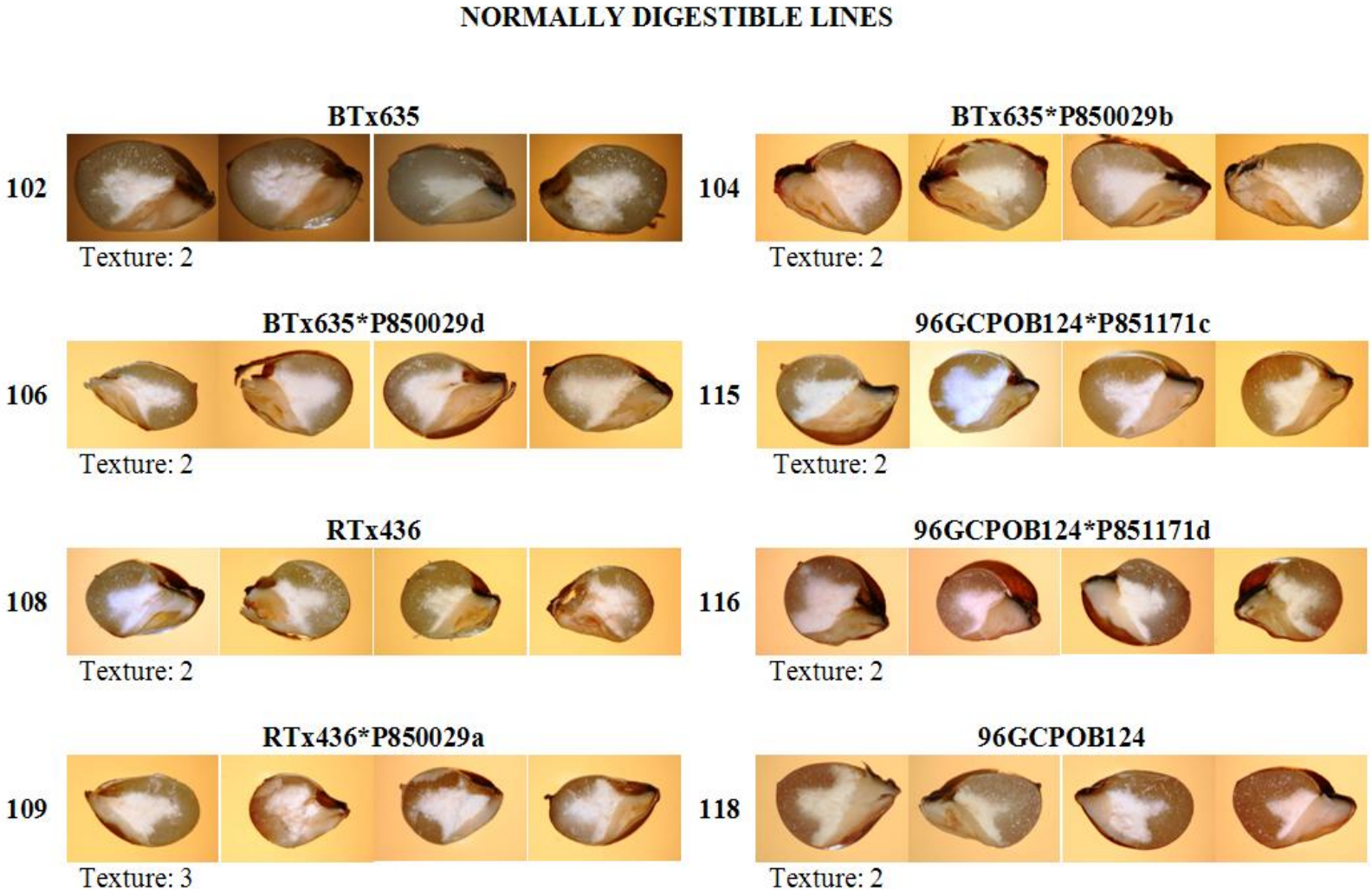

Figure 2: Continued.

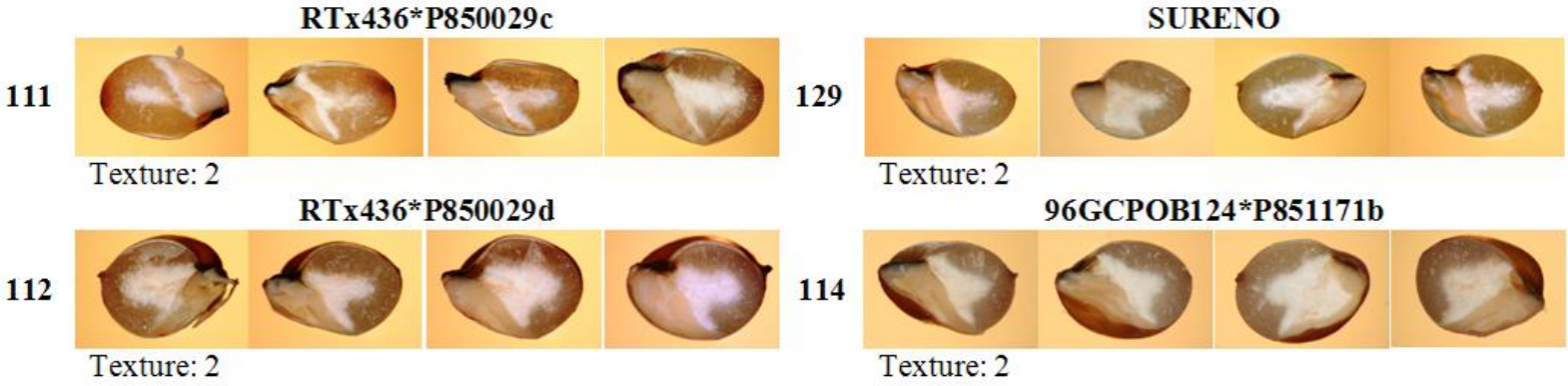

Table III: Means squares from contrast of digestible groups based on seed hardness index, starch content, thousand kernel weight and endosperm texture index.

Source	Seed hardness index	Starch content	Thousand kernel weight	Endosperm texture index.
HD vs. MD	39573.0148**	349.818561**	123.460729**	210.3582090**
HD vs. ND	259285.3733**	1389.080333**	1787.203597**	963.0271084**
MD vs. ND	1479.9379**	2.076907	77.207483**	0.1730769

Notes: **significant at the 0.01 level.

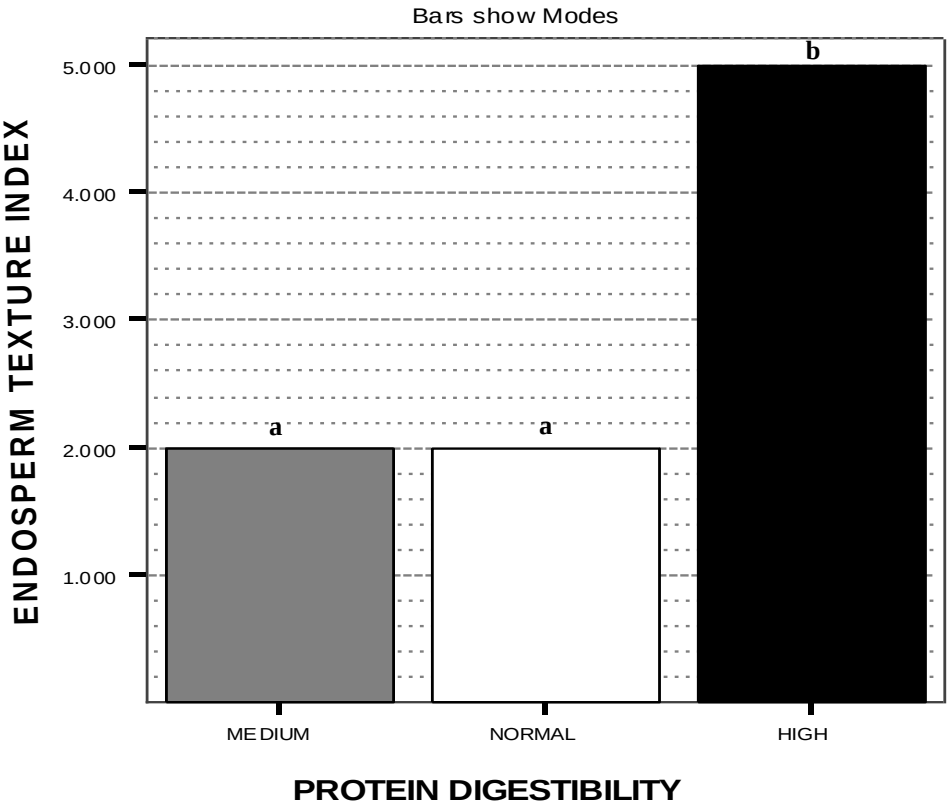

Figure 3: Endosperm texture index per digestible group.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

Table IV: Correlations of the recombinant inbred lines' grain physical characteristics across environments.

	Turbidity at 60 min	Starch (%)	Germination (%)	Thousand kernel weight (g)	Hardness Index	Endosperm Texture Index	Threshed Grade Score	Fungal Incidence
Turbidity at 60 min	1	0.565(**)	-0.072	0.550(**)	0.792(**)	-0.914(**)	-0.229(**)	-0.074
Starch (%)		1	-0.078	0.362(**)	0.638(**)	-0.591(**)	-0.076	0.101(*)
Germination (%)			1	0.073	0.195(**)	0.014	-0.489(**)	-0.120(**)
Thousand kernel weight (g)				1	0.478(**)	-0.520(**)	-0.193(**)	-0.065
Hardness index					1	-0.884(**)	-0.418(**)	-0.123(**)
Endosperm texture index						1	0.228(**)	0.078
Threshed grade score							1	0.423(**)

Notes: **Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

Single Kernel Hardness Test (SKHT). The SKHT was used to determine the grain hardness of the RILs and DGs. The RILs combined analysis (fixed model) across seven environments revealed highly significant genotype, environment, and genotype*environment effects (Table V). Tukey HSD grouped the RILs into significant difference groups (Figure 4). The DGs combined analysis revealed highly significant DG and environment effects, but no significant DG*environment effect was detected suggesting that the DGs' grain hardness does not change across environments (Table VI). Tukey HSD grouped them as follow: ND > MD > HD (Figure 5). This outcome was corroborated via contrast among the DGs (Table III). According to these results, the ND RILs have the hardest grains, the MD RIL is intermediate grain hardness and the HD RILs have the softest grains suggesting that as the protein digestibility increases the grain hardness decreases. This hypothesis seems to be supported by the highly significant correlation (0.792**) between the level of turbidity and the hardness index of the RILs under study (Table IV). Finally, it could be hypothesized that the softness of the HD RILs occurs as a consequence of the highly significant correlation (-0.914**) between the level of turbidity and the endosperm texture index (Table IV). Since the HD RILs have the least densely packed endosperm matrix, they also have the softest grains.

Table V: Mean squares from ANOVA combined analysis of recombinant inbred lines based on seed hardness index, starch content and thousand kernel weight.

Source	Hardness index	Starch content	Thousand kernel weight
RIL	14894.436**	130.967**	263.683**
Environment	4041.099**	59.614**	125.947**
RIL * Environment	169.926**	5.093**	7.443**
Error	4.763	1.078	0.552
Adjusted R^2	0.993	0.859	0.960

Notes: **significant at the 0.01 level.

Table VI: Mean squares from ANOVA combined analysis of digestible groups based on seed hardness index, starch content and thousand kernel weight.

Source	Hardness index	Starch content	Thousand kernel weight
DG	118201.468**	612.312**	744.913**
Environment	1584.597**	16.464**	54.680**
DG * Environment	132.773	5.149	5.960
Error	91.861	4.106	8.883
Adjusted R^2	0.862	0.463	0.352

Notes: **significant at the 0.01 level.

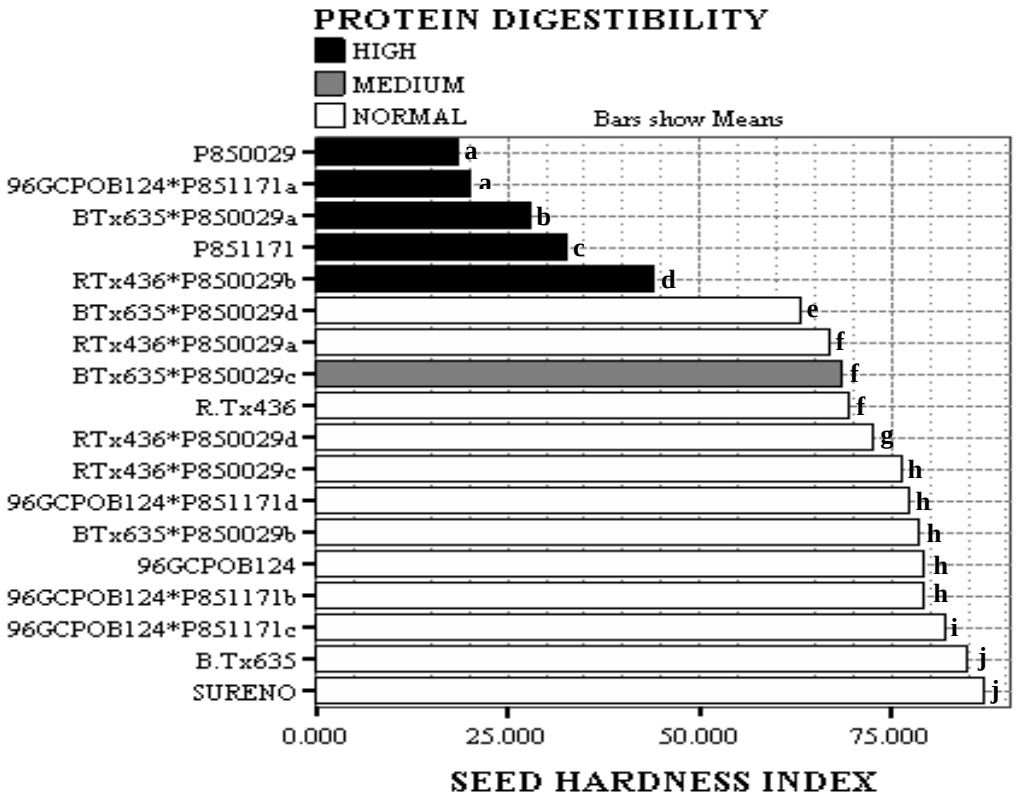

Figure 4: Single kernel hardness test per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

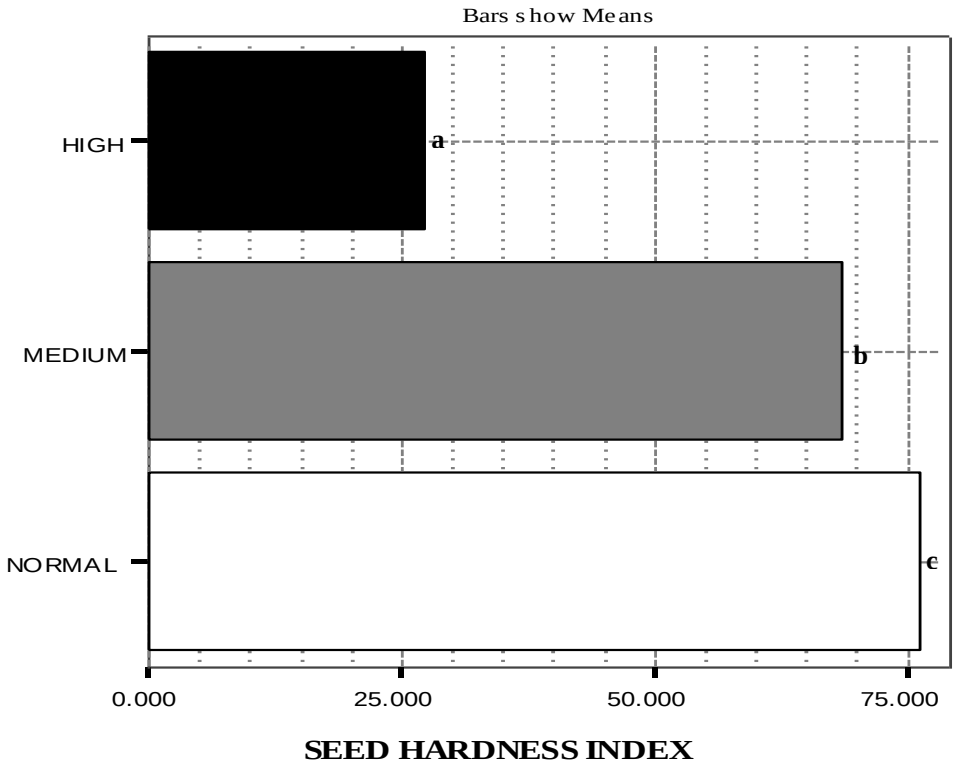

Figure 5: Single kernel hardness test per digestible group.
- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

Near-Infrared Reflectance Spectrophotometry. An NIR analysis was used to estimate the starch content of the RILs and DGs. The RILs combined analysis (fixed model) across seven environments revealed significant genotype, environment and genotype*environment effects (Table V). Tukey's HSD grouped them into significant difference groups (Figure 6). The DGs combined analysis revealed significant DG and environment effects, but no significant DG*environment effect was detected (Table VI)

suggesting the DGs' starch content does not change across environments. Tukey's HSD grouped them as follow: MD > ND > HD (Figure 7). This outcome was corroborated via contrast between DGs (Table III). These results show that HD RILs have the lowest starch content suggesting that as the protein digestibility increases the starch content decreases. This hypothesis is supported by the significant correlation (0.565**) between the level of turbidity and the starch content of the RILs (Table IV).

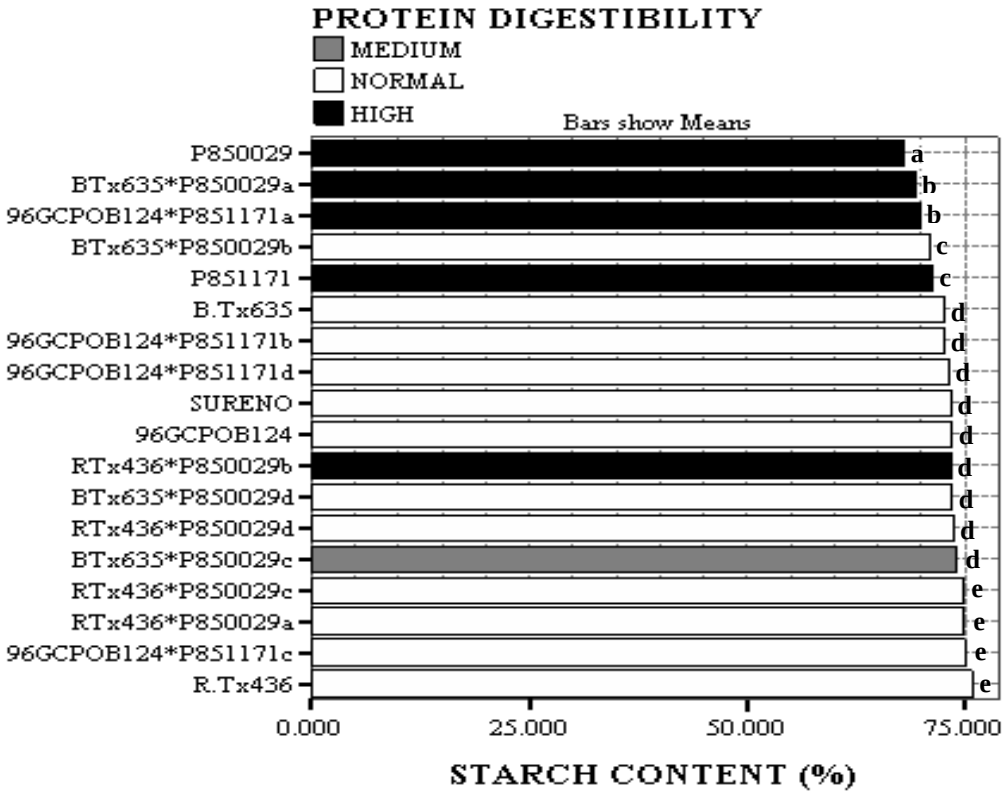

Figure 6: Starch content per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

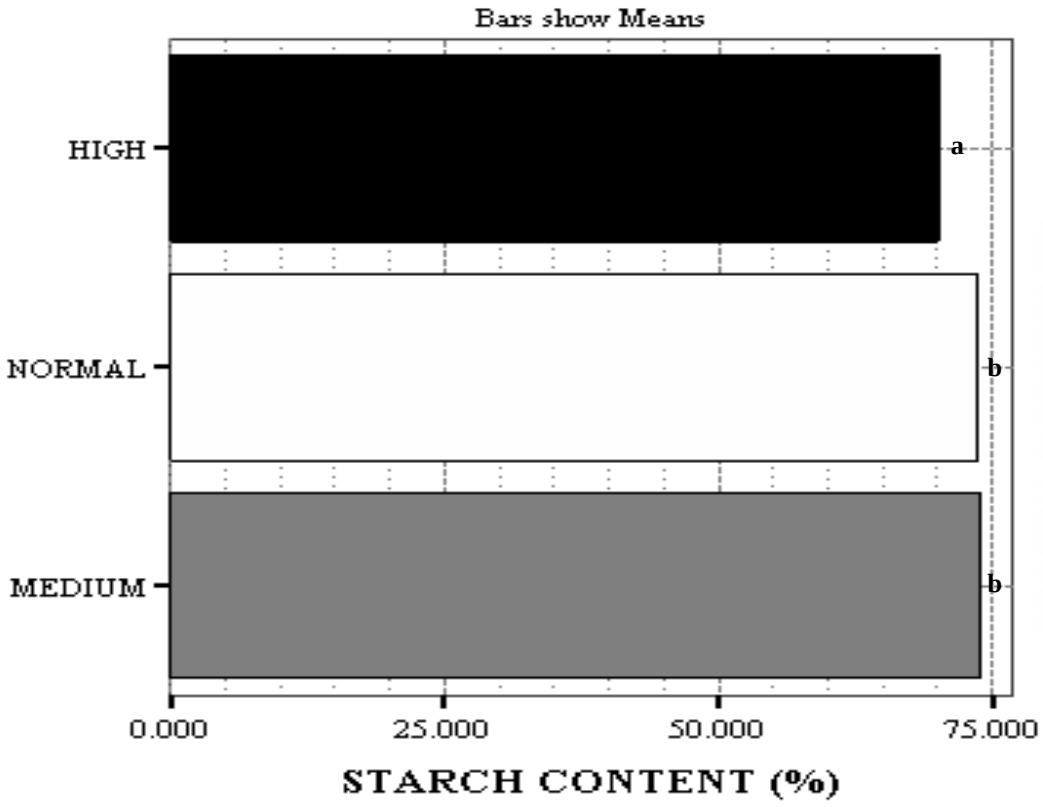

Figure 7: Starch content per digestible group.
- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

Thousand Kernel Weight Test. The SKHT was used to determine the average thousand kernel weight of the RILs and DGs. The RILs combined analysis (fixed model) across seven environments revealed significant genotype, environment, and genotype*environment effects (Table V) and Tukey HSD significant difference groups (Figure 8). The DGs combined analysis revealed significant DG and environment

effects, but no significant DG*environment effect was detected suggesting that the DGs' thousand kernel weight does not change significantly across environments (Table VI). Tukey HSD grouped them as follow: ND > MD > HD (Figure 9). This outcome was corroborated via contrast between DGs (Table III). These results show the HD RILs have the lowest thousand kernel weight which suggests that as the protein digestibility increases the thousand kernel weight decreases. This hypothesis is supported by the significant correlation (0.550**) between the turbidity and the thousand kernel weight of the RILs (Table IV). Finally, it could be hypothesized that the low thousand kernel weight of the HD RILs occurs as a consequence of the significant correlation (0.362**) between the starch content and the thousand kernel weight (Table IV). Since the HD RILs have the lowest starch content consequentially they also have lowest thousand kernel weights.

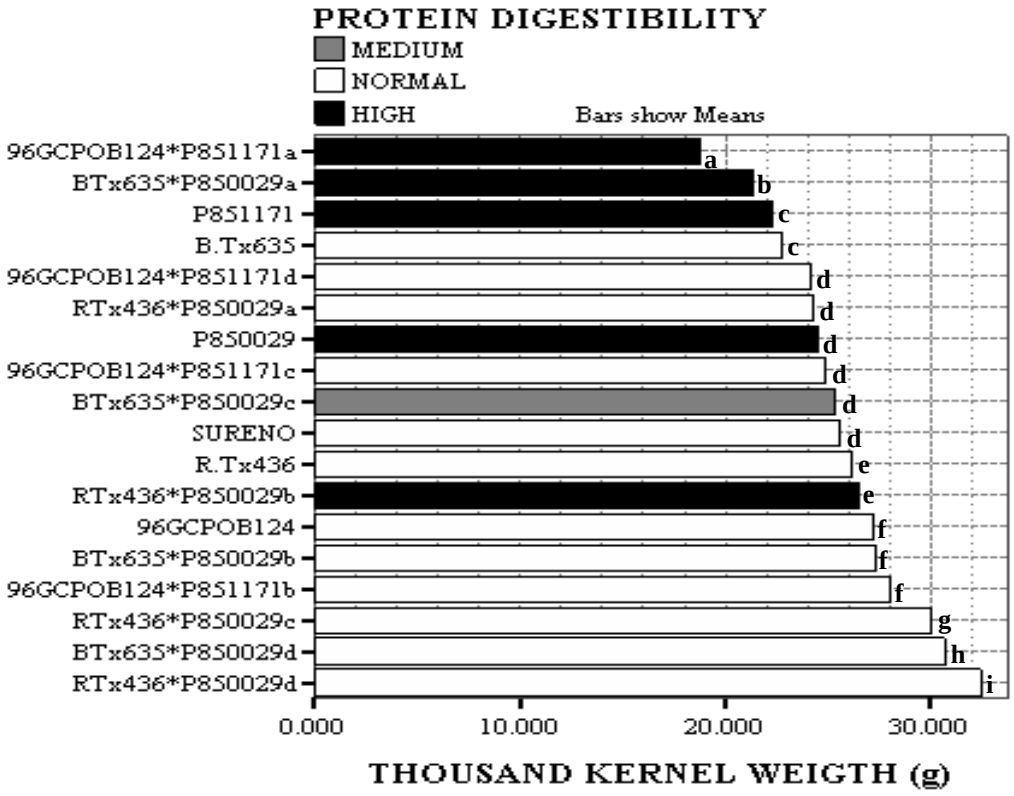

Figure 8: Thousand kernel weight per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

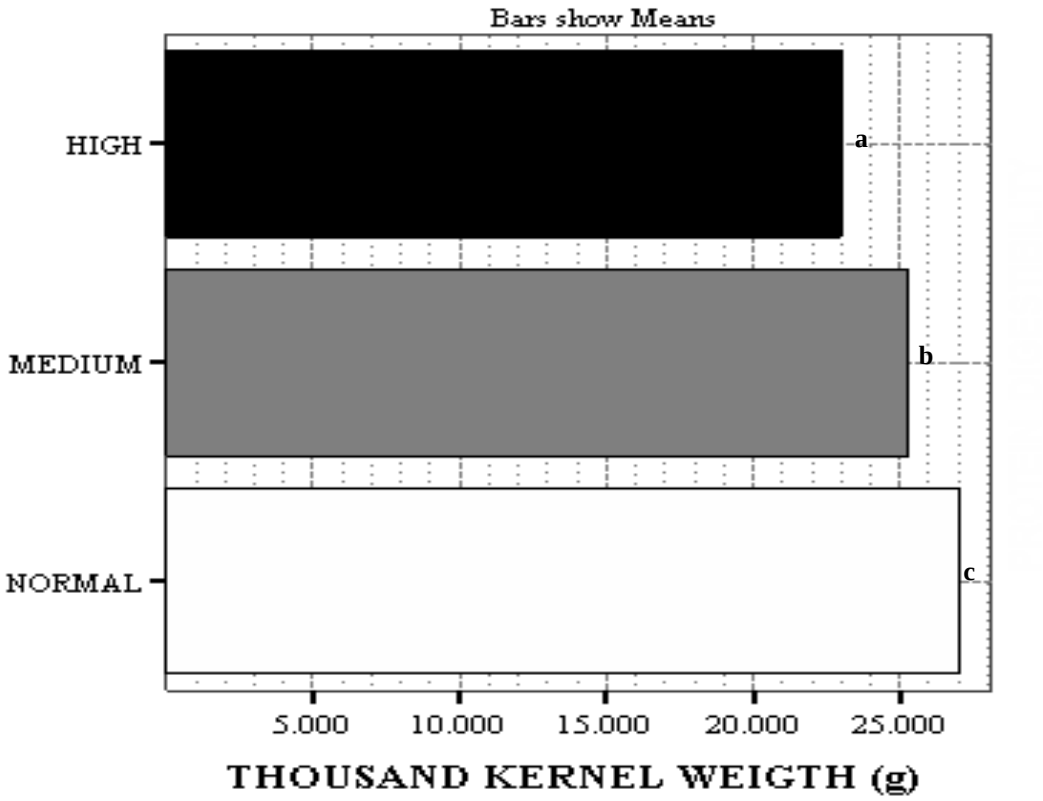

Figure 9: Thousand kernel weight per digestible group.
- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

3.3. Evaluation of the level of susceptibility to GMDC.

The Threshed Grade Score. The RILs combined analysis (fixed model) revealed significant environment effects, but no significant genotype and genotype*environment effects were detected suggesting that all the RILs were equally affected by the GMDC (Table VII). The DGs combined analysis, however, revealed significant DG and

environments effects, but no significant DG*environment effect was detected (Table VIII). This suggests the DGs' threshed grade score does not change significantly across environments. Tukey HSD grouped them as follow: HD > MD & MD > ND (Figure 10). This outcome was corroborated via contrast among the DGs (Table IX). These results show that the HD RILs have the highest scores suggesting that as the protein digestibility increases the level of susceptibility to the GMDC increases. This hypothesis is supported by the highly significant correlation (-0.229**) between the level of turbidity and the threshed grade score of the RILs (Table IV). Finally, it could be hypothesized that the higher susceptibility of the HD RILs to grain molds occurs as a consequence of their floury endosperms due to the significant correlation (0.228**) between the endosperm texture index and the threshed grade score which suggest that if the endosperm becomes softer the susceptibility to grain molds increases (Table IV).

Table VII: Mean squares from ANOVA combined analysis of recombinant inbred lines based on threshed grade score, mycoflora analysis and germination.

Source	Threshed grade score	Mycoflora analysis	Germination (%)
RIL	2.331	30.698**	2234.935**
Environment	54.667**	1792.718**	10399.111**
RIL * Environment	0.678	14.849**	369.374**
Error	0.750	4.625	104.394
Adjusted R^2	0.793	0.518	0.718

Notes: **significant at the 0.01 level.

Table VIII: Mean squares from ANOVA combined analysis of digestible groups based on threshed grade score, mycoflora analysis and germination.

Source	Threshed grade score	Mycoflora analysis	Germination (%)
DG	6.314**	69.118**	415.255
Environment	33.900**	636.218**	3448.362**
DG * Environment	0.914	3.785	466.393*
Error	0.887	5.253	231.813
Adjusted R^2	0.755	0.452	0.374

Notes: **significant at the 0.01 level.
*significant at the 0.05 level.

Table IX: Means squares from contrast of digestible groups based on threshed grade score, mycoflora analysis and germination.

Source	Threshed grade score	Mycoflora analysis	Germination (%)
HD vs. MD	8.47302231	32.0468900	1127.421310
HD vs. ND	45.41613557**	216.3770415**	197.546709
MD vs. ND	0.07487267	1.1852564	787.375888

Notes: **significant at the 0.01 level.

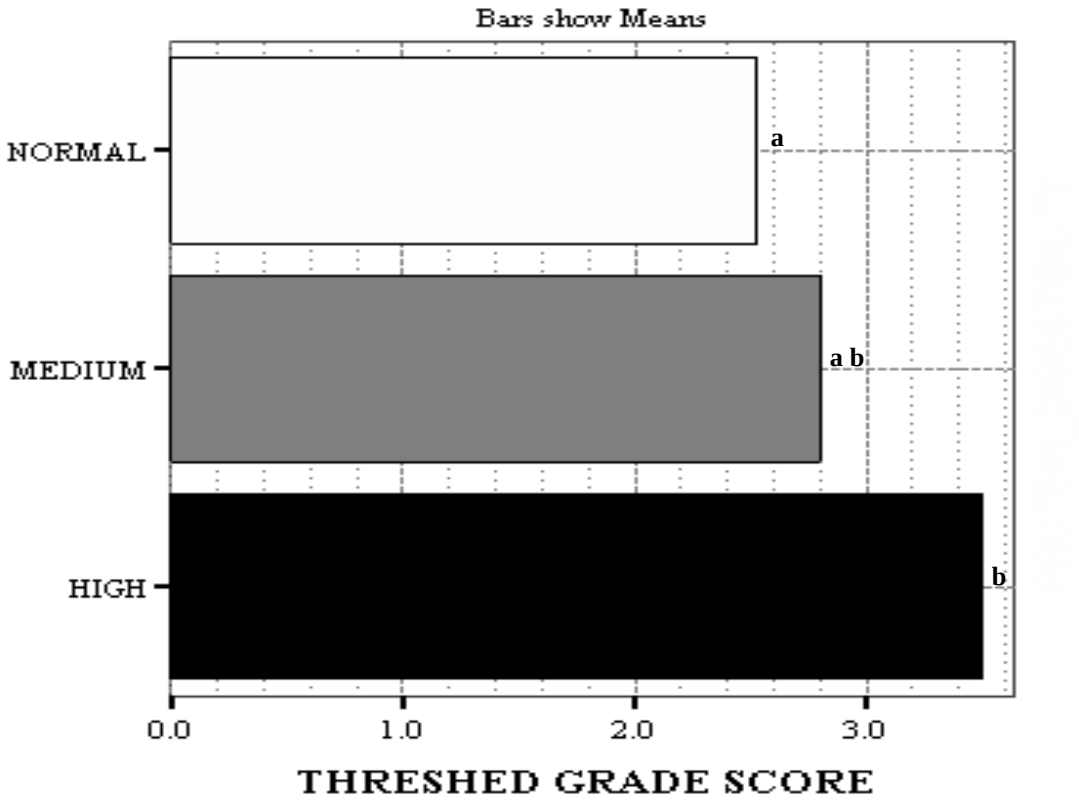

Figure 10: Threshed grade score per digestible group.
- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

The Mycoflora Analysis. The RILs combined analysis (fixed model) revealed significant genotype, environment and genotype*environment effects (Table VII). Tukey HSD grouped them into significant difference groups (Figure 11). The DGs combined analysis revealed significant DG and environments effects, but no significant DG*environment effect was detected suggesting that the DGs' fungal incidence does not change

significantly across environments (Table VIII). Tukey HSD grouped them as follow: HD > MD > ND (Figure 12). However, this outcome was not completely supported by the contrast analysis among the DGs (HD, MD, & MD, ND) (Table IX). These results show the HD RILs have the highest fungal incidences suggesting that as the protein digestibility increases the level of susceptibility to the GMDC increases. However, there is evidence against this hypothesis because two HD RILs (RTx436*P850029b and P850029) were found among the most resistant cultivars, there is not a significant correlation between the level of turbidity and the fungal incidence (Table IV), and the germination potential of the HD RILs was not significantly different to other DGs.

The Germination Test. The RILs combined analysis (fixed model) revealed significant genotype, environment and genotype*environment effects (Table VII). Tukey HSD grouped them into significant difference groups (Figure 13). The DGs combined analysis revealed no significant DG effect, but significant environment and DG*environment effects were detected suggesting the DGs' germination potential changes significantly across environments (Table VIII). Tukey HSD grouped them as follow: HD > ND > MD (Figure 14). This outcome was supported by the contrast analysis among the DGs (Table IX). These results show that all the RILs have the same germination potential suggesting the HD RILs are not affected to a greater extent by grain molds. This hypothesis is supported by the lack of a significant correlation between the turbidity and the germination rate of the RILs (Table IV).

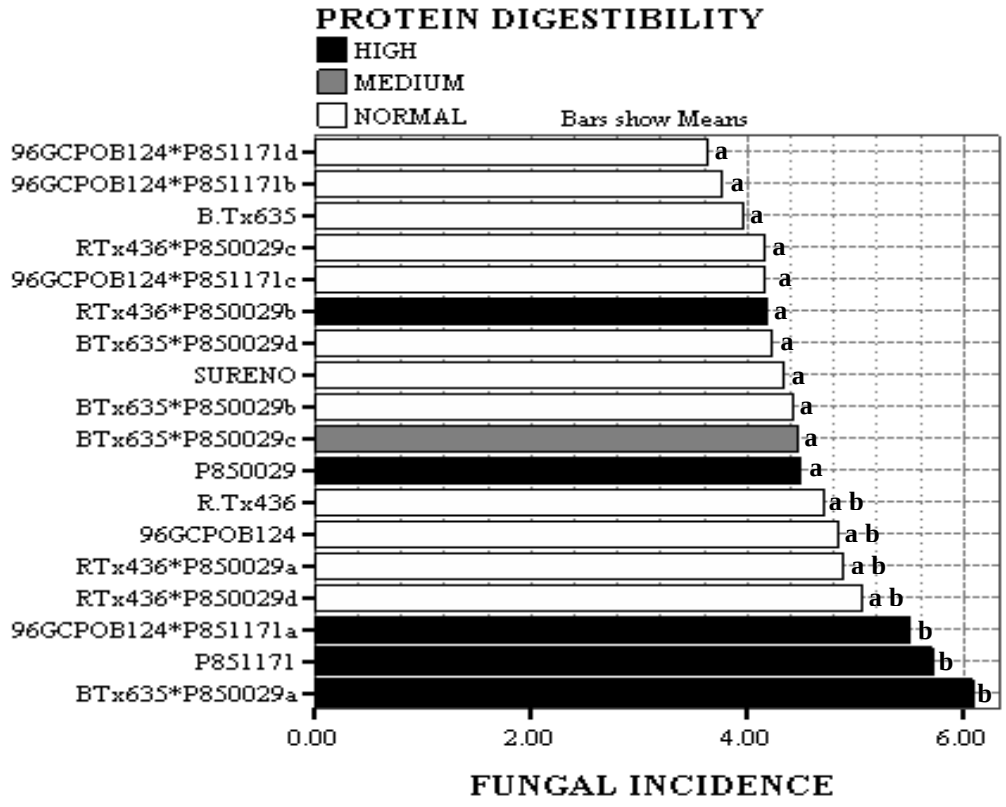

Figure 11: Mycoflora analysis per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

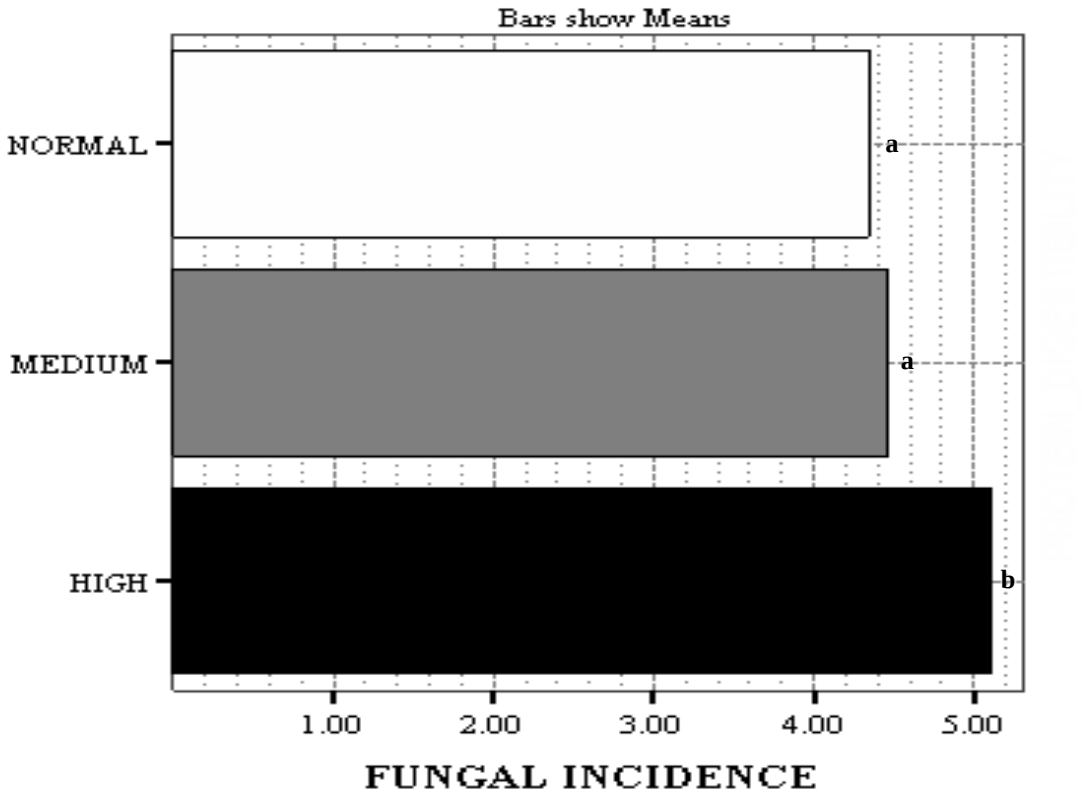

Figure 12: Mycoflora analysis per digestible group.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

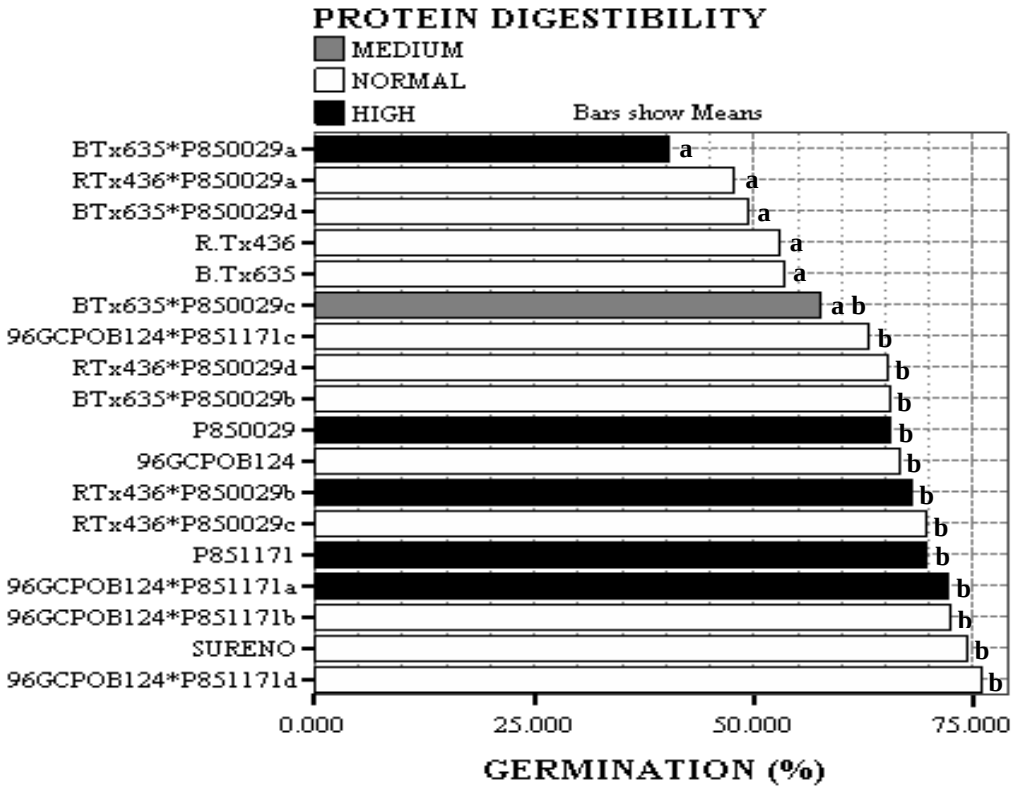

Figure 13: Germination rate per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

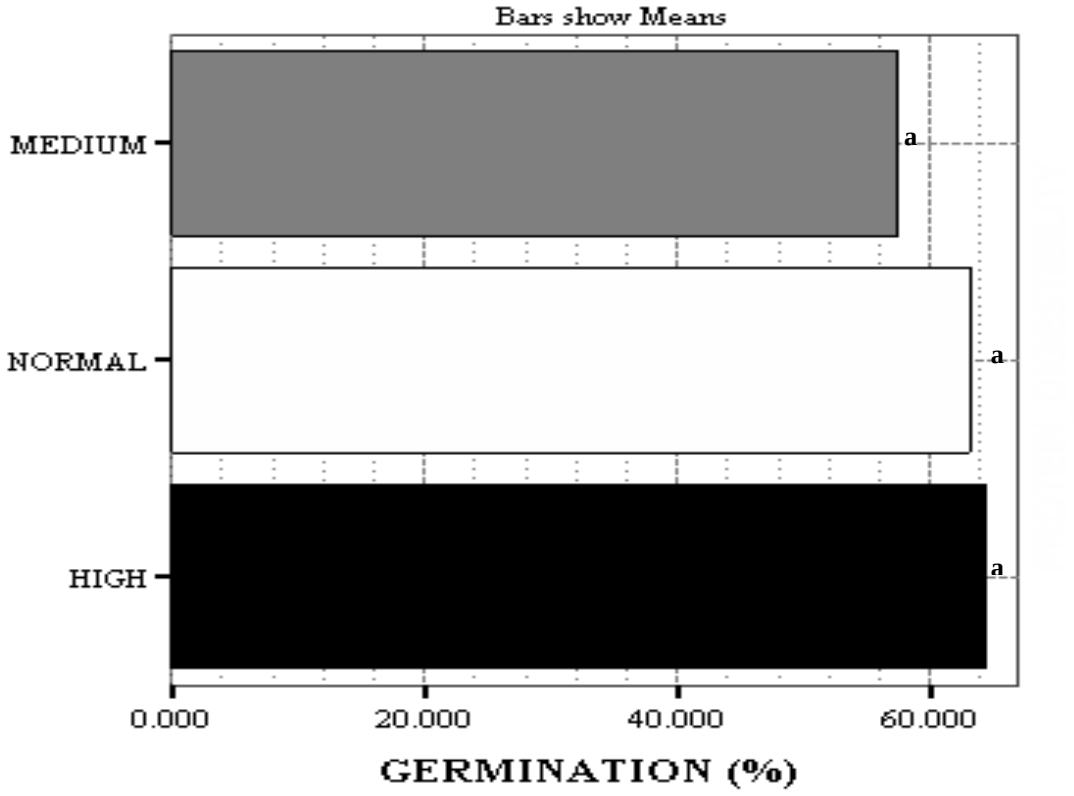

Figure 14: Germination rate per digestible group.

- Digestible groups defined by color of bars.
- According to Tukey's HSD, digestible groups with the same letter are not different at a 0.05 level of significance.

4. Discussion.

The analysis of turbidity of the breeding population revealed that the improvement of the RILs' protein digestibility is a random variable which accounts for the classification of the sorghum lines into DGs. The analysis of the grain physical attributes revealed the presence of a linear association between the level of protein digestibility and the kernel

physical characteristics normally associated with the GMDC. This association shows that the HD RILs have softer grains, floury endosperms, low starch contents and low thousand kernel weights. One exception to these trends was RTx436*P850029b which among the HD lines displays the highest seed hardness, and has a starch content and thousand kernel weight not significantly different to many ND RILs.

In general, the mycoflora analysis of DGs and RILs and the threshed grade scores of DGs revealed that the HD lines are more susceptible to grain molds. The combined effect of the above mentioned kernel physical characteristics could explain the HD RILs higher susceptibility to fungal attack. The germination test shows that there were no significant differences between the RILs' germination potential suggesting that although the HD RILs tend to be more easily infected by grain molds that does not translate into greater damage of kernel micro-structures (embryo). The lack of a significant correlation between the level of turbidity and the germination rate of the RILs provides evidence that the HD lines do not have an inherently lower germination potential. Additionally, these conclusions are valid only for the breeding population on which the experiments were performed due to the fixed model employed to analyze the collected data.

An exception to these results was RTx436*P850029b which has fungal incidences not significantly different to the rest of the ND RILs. The RTx436*P850029b higher resistance to mold infection could be attributed to its harder endosperm. This hypothesis is supported by the significant negative correlation between the hardness index and the mycoflora analysis and the threshed grade scores. Based on this,

RTx436*P850029b could be a suitable parental line for the development of new HD breeding populations with higher grain mold resistance.

Finally, a possible future approach to determine the HD sorghum lines level of susceptibility to grain mold is the measurement of ergosterol content in sorghum flours which would provide an estimation of the fungal biomass located inside the kernels.

CHAPTER III

IDENTIFICATION OF GMDC FUNGI, THEIR IMPORTANCE & PREFERENTIAL PATHOGENICITY TOWARDS HD SORGHUM LINES BASED ON CARYOPSIS QUALITY AND GERMINATION

1. Introduction.

In sorghum, many saprophytic and/or facultative parasitic fungal genera and species are associated with the internal mycoflora of sorghum grains and are thought to contribute to the GMDC development. Some of the reported pathogens are *Curvularia lunata* and *Fusarium thapsinum* (Prom, 2003), *Fusarium semitectum* (Erpelding et al., 2006), *Alternaria sp.*, *Colletotrichum sublineolum* (Gwary D.M. et al., 2006), *Penicillium sp*., *Phoma sorghina*, *Aspergillus flavus*, and *Aspergillus niger* (González H.H.L. et al., 1997).

The identification of fungal genera and species associated with the internal mycoflora of sorghum grains is important since it allows the classification of molds based on roles and the identification of germplasm sources of resistance for the breeding of cultivars with higher tolerance to pathogens responsible for the initial infection and development of the GMDC. However, previous reports provide no information about the fungal agents that infect HD RILs grains or the susceptibility of the HD RILs to specific fungal pathogens.

The objectives of this study included the identification of the fungi (genera and species) responsible for the GMDC in stored grains based on caryopsis deterioration and

germination reduction and the measurement of preferential pathogenecities of GMDC fungi towards HD RILs.

2. Statistical analysis and interpretation.

The incidence of 10 fungal genera (*Alternaria sp., Aspergillus sp., Bipolaris sp., Curvularia sp., Fusarium spp., Nigrospora sp., Phoma sp., Penicillium sp., Rhizopus sp. and Mucor sp.)* and 2 species (*Fusarium semitectum. and Fusarium thapsinum.)* was recorded during the development of a mycoflora analysis. SPSS and SAS were used to estimate correlations (Pearson product moment correlations), compare the means of the DGs (Contrast), detect significant differences between RILs, and DGs via analysis of variance (ANOVA) and test for significance by Fisher's LSD.

2.1. The identification of the fungi (genera and species) responsible for the GMDC in stored grains based on caryopsis deterioration and germination reduction. The fungal incidences recorded across 7 environments (Beeville 2005, College Station 2005, College Station 2006, Corpus Christi 2005, Halfway 2005, Weslaco 2005 and Weslaco 2006) were analyzed using Pearson correlations to identify pathogens responsible for GMDC based on associations with caryopsis characteristics linked to grain mold damage under the following hypothesis:

$\rho_{xy} = 0$

$\rho_{xy} \neq 0$

Where X= pathogen.

Y= germination (%); thousand kernel weight (g), starch content (%), and seed hardness index.

2.2. The measurement of preferential pathogenecities of GMDC fungi (genera and species) towards HD sorghum RILs.

a. ANOVA factorial designs were used to reveal specific GMDC pathogens' infecting patterns within the breeding population. A RCBD with 15 replications per RIL was used and the combined analysis performed on the data collected from 5 environments (Beeville 2005, College Station 2006, Corpus Christi 2005, Halfway 2005 and Weslaco 2005) based on consistency of results. The significant differences between the RILs and DGs based on selected pathogens' incidences were estimated by an all fixed model under the following hypothesis:

H_o= the mean of all RILs/DGs are equal.

H_a= at least one RIL/DG is different.

Model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha_i*\beta_j + error_{ij}$

Where Y = pathogen incidence (sum of fungal colonies).

μ = intercept.

α = RIL/DG effect.

β = environment effect.

$\alpha_i*\beta_j$ = RIL*environment interaction effect.

DG*environment interaction effect.

A Fisher's LSD test was used to identify the significantly different RILs and DGs

b. Contrast analyses on the data collected from the five previously mentioned environments identified the significant differences between DGs based on selected pathogens' incidences. The contrasts were tested under the following hypotheses:

HD vs. MD

H_o: $l = \mu_1 - \mu_2 + 0\mu_3 = 0$

H_a: $l = \mu_1 - \mu_2 + 0\mu_3 \neq 0$

HD vs. ND

H_o: $l = \mu_1 + 0\mu_2 - \mu_3 = 0$

H_a: $l = \mu_1 + 0\mu_2 - \mu_3 \neq 0$

MD vs. ND

H_o: $l = 0\mu_1 + \mu_2 - \mu_3 = 0$

H_a: $l = 0\mu_1 + \mu_2 - \mu_3 \neq 0$

Where: μ_1= HD $_{\text{pathogen incidence mean.}}$

μ_2= MD $_{\text{pathogen incidence mean.}}$

μ_3= ND $_{\text{pathogen incidence mean}}$

3. Results and discussion.

3.1. Identification of GMDC pathogens. The analysis of the grain internal mycoflora showed that *Alternaria sp.* accounted for 41.9% of the total fungal species isolated from naturally infected grains. Other fungal genera and species included *Fusarium semitectum* (12.6%), *Bipolaris sp*. (9.6%), *Fusarium spp.* (6.1%), *Phoma sp.* (5.9%), *Curvularia sp.* (5.3%), *Fusarium thapsinum* (2.9%), *Aspergillus sp.* (2.2 %), *Penicillium sp.* (1.2%), *Nigrospora sp.* (0.42%), and *Rhizopus sp / Mucor sp*. (0.39%) (Figure 15).

The correlation analyses revealed that only *Aspergillus sp., Curvularia sp., Fusarium spp., Fusarium semitectum, Fusarium thapsinum, Nigrospora sp., and Rhizopus sp / Mucor sp*. were significantly and meaningfully associated to seed germination, starch content, thousand kernel weight and seed hardness index (Table X).

In general, the correlations denote an inverse association between these molds and the selected grain traits. Additionally, the efficiency of the threshed grade score and the mycoflora analysis as estimators of the level of incidence of these pathogens was also recorded. In most cases, the higher the mycoflora and/or threshed grade scores the higher the incidence of the selected molds (Table X).

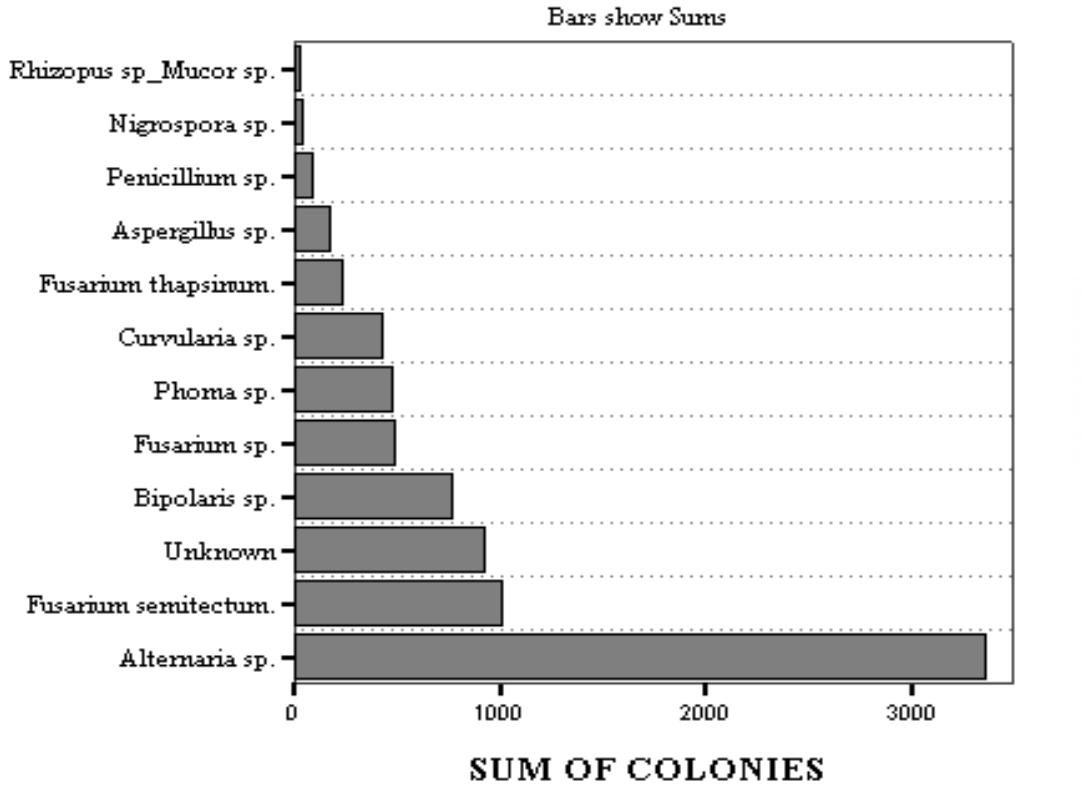

Figure 15: Fungal incidence of genera and species isolated from mycoflora analysis.

Table X: Correlations between pathogens and grain traits linked to grain mold damage.

		Turbidity (60 min)	Starch (%)	Germination (%)	Thousand kernel weight	Hardness index	Endosperm texture index	Threshed grade score	Fungal incidence
Alternaria sp.	Pearson Correlation	-0.035	0.098(*)	0.374(**)	0.002	0.140(**)	0.003	-0.155(*)	0.576(**)
	Sig. (2-tailed)	0.429	0.029	0.000	0.956	0.002	0.939	0.010	0.000
	N	519	501	519	504	504	519	273	519
Aspergillus sp.	Pearson Correlation	0.035	0.091(*)	-0.350(**)	-0.110(*)	-0.144(**)	-0.036	0.765(**)	0.268(**)
	Sig. (2-tailed)	0.428	0.041	0.000	0.014	0.001	0.408	0.000	0.000
	N	519	501	519	504	504	519	273	519
Bipolaris sp.	Pearson Correlation	-0.066	0.117(**)	0.024	0.069	0.002	0.017	0.021	0.360(**)
	Sig. (2-tailed)	0.136	0.009	0.581	0.121	0.966	0.704	0.729	0.000
	N	519	501	519	504	504	519	273	519
Curvularia sp.	Pearson Correlation	0.041	0.112(*)	-0.125(**)	0.097(*)	-0.034	-0.026	0.023	0.337(**)
	Sig. (2-tailed)	0.351	0.012	0.004	0.029	0.446	0.556	0.704	0.000
	N	519	501	519	504	504	519	273	519
Fusarium spp.	Pearson Correlation	-0.142(**)	-0.054	-0.158(**)	-0.093(*)	-0.202(**)	0.187(**)	-0.080	0.364(**)
	Sig. (2-tailed)	0.001	0.231	0.000	0.038	0.000	0.000	0.186	0.000
	N	519	501	519	504	504	519	273	519
Fusarium semitectum.	Pearson Correlation	-0.020	-0.137(**)	-0.280(**)	-0.035	-0.131(**)	0.097(*)	-0.106	0.162(**)
	Sig. (2-tailed)	0.656	0.002	0.000	0.437	0.003	0.028	0.081	0.000
	N	519	501	519	504	504	519	273	519
Fusarium thapsinum.	Pearson Correlation	-0.021	-0.170(**)	-0.252(**)	-0.053	-0.118(**)	0.037	-0.121(*)	0.162(**)
	Sig. (2-tailed)	0.631	0.000	0.000	0.237	0.008	0.406	0.046	0.000
	N	519	501	519	504	504	519	273	519
Nigrospora sp.	Pearson Correlation	-0.018	0.000	-0.107(*)	0.079	-0.058	0.005	0.351(**)	0.151(**)
	Sig. (2-tailed)	0.687	1.000	0.015	0.077	0.194	0.906	0.000	0.001
	N	519	501	519	504	504	519	273	519
Phoma sp.	Pearson Correlation	-0.045	0.068	-0.005	0.041	-0.059	0.063	-0.067	0.344(**)
	Sig. (2-tailed)	0.309	0.126	0.904	0.361	0.183	0.151	0.269	0.000
	N	519	501	519	504	504	519	273	519

Table X: Continued.

		Turbidity (60 min)	Starch (%)	Germination (%)	Thousand kernel weight	Hardness index	Endosperm texture index	Threshed grade score	Fungal incidence
Penicillium sp.	Pearson Correlation	0.002	-0.037	-0.060	0.045	-0.025	0.039	-0.083	-0.108(*)
	Sig. (2-tailed)	0.970	0.402	0.170	0.309	0.577	0.375	0.170	0.013
	N	519	501	519	504	504	519	273	519
Rhizopus sp / Mucor sp.	Pearson Correlation	-0.015	0.008	-0.194(**)	-0.133(**)	-0.138(**)	0.035	0.734(**)	0.213(**)
	Sig. (2-tailed)	0.737	0.851	0.000	0.003	0.002	0.428	0.000	0.000
	N	519	501	519	504	504	519	273	519
Unknown.	Pearson Correlation	-0.113(*)	-0.054	-0.111(*)	0.001	-0.141(**)	0.106(*)	0.097	0.327(**)
	Sig. (2-tailed)	0.010	0.225	0.012	0.978	0.002	0.015	0.111	0.000
	N	519	501	519	504	504	519	273	519

Notes: **Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

3.2. Analysis of GMDC pathogens contagious patterns. The individual RILs combined analyses (fixed models) across five environments revealed the following:

Aspergillus sp.: Significant environment and genotype*environment effects; however, no significant genotype effect was detected which indicated that although the RILs were equally infected the contagious pattern changed across environments (Table XI).

*Nigrospora sp.: S*ignificant environment effect; however, no significant genotype or genotype*environment effects were detected which indicated that the RILs were equally infected and the contagious pattern did not change across environments (Table XI).

*Rhizopus sp. / Mucor sp.: S*ignificant genotype and environment effects; however, no significant genotype*environment effect was detected which indicated that although the RILs were not equally infected the contagious pattern did not change across environments (Table XI and Figure 16).

Curvularia sp., Fusarium semitectum, Fusarium thapsinum, and *Fusarium spp.:* Significant genotype, environment and genotype*environment effects were detected which indicated the RILs were not equally infected and the contagious patterns changed across environments (Table XI and Figures 17, 18, 19, 20 respectively).

Table XI: Mean squares from ANOVA combined analyses of recombinant inbred lines based on *Aspergillus sp.*, *Curvularia sp.*, *Fusarium spp.*, *Fusarium semitectum*, *Fusarium thapsinum*, *Nigrospora sp.* and *Rhizopus sp / Mucor sp.*

Source	*Aspergillus sp.*	*Curvularia sp.*	*Fusarium spp.*	*Fusarium semitectum*	*Fusarium thapsinum*	*Nigrospora sp.*	*Rhizopus sp / Mucor sp.*
RIL	0.431	0.675**	1.692**	13.738**	1.143**	0.013	0.064**
Environment	4.130**	12.976**	17.117**	25.927**	11.575**	0.196**	0.126**
RIL*Environment	0.514**	0.475*	1.202**	3.251**	0.680**	0.020	0.039
Error	0.279	0.340	0.388	1.326	0.236	0.021	0.032
Adjusted R^2	0.071	0.115	0.210	0.177	0.175	0.025	0.020

Notes: **significant at the 0.01 level.
*significant at the 0.01 level.

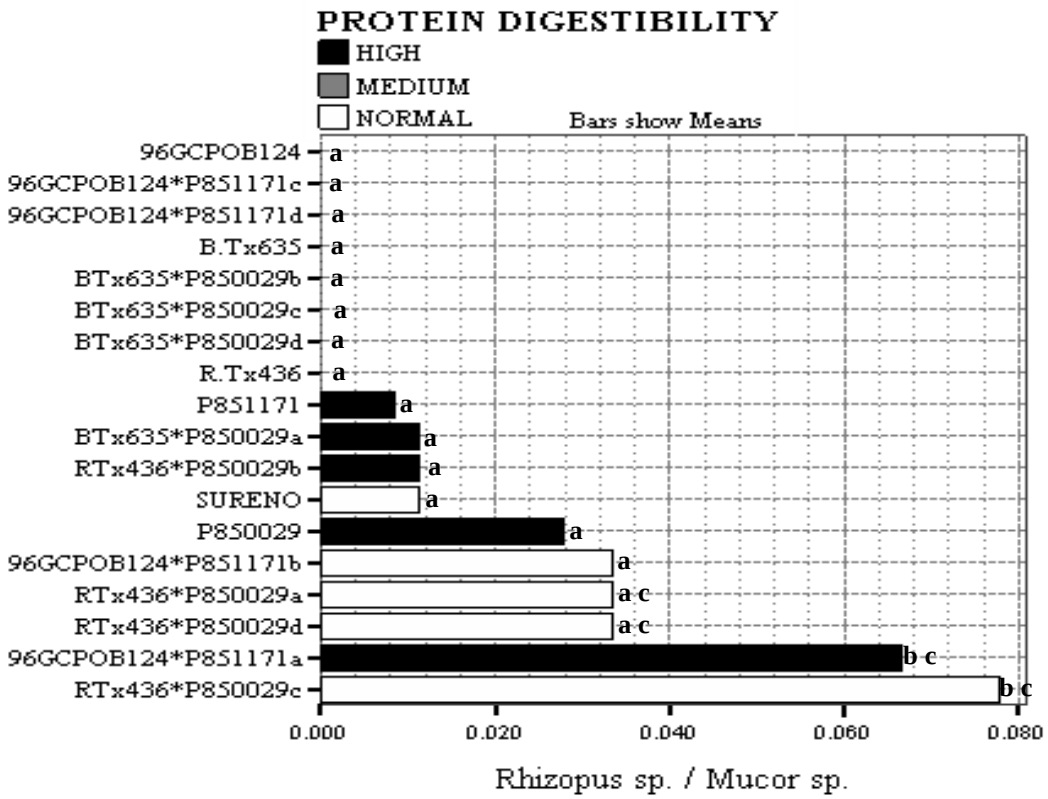

Figure 16: *Rhizopus sp. / Mucor sp.* pathogenic pattern per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

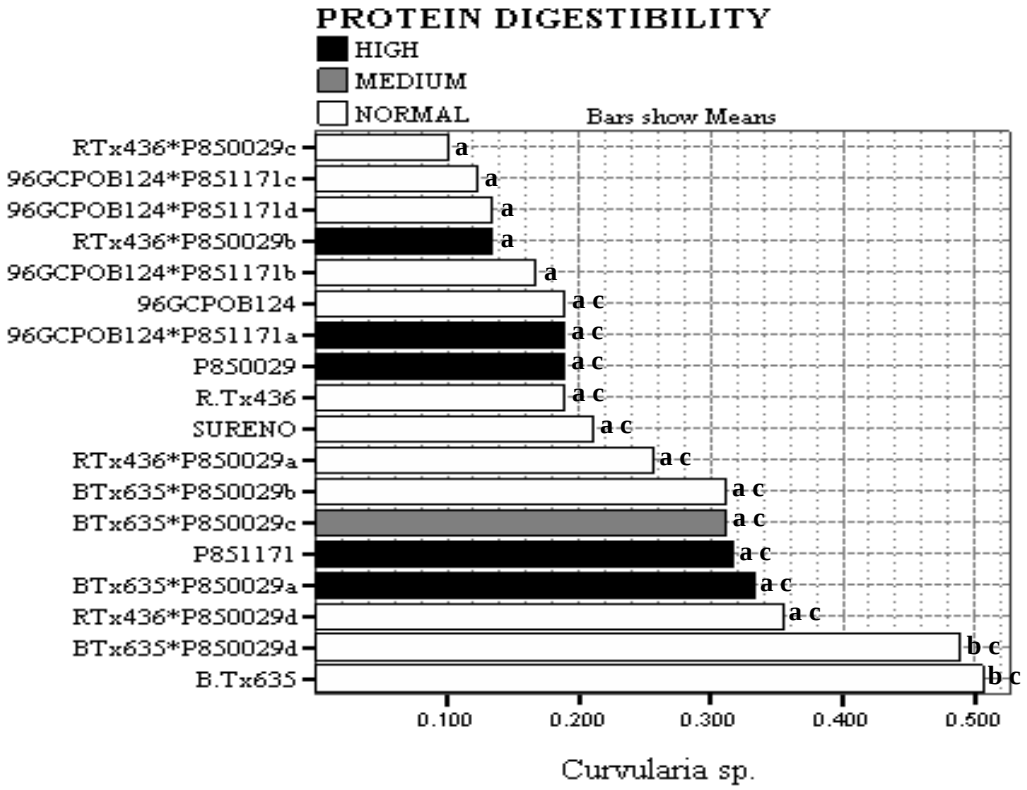

Figure 17: *Curvularia sp.* pathogenic pattern per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

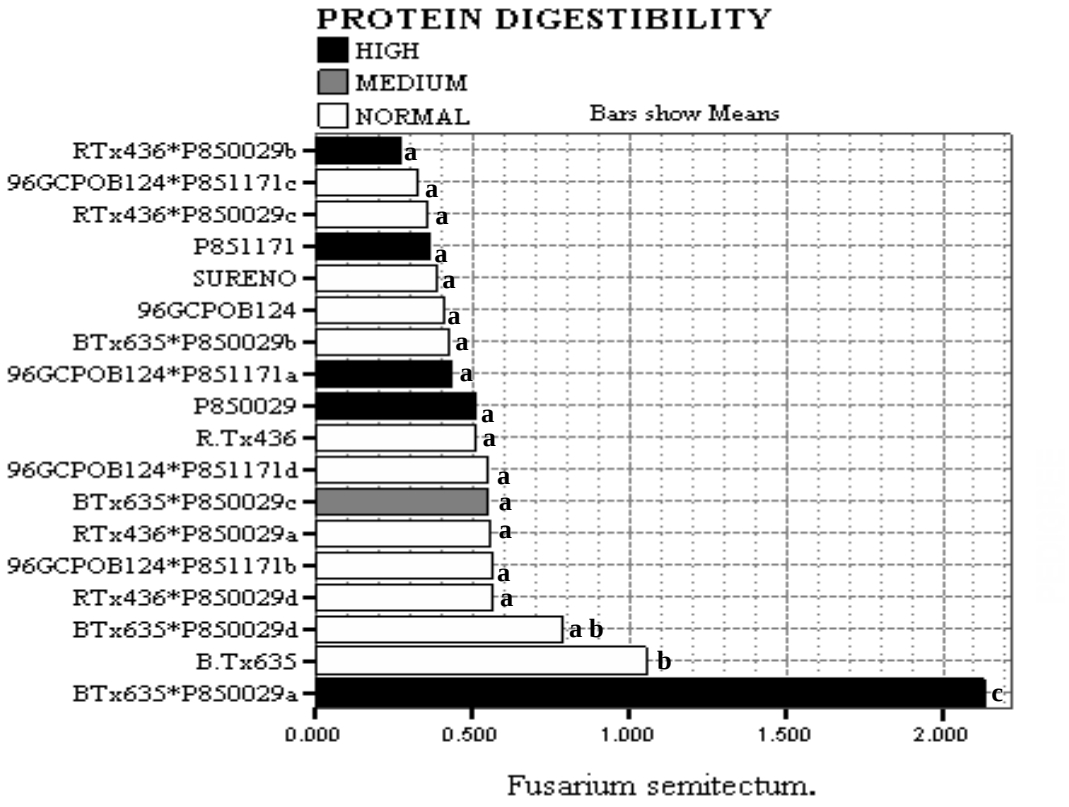

Figure 18: *Fusarium semitectum* pathogenic pattern per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

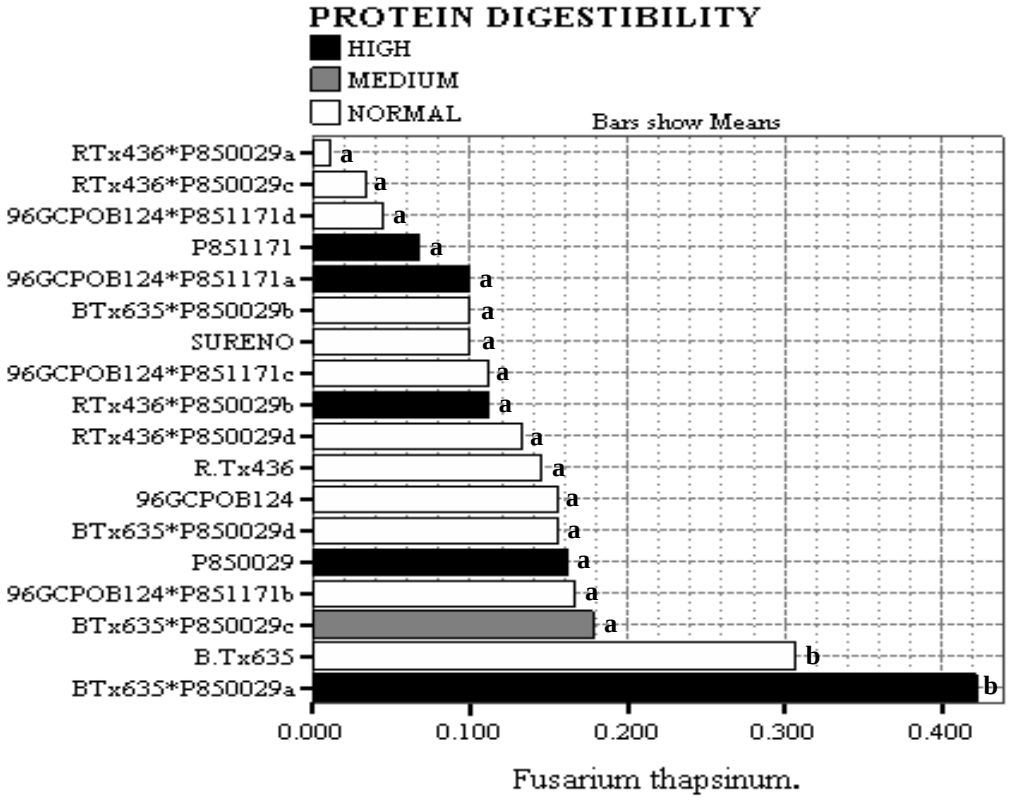

Figure 19: *Fusarium thapsinum* pathogenic pattern per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

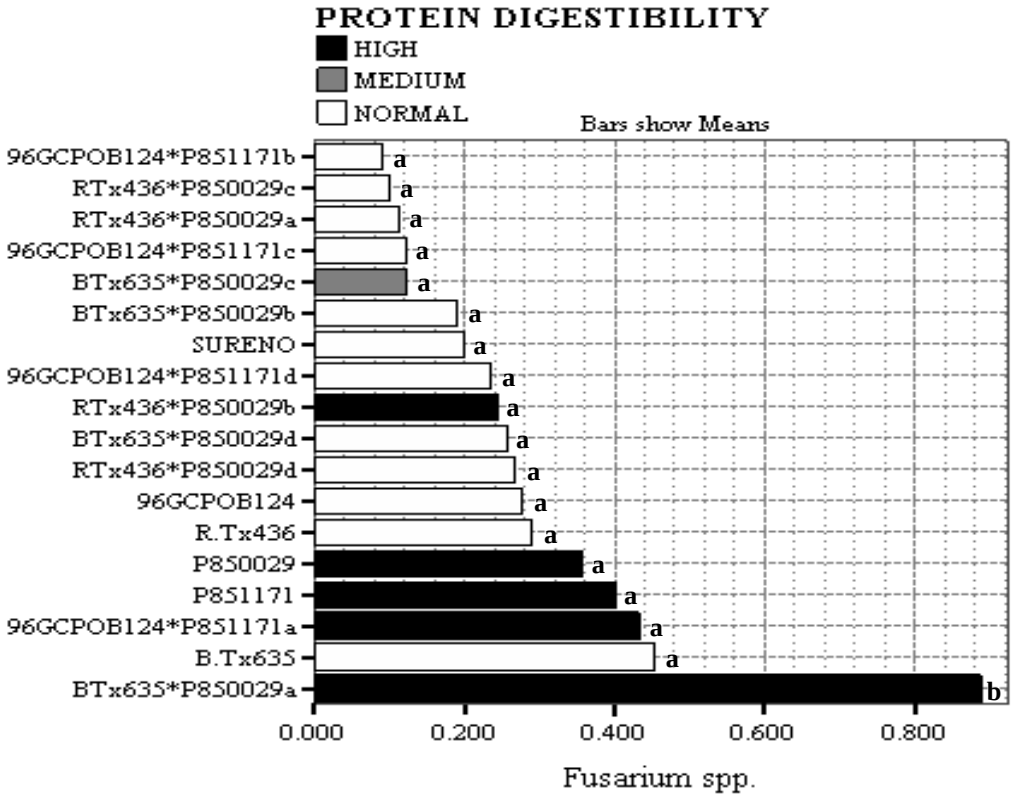

Figure 20: *Fusarium spp.* pathogenic pattern per recombinant inbred line.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, recombinant inbred lines with the same letter are not different at a 0.05 level of significance.

The DGs combined analysis (fixed model) across five environments revealed:

Aspergillus sp., Curvularia sp., Fusarium thapsinum: Significant environment effect; however no significant DG and DG*environment effects were detected which indicated that the DGs were equally infected and the contagious patterns did not change across environments (Table XII). These outcomes were supported by the contrast between DGs (Table XIII).

Nigrospora sp., Rhizopus sp. / Mucor sp.: No significant effects were detected indicating the DGs were equally infected and the contagious patterns did not change across environments (Table XII). These outcomes were supported by the contrast between DGs (Table XIII).

Fusarium spp.: Significant DG, environment and DG*environment effects were detected which indicated the DGs were not equally infected and the contagious pattern changed across environments (Table XII). Fisher's LSD grouped them as follows: HD > ND > MD (Figure 21). These outcomes were supported by the contrast between DGs (Table XIII).

Fusarium semitectum: Significant environment effect, but no DG and DG*environment effects were detected which indicated the DGs were equally infected and the contagious pattern did not change across environments (Table XII). These outcomes, however, were not supported by the contrast between DGs (Table XIII).

Table XII: Mean squares from ANOVA combined analyses of digestible groups based on *Aspergillus sp., Curvularia sp., Fusarium spp., Fusarium semitectum, Fusarium thapsinum, Nigrospora sp.* and *Rhizopus sp / Mucor sp.*

Source	*Aspergillus sp.*	*Curvularia sp.*	*Fusarium spp.*	*Fusarium semitectum*	*Fusarium thapsinum*	*Nigrospora sp.*	*Rhizopus sp. / Mucor sp.*
DG	0.441	0.347	4.394**	1.996	0.562	0.013	0.028
Environment	0.912**	5.368**	6.279**	13.206**	5.855**	0.040	0.022
DG*Environment	0.229	0.327	2.837**	2.468	0.241	0.009	0.022
Error	0.291	0.353	0.424	1.545	0.259	0.021	0.033
Adjusted R^2	0.032	0.081	0.136	0.041	0.093	0.019	0.004

Notes: **significant at the 0.01 level.

Table XIII: Means squares from contrast analyses of digestible groups based on *Aspergillus sp., Curvularia sp., Fusarium spp., Fusarium semitectum, Fusarium thapsinum, Nigrospora sp., Rhizopus sp / Mucor sp.*

Source	*Aspergillus sp*	*Curvularia sp.*	*Fusarium spp.*	*Fusarium semitectum*	*Fusarium thapsinum*	*Nigrospora sp.*	*Rhizopus sp. / Mucor sp.*
HD vs. MD	0.45616693	0.51358320	8.040935**	1.55672515	0.01286550	0.02392344	0.04688995
HD vs. ND	0.26736216	0.13405807	19.92428**	8.6495895*	0.77418407	0.00925003	0.02745357
MD vs. ND	0.88799667	0.32193972	0.67196106	0.01024531	0.28428348	0.04214377	0.02114505

Notes: **significant at the 0.01 level.
*significant at the 0.01 level.

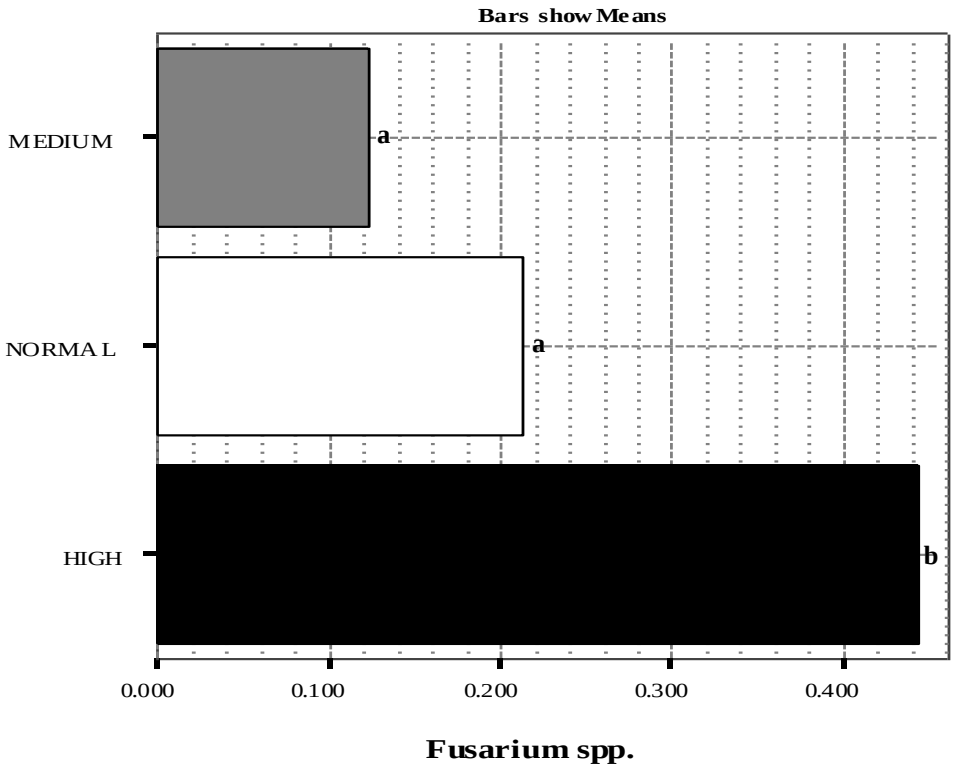

Figure 21: *Fusarium spp.* pathogenic pattern per digestible group.

- Digestible groups defined by color of bars.
- According to Fisher's LSD, digestible groups with the same letter are not different at a 0.05 level of significance.

Table XIV: Means squares from contrast analysis of digestible groups based on *Aspergillus sp., Curvularia sp., Fusarium spp., Fusarium semitectum, Fusarium thapsinum, Nigrospora sp.,* and *Rhizopus sp / Mucor sp.*

Source	Mycoflora analysis
HD vs. MD	18.48899522*
HD vs. ND	61.29052720**
MD vs. ND	0.56417379

Notes: **significant at the 0.01 level.
*significant at the 0.01 level.

Table XV: Mean squares from ANOVA combined analyses of digestible groups based on fungal incidence, germination and *Fusarium spp*.

Source	Fungal incidence	Germination (%)	*Fusarium spp.*
DG	24.883**	1545.626**	1.416*
Environment	631.304**	3462.008**	4.987**
DG * Environment	4.842	409.967*	1.535**
Error	5.242	200.619	0.364
Adjusted R^2	0.454	0.420	0.108

Notes: Analysis performed subtracting BTx635*P850029a from the population.
**significant at the 0.01 level.
*significant at the 0.01 level.

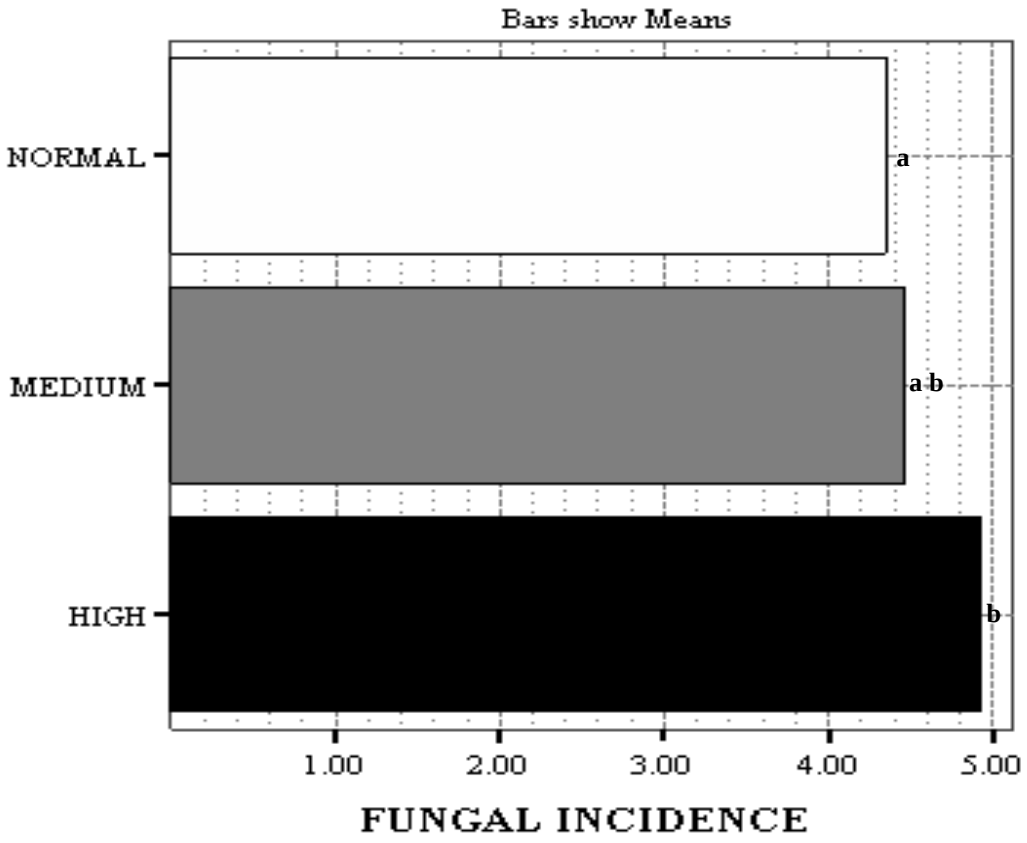

Figure 22: Mycoflora analysis per digestible group - BTx635*P850029a.

- Digestible groups defined by color of bars.
- Significant groups calculated subtracting BTx635*P850029a from the population.
- According to Fisher's LSD, digestible groups with the same letter are not different at a 0.05 level of significance.

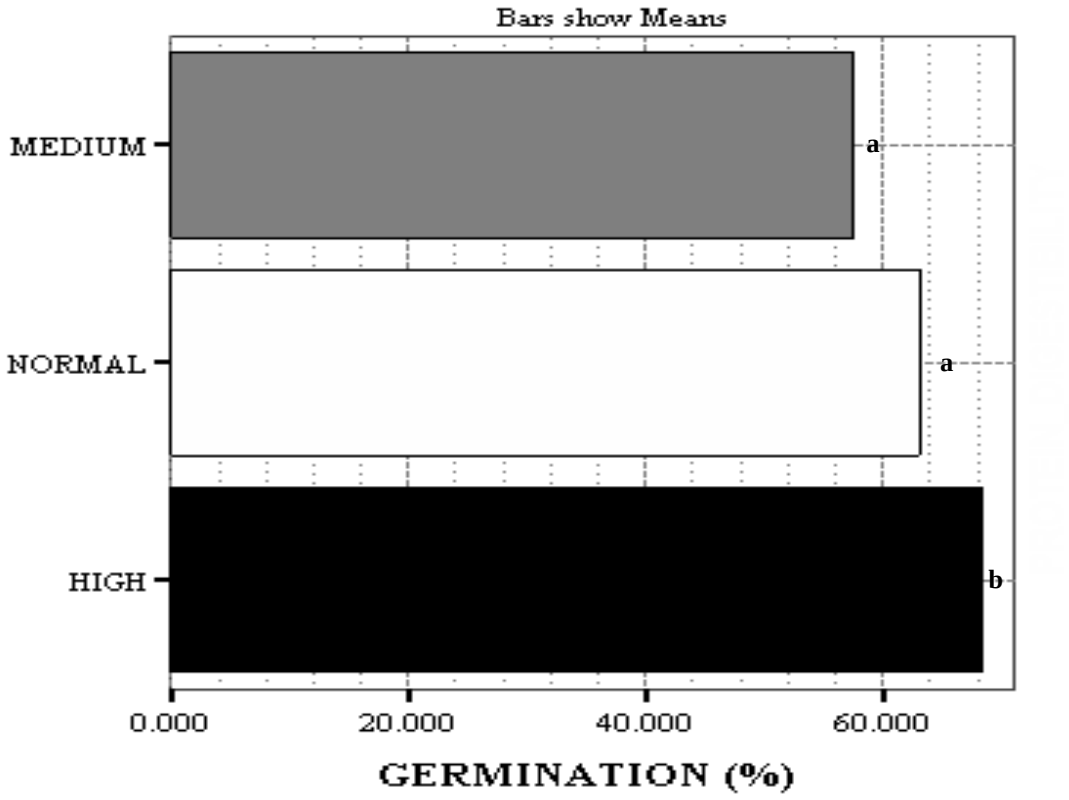

Figure 23: Germination rate per digestible group - BTx635*P850029a.

- Digestible groups defined by color of bars.
- Significant groups calculated subtracting BTx635*P850029a from the population.
- According to Fisher's LSD, digestible groups with the same letter are not different at a 0.05 level of significance.

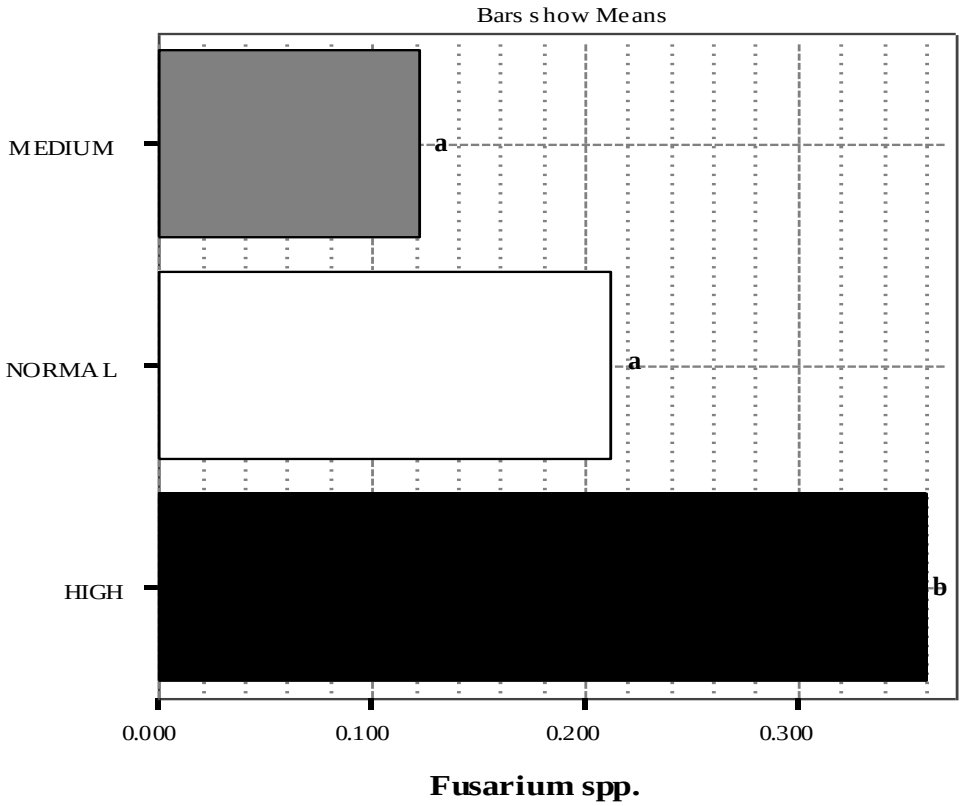

Figure 24: *Fusarium spp.* pathogenic pattern per digestible group - BTx635*P850029a.

- Digestible groups defined by color of bars.
- Significant groups calculated subtracting BTx635*P850029a from the population.
- According to Fisher's LSD, digestible groups with the same letter are not different at a 0.05 level of significance.

Table XVI: Correlations between pathogens.

		Aspergillus sp.	*Bipolaris sp.*	*Curvularia sp.*	*Fusarium spp.*	*Fusarium semitectum*	*Fusarium thapsinum*	*Phoma sp.*	*Penicillium sp.*	*Rhizopus sp. Mucor sp.*
Alternaria sp.	Correlation	-0.245(**)	0.013	0.009	-0.022	-0.138(**)	-0.013	0.017	-0.072(**)	-0.120(**)
	Sig. (2-tailed)	0.000	0.503	0.633	0.267	0.000	0.519	0.382	0.000	0.000
	N	2595	2595	2595	2595	2595	2595	2595	2595	2595
Aspergillus sp.	Correlation	1	-0.059(**)	-0.039(*)	-0.103(**)	-0.070(**)	-0.077(**)	-0.063(**)	-0.020	0.405(**)
	Sig. (2-tailed)		0.003	0.049	0.000	0.000	0.000	0.001	0.306	0.000
	N		2595	2595	2595	2595	2595	2595	2595	2595
Bipolaris sp.	Correlation		1	0.033	0.003	-0.076(**)	-0.026	0.034	-0.031	-0.020
	Sig. (2-tailed)			0.092	0.891	0.000	0.190	0.085	0.116	0.299
	N			2595	2595	2595	2595	2595	2595	2595
Curvularia sp.	Correlation			1	0.108(**)	0.002	-0.029	0.043(*)	-0.009	-0.058(**)
	Sig. (2-tailed)				0.000	0.921	0.136	0.029	0.648	0.003
	N				2595	2595	2595	2595	2595	2595
Fusarium spp.	Correlation				1	0.134(**)	0.025	0.087(**)	-0.020	-0.091(**)
	Sig. (2-tailed)					0.000	0.199	0.000	0.299	0.000
	N					2595	2595	2595	2595	2595
Fusarium semitectum	Correlation					1	0.038	0.023	0.016	-0.084(**)
	Sig. (2-tailed)						0.053	0.235	0.416	0.000
	N						2595	2595	2595	2595
Fusarium thapsinum	Correlation						1	-0.023	-0.021	-0.059(**)
	Sig. (2-tailed)							0.244	0.290	0.003
	N							2595	2595	2595
Phoma sp.	Correlation							1	-0.033	-0.072(**)
	Sig. (2-tailed)								0.090	0.000
	N								2595	2595
Penicillium sp.	Correlation								1	-0.025
	Sig. (2-tailed)									0.204
	N									2595

Notes: **Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

CHAPTER IV

CONCLUSIONS

The correlation analyses between the pathogens and the grain mold assessment methods (threshed grain score and mycoflora analysis) showed the mycoflora analysis is more efficient estimating the pathogens incidence due to the highly significant correlations (Table X).

Despite the pathogens level of incidence, not all the molds were associated with grain damage *per se*. The fungal genera and species responsible for the development of the GMDC were identified as follows: *Aspergillus sp., Curvularia sp., Fusarium spp., Fusarium semitectum, Fusarium thapsinum, Nigrospora sp.,* and *Rhizopus sp/ Mucor sp.*

Most individual pathogens combined analyses revealed significant differences between RILs (Table XI). Based on Fisher's LSD, the majority of RILs have similar levels of susceptibility except for HD RIL BTx635*P850029a which has the highest susceptibility especially when infected by *Curvularia sp., Fusarium semitectum, Fusarium thapsinum,* and *Fusarium spp.* (Figures 17, 18, 19, 20 respectively). On the contrary, RTx436*P850029b showed the lowest mold susceptibility.

Most individual pathogen combined analyses did not reveal significant differences between DGs (Table XII) indicating the DGs show similar susceptibility to mold infection. The exception was *Fusarium spp.* which revealed significant DGs effects (Figure 21) providing evidence of the HD RILs higher susceptibility towards this pathogen (HD > ND > MD). These results were corroborated via contrast analysis of

DGs with the exception of *Fusarium semitectum* which also revealed significant differences between HD and ND RILs (HD MD & MD ND) (Table XIII).

In contrast, the DGs contrast analysis restricted to pathogens significantly associated to grain mold damage indicated the HD lines are significantly different to the MD and ND lines (Table XIV). Fisher's LSD grouped them as follows: HD > ND > MD indicating that the HD lines had the highest fungal incidences.

When HD RIL BTx635*P850029a was subtracted from the population, the ANOVA of DGs across environments based on fungal incidence revealed significant DG, environment effects, but no significant DG * Environment effect was detected (Table XV) indicating the fungal infecting pattern did not change across environments. Fisher's LSD grouped them as follow: HD >MD & MD >ND (Figure 22). The DGs combined analysis based on germination rate revealed significant DG, environment and DG * Environment effects (Table XV). Fisher's LSD grouped them as follow: HD >ND >MD (Figure 23). These results provide evidence that with the exception of BTx635*P850029a the HD lines were not affected to a greater extent by their higher internal mycoflora when compared to other DGs. Additionally, it could be hypothesized that the HD significantly higher germination occurs due to their modified protein matrix. The invaginated conformation of endosperm protein bodies may provide to glycolytic endogenous enzymes (α-Amylase and β-Amylase) better access to the endosperm starch granules which would translate into a higher bioavailability of carbohydrates for the embryo during germination. Finally, the DGs combined analysis based on the incidence of *Fusarium spp*. revealed significant DG, environment, and DG * Environment effects

(Table XV). Fisher's LSD grouped them as follow: HD >ND >MD (Figure 24). These results provide evidence that *Fusarium spp*. has a significant pathogenetic preference for the HD RILs.

In conclusion, from a statistical point of view the HD RILs are significantly more susceptible to grain mold infection; however, their significantly higher vulnerability to grain mold damage can be attributed to BTx635*P850029a and to the preferential HD pathogenicity of *Fusarium spp.* Additionally, these conclusions are valid only for the breeding population on which the experiments were performed due to the fixed models employed to calculate the main and interactions effects of the collected data.

Finally, future research approaches may include the breeding of HD lines with higher resistance to pathogens significantly associated to grain mold damage (specifically *Fusarium spp*.). A closer examination of the antagonistic relationship between fungi may reveal the mechanisms by which some genera/species inhibit the incidence of other fungi as in the case of *Penicillium sp.* which as a result of its significant inverse association with *Alternaria sp.* (Table XVI) has been correlated to a significant reduction of the overall fungal incidence (Table X).

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