Effect of Irrigation on Fiber Concentration and In-Vitro Fiber Digestibility of Corn Plant Tissues

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Authorization to Submit Thesis

This thesis of Lani L. Martin submitted for the degree of Master of Science with a Major in Animal and Veterinary Science and titled "Effect of Irrigation on Fiber Concentration and In-Vitro Digestibility of Corn Plant Tissues" has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

The objective of this study was to determine the effect of irrigation on neutral detergent fiber (NDF) and lignin (LIG) concentrations and on *in-vitro* apparent dry matter digestibility (IVDMD), and *in-vitro* neutral detergent fiber digestibility (IVNDFD) of stems, leaf-sheaths, and leaf-blades of corn. Five commercial corn hybrids for silage (one of them showing the brown midrib phenotype) were planted in a split-plot setting within a randomized complete block design (4 replicates). Treatments consisted of a control treatment with furrow irrigation at planting and 3 more times during crop growth (IRRIGATED) and a non-irrigated treatment with furrow irrigation only at planting (NON-IRRIGATED). When the corn was between ¼ and ¾ milk-line stage of maturity, 5 plants from each plot were cut by hand, and stems, leaf-sheaths, and leaf-blades from the second phytomer below (LOWER) and the second phytomer above (UPPER) the ear insertion were dissected and frozen for analysis. Tissues were analyzed for NDF concentration, IVDMD, and IVNDFD. Data were analyzed using Proc Mixed of SAS, and the model included the effects of block (random, $df = 3$), treatment (fixed, $df = 1$), whole-plot error (random, $df = 3$), hybrid (fixed, $df = 4$), treatment by hybrid interaction (fixed, $df = 4$), and the residual or split-plot error (random, $df = 25$). IRRIGATED contained lower NDF concentrations ($P < 0.01$; 64.6 vs. 67.6% NDF) and greater IVDMD than NON-IRRIGATED plots ($P < 0.05$; 56.7 vs. 54.8% IVDMD). IVNDFD tended to be greater for IRRIGATED than for the NON-IRRIGATED plots ($P < 0.10$; 51.7 vs. 50.1% IVNDFD). Irrigation did not affect LIG concentration in the cell wall ($P > 0.1$), which averaged 19.9%. UPPER phytomers had a lower NDF concentration ($P < 0.01$; 64.4 vs. 67.7%) and a greater IVNDFD than LOWER phytomers ($P < 0.01$; 52.8 vs. 49.0%). In conclusion, under the conditions of this study, limited water supply does not affect lignin concentration in the cell wall and does not increase the in vitro digestibility of fiber in corn

for silage. The latter observation is contrary to the general industry belief that water-stress increases fiber digestibility in forages.

Drought, Fiber digestibility, Environment

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Lastly, I would like to thank Amin Ahmadzadeh, for being an inspiration, a mentor, an educator and most importantly a dear friend for the past five years.

Dedication

This thesis is dedicated to Mike, for more reasons than I can count. He stayed by my side while I pursued my degrees and brought joy to my life for the past five years.

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Chapter 1: Literature Review

Impact of Drought on Plants

Due to the uncontrollable nature of drought and the fact that it limits the most important nutrient for plant development and growth, drought is one of the most environmentally impactful stresses for crops. It impairs plant growth and development, limits plant production and the performance of crop plants, more than any other environmental factor (Shao et al., 2009). The effect of drought on the dairy industry has been historically catastrophic, for instance, a severe nationwide drought in the United States in 2012 reduced corn silage yield by 16.3% compared to the year prior. The crop losses costed the dairy industry $1 billion (USDA, 2013).

Ferraretto et al. (2018) found that optimizing harvest maturity, kernel processing, theoretical length of cut, and cutting height improved or maintained the nutritive value of corn silage and thus milk production of lactating dairy cows. They stated that technological advancements have been developed to help dairy producers and corn growers to enhance fiber and starch digestibility of whole-plant corn silage. Drought stress may influence digestibility factors in certain hybrids and may also affect harvest time regarding optimal digestibility of the corn plant. Some known impacts of drought include: stunted growth; lowered yield; altered membrane integrity, pigment content, osmotic adjustment water relations, and photosynthetic activity (Benjamin and Nielsen, 2006; Praba et al., 2009).

The susceptibility of plants to drought stress varies depending on the degree of stress, other additional stress factors, plant species, and their developmental stages (Demirevska et al., 2009). Many different events can cause plants to acclimate to water deficit, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant

structure, growth rate, tissue osmotic potential and antioxidant defenses (Duan et al., 2007). Additionally, temperature typically has large influences on forage quality. "Plant temperature is the result of complex interactions between plants and their environment and is influenced by radiation flux density, heat conduction, heat convection, latent heat, as well as morphological and anatomical features of plants" (Fahey, 1994).

Once temperatures rise above the optimum temperature, increasing thermal motion may interfere with enzyme-substrate binding by changing the three-dimensional conformation of the enzymes. Other plant processes that are also affected by temperature include membrane phase changes and changes in viscosity and rates of diffusion and translocation. A change in temperature will result in a shift in carbon partitioning among alternate pathways. There is limited information available about the direct effects of temperature on reactions leading to the formation of plant cell walls, but there is a generally positive relationship between heat and concentrations of structural material (partitioning of photosynthate between cell walls and cell contents). High growth temperatures decrease stem diameter and increase rate of maturation and lignification (Fahey, 1994).

In a study conducted by Deinum et al. (1968), the effects of temperature, solar radiation and N nutrition on ryegrass digestibility were tested. Out of these three factors, temperature consistently had the greatest negative correlation with apparent digestibility. Deinum et al. (1968) also stated, "High growth temperatures also promote stem development over leaf development and consequentially will lower leaf/stem rations in herbage." Heat stress may affect the nutritional composition of corn silage (including NDF) even in crops that have been adequately watered. Ferreira (2015) observed high concentrations of NDF for corn silage that came from a corn field that was irrigated but suffered from heat stress

immediately after pollination (unpublished observations), suggesting that silage quality is not assured exclusively by water status.

Studies revealed the effects of drought on kernel development and whole plant composition. If drought occurs early on during the vegetative state, kernel development is not affected nor is potential nutritional value. Late set drought (after silking) causes lowered grain yield on the ear (Ferreira and Brown, 2015). Khan et al. (2004) conducted a study comprising of six treatments, namely, control (six irrigations), five, four, three, two and one irrigation in maize. It was concluded that plant height, stem diameter, and leaf area decreased noticeably with increasing water stress. The reduction in plant height could be attributed to a decline in the cell enlargement and more leaf senescence in the plant under water stress (Manivannan et al., 2007). Furthermore, Kamara et al. (2003) revealed that water deficit imposed at various developmental stages of corn reduced total biomass accumulation at silking by 37%, at grain-filling period by 34% and at maturity by 21%. Cell growth is considered one of the most drought sensitive physiological processes due to the reduction in turgor pressure (Nonami, 1998).

Cell Wall Characteristics

Fiber is typically composed of the cell wall, chich consists of hemicellulose, cellulose, lignin, silica and some proteins (heat damaged) (Van Soest, 1993). The cell wall includes beta 1-4 linked polysaccharides that are not able to be fermented by microorganisms, into absorbable sugars, in non-ruminant mammalian species. Ruminants, however, can utilize the cross-linked polysaccharides as nutrients because of the microbes in the rumen (Van Soest, 1993).

The growing cell wall consists mainly of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins, proteins and phenolics (Moore and Hatfield, 1994). Cellulose microfibrils are composed of β-1,4-linked D-glucose units arranged into crystalline and non-crystalline regions through hydrogen bonds. The cell wall must be able to grow. It starts as a primary wall and elongates because the wall polymers are not yet cross-linked (Iiyama et al. 1993).

The cell wall of the typical monocot species, such as corn, contains; 25% cellulose, 55% hemicellulose (arabinoxylans, xyloglucan and mixed-linked glucans) and 10% pectin. The xylans are substituted with arabinose and glucuronic acid side chains and are called arabinoxylan (AX) or glucuronoarabinoxylans (GAX). A proportion of the arabinose side chains are further substituted with phenolic acid residues, mainly ferulic (4-hydroxy-3-methoxycinnamate), p-coumaric (4-hydroxycinnamate) and 4-O-methylglucuronic acids. When maturation occurs after the growing phase, secondary wall deposition begins with lignification (Terashima et al. 1993). The arabinoxylans in grasses serve a major structural role by binding to cellulose microfibrils and becoming oxidatively cross-linked with each other and with lignin by phenolics. Later in plant development, the lignin polymer becomes a significant constituent of the cell wall (Santiago et al., 2013). Essentially, as the cell matures, the newer polysaccharides in the wall are not lignified, then lignification follows (Jung, 1998). A study by Riboulet et al., (2008) compared cell wall composition of two corn hybrids, one that is known to be more digestible and one that is known to be less digestible. As a result of faster lignification and cell wall thickening, the less digestible hybrid showed thicker cell walls and higher levels of lignification than the more digestible hybrid at maturity. After maturity there were not significant changes in cell wall and lignin.

The observation that consistent negative relationships of Klason-lignin concentration with cell-wall polysaccharide degradability agrees with the view that lignin is the primary limitation to polysaccharide removal from forage cell walls (Jung and Deetz, 1993). Even though the complex matrix interactions in cell‑wall organization make it difficult to identify any single cell‑wall component, or simple combination, that can accurately predict degradability of maize cell walls, it is likely that lignin concentration and phenolic acid content are the main predictors of corn cell-wall degradability (Jung and Buxton, 1994). This is reflected in the consistent negative relationships of Klason-lignin concentration with cell-wall polysaccharide degradability (Jung and Deetz, 1993).

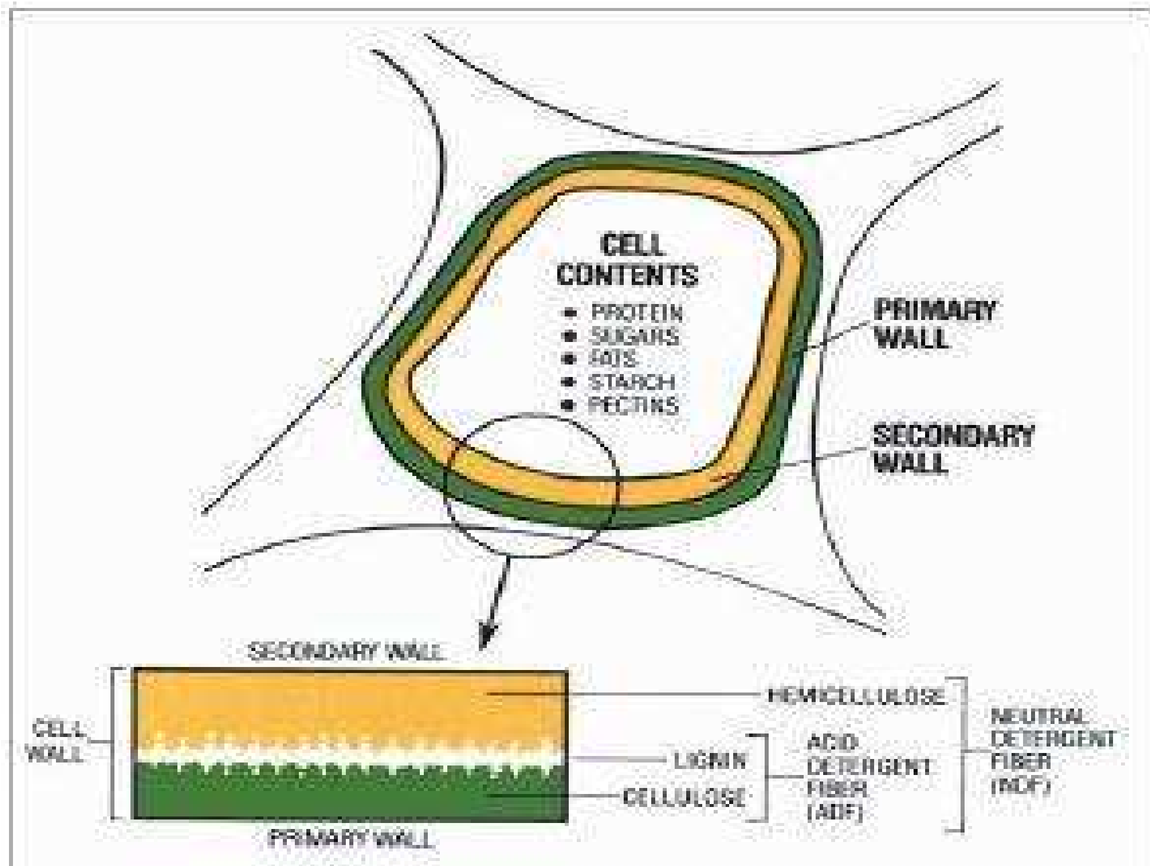

Figure 1.1 Diagram of a plant cell showing cell-wall structure (from Advanced Forage Management 1999)

Chapter 2: Effect of Drought on Corn Plant Tissue Digestiblity

Introduction

Corn silage is a commonly used feedstuff in dairy cattle operations in the United States (Mowrey and Spain, 1999). It produces more digestible energy per acre than any other forage, because of energy available from fiber digestibility in the stover and starch digestibility in the grain (kernel) (Martin et al., 2007). Digestible energy availability also depends on the stover to grain ratio. Water stress causes severe constraint on corn plant productivity and can cause: lowered yields, decreased water-use efficiency and reduced leaf size, stem extension and root proliferation (stover to grain ratio) (Farooq et al., 2009).

Little is known about the effects of water stress on corn plant cell wall composition, particularly lignification. Corn cell wall composition is directly correlated with digestibility in dairy cattle (Jung and Allen, 1995). Lignin is the key element that limits cell-wall digestibility. High lignin concentration in the cell-wall is negatively correlated to forage intake of ruminants (Jung and Allen, 1995). Corn silage with higher Neutral Detergent Fiber (NDF) digestibility increases feed intake and 4% fat corrected milk (FCM) production (Ivan et al., 2005). Forage intake is a key indicator of animal performance because the indigestible portions of forage are responsible for physical fill which is the main determinant of feed intake and feed intake is directly correlated with milk production (Waldo, 1986). Despite continual improvements in total dry matter and kernel productivity of corn hybrids, there have been few advancements in nutritional quality of whole plant corn silage in the last century (Lauer et al., 2001; Ferraretto and Shaver, 2015). The cell contents are uniformly and completely digested, but the CW is not (Van Soest, 1993). Dry matter digestibility is a function of cell wall concentration because dry matter digestibility decreases as cell wall

concentration increases. Alternatively, cell wall digestibility is a function of cell wall composition, particularly lignin (Ferreira and Brown, 2017).

A common belief in the dairy industry is that feeding water-stressed corn silage (after a drought) increases productivity in dairy cows (Ferreira and Brown, 2015). The goal of this study was to identify how water-stress affects cell wall composition and concentration of corn plant tissues. Cell wall composition and concentration help to understand total plant digestibility which is important because increased digestibility is correlated with greater production.

The focus of this study was to understand the digestibility of "corn plant tissues". The tissues that were analyzed were the stems, stem sheaths, and blades from an upper and lower phytomer of the plant. Since corn is a monocot, it develops a structure of cells that have parallel veins, particularly in the foliage. The stiff, rigid tissues of the central stems are rich in sclerenchyma cells that provide the strength and overall structure to the plant (Wright et al, 2017). Leaves also contain structural cells which help them maintain enough rigidity to create a surface area to catch rays of sun for photosynthesis, but there are also more vascular tissues. Vascular tissues, xylem and phloem, are found in the roots, stems and leaves. Xylem tissues carry water, hormones and liquids upward in the plant while downward movement of the same occurs in phloem tissues. Cells that actively conduct photosynthesis are primarily oriented in the upper layers of leaves while leaf undersides are full of air space and stomata, which open and close for gas exchange and to release or retain water vapor (Wright et al., 2017). Leaves and stem sheaths are more digestible than stems (Weaver et al., 1976) This is likely due the higher number of structural cells in the more rigid stem tissues. Upper

phytomers tend to be more digestible in corn plants since they often contain a higher ratio of blades/sheaths: stems. (Ferreira and Brown, 2017).

The *in-vitro* technique is an anaerobic fermentation of a sample substrate with medium and filtered rumen contents followed by an end point measurement. The medium is a buffer that mimics ruminant saliva (Van Soest, 1982). This buffer allows maintenance of a desirable pH for extended incubation times (McDougall, 1948). Another important aspect of creating an ideal *in-vitro* rumen environment is the time at which rumen fluid is collected should be constant relative to feeding time of each animal used for fluid collection. Multiple feedings per day will also help to allow for optimal enzymatic activity in the rumen fluid (Weiss, 1992). This method can be very useful in estimating apparent DM digestibility, but has some limitations. Apparent *in-vitro* digestibility is a direct measure of DM loss, however it is not an indicator of true digestibility because it does not measure whole tract DM losses in a dairy animal. When using a porous bag in a simulated rumen environment that utilizes rumen fluid, dry matter disappearance is what is measured, but this technique does not consider that the final weight may include bacteria and not just undigested substrates. This error is more prevalent in high-fiber feeds (Sauvant et al., 1985)

In-vitro true dry matter digestibility is calculated by correcting for endogenous losses, apparent digestion does not. Endogenous losses include sloughed off intestinal cells, digestive juices (enzymes), and microbial matter. The process is quantified by measuring fecal output of fasted animals (Van Soest et al., 1966)

Neutral detergent fiber is the measure of the chemical compounds generally considered to comprise fiber (cellulose, hemicellulose and lignin). Within a specific feedstuff, concentrations of NDF, ADF and crude fiber are highly correlated. On average

NDF is less digestible than non-fiber carbohydrates, therefore it is negatively correlated with energy concentration (NRC, 2001).

Lignin is the key element that limits cell-wall digestibility, which means that it is the main factor which limits utilizable cell wall components available for animal herbivores and anaerobic digestion systems (Van Soest,1982). High lignin concentration in the cell-wall is negatively correlated with forage intake of ruminants (Jung and Allen, 1995). Lignin is a polymer formed from monolignols derived from the phenylpropanoid pathway in vascular plants. It develops in cell walls as a result of plant maturity. Later in plant development, the lignin polymer becomes a significant constituent of the cell wall (Santiago et al., 2013). According to Moore et al. (2001), "Lignin is considered an anti-quality component in forages because of its negative impact on the nutritional availability of plant fiber." Higher rates of lignification are found in structural tissues such as xylem and sclerenchyma (greater in stems than blades and sheaths) (Moore et al. 2001).

There are several different processes that can be used as tools to better understand digestibility and to obtain estimations of apparent and true digestibility of plant fiber. This study was designed to utilize as many of these processes as possible to create the most accurate representation of digestibility of the pant tissue. It is important to understand the complete potential of plant digestibility using laboratory techniques so that feeding methods can be more accurate and efficient at the farm. The best way to understand total plant digestibility is to understand cell wall composition and concentration. This knowledge can help dairy farmers and nutritionists create more balanced rations for better production of dairy cattle.

Materials and Methods: Study Field Planting and Irrigation

The corn used for the study was planted, irrigated and harvested at the University of Idaho Research and Extension Center. The soil was a surface-irrigated Portneuf silt loam (coarse-silty, mixed, mesic, Durinodic Xeric Haplocalcid) near Kimberly, Id, USA (42º30' N and 114º8' W, elevation 1200 m) On May 12, 2018 a total of fifty-six plots were planted in four blocks containing 4 irrigated rows and 4 non-irrigated rows of corn each. Within each block there were seven irrigated plots and seven non-irrigated plots. Each plot was 8.5 m long with a 2 m alley between each plot. In between each row was a furrow for irrigation and the rows were 76 cm apart. Seeds were planted 3.8 cm deep with a John Deere 30-20 tractor and an Almaco Twin Plate 2 Planter. The planting population was 40,000 plants per acre. Four rows of corn were planted on all borders of the study field to protect the study from cross pollination and irrigation seepage from surrounding areas. These rows were not included for data collection in this study.

The irrigation ditch was located on the West end of the field. The entire field was irrigated at the time of planting. Following the initial irrigation, one furrow in each plot was not irrigated for the rest of the study. Two contiguous furrows within each plot were irrigated every 10 days. These furrows were flagged, and irrigators manually diverted water with tubing into the specified furrows. The treatments in blocks one through three were assigned irrigated and drought, respectively. The treatments in block four were assigned drought and irrigated, respectively.

Materials and Methods: Hybrids

Seven commercial hybrids were used in this study. For statistical accuracy, they were

planted in the fifty-six-plot field, based on a randomized split-plot design. The hybrids used were: Pioneer P1449AMX which originated in Indiana, US. It had a relative maturity of 115-days. This variety expressed the Brown Mid-Rib phenotype; Caverndale Farms CF753 and it originated in Iowa, US. It has a relative maturity of 108 days; Caverndale Farms CF664 and it originated in Indiana, US. It had a relative maturity of 102 days; Dyna-Gro 1720349, originated in Michigan, US. It had a relative maturity of 107 days. Dyna-Gro 1069114 originated in Iowa, US. It had a relative maturity of 105 days; MastersChoice MCT4934 which originated in Illinois, US. It had a relative maturity of 99 days; and MastersChoice MCT4632 which originated in Illinois, US. It had a relative maturity of 96 days.

Materials and Methods: Harvest

Ten plants from each plot were cut by hand 15 cm above the ground, gathered and weighed. Then, they were chopped using a standard Troy-Bilt (2") 208cc Chipper Shredder to make a representative chopped sample. Fifty grams of each sample were dried in a forced air oven at 55ºC for 48 hours. The samples were re-weighed after drying and dry matter (DM) percentages were calculated and recorded.

Three additional plants were collected from each plot. Using a three-inch knife blade, a sample of the leaf-blades, leaf-sheaths and stems was collected from each plant. Samples were collected from the lower and upper nodes of each corn plant. The samples were dried in a forced-air oven at 55ºC for 48 hours. After drying, each tissue from each node of the three plants were ground together through a 4 mm Wiley mill to form a composite representation from each plot (Upper stems, upper sheaths, upper blades, lower stems, lower sheaths and lower blades from each plot). Samples were transported to the Dairy Nutrition Laboratory at

Virginia Tech, where they were ground through a 1 mm Wiley Mill and prepared for various laboratory procedures.

Materials and Methods: In-Vitro Apparent and True Dry Matter Digestibility

The *in-vitro* apparent digestibility procedure was performed at the Dairy Nutrition Laboratory at Virginia Tech, Blacksburg, Virginia, using a protocol developed by Gonzalo Ferreira. Empty Ankom F57 filter bags were placed in a 500-mL glass beaker. They were then covered in acetone and soaked for five minutes. The bags were removed from the acetone and air-dried on a cloth for 24 hours. The bags were labeled with an acetone-resistant permanent marker, according to a numerical assignment of each sample (see Appendix table 1). The bags were dried in a forced-air oven at 100°C for two hours. After drying, the bags were weighed, and weights were recorded. The empty bags were then filled with 0.2500 grams of sample, then the bag and sample were weighed, and weights were recorded. The bags were double heat-sealed to ensure that samples would not spill.

At least 12 hours before the incubation began, eight 1-gallon glass jars were prepared by inserting a permeable plastic separator into each one. Twenty-six samples were placed in each jar, such that the original sample and its duplicate were on opposite sides of the separator. The Daisy Incubator and 2 water baths, which were set for 39°C, were turned on.

Two hours before incubation began a media was prepared by placing a 20 L carboy on a magnetic stir-plate. Trypticase (34 grams), water (6,800 mL), micro-mineral solution (1.7 mL), buffer solution (3,400 mL), macro-mineral solution (3,400 mL) and Resazurin (0.1% w/v) (17mL) was added to the carboy and mixed for 5 minutes. Using a 2,000-mL graduated cylinder, 1,200 mL of the media solution was added to each of the eight 1-gallon

glass jars. Then, 3,360 mL of the media was added to a 4-L flask labeled “Flask A”. Flask A was placed in one water-bath and the eight incubation jars were placed in the second water-bath, both the flask and jars were purged with CO2.

One hour before incubation the reducing agent, Cysteine HCL (4.576 g), water (704 mL), 1 N NaOH (28.6 mL) and Sodium Sulfide (4.576 g), was prepared in a 1 L flask labeled “Flask B”. Using a 100-mL graduated cylinder, 60 mL of reducing agent was added to each incubation jar and 168 mL was added to Flask A (a few minutes after adding the reducing agent, the solution turned from a pink to a grayish color). A 4-L flask labeled “Flask C” was placed in the water-bath and purged with CO2. A funnel was placed in the opening of the flask and a 2-layer cheesecloth was placed in the funnel. While the media and reducing agent were being prepared, two 2-L coolers of rumen fluid was collected from three cannulated Holstein cows at the Kentland Farm Dairy Complex and transported back to the Dairy Nutrition Laboratory. A 1-layer cheesecloth was placed in a funnel which was then placed in the opening of a 2-L graduated cylinder. A professional-grade steel blender was filled with hot water and purged with CO2.

The pH of the rumen fluid from one cooler from each cow was recorded. Then, the top layer (1 inch) of rumen solids was removed and discarded from each of the three coolers. The remaining rumen contents (both liquid and solid) were poured into the 2-L graduated cylinder. Using the cheesecloth layer that was placed in the funnel in the cylinder, the liquid portion of the rumen contents was separated from the solid portion. Using a 1-L flask 550 mL of the liquid portion from each of the three coolers was poured into Flask C (total volume of rumen fluid in Flask C was 1,650 mL). 280 g of the solid portion of the rumen contents from each of the 3 coolers (total of 840 g) was added to the blender along with 1,680 mL of

the pre-warmed media from Flask A and blended on the low setting for 15 seconds and on the high setting for 45 seconds. The blended contents were then poured into Flask C through 2 layers of cheesecloth. Flask C was swirled for 20 seconds then using a 1000-mL graduated cylinder, 800 mL of the inoculum was added to 4 of the eight incubation jars in two 400 mL increments (a small sample of the inoculum was collected, and the pH of the sample was recorded). The same procedure was repeated with the remaining three coolers of rumen fluid and added to the remaining four incubation jars. The incubation jars were placed in the Daisy incubator for 30 hours.

Temperature and pH from each jar were immediately taken and recorded after incubation. The fluid from each jar was discarded and the sample bags were immediately placed in an ice and cold-water bath. The ice bath was then stirred for 2 minutes. Without removing any of the sample bags, the ice and water were discarded and replaced with clean ice and water. These steps were repeated until the water was clear (4 times). After rinsing, the bags were air-dried on a clean cloth for 24 hours, then moved to a forced-air oven and dried at 55°C for 24 hours. The bags were weighed and recorded. The initial bag weights were subtracted from the final bag and sample weights to obtain the final sample weights. Then the final sample weights were subtracted from the initial sample weights to obtain apparent In-Vitro digestibility of each sample. These procedures were performed twice in order to process all samples and their duplicates.

Materials and Methods: Neutral Detergent Fiber

The Neutral Detergent Fiber procedure was performed at the Dairy Nutrition Laboratory at Virginia Tech, using the protocol and instruments provided by Ankom Technologies. The procedure was performed on the initial dry, ground samples. It was also

performed on the samples and their duplicates that had been analyzed in the apparent *in-vitro* digestibility procedure, in order to obtain true *in-vitro* digestibility data for the samples.

Reagents for the procedure were prepared prior to performing the Neutral Detergent Fiber procedure on the previously indicated samples. For more information on the reagents used refer to Appendix Table 2. For the initial dry, ground samples, empty Ankom F57 bags were placed in a laboratory-safe canning jar. The bags were labeled with an acetone-resistant sharpie, according to a numerical assignment of each sample (see appendix table 3). The bags were dried in a forced-air oven at 100°C for two hours. After drying, the bags were weighed, and weights were recorded. The empty bags were then filled with 0.450-0.50 grams of sample, then the bag and sample were weighed, and weights were recorded. The bags were double heat-sealed to ensure that samples would not spill. Bag preparation was not necessary for the samples from the apparent *in-vitro* digestibility procedure. The instrument used for this procedure was an Ankom^{2000}. This instrument could perform the digestion at $100 \pm 0.5^{\circ}$C and maintaining a pressure of 10-25 PSI. It could create a similar flow around each sample to ensure uniformity of extraction with 65 RPM agitation. Twenty-three sample bags and one empty filter bag were shaken to evenly disperse the sample throughout the bag, and then arranged by threes in the eight Bag Suspender Trays. The trays were then stacked on the center post of the Bag Suspender with each level rotated 120 degrees in relation to the tray below it. One empty tray was placed on top. An exhaust hose was connected to the instrument and positioned into the drain. The Bag Suspender with the bags was placed into the Vessel and the Bag Suspender Weight was placed on top of the empty ninth tray to keep the Bag Suspender submerged in the instrument. Using a 2-L graduated cylinder, 2000 mL of ambient temperature Neutral Detergent solution and 20 grams of sodium sulfite and 4.0 mL

of alpha-amylase were added to the fiber analyzer vessel. Once the vessel lid was closed and secured to ensure that pressure was maintained inside of the vessel, the heat and agitate cycles were turned on for 75 minutes. During incubation, enough water for the rinse cycles was boiled. After incubation the instrument was turned off and the drain valve was slowly opened, and the solution was discarded down the drain. The valve was then closed, and the Vessel Lid was opened. Using a 2-L graduated cylinder, 2000 mL of 90°C distilled water and 4.0 mL of alpha-amylase were added to the Vessel and the agitate cycle was turned on for 5 minutes. After 5 minutes, the agitate cycle was turned off and the rinse solution was drained. The rinse procedure was repeated 4 times (the last 2 rinses did not contain the alpha-amylase).

After completing the rinsing cycles, the filter bags were removed from the vessel. Excess water was gently squeezed out by placing the samples between two paper towels and gently applying pressure. Then the samples were placed in a 250 mL glass beaker and enough acetone to cover the bags was added to the beaker. The bags soaked in the acetone solution for 5 minutes. The bags were then removed from the acetone and placed on a cloth, to be air dried overnight. Then the bags were placed in a forced-air oven for 2 hours at 100°C.

The bags were weighed, and the weights were recorded. The initial bag weights were subtracted from the final bag and sample weights to obtain the final sample weights. Then the final sample weights were subtracted from the initial sample weights to obtain Neutral Detergent Fiber of each sample. (For the bags that had already been processed in the *in-vitro* procedure, the final NDF bag weight was subtracted from the post *in-vitro* bag to determine True *in-vitro* digestibility of the samples.

Materials and Methods: Cell Wall Extraction

The cell wall isolation procedure was performed at the Dairy Nutrition Laboratory at Virginia Tech, using a protocol developed by Alston Brown and Gonzalo Ferreira from a protocol obtained from Ron Hatfield. Dried samples were used after being ground through the 1 mm Wiley mill. Duplicate samples were not used for this procedure. Reagents for the procedure were prepared prior to performing the Cell Wall Extraction procedure on the previously indicated samples. For more information on the reagents used refer to Appendix Table 4.

Prior to beginning the procedure 28, 35-mL Oakridge centrifuge tubes were labeled with a permanent marker, according to a numerical assignment of each sample (see Appendix Table 5) and were dried overnight in a forced-air oven at 55°C. The samples were also placed in the oven overnight to ensure that no moisture was absorbed in storage. After drying the Oakridge Centrifuge tubes were weighed and the weights were recorded. One to two grams of each sample was weighed into the Oakridge centrifuge tube and the weights were recorded.

Using a bottle-top dispenser, 10mL of 50mM Tris-Acetate buffer was added to each sample. The samples were then incubated at 4°C for 24 hours. During incubation a water bath was prepared at 100°C and a second water bath was prepared at 55°C. After incubation, the following steps were performed three times. 10mL of 50mM Tris-Acetate buffer was added to each sample, caps were secured on the Oakridge tubes and they were centrifuged at 20,000 x g for 20 minutes at 20°C. Then the supernatant was removed from each sample by vacuum. 10mL of 50mM Tris-Acetate buffer was added to each sample, then the tubes were capped and placed in the water bath at 100°C for 2 hours. During incubation, in a 500-mL glass beaker, the Tris-acetate buffer with 20 U Amylase and 40 U amyloglucosidase was prepared. After incubation the tubes were removed from the water bath and the supernatant was removed by vacuum. Using a 10 mL pipette, 10mL of Tris-acetate buffer with 20 U

Amylase and 40 U amyloglucosidase was added to each sample and the samples were then incubated in a water bath at 55°C for 2 hours. While the samples were incubating the 80% Ethanol solution was prepared in a 1-L orange capped glass bottle. Immediately after incubation the samples were centrifuged at 1,000 x g for 20 minutes and the supernatant was removed by vacuum. The following steps were performed three times for the stem and sheath samples and five times for the leaf samples (the purpose of this step was to remove all chlorophyll from the samples): using a bottle-top dispenser 20 mL of 80% ethanol was added to each sample and was mixed for 20 minutes on an orbital shaker set on “High” , then the samples were centrifuged at 2,000 x g for 15 minutes and the supernatant was removed by vacuum. Using a bottle top dispenser, 20 mL of Acetone was added to each sample. The samples were them incubated overnight at 4°C. The following morning the samples were mixed on an orbital shaker set on “High” for 20 minutes. During shaking a 2:1 mixture of Chloroform and methanol was prepared in a 500-mL, orange-capped glass bottle. Using a bottle top dispenser, 20 mL of the chloroform: methanol solution was added to each sample. The samples were then incubated for 20 minutes while being shaken, then centrifuged at 2,000 x g for 15 minutes. The supernatant was removed from each sample by vacuum. The following steps were performed two times: Using a bottle-top dispenser, 20 mL of acetone was added to each sample and incubated for twenty minutes while being shaken. The supernatant was removed from each sample by vacuum. The samples were then dried at room temperature for 48 hours, then dried in a forced-air oven at 55°C for 24 hours. The samples in the tubes were weighed and the weights were recorded. To calculate cell wall concentration, the tube weight was subtracted from the final tube and sample weight, then the final sample weight was subtracted by the initial sample weight. The Cell Wall isolation procedure performed 9 times to accommodate for all the samples that were analyzed.

Materials and Methods: Cell Wall Lignin Extraction with Acetyl Bromide

The cell wall Lignin- Extraction procedure was performed at the Dairy Nutrition Laboratory at Virginia Tech, using a protocol developed by Alston Brown and Gonzalo

Ferreira from a protocol obtained by Ron Hatfield. The samples analyzed were the final products of the Cell Wall Isolation procedure. Reagents for the procedure were prepared prior to performing the Cell Wall Lignin- Extraction procedure on the previously indicated samples. For more information on the reagents used refer to Appendix Table 6.

Before extraction began the samples were dried in a forced-air oven at 55°C for 24 hours, then 10-30 grams of each sample was weighed and placed in capped glass tubes which were labeled according to a numerical assignment of each sample (see Appendix Table 7) and the weights were recorded. A dry heat block was warmed to 50°C just prior to beginning. Under the hood and using a repeating pipette, 2.5 mL of 25% acetyl bromide was added to each tube and the tubes were tightly capped. The tubes were then mixed by gently swirling each one for about 5 seconds, then incubated for 2 hours on the dry heat block. The samples were mixed by being gently swirled every 30 minutes. During incubation: the matrix solution was prepared; the scintillation vials and microcentrifuge tubes were labeled according to the aforementioned numerical assignment system; 9.5 mL of matrix solution, for spectrophotometry, was added to the scintillation vials; and an ice-water bath was prepared. After Incubation the tubes were removed from the dry heat block and placed in the ice-water bath. Under the hood, 1.5 mL of the reaction mixture (sample) was decanted into the corresponding labeled microcentrifuge tubes. The microcentrifuge tubes were then capped and centrifuged at 12,000 x g for 3 minutes at room temperature, then 0.5 mL of the supernatant were transferred to the matrix solution in the corresponding labeled scintillation vials using a 1-mL pipet. The scintillation vials were mixed by gentle swirling.

About 1 mL of solution from each scintillation vial was then transferred into a quartz UV cuvette with a Pasteur pipette. The cuvette was then placed in the Spectrophotometer and

the absorbance was read at 230 to 350 nm (using a pre-made file, that was saved to the spectrophotometer computer software, called: Acetyl Bromide Lignin protocol). After the absorbance reading was recorded, the waste from the cuvette was discarded and the cuvette was cleaned with distilled water and the next sample was then transferred to the cuvette for absorbance reading. The highest absorbance (280 nm) and the appropriate extinction coefficient was used to calculate the concentration of lignin.

Statistical Analysis

The experiment involved a factorial treatment structure, therefore, a split-plot design was implemented. This design allows for more than one randomization when assigning treatments. It is also the ideal design when each one factor has different experimental units that the other factors. Data were analyzed using Proc Mixed of SAS, and the model included the effects of block (random, df = 3), treatment (fixed, df = 1), whole-plot error (random, df = 3), hybrid (fixed, df = 4), phytomer (fixed, df= 1), tissue (fixed, df= 2), treatment by hybrid interaction (fixed, df = 4) and the residual or split-plot error (random, df = 23).

Results

The effect of irrigation treatment on the percentage of dry matter (DM) in the corn plant tissues was statistically significant, with the non- irrigated corn plants showing a higher percentage of DM than the irrigated corn plants ($P < 0.01$; 36.8% vs. 30.3% DM: Figure 2.1). These results prove that the non-irrigated corn received less water than the irrigated plants, this allows the non-irrigated treatment to be identified as “water-stressed” compared to the irrigated treatment.

There was a statistical difference in percentage of DM amongst the hybrids (P< 0.01), with Hybrid 1 (showing the BMR phenotype) containing the lowest percent DM and Hybrids 4 and 5 showing the highest percent DM. (P< 0.01; 29.1% vs. 32.4% vs. 34.0% vs. 36.2% vs. 36.2%: Figure 2.2)

Irrigation treatment did not significantly affect overall DM yield (P> 0.22). The effect of hybrid was a trend for hybrids 1,2,3,4, and 5 (P= 0.06; 15520 lb. vs. 17031 lb. vs. 16821 lb. vs. 16046 lb. vs. 17973 lb.: Figure 3.4). These results of DM yield may not accurately represent yield potential, because the plot size may not have been large enough to accurately calculate plants per hectare.

The effect of irrigation on the *in-vitro* true dry matter digestibility (IVTDMD) was statistically significant. Non-irrigated samples showed lower IVTDMD than irrigated samples (P< 0.05; 66.5 vs. 69.2%: Figure 2.3). When exposed to a rumen-like environment for 30 hours, the irrigated treatment samples were more digestible.

Hybrid had a significant effect on IVDMD (P< 0.05) and IVTDMD (P< 0.05; 73.2% vs. 68.1% vs. 67.5% vs. 65.5% vs. 65.0%: Figure 2.4). Hybrid 1 (BMR phenotype) had the highest IVTDMD and hybrid 5 had the lowest IVTDMD. Although there was not a significant difference between hybrids 2, 3, 4 or 5, these results show that the BMR hybrid had a higher percent of DM loss after the *in-vitro* incubation period and thus continues to characterize BMR as a more digestible phenotype in corn plants.

Phytomer also had a significant effect on IVTDMD. The upper phytomer had higher IVTDMD than the lower phytomer (P< 0.05; 69.9% vs. 65.8% Figure 2.5). A higher percentage of DM losses in the upper phytomer indicates higher true digestibility than in the lower phytomer.

The effect of tissue was significant on IVTDMD (Figure 2.6). Blades were more digestible than sheaths and stems.

The effect of irrigation treatment on concentration of Neutral Detergent Fiber (NDF) was statistically significant. The irrigated treatment samples had lower concentrations of NDF than the non-irrigated treatment (P<0.01; 64.6% vs. 67.6%). There was a statistically significant difference in NDF concentration amongst hybrids (P< 0.01; 62.2% vs. 66.0% vs. 65.7% vs. 67.8% vs. 68.7%), with hybrid 1 showing the lowest concentration of NDF and Hybrids 4 and 5 showing the highest concentrations of NDF. These results reflect that BMR is a more digestible hybrid of corn, because a lower NDF concentration is negatively correlated with apparent digestibility. The effect of Phytomer on concentration of NDF was also statistically significant. Lower phytomers showed higher concentrations of NDF than upper phytomers (P< 0.01; 67.7% vs. 64.4%). Data from this study shows higher NDF concentrations in the lower phytomers which is negatively correlated with apparent digestibility. The effect of tissue on NDF was statistically significant. Sheaths had the highest concentration of NDF, followed by blades, then stems (P< 0.01; 76.4% vs. 62.0% vs. 59.8%:). The interaction between irrigation treatment and tissue on NDF concentration was significant. (P< 0.01)

The effect of irrigation treatment on in-vitro neutral detergent fiber digestibility (IVNDFD) showed a trend where irrigated samples had higher IVNDFD than the non-irrigated samples (P< 0.1; 51.7% vs. 50.1%: Figure 2.7). These data suggest that irrigated samples are more digestible than non-irrigated samples, which is consistent with other findings in this experiment. Hybrid had a significant effect on IVNDFD (P< 0.01; 56.3% vs. 51.1% vs. 49.5% vs. 48.7% vs. 48.9%: Figure 2.8). Hybrid 1 (BMR phenotype) had the

highest IVNDFD and hybrids 4 and 5 had the lowest IVNDFD. This data is consistent with the literature findings that suggest that BMR is a more digestible hybrid. Phytomer also had a significant effect on IVNDFD. Upper phytomers showed higher IVNDFD than lower phytomers (P< 0.1; 52.8% vs. 49.0%; Figure 2.9). This data suggests that upper phytomers are more digestible than lower phytomers in the rumen. Tissue had a significant effect on IVNDFD (P< 0.01; Blades: 53.7% vs. Sheaths: 58.4% vs. Stems: 40.6%: Figure 2.10). Blades and sheaths had higher IVNDFD than the stems. This observation reflects the common characteristic of higher digestibility in blades and sheaths than in stems in corn plants. And in general plant anatomy, metabolic tissues like the blades tend to be much more digestible than support tissues like the stems.

Treatment had no statistically significant effect on the concentration of lignin in the samples (P> 0.1: Avg. 19.2%). Hybrid had a significant effect on the concentration of lignin (P< 0.01; 18.0% vs. 19.9% vs. 19.0% vs. 19.0% vs. 20.5%: Figure 2.11). Hybrid 1 had the lowest concentration of lignin and hybrid 5 had the highest content of lignin. These data further confirm that BMR corn is more digestible because lignin concentration is inversely correlated with digestibility. There was a statistical difference in lignin concentration amongst the phytomers. The lower phytomer had higher concentrations of lignin than the upper phytomers (P< 0.01; 19.9% vs. 18.6%: Figure 2.12). There was a statistical difference in concentration of lignin amongst different tissue types. Blades had a slightly lower lignin concentration than sheaths. Stems had a drastically higher lignin concentration than the blades and sheaths (P< 0.01; 14.4% vs. 17.3% vs. 26.1%: Figure 2.13). These results strongly indicate that stems are a less digestible tissue, because lignin concentration is negatively correlated with digestibility.

Discussion

A general industry belief is that forage crops from drought years tend to have higher quality than normal years (Deetz et al., 1996). The findings of this study do not support this general industry hypothesis. Based on the results it can be concluded that the irrigated treatment plots had lower concentrations of dry matter and neutral detergent fiber than the irrigated treatment plots, and irrigated treatment plots had higher in-vitro dry matter digestibility, in-vitro true dry matter digestibility, and in-vitro neutral detergent fiber digestibility than the non-irrigated treatment plots. A higher concentration of NDF in the non-irrigated treatment is an initial indicator that water stress decreases apparent digestibility in corn plant tissues, because it is the measure of all fibrous components of the plant and is highly, negatively correlated with total energy (NRC, 2001). The main reason for this is that NDF includes lignin, the key indicator of true digestibility, however NDF is not an accurate measure of true digestibility because it also includes the fibrous components cellulose and hemicellulose which are digestible by rumen microbes (NRC, 2001). There was no difference in lignin concentrations between the irrigated and non-irrigated treatment plots. A study conducted by Deetz et al. (1996) that evaluated the effect of drought on cell wall composition of alfalfa also found that lignification was not significantly affected by water status. Based on these results it can be concluded that the irrigated treatment plots tended to have higher overall digestibility than the non-irrigated treatment plots, however since lignin is the main indicator of true digestibility (Jung and Buxton, 1994) it cannot be concluded that irrigation has an effect on overall digestibility of the stover portion of corn silage. These results do not reflect the general belief that drought increases quality (digestibility) of forage crops. The general trend of the results of this study would suggest that water-stress decreases

digestibility due to cell wall composition. The results of the study conducted by Deetz et al. (1996) reflect similar conclusions. In that study, improvement in alfalfa forage quality during periods of water deficit was not attributed to improved cell-wall degradability but was likely the result of delayed maturity and decreased cell-wall concentration.

Digestibility was significantly affected by hybrid. The main conclusions that can be drawn is that hybrid 1, which exhibited the brown mid-rib phenotype had a lower dry matter percentage and lower NDF concentration than hybrids 2-5, with hybrids 4 and 5 having the highest dry matter percentage and NDF concentration. Hybrid 1 had significantly higher in-vitro dry matter digestibility, in-vitro true dry matter digestibility and in-vitro neutral detergent fiber digestibility than hybrids 2-5, with hybrids 5 and 4 tending to have the lowest digestibility. Hybrid 1 also had a significantly lower concentration of lignin than hybrids 2-5, with hybrid 5 having the highest concentration of lignin. These results are comparable to other studies. A study conducted by Barnes et al. (1971) compared normal corn to three BMR hybrids. The experiment also compared digestibility of tissues. IVDMD of the whole plant normal, and BMR 1, BMR 2 and BMR 3 was 68.3, 72.0, 75.5 and 77.8%, respectively, for plants harvested 35 days post-silk. IVDMD values for leaf, sheath, cob, and tassel from the same harvest were higher ($P<.05$) for all mutants compared to the normal counterpart. The IVDMD of two of the BMR hybrid's stem tissue from the same harvest were higher ($P<.01$) than the stem tissue of the normal corn hybrid. The IVDMD of two of the BMR hybrids whole plants were greater than the normal counterparts in all harvests.

The Brown Mid Rib (BMR) phenotype is characterized by being more digestible, but usually tends to be lower in DM yield than hybrids that do not express the BMR phenotype (Rook et. al, 1977). These characteristics are reflected in the data from this study.

Phytomer is also significantly affected by digestibility. There was no difference in dry matter percentage between the upper and lower phytomers. Neutral detergent fiber concentrations were higher in the lower phytomer than in the upper phytomer. The upper phytomer had higher *in-vitro* dry matter digestibility, *in-vitro* true dry matter digestibility and *in-vitro* neutral detergent fiber digestibility were all significantly higher in the upper phytomers than in the lower phytomers. Lower phytomers had a significantly higher concentration of lignin than the upper phytomers. These results indicate that the upper phytomer portion of the stover of corn plants is more digestible than the lower phytomer portion of the stover of corn plants. These findings were consistent with data from Ferreira and Brown (2017) who saw significant differences in cell wall concentration and composition of an upper, middle and lower phytomer of 8 different corn hybrids. Their general findings were that cell wall concentration tended to be higher in lower nodes and decrease in middle and upper. Scobbie et al. (1993) found that the cell wall composition, and maturity of the cells, changed from the lower sections to the upper sections in the same internode. The cell wall of the lowest internode section was like what would be seen in a primary cell wall profile. Cell wall components increased substantially in the upper half of the internode (68.6 to 72.8% of dry matter), and 1,4 linked GLU and xylans made up most of the cell wall. Scobbie et al.(1993) also identified significant differences between lower phytomers and upper phytomers.

Tissue type was also a significant factor in digestibility. There was no difference in dry matter % between the stems, sheaths or blades. The neutral detergent fiber concentration in the sheaths was lower than that in the blades and stems. In *in-vitro* dry matter digestibility, *in-vitro* true dry matter digestibility and *in-vitro* neutral detergent fiber digestibility, blades

were significantly higher than stems and sheaths tended to be lower than blades and higher than stems. Stems had a significantly higher concentration of lignin than sheaths and sheaths had a significantly higher concentration of lignin than blades which is consistent with findings by Ferreira and Brown (2017) which found that the concentration of lignin increased from the leaf blade (10.8% CW) to the leaf sheath (13.1% CW) to the stem (17.3% CW). These results indicate that blades were the most digestible tissue and stems were the least digestible tissue in the stover of the corn plant. Studies that compared tissue digestibility under low moisture conditions in alfalfa had results that agreed with the findings in this study. Vough and Marten (1971) reported that moisture stress had a positive effect on stem IVDDM of alfalfa harvested at the first-flower stage. Snaydon (1972) reported similar results with alfalfa, but the growth stage at harvest was not specified. Halim et al. (1985) observed that continuous water stress delayed phonological development, increased the proportion of stems, and increased the IVDDM concentration of stems. Albrecht et al. (1987) suggested that low moisture availability may have a negative effect on stem IVDMD after alfalfa reaches the midflower stage (3 weeks after early-bud).

The interactions effects of the irrigation treatment and the phytomers and the interactions of the irrigation treatment and the tissues, suggested that irrigation had a greater effect on tissues that tend to be less digestible.

Conclusions

The findings of this study agree with the literature that suggests BMR is a more digestible hybrid, leaves are more digestible than stems and upper phytomers are more digestible than lower phytomers, based on the observations in this study that showed higher concentrations of cell wall and higher concentrations of NDF and lignin within the cell walls

of the hybrids that did not express the BMR phenotype, the stems and the lower phytomers. The results of this study, however, do not support the general industry belief that drought increases digestibility of the corn stover, because the non-irrigated plants had lower NDF digestibility, IVTDMD and IVNDFD as well as higher concentrations of cell-wall. Most of the cell wall characteristics that were observed suggest less digestible traits in the non-irrigated plants, however there was not a significant difference in lignin concentration between the irrigation treatments.

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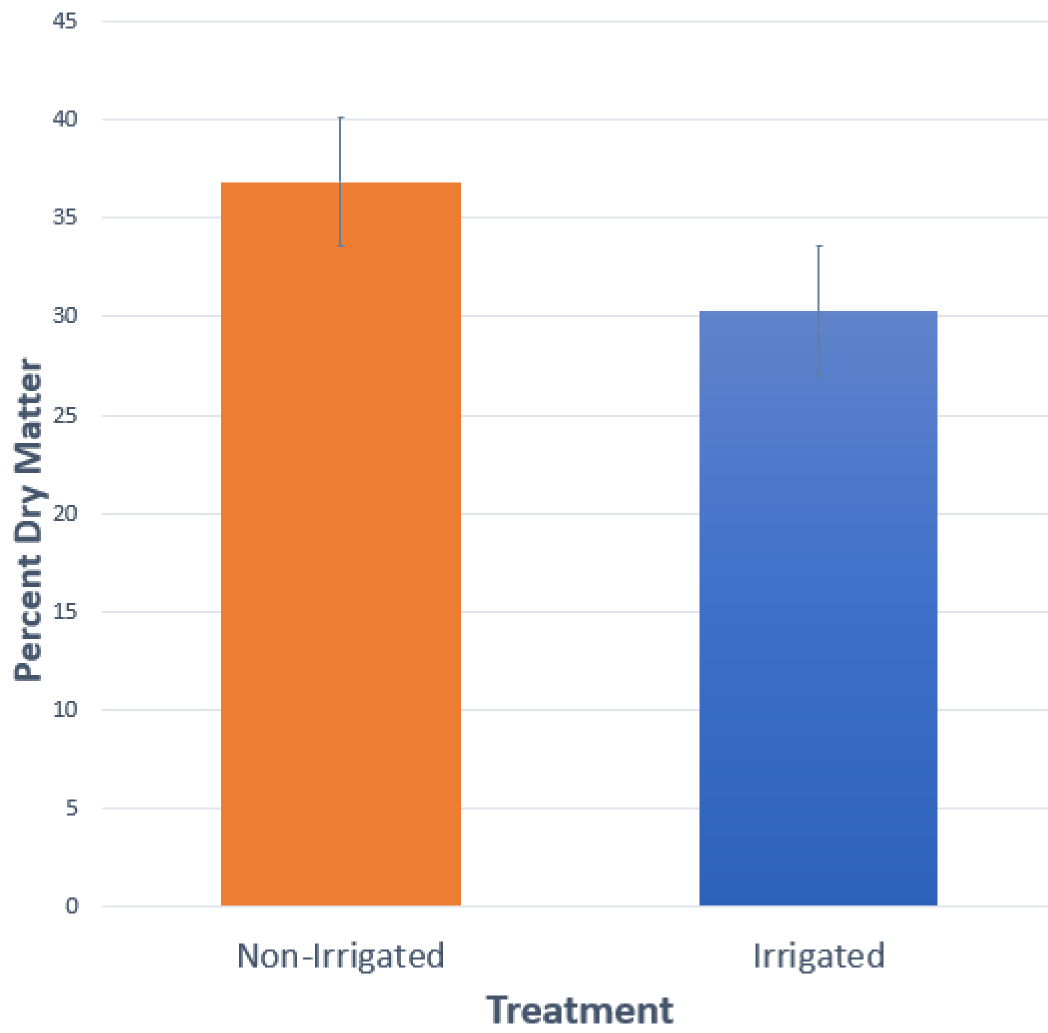

FIGURE 2.1: Irrigation Treatment Dry Matter (P< 0.01)

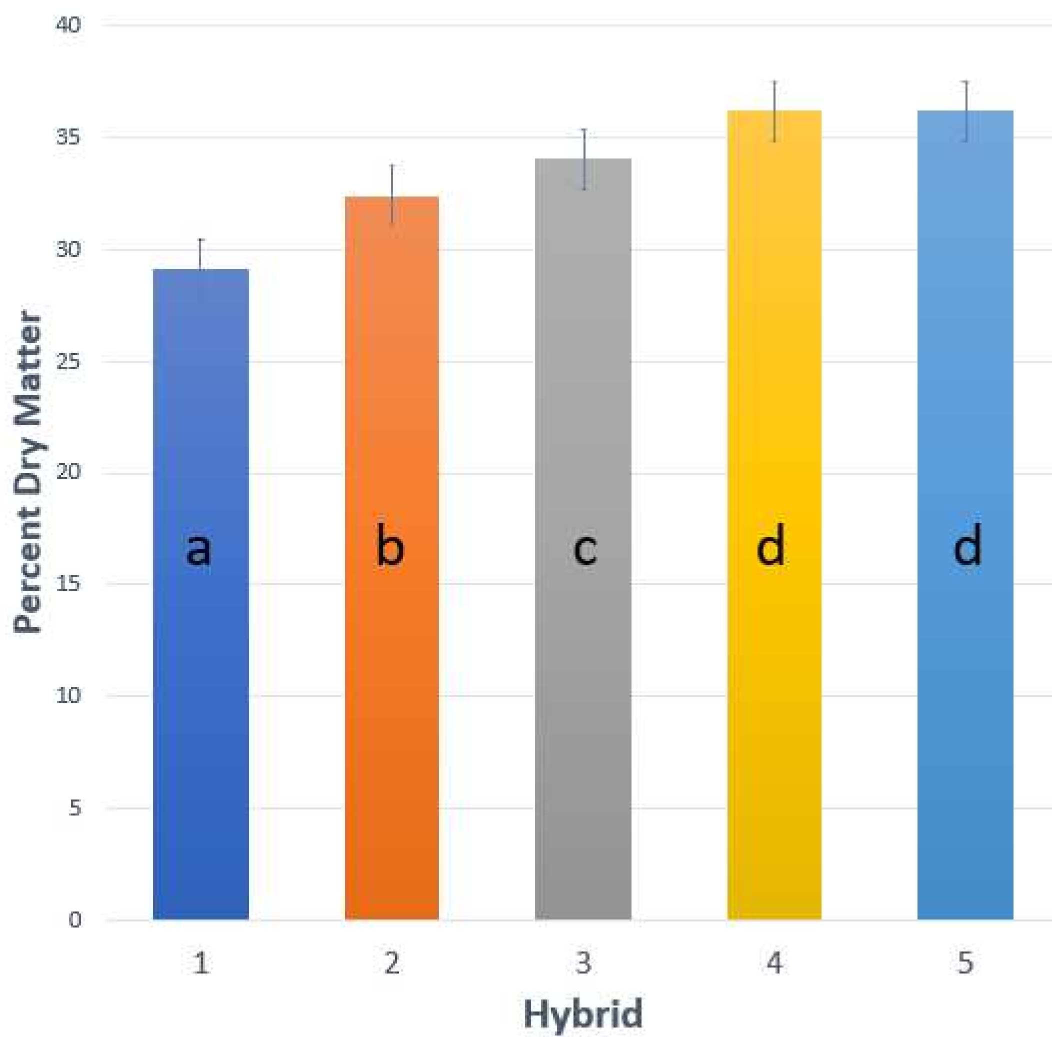

FIGURE 2.2: Hybrid Dry Matter Percentage (P< 0.01)

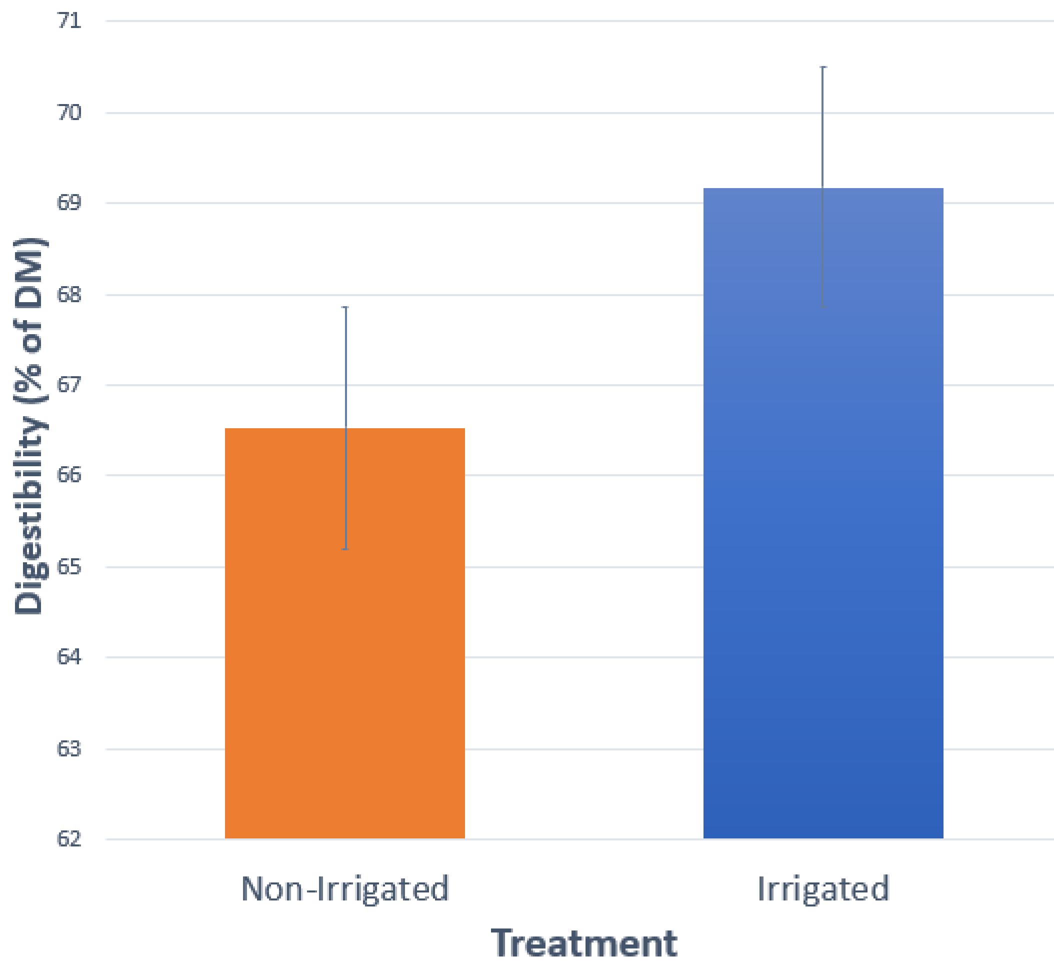

FIGURE 2.3: Irrigation Treatment *In-Vitro* True Dry Matter Digestibility (P= 0.02)

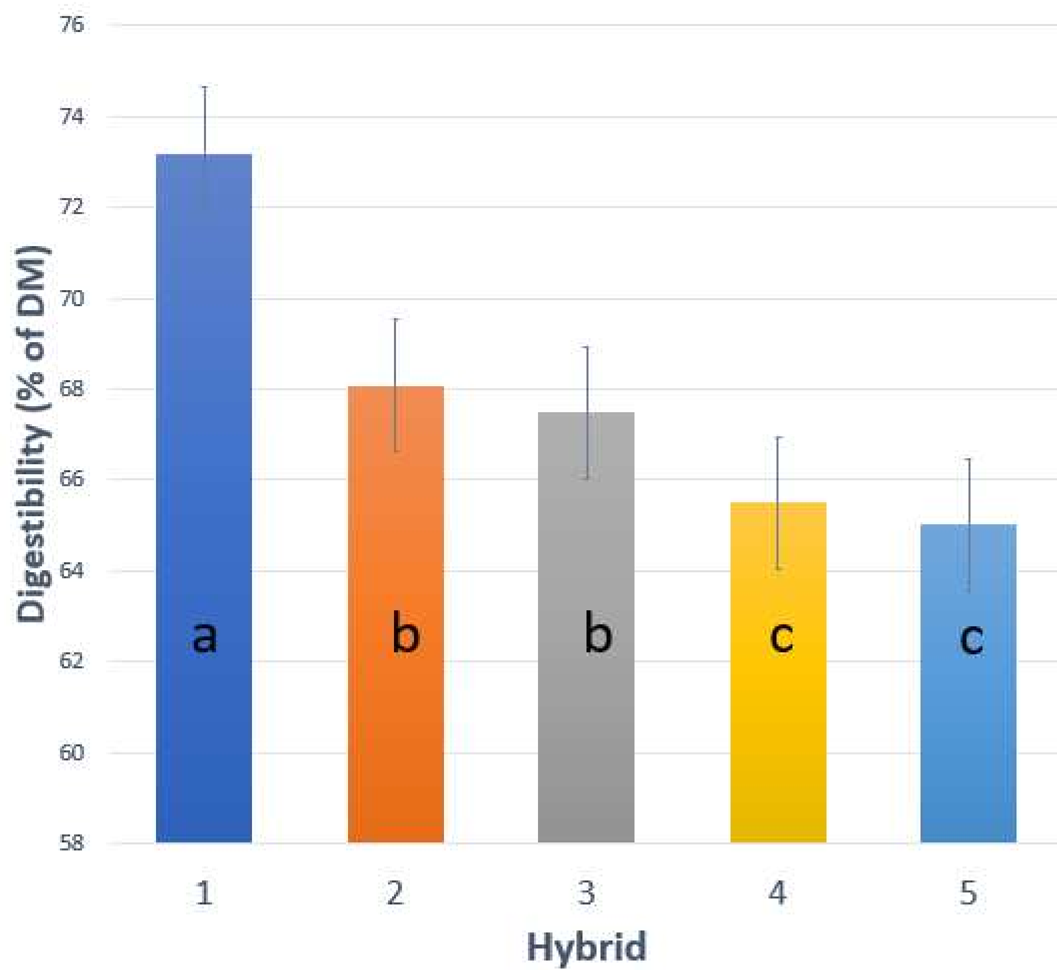

FIGURE 2.4: Hybrid *In-Vitro* True Dry Matter Digestibility (P< 0.01)

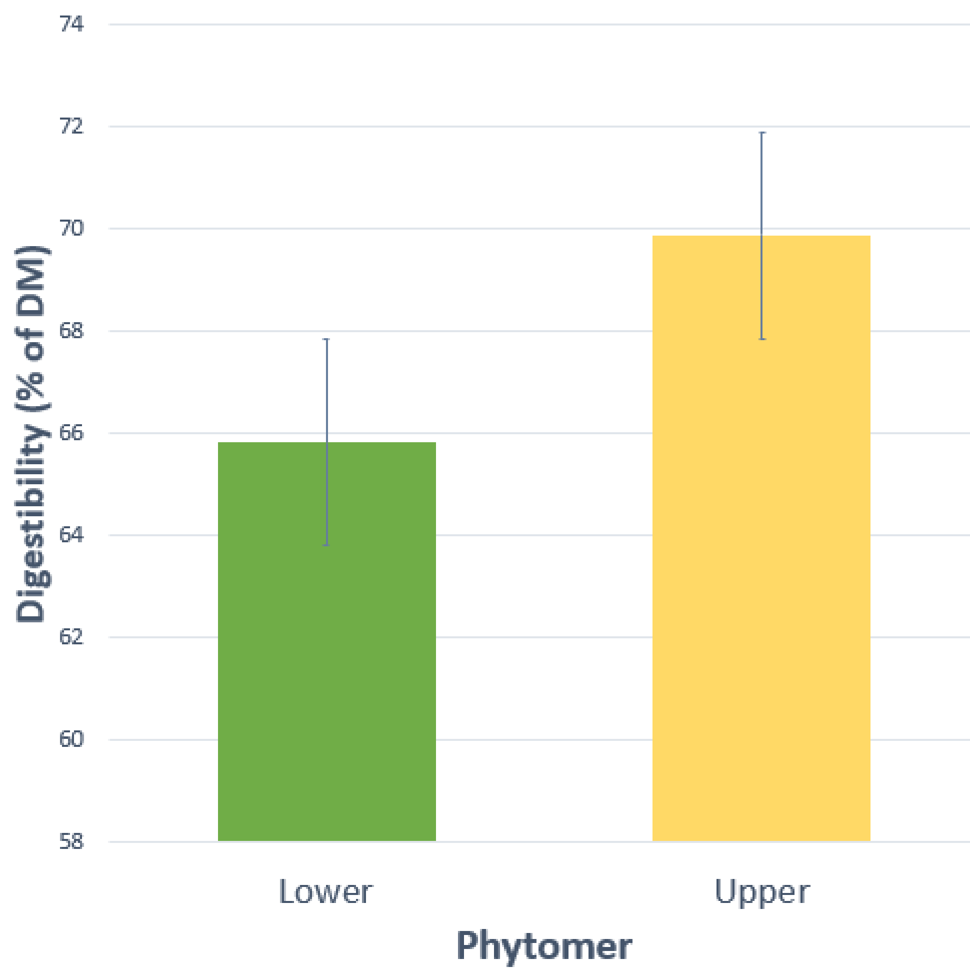

FIGURE 2.5: Phytomer *In-Vitro* True Dry Matter Digestibility (P< 0.01)

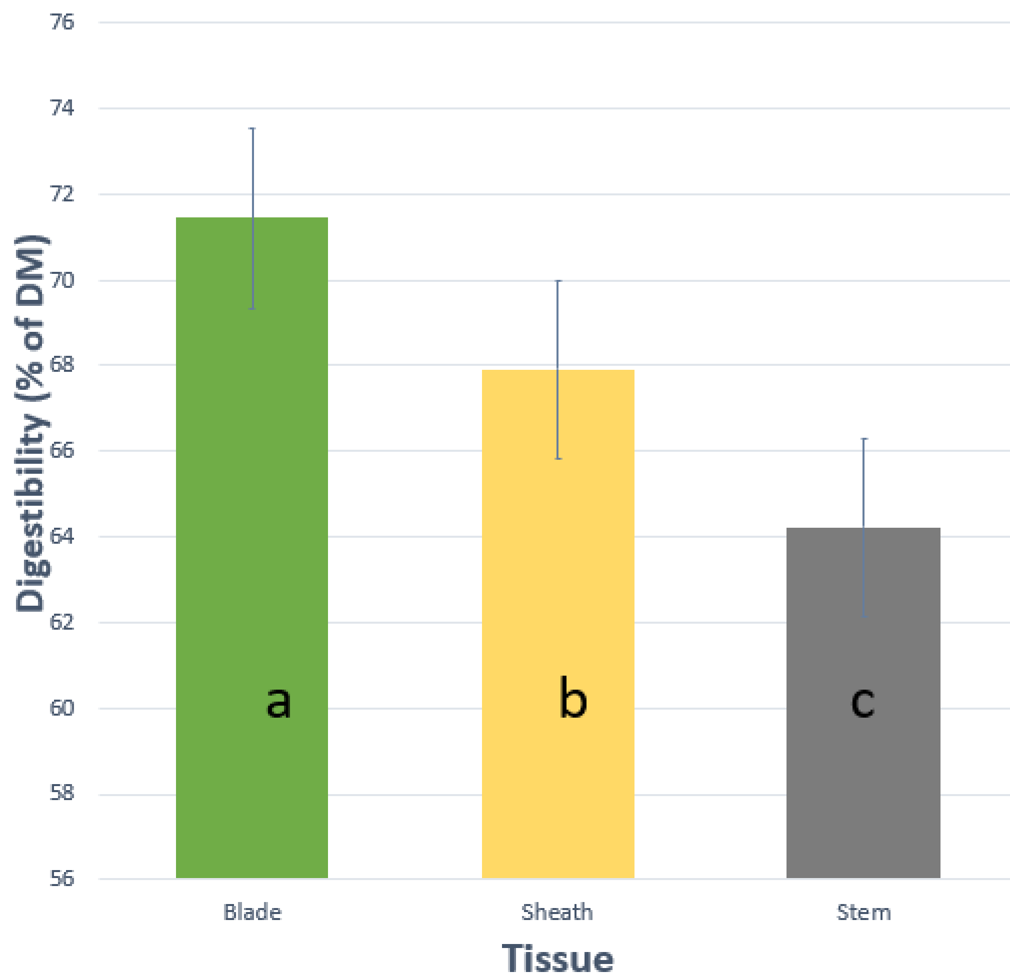

FIGURE 2.6: Tissue *In-Vitro* True Dry Matter Digestibility (P< 0.01)

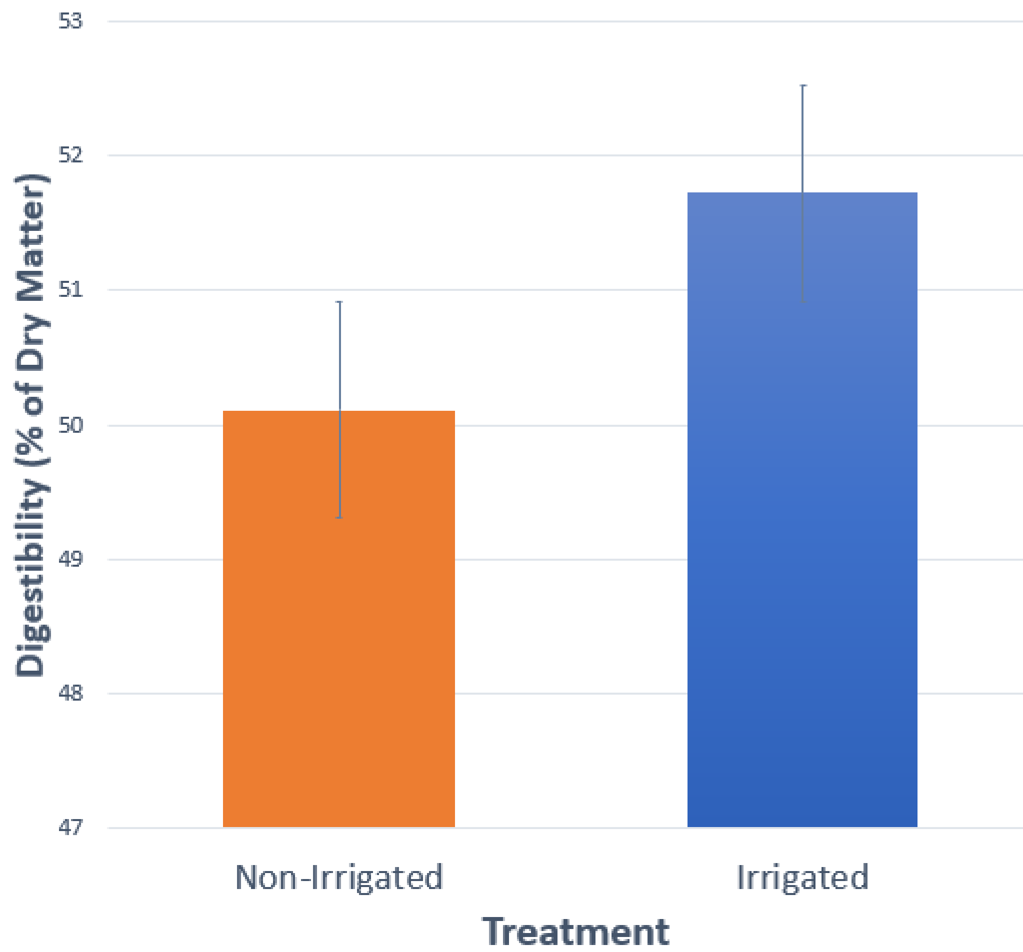

FIGURE 2.7: Irrigation Treatment *In-Vitro* Neutral Detergent Fiber Digestibility ($P < 0.1$)

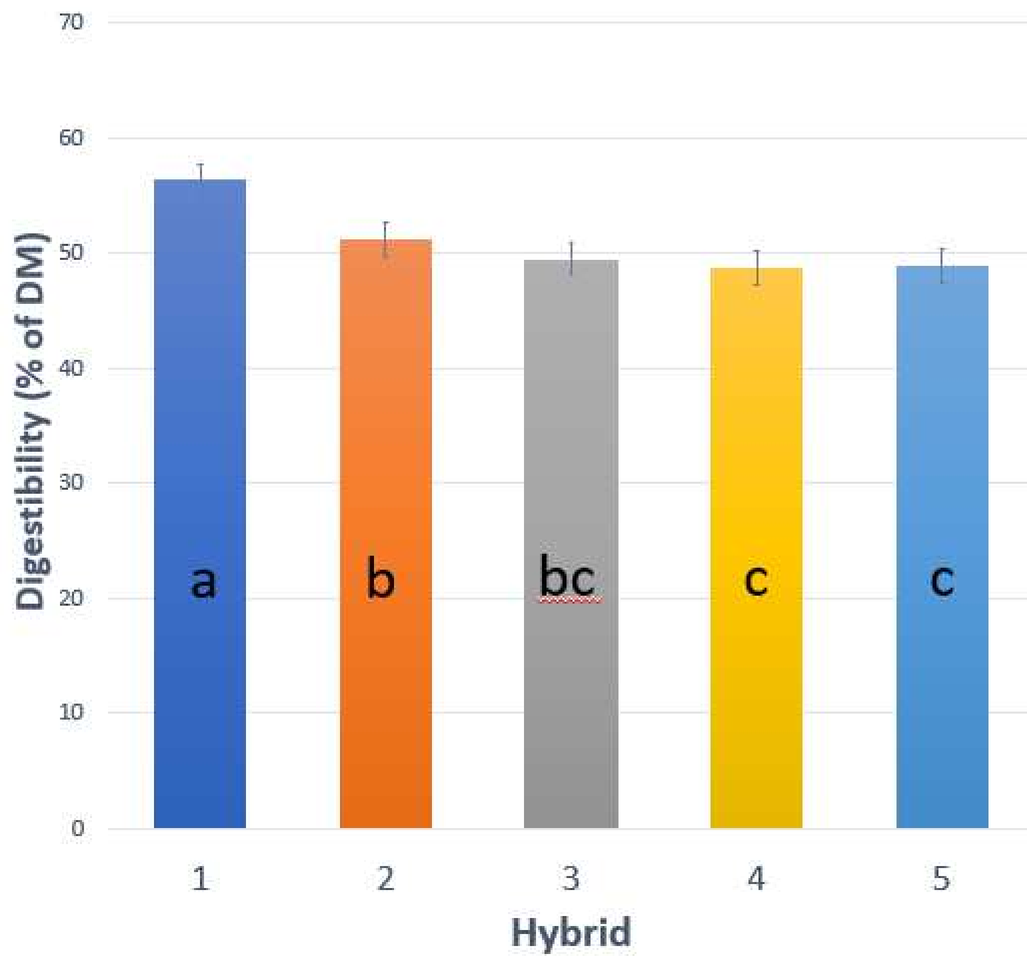

FIGURE 2.8: Hybrid *In-Vitro* Neutral Detergent Fiber Digestibility (P< 0.05)

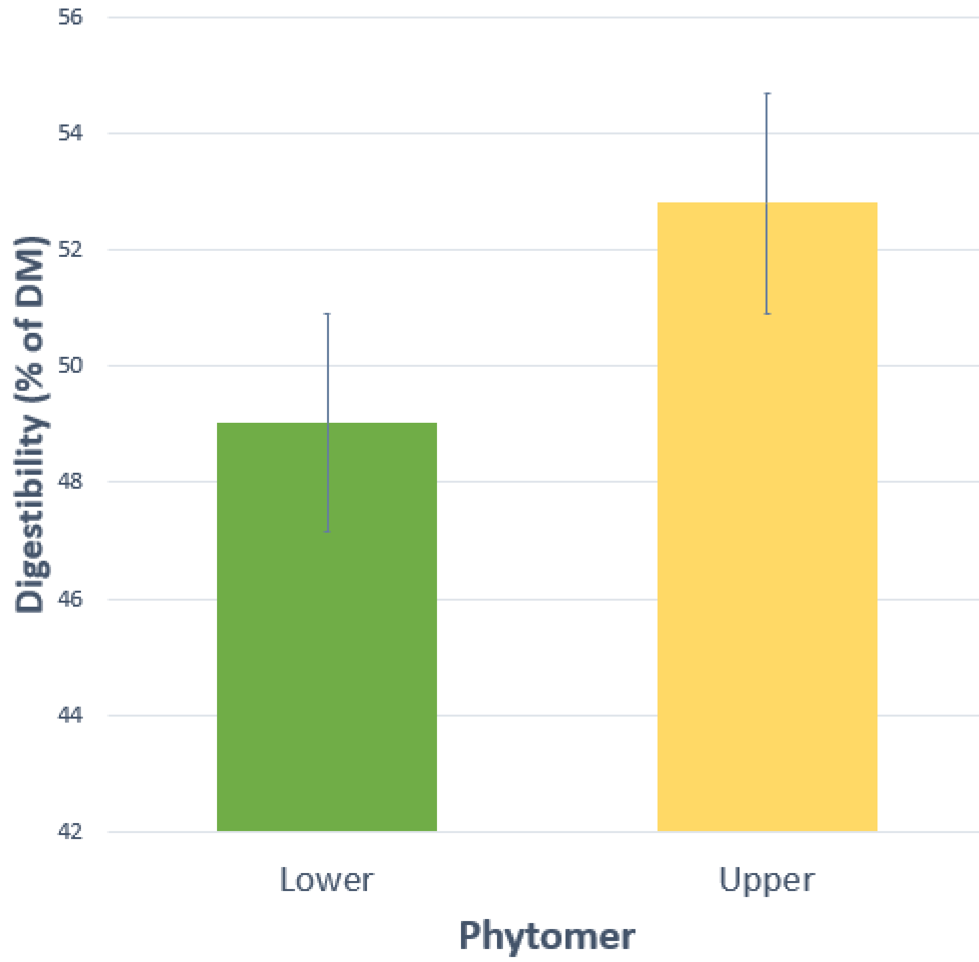

FIGURE 2.9: Phytomer *In-Vitro* Neutral Detergent Fiber (P< 0.01

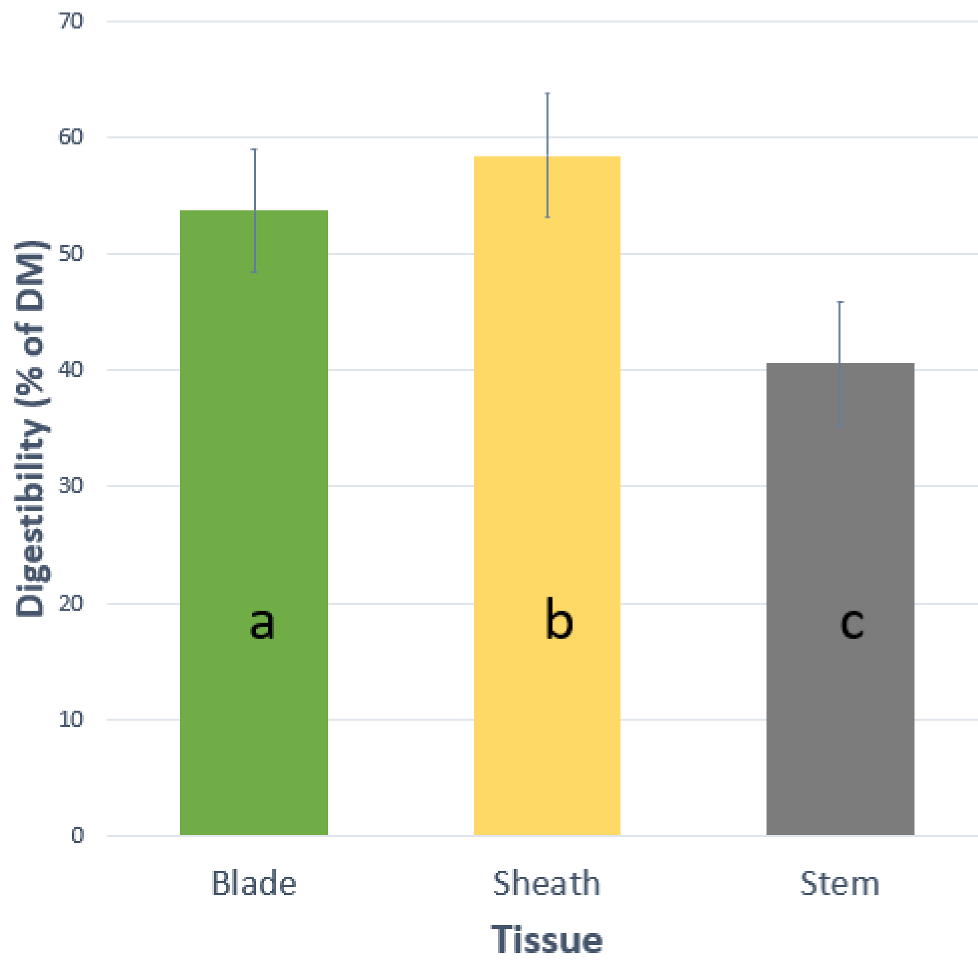

FIGURE 2.10: Tissue *In-Vitro* Neutral Detergent Fiber (P< 0.01)

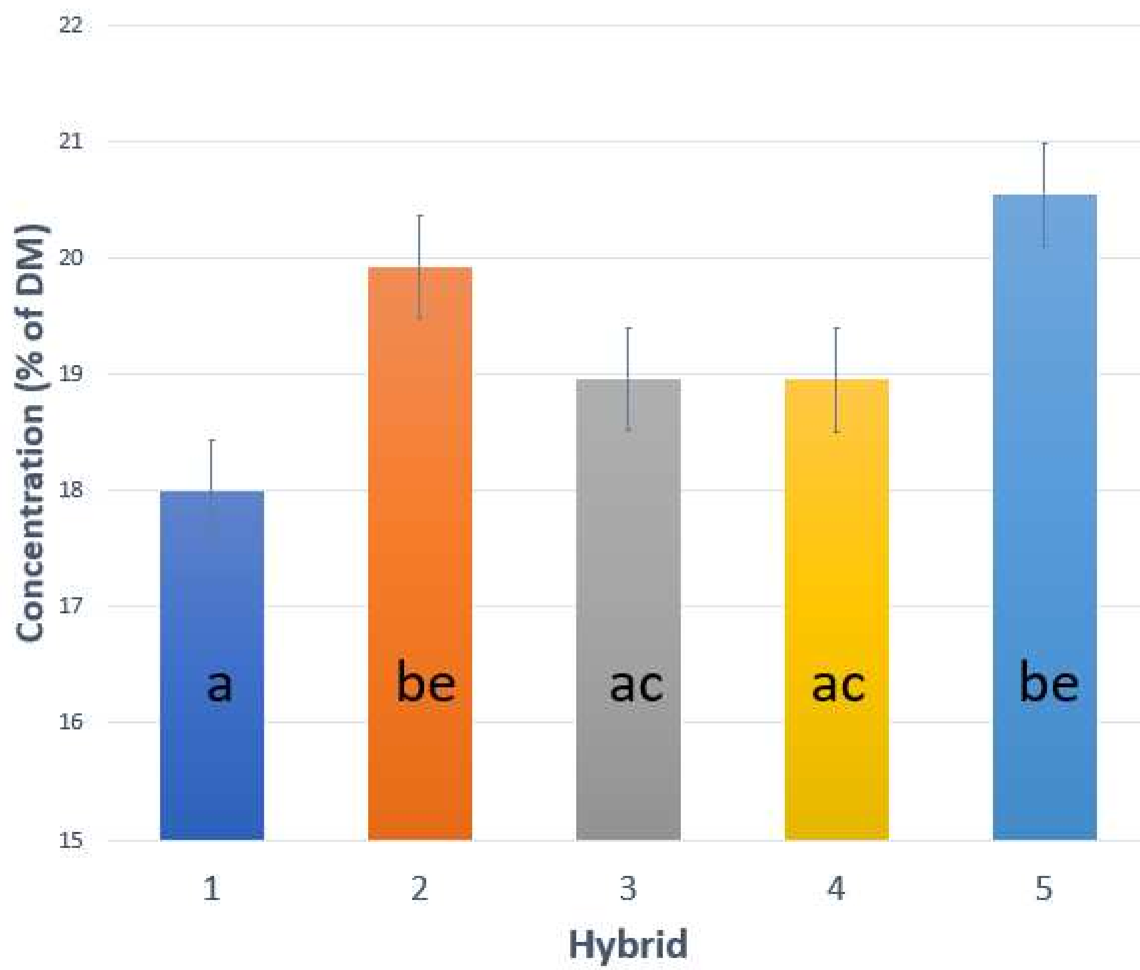

FIGURE 2.11: Hybrid Lignin Concentration (P< 0.01)

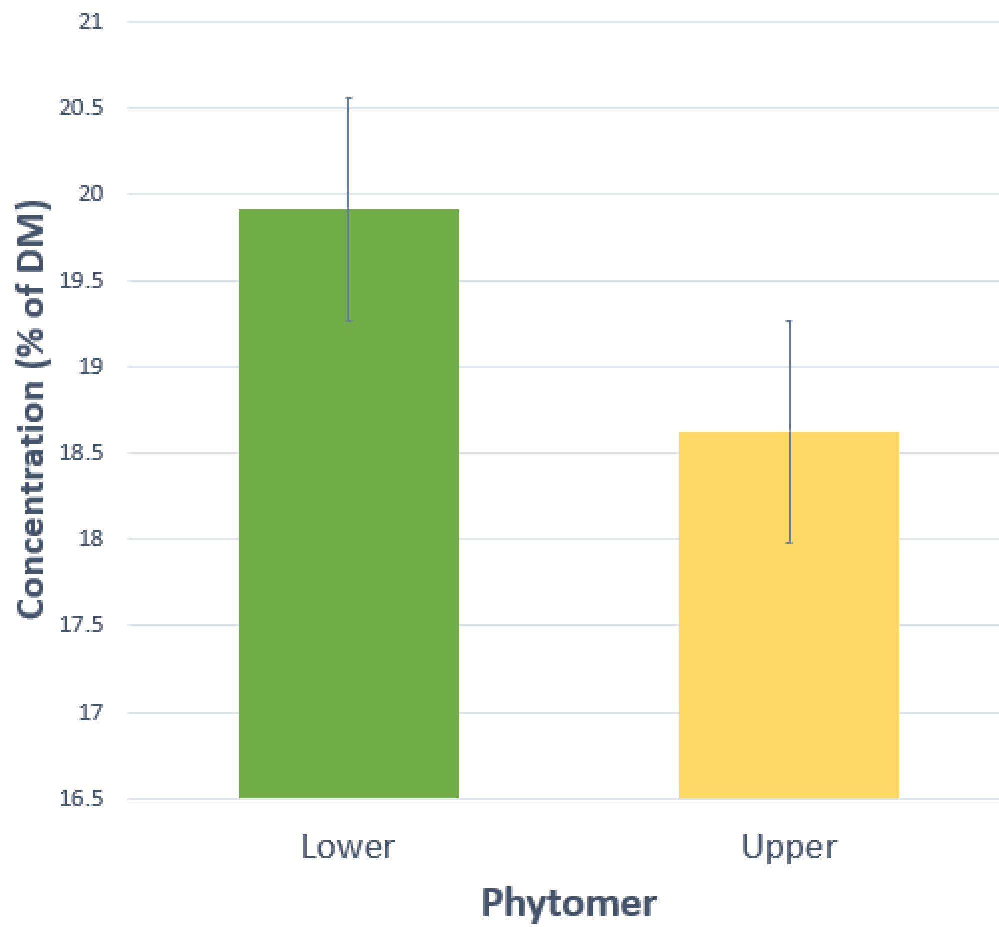

FIGURE 2.12: Phytomer Lignin Concentration (P< 0.01)

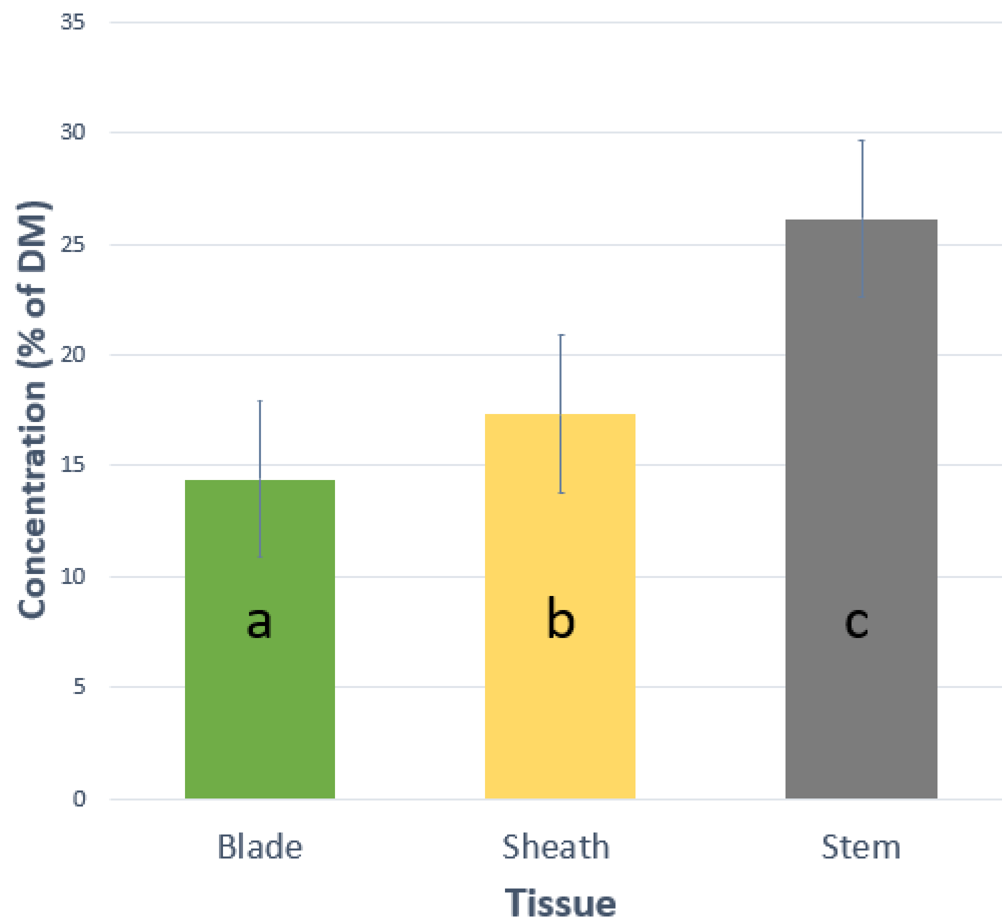

FIGURE 2.13: Tissue Lignin Concentration (P< 0.01)

Appendix A - Bag Labeling -In-Vitro Procedure

Bag	Plot	Sample	Replicate	Phytomer	Tissue	Hybrid	IV Run	Jar
1	1	1	1	Lower	Stem	5	1	1
2	1	1	2	Lower	Stem	5	1	1
3	1	2	1	Lower	Sheath	5	1	1
4	1	2	2	Lower	Sheath	5	1	1
5	1	3	1	Lower	Blade	5	1	1
6	1	3	2	Lower	Blade	5	1	1
7	1	4	1	Upper	Stem	5	1	1
8	1	4	2	Upper	Stem	5	1	1
9	1	5	1	Upper	Sheath	5	1	1
10	1	5	2	Upper	Sheath	5	1	1
11	1	6	1	Upper	Blade	5	1	1
12	1	6	2	Upper	Blade	5	1	1
…	…	…	…	…	…	…	…	…
…	…	…	…	…	…	…	…	…

Appendix B - Neutral Detergent Fiber Reagents

1.) Neutral Detergent Solution: Add 30 g Sodium dodecyl sulfate (USP), 18.61 g Ethylenediaminetetraacetic disodium salt (dehydrate), 6.81 g Sodium borate, 4.56 g Sodium phosphate dibasic (anhydrous), and 10.0ml Triethylene glycol to 1 L distilled H2O. Check that pH is from 6.9 to 7.1. Agitate and heat to aid solution. CAUTION: Powdered chemicals will irritate mucous membranes. A dust mask and gloves should be worn when handling these chemicals.

2.) Alpha-amylase—Heat-stable bacterial alpha-amylase: activity = 17,400 Liquefon Units / ml (FAA, ANKOM Technology).

3.) Sodium sulfite—Na2SO3, anhydrous (FSS, ANKOM Technology)

Appendix C - Neutral Detergent Fiber Bag Labels

Bag	Plot	Sample	Phytomer	Tissue	Hybrid
1	1	1	Lower	Stem	5
2	1	2	Lower	Sheath	5
3	1	3	Lower	Blade	5
4	1	4	Upper	Stem	5
5	1	5	Upper	Sheath	5
6	1	6	Upper	Blade	5
…	…	…	…	…	…
…	…	…	…	…	…

Appendix D - Cell Wall Isolation Procedure Reagents

1.) 50 mM Tris-acetate Buffer: Using a graduated cylinder, pour 900 mL of water into a 1-liter beaker containing a stir bar. Weigh 6.05 g Tris base, and transfer to the beaker while stirring under the hood. Adjust pH to 6.2 by adding acetic acid. Transfer the solution to a 1-Liter volumetric flask and raise to volume by adding water.

2.) 80% Ethanol: In a large bottle, add 840 mL of 95% ethanol. Then add 160 mL of distilled water. Mix by shaking.

Appendix E - Cell Wall Isolation Tube Labels

Tube	Sample	Tissue
1	1	Stem
2	4	Stem
3	7	Stem
…	…	…
…	…	…
28	130	Stem

Tube	Sample	Tissue
1	2	Sheath
2	5	Sheath
3	8	Sheath
…	…	…
…	…	…
28	131	Sheath

Tube	Sample	Tissue
1	3	Blade
2	6	Blade
3	9	Blade
…	…	…
…	…	…
28	132	Blade

Appendix F - Cell Wall Lignin Extraction with Acetyl Bromide Reagents

1.) 25% (v/v) Acetyl Bromide: Using a graduated cylinder, pour 100 mL of glacial acetic acid into a 250- mL volumetric flask. Using a graduated cylinder, pour 62.5 mL of acetyl bromide into the same volumetric flask. Raise the volume to 250 mL by adding glacial acetic acid. Transfer the solution to an amber, capped- bottle.

2.) 0.5 M Hydroxylamine: Pour approximately 25 mL of water into a 50 mL volumetric flask. Pipet 1.67 mL of 50% (w/v) hydroxylamine into the same volumetric flask. Raise to 50 mL by adding water. Transfer to a capped and labeled glass tube.

3.) Matrix Solution: (The following is preparation for 1 sample, adjust according to the number of samples being run). Degas 2 M NaOH by placing a bottle under a nitrogen stream for at least 30 minutes. Under the hood, mix in an orange-capped bottle; 2 mL of 2 M NaOH, 7.15 mL of glacial acetic acid, and .35 mL of 0.5 M hydroxylamine.

Appendix G - Cell Wall Lignin Extraction with Acetyl Bromide Labels

Vial	Sample	Tissue
1	1	Stem
2	1	Stem
3	4	Stem
4	4	Stem
…	…	…
157	3	Blade
158	3	Blade
159	6	Blade
160	6	Blade
…	…	…
293	2	Sheath
294	2	Sheath
295	5	Sheath
296	5	Sheath
…	…	…