

ABSTRACT

SMITH, MATTHEW WOORE. Genetic Variation for Growth Regulator Response and for Grain Protein and Phenylalanine Contents in Diverse Maize (Under the direction of Dr. James B. Holland).

Maize is a globally significant crop and culturally important food plant. Plant breeders have long worked to improve both maize yield and nutrition, but often in separate programs. Maize growth and development are affected by various growth regulating chemicals, including strobilurin and triazole fungicides, which may help to improve overall yields. Both aforementioned classes of fungicide have been implicated in delayed leaf senescence in maize. This delayed senescence sometimes accompanies increased yields, although both yield and delayed senescence have been shown to vary by genotype. If the response to growth regulators is a heritable trait, it may be worthwhile to breed for an exaggerated response that might induce consistent yield benefits with strobilurin and/or triazole growth regulator treatment. Regarding nutrition, maize has long been the subject of efforts to improve kernel protein quality. Certain human metabolic conditions, however, require reduced protein or reduced levels of specific amino acids. Given that breeders have succeeded in producing maize plants with improved kernel protein quality, it may be possible to breed for low kernel protein content or even to target a specific amino acid for reduction. Four experiments were planted at multiple locations across two years to examine a broad array of maize hybrid and inbred genotypes for variation in response to growth regulator treatment and, separately, for protein content and amino acid composition. Results showed no effect of growth regulator treatment on yield and no correlation between delayed leaf senescence and improved yield or yield components. The interaction of genotype by growth regulator treatment was not significant, and where individual genotypes showed any responses to treatment they were not related to improved yield or yield components and followed no predictable pattern based on pedigree groupings. However, the protein content of maize kernels was found to vary widely by genotype and by the hybrid or inbred condition of the plants. This variability was found to be highly heritable. Further testing found a smaller range of changes in amino acid composition, but these too were highly heritable. While breeding maize for response to growth regulator treatment is not expected to result in genetic gain, breeding for reduced kernel protein and altered amino acid composition should be successful.

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Genetic Variation for Growth Regulator Response and for Grain Protein and Phenylalanine Content in Diverse Maize.

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DEDICATION

This is for Dad. I love you. Thanks for everything.

BIOGRAPHY

Born in Baltimore, Matthew grew up in the suburban wilderness of Jacksonville, Florida. He earned a degree in architecture from Clemson University in 1999, and spent 15 years in his own personal wilderness, working as a political campaign manager, reporter, U.S Air Force officer and pilot, FedEx delivery driver, and BMW assembly technician, among other things, before returning to school at NC State University in 2013. Following acceptance of the Master of Science he plans to continue work towards a PhD in plant breeding.

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Chapter One: Literature Review

Maize (*Zea mays* L.) was domesticated in Meso-America, but has spread to become the most important single source of food and feed worldwide (Rosegrant et al 2009). Maize production is a \$50 billion industry in the United States (NASS 2018). Global maize consumption comes to approximately 19 kg per year per person, although this is higher in developing countries and lower in developed ones. Global estimates project demand for cereals to increase by nearly a third by 2050 (Alexandratos & Bruinsma 2012), leading to numerous research efforts to improve maize yield and nutritional value.

Plant growth regulators and maize

A wide variety of chemicals have been used in agriculture as plant growth regulators (PGR), designed to improve harvestability, increase yield, or otherwise affect plant growth in an effort to manage agronomic characteristics. These include such items as mepiquat chloride for limiting foliar growth in cotton (York 1983), and maleic hydrazide for topping and suckering in tobacco (Naylor 1950). Various synthetic plant hormones have been used on maize among other crops to control plant size or induce reproductive growth to begin earlier or later than it might naturally. Some plant growth regulators also affect the growth of bacteria or fungi.

A class of plant growth regulators that affect organisms other than plants are the triazoles. Triazoles are a class of sterol-synthesis regulators and have demonstrated effects on sterol synthesis across kingdoms. The triazoles act most strongly against biosynthesis of ergosterol, which is the sterol found in all fungal cell membranes, but which is not found in plants or higher animals (Klemptner et al 2014). It has less effect against biosynthesis of the plant sterols, but this action has allowed the triazoles to find uses as both fungicides and plant growth regulators (Schwinn 1983).

Inhibition of sterol biosynthesis is accomplished by interference with sterol 14 α -demethylase cytochrome p450 (CYP51), an enzyme found in all biological kingdoms that converts lanosterols to functional sterols. In fungi the preferred lanosterol is 24-methylenedihydrolanosterol (24-MDL), while plants produce sterols from a number of lanosterols in addition to 24-MDL. CYP51 has high sequence conservation across kingdoms but the substrates and products of its reactions vary enough that the same CYP51 inhibitor will

have different degrees of effect in different organisms. Triazoles inhibit CYP51 by preventing substrate binding, and are specifically effective against 24-methylenedihydrolanosterol and thus against fungal ergosterol biosynthesis (Lepesheva & Waterman 2007).

Triazoles have also been found to restrict biosynthesis of both gibberellic acid (Rademacher et al 1987) and brassinosteroids (Hartwig et al 2012). Gibberellic acid (GA) and brassinosteroids (BR) are plant hormones that have important effects on cell elongation in stem and leaf tissues and thus leaf dimensions, internode length, and plant size, and also have roles in the transition to reproductive growth; restriction of GA *in vivo* results in stunting or dwarfing of plants with reduction of side shoots and a delay in maturation, but does not eliminate reproduction (Evans & Poethig 1995). Some triazoles (e.g., paclobutrazole) are primarily used as PGRs for this growth-inhibiting property, while others (e.g., propiconazole) are primarily fungicides with some attendant plant growth regulating effects. All triazoles exhibit some degree of both fungicidal and plant growth regulatory properties (Fletcher et al 1986).

Used as growth regulators, triazoles have been found to increase the apparent green pigmentation in maize leaves, but this may be due to unchanged pigment quantity concentrated into smaller leaves, rather than an actual increase in the quantity of photosynthesizing pigments (Khalil et al 1990). It has, however, been suggested that this concentration of pigment in smaller plant tissues may lead to greater water use efficiency and improved drought stress tolerance, a potential source of yield gains, as well as that yield gains result from alteration in the balance of plant hormones rather than any effects on sterol biosynthesis (Fletcher et al 2000).

Triazoles are not the only fungicides with plant growth regulating properties. The strobilurins are a newer class of fungicides with a different mode of action than triazoles but which present similar growth regulating properties. The first strobilurin, Strobilurin A, was isolated from the mycelia of *Strobilurus tenacellus* (Pers.) Singer, a small wood-rotting Old World fungus, for which the compound acts as a local defense against fungal competitors (Anke et al 1977). Numerous similar compounds have been isolated from other species.

All strobilurins act by inhibiting mitochondrial respiration in fungi. They are generally nontoxic to bacteria and have low toxicity in mammalian systems (although having some effect against tumors), but in

fungi provide very strong interference in the function of cytochrome bc₁ in the mitochondrial membrane. Specifically, the strobilurins bind reversibly at the cytochrome b quinone oxidation center (Q_o), where ubiquinol binds to be oxidized before moving to cytochrome c. Ubiquinol is still able to bind at Q_o in the presence of a strobilurin, but it is not oxidized and thus the ATP-production process is halted (Clough 1993). All such fungicides are referred to as quinone outside inhibitors (QoI).

Cytochrome bc₁ occurs in mitochondria across all eukaryotes, raising the question of why strobilurins effectively kill fungi but are not particularly toxic toward plants and animals. Roehl & Sauter (1993) tested the efficacy of strobilurin A in blocking respiration at cytochrome bc₁ in mitochondrial solutions prepared from five different species, and found that in fact the compound has the same capacity for respiration inhibition in all species (including mouse, fly, and maize) and therefore some combination of other cellular processes, such as uptake and transport, are responsible for the differential toxicity in fungi and other kingdoms.

Thus any growth-regulating effects of strobilurins on plants are not related to inhibition of mitochondrial respiration. Much research has been carried out in small grains on the ability of various commercial strobilurins to enhance grain quality and yield in the absence of or beyond what would be expected from disease suppression. Numerous studies of strobilurins in comparison with triazoles in small grains have shown that when both fungicide classes provide equivalent disease suppression as for example with *Septoria tritici*, the strobilurin-treated plants nonetheless produce greater yield than the triazole-treated plants (Jones & Bryson 1998). A variety of theories have been put forward to explain this.

Grossman & Retzlaff (1997) first suggested that strobilurins were increasing the levels of certain cytokinins in plant tissue, but not of auxins, and not consistently across species. However, they did determine that 1-aminocyclopropane-1-carboxylic acid (ACC) was significantly reduced in treated tissue. ACC is the biological precursor to ethylene, which is the key plant hormone determining the start of leaf senescence. Further studies have suggested enhanced chlorophyll content of treated tissues, greater water use efficiency, and reduced abscisic acid production due to strobilurin treatment (Bartlett et al 2002). Wu and von Tiedemann (2001) showed reduced levels of reactive oxygen species (free radicals) in plant tissue treated with strobilurins and suggested this may be more significant than ethylene suppression.

Wu and von Tiedemann (2002) later showed further evidence that wheat and barley plants treated with strobilurins, and to a lesser degree triazoles, experienced notably delayed leaf senescence. Senescing leaves may be more accurately defined by protein and electrolyte loss to other plant tissues; treatment with the growth regulators led to not only higher protein content and electrolyte levels in older plant tissues, but also significantly increased activity of superoxide dismutase and other anti-oxidant enzymes in the tissue. This increase in enzymatic activity appeared to be of primary significance in the delayed senescence of leaf tissues in these plants, with reduced ACC and ethylene biosynthesis a consequence of reduced oxidative stress rather than a direct action of the growth regulator.

Keeping leaves of domesticated small grain plants green can help to improve yield (Gooding et al 2000). As long as leaf tissues are actively fixing carbon, more photosynthate can be stored in the developing kernels; as leaves senesce prior to final grain maturity, nitrogen, minerals, and metabolites are remobilized from vegetative tissue to the maturing kernels. It is worth noting that studies in small grains focus especially on the delayed senescence of the flag leaf, which provides 45% or more of the photosynthate that ultimately reaches the kernel; however, maize lacks such a dominant flag leaf (Lupton 1972). Domesticated wheat contains an allele of the *Gpc-B1* locus that causes delayed leaf senescence compared to wild relatives, as well as reduced kernel protein and micronutrient content (Uauy et al 2006). The gene does not affect kernel size or weight, however, suggesting that delayed senescence permits the plant to store additional photosynthate starch in the kernels but, by not also delaying kernel maturity, the plant is unable to remobilize nitrogen and micronutrients at the same rate.

Uauy et al's (2006) findings suggest that the biochemical processes behind leaf senescence and kernel maturity are controlled via distinct mechanisms; merely delaying senescence without also delaying maturity may thus generally alter kernel composition more than kernel weight and yield. Yield gains are consistently found in small grains with the application of strobilurin fungicides, although not always to an economically beneficial level (Bayles & Hilton 2000), suggesting that some additional process is at work beyond just delayed senescence. No clear evidence has identified the source of this yield boost.

Nason et al (2007) found that strobilurins applied to drought-stressed wheat and barley in fact decreased water use efficiency in drought stressed plants, and led to reduced rates of photosynthesis in all

treated plants. Blandino et al (2012), in a large study of combined strobilurin/triazole use on maize at a variety of different spray timings, found no change in stalk diameter or leaf chlorophyll content, but nonetheless identified a yield gain at one specific spray timing; the authors suggested that the growth regulator at this single timing was improving photosynthetic efficiency to produce the yield gain (Blandino et al 2012). Other studies have suggested that the yield boost may come from greater ear tip fill (Nelson & Meinhart 2011) or reduced lodging at maturity (Mallowa et al 2015). None of these processes has definitively been shown to cause yield gains.

Most early work in yield increase and stay-green traits was done in small grains, where the effects seem to be most pronounced. Recently, similar testing has been carried out in maize. Application of strobilurins was shown to delay leaf senescence without delaying physical maturity of kernels, meaning that while leaves stayed green longer when treated, there was no increase in grain-fill period (Byamukama et al 2013). Treated plots were found to produce higher yield only at statistically non-significant levels. Kalebich et al (2017) similarly found delayed leaf senescence with strobilurin treatment, but no changes in ear size or nutritional composition, while a silage corn variety was found to have higher lignin content in stalks when treated.

Many of the cited studies have been carried out using only a single or handful of varieties of any particular species. Nafziger et al (1986) were the first to note that growth regulators do not affect all cultivars of a crop species in the same way, and this may also be behind the inconsistent yield gain findings. Bayles & Hilton (2000) reported results of a study of 28 wheat and barley cultivars and noted that while all varieties responded to strobilurin sprays with delayed senescence and increased yield, not all varieties did so at the same rate.

One test of strobilurin use in maize involved 20 commercial hybrids in a variety of locations in Brazil (da Costa et al 2012). In locations with high disease pressure, strobilurin treatment provided a significant yield boost in all varieties, although not the same amount of boost. In low disease conditions, yield increases were inconsistent. This result may be due to varieties differing in their susceptibility to disease and thus in the impact a fungicide may have on any given variety. It is conceivable that genetic variation may impact the way a plant responds to both a pathogen and the treatment for it.

While strobilurins and triazoles have led to yield boosts when used as growth regulators, particularly in small grains, the decision to spray a growth regulator of any kind is an economic one. If there is not enough of a yield boost to recover the cost of the chemistry then growers will not be interested. To that end Weisz et al (2011) analyzed a large number of trials of strobilurins on wheat, noting any yield increases, and by comparing the cost of treatment and the price available for wheat were able to assert that in the absence of disease, the odds favor losing money with strobilurin sprays rather than increasing profit. A similar analysis carried out in maize showed even higher likelihood of loss and lower chance of profit when spraying strobilurin or triazole fungicides in low-disease conditions (Paul et al 2011).

Recently these results have been called into question. Most of the research on strobilurin and triazole use as growth regulators has been done at small scales on research farms. Paul et al (2011) had already found that when yields were already high, the addition of fungicide had less effect on yield than when yields were low, regardless of disease pressure. Vincelli & Lee (2015) first reported an interaction between fungicide-induced yield gain and location within a plot, with respect to distance from end-of-plot alleys, a common feature of research plots in maize. Maize growing closer to alleys and plot edges yielded more than plants in the center of the same plot regardless of the treatment received. This suggests that the yield boosting effects of strobilurins and triazoles may be masked, at least in maize, by the small size of research plots, although without suggesting that prior research is invalid.

Tedford et al (2017) conducted a large-scale study over multiple years and 26 locations, testing for yield benefits from strobilurin and triazole application on maize at varying plot sizes. In small plots, yield gains were least consistent and least significant, while in large-scale strip trials (8.1 ha per trial) yield gains were much more consistent and sometimes economically justifiable. Problematically, this study only noted disease pressure in the small plots, not the large plots or strip trials. Since the idea of using these fungicide classes as growth regulators assumes that they would not be used otherwise due to lack of fungal disease pressure, it is impossible to compare the small plot results with the large plot and strip trial results on an equal basis.

Problems aside, the Tedford et al (2017) results suggest that maize may benefit from these growth regulators in commercial field conditions. It is also possible that, following the findings of da Costa et al

(2012) and Bayles & Hilton (2000), the effect of the PGRs may vary from variety to variety, and the contradictory findings of numerous researchers could result in part from which variety of which crop is being tested. Further work is needed to isolate PGR effects at low-disease conditions in large plots, as well as in testing varieties for strength of response. Variety by treatment interactions may appear at smaller scale, and aid both identifying ideal testing varieties for future large-scale research and possibly permit breeding of high-response materials to further increase yields in commercial fields.

Breeding for nutritional value of maize grain

Given the importance of maize in human caloric intake, yield cannot be the sole focus of breeding and research; boosting yield alone will have limited effects on human nutrition if the nutritional quality of maize is not itself also improved. Maize, like most other grains, is low in lysine (Lys) and tryptophan (Trp) so that humans who receive the majority of their daily calories from maize tend to suffer from deficiencies of these key amino acids. Breeding a nutritionally superior maize has long been an aim of breeders in the developing world (Prasanna et al 2001).

The *opaque 2* mutation causes a shift in the balance of storage protein bodies in the maize endosperm (Mertz et al 1964). The discovery of a mutation that alters the amino acid composition of the maize kernel opened the door to initiatives to improve the nutritional value of maize. Now known as the Quality Protein Maize (QPM) initiative, breeders around the world have worked on harnessing the improved amino acid profile found in *o2* and other maize mutants while working to get around the negative agronomic traits associated with these kernel mutations. Progress has been mixed; while *o2* mutants with higher Lys and Trp have been produced, the mutation results in soft kernels that are easily damaged, lost, or infected with disease, so combining the improved amino acid profile with market-acceptable food quality has been difficult. Some notable successes have been achieved; as of 1999, as much as half of all maize acreage in Ghana was planted in a single CIMMYT-released QPM variety, which had achieved wide market acceptance (Vasal 2000). Nonetheless, researchers have had more luck developing QPM varieties than encouraging their adoption.

A recent study (Flint-Garcia et al 2009a) demonstrated that there is significant variation in the amino acid composition of maize, and that this may be sufficient to permit breeding for amino acid content, presumably beyond the current work with Lys and Trp. If borne out, this compositional flexibility opens the possibility of breeding maize with selectively altered amino acid composition to permit its inclusion in restrictive diets for patients with inborn errors of amino acid metabolism.

Inborn errors of amino acid metabolism are genetic conditions resulting from recessive mutations to enzymes that play key roles in amino acid metabolism. Such conditions exist in humans for most of the common amino acids, but the most common by far are phenylketonuria (PKU) and maple-syrup urine disease (MSUD). These two conditions require lifelong restrictive diets (Vernon 2015).

All enzymes and proteins in all kingdoms of life are made from 20 amino acids directly encoded by DNA, and a handful of additional amino acids (e.g. selenocysteine) that must be manufactured in the cell post-translation. Although all plants are capable of synthesizing all 20 of the amino acids, most animals have lost the ability to synthesize some of them; those that cannot be directly synthesized in the body are referred to as “essential” for a given species (Barrett 1985). Nine amino acids are essential for humans (Table 1.1) (Salway 2013).

The branched chain amino acids (BCAA), valine (Val), leucine (Leu), and isoleucine (Ile) share certain structural similarities, namely a simple branched side chain. All are essential for humans and have similar metabolic activity in the human body. BCAAs are metabolized in the skeletal muscle into α -ketoacids, and these are further catabolized by the branched-chain α -ketoacid dehydrogenase enzyme complex (BCKAD) ultimately into acetyl CoA and thereby into the glutamate synthesis pathway. In MSUD, a subunit of the BCKAD is not functional, leading to buildup of BCAAs, restriction of glutamate synthesis, and neurological symptoms including encephalopathy (Blackburn et al 2017).

Phenylalanine (Phe) is an aromatic amino acid consisting of a benzene ring (called a phenyl group) attached to the simpler alanine (Ala) amino acid. In humans, ingested Phe is directly used in protein assembly, or catabolized into the amino acid tyrosine (Tyr), from which several key neurological compounds, including dopamine and epinephrine are produced. Because humans can metabolise Tyr only from Phe, Tyr is

sometimes referred to as “conditionally essential,” since a deficiency in Phe intake would lead to downstream deficiencies in Tyr as well. (Salway 2013).

The first step in metabolizing Phe into Tyr is enabled by the enzyme phenylalanine hydroxylase (PAH). PKU is caused by nonfunctional or partially functional PAH. While many different mutations can cause PKU, the result is that Phe cannot be converted to Tyr or the downstream neurotransmitters. Tyr is thus essential for dietary intake in PKU patients, but more significantly, excess Phe from the diet is not catabolized by PAH and as it cannot be eliminated directly through the kidneys it builds up in the body. Because of its structural similarity to neurotransmitters like dopamine, Phe can penetrate the blood-brain barrier and occupy the cellular receptors for these neurotransmitters. Left untreated, this condition can lead to substantial neurological deficits (Dennison 2005).

The negative effects of PKU can be avoided with life-long maintenance of a very strict low-Phe diet, with supplementation of other essential amino acids; infants in most of the world are tested for PKU and MSUD shortly after birth so that diet restrictions can be implemented immediately if needed (de Baulny et al 2007). This restrictive diet, which is necessarily meat- and dairy-free, also excludes most grains, maize among them. Maize can be consumed in limited quantities as sweet corn; harvested before maturity, sweet corn has approximately half the protein content of food products from mature maize, ~3 g protein per 100 g serving vs ~9 g for a 120 g serving of maize meals or grits, and 7-9 g for 100 g of maize flours (USDA 2015). This is unfortunate, because in much of the world maize is a significant contributor to the diet, through mashes such as polenta, grits, and mielie pap, to simple breads like tortillas and cornbread, as well as in snack foods like chips and popcorn. Given that maize with altered amino acid composition exists and has been deliberately bred, it is possible that breeding for reduced levels of Phe, or the BCAAs, might be successful and permit this staple food to be included in the prescribed diet.

Some previous studies that have examined the amino acid composition of maize grain have found Phe and Leu (a major component of maize protein) to be highly correlated with total kernel protein (TKP). Keeney (1970) examined amino acid composition among multiple maize varieties with differing fertilizer regimes, and reported that Phe as a proportion of TKP tends to remain consistent even as TKP varies among

varieties—in other words, Phe as a component of total protein is stable, and total Phe rises and falls in concert with TKP.

Baudet et al (1986) analyzed amino acid content, gross protein, and total kernel nitrogen (N) in several maize varieties and concluded that amino acid content as a proportion of total grain weight varied according to a set of specific equations for each amino acid. Although not all N in the kernel is bound in protein, the proportion of N that is not protein-bound is roughly invariable, so that if either total kernel N or total kernel protein can be determined, the amino acid composition could be worked out mathematically. These results suggested that any action that manipulates total kernel N will have a predictable and invariable effect on amino acid composition, and that neither agronomic management nor genetic variation can alter this.

Flint-Garcia et al (2009b) tested a very broad selection of maize and teosinte varieties and found significant variation in both total grain protein and amino acid composition, and indeed in the distribution of types of storage proteins found in the kernels of different maize and teosinte varieties. This contradicts the assertion of Baudet et al (1986) that amino acid composition is not genetically variable. Keeney (1970), similarly, found that N application caused different amino acids to vary in different ways. Keeney (1970) and Flint-Garcia et al (2009b) both noted, however, that Phe as a proportion of TKP was notably consistent among the amino acids. Despite these disagreements it does appear that Phe content, at least, is predictable as a function of TKP. This means both that TKP can be used as a proxy for kernel Phe content, and that breeding for reduced Phe could be accomplished by breeding for reduced protein overall.

Determination of the total protein or N content of grain can be accomplished in several ways. The two standard methods, per the Association of Official Analytical Chemists (2012), are called the combustion method and the Kjeldahl method. These are the standards for determining total N and crude protein content in a variety of grain and other food sources, but both have the drawback that samples are destroyed in the analysis. This has induced the development of less costly methods, particularly the use of near-infrared spectrometry, to estimate macronutrient profiles of grains, feeds, and food (Davies & Grant 1987). Karn et al (2017) developed an algorithm for estimating total kernel macronutrient makeup (starch, protein, and oil) in a range of modern maize varieties. Karn et al (2017) used relatively large samples of maize grain for

analysis, but other researchers have made progress at using Near Infrared Spectrometry (NIRS) to estimate protein content on significantly smaller samples, even as small as a single kernel (Baye et al 2005).

Few researchers are actively attempting to depress protein content in any crop species. In wheat, asparagine (Asp) is the precursor to acrylamide, a carcinogen formed in bread, and some effort has been made at depressing Asp content specifically within wheat kernels, but this research is in its early stages (Rapp et al 2018). No kernel composition mutations have been identified in maize or other grains with specific action against Phe or BCAA accumulation.

Maize kernels first and foremost are seeds, from which new plants will grow. They thus contain an embryo and stored nutrients. The embryo itself is not a reasonable target for amino acid suppression since that would imply a growing plant that was deficient in the suppressed amino acid, possibly because it could not synthesize it, and such mutants would likely be lethal if they existed. However, the vast majority of the weight of a maize kernel is in stored nutrients including starch and protein, not in the embryo (Baudet et al 1986).

In maize kernels, the primary protein storage molecules are called zeins. Zein molecules are produced by the endosperm and folded into protein storage bodies in the kernel. There are four classes of zeins which are found in varying amounts in the kernel, with the largest constituent being α -zein, of which there are two types. The α -zeins contain no Lys or Trp, which is the source of maize's deficiency in these two amino acids. Two γ -zeins are the next most common storage proteins, while β - and δ -zein make up minor fractions, along with a handful of non-zein storage proteins. The γ -zeins are also deficient in Lys and Trp; of special interest here, the 27 kDa γ -zein has very little Phe (2 residues out of ~ 250), while the 16 kDa γ -zein and the α -zeins are all much higher in Phe. β -zein has no Phe residues at all (Coleman & Larkins 1999).

The previously discussed *o2* mutation reduces the fraction of zeins in the endosperm; *o2* plants compensate for this by increasing production of other storage proteins, in particular glutelins, which are higher in Lys and Trp. In this way the amino acid composition of *o2* mutants is altered. In the years since the *o2* mutation was first reported, numerous modifiers of the mutation have been found that adjust the amounts of specific zeins and glutelins produced in the endosperm (Hasjim et al 2009). In fact, certain modifiers of

the $\alpha 2$ gene are known to enhance production specifically of the 27 kDa γ -zein; this has been exploited in QPM work (Geetha et al 1991). It is theoretically possible therefore that an as-yet unidentified modifier to the $\alpha 2$ mutation could enhance production of both the 27 kDa γ -zein and the β -zein; these are the only two of the six zein molecules encoded by single genes and should be easily exploited. Such a modifier, if it could be found or engineered, might lead to a very low-Phe kernel with otherwise normal protein content.

Until such a modifier is found or engineered, attempts to produce a low-Phe or low-BCAA maize must start with traditional breeding of low-protein parents, combined with testing for any unusually low-Phe or low-BCAA variants not heretofore identified. The ongoing Illinois Long Term Selection Experiment (ILTSE) has succeeded in significantly increasing or decreasing average kernel protein content (also kernel oil content) in a population derived from a single open-pollinated strain, Burr's White. This experiment has demonstrated the wide range of potential kernel nutrient compositions within a single population, and suggests that examination and breeding of diverse maize varieties for low protein is likely to succeed (Below et al 2004).

Studies of the ILTSE have found that low TKP acts in a dominant manner over high TKP, with hybrids of high- and low-protein lines having protein levels equal to or lower than the low-protein parent, a trend that continues in future generations including backcrosses to the high-protein parent (Frey 1949). This may be explained in part by dominance of low-protein traits, but also by the fact that hybrid vigor leads to kernels higher in starch than those of inbred parents (Frey 1949). Letchworth & Lambert (1998), working with the ILTSE materials, determined that protein concentration was maternally determined, with little or no effect from the pollen genotype. Taken together, these findings suggest that a breeding program would best reduce protein content by selecting maternal parents for low protein, and then producing hybrid seed with male parents with acceptable food production quality traits.

Table 1.1 The 20 amino acids

Amino Acid	Abbr	Human Essential
Alanine	Ala	
Asparagine	Asn	
Aspartic Acid	Asp	
Arginine	Arg	
Cysteine	Cys	
Glutamine	Gln	
Glutamic Acid	Glu	
Glycine	Gly	
Histidine	His	Yes
Isoleucine	Ile	Yes
Leucine	Leu	Yes
Lysine	Lys	Yes
Methionine	Met	Yes
Phenylalanine	Phe	Yes
Proline	Pro	
Serine	Ser	
Threonine	Thr	Yes
Tryptophan	Trp	Yes
Tyrosine	Tyr	
Valine	Val	Yes

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Chapter Two: Variation in Maize for Response to Growth Regulators

Abstract

Fungicides of the quinone outside inhibitor class (strobilurins) and of the CYP51 inhibitor class (triazoles) are known to have growth regulating effects on maize and other crop plants, including delayed leaf senescence. When applied to maize in the field, these growth regulating effects coincide with increased yield, although the yield increases are not consistent and may not economically justify the use of the fungicides in absence of disease. The response of maize plants to these growth regulators is not uniform across genotypes or environments. It may therefore be possible to breed selectively for high rates of response to growth regulator treatment in order to isolate and improve the consistency and margin of yield increases. To test this hypothesis, diverse samples of maize inbred lines and hybrids were evaluated across three locations in two years for response to treatment with a combined strobilurin and triazole fungicide, applied at V5/6 and VT growth stages. Response to growth regulator treatment and the interaction of treatment by genotype were evaluated separately in hybrids and inbreds. Agronomic traits, including grain yield and several yield components, lodging and delayed leaf senescence, were evaluated. Yield was not significantly affected by treatment, either on average across varieties nor for any variety individually. Leaf senescence in hybrids was delayed significantly by treatment, but the interaction of treatment and genotype was not significant and was not correlated with improved yield or yield components. The interaction of inbred with treatment was significant ($p < 0.04$) for leaf senescence, but the genotypes responsive to treatment in the inbred study were not parents of more responsive hybrids, nor did they group according to known pedigree and genetic relationships. These results suggest that breeding to enhance the response to strobilurin and triazole treatment is not likely to be effective in maize.

Introduction

Growth regulators are a class of chemical with defined effects on plant growth, such as flowering induction or delayed senescence, and represent a potential mechanism for yield increases, in addition to genetic improvement through breeding and other agronomic production improvements. Several fungicide classes have growth-regulating effects on plants, and studies have shown measurable yield increases from the application of certain fungicide classes to grain crops in the absence of fungal infection (Wu & von Tiedemann 2001, Blandino et al 2012, Paul et al 2011).

Strobilurins are a class of fungicide first developed in the 1990s as synthetic mimics of the toxin produced by the fungal genus *Strobilurus* among others (Anke et al 1977). Strobilurins act as quinone-oxidoreductase-inhibitors, blocking electron transport at the Cytochrome bc₁ complex in the mitochondrial inner membrane (Becker et al 1981). The first commercial strobilurins were brought to market in 1996 (Bartlett et al 2002) and numerous studies since then have identified the effects of strobilurin on wheat, in particular the tendency of strobilurin-treated plant tissue to remain green after untreated tissue has senesced (Grossmann & Retzlaff 1996, Jones & Bryson 1998, Wu & von Tiedemann 2001). This “stay-green” trait was later identified in other small grain crop species in the field (Bayles & Hilton 2000), as well as in maize (Byamukama et al 2013), with several different strobilurin fungicides the subject of these studies.

Venancio et al (2003) suggested that the physiological effects of strobilurins might justify their use in maize as growth regulators in the absence of disease. Early studies generally found that there was insufficient evidence to suggest that strobilurin application in small grains was apt to be profitable in the absence of disease (Weisz et al 2011). More recent studies have attempted to better replicate commercial farming operations, testing for economic yield boosts at larger scales, with results more suggestive that strobilurin sprays for yield boost are in fact financially justified, but that the effects on yield are difficult to measure on small experimental plots (Vincelli & Lee 2015; Tedford et al 2017).

The triazoles, another fungicide class, have been shown to reduce production of gibberellic acid (Rademacher et al 1987). While gibberellic acid (GA) is known to regulate stem length and plant maturation, it is only one element in the regulation of these traits (Evans & Poething 1995); however, GA is important in signaling the start of leaf senescence (Fletcher et al 2000). Wu and von Tiedemann (2001) showed that

triazole application, particularly as plants near maturity, aids in retarding leaf senescence, and suggested that this stay-green effect may be a result of the triazole's suppression of GA. Many tests of the use of fungicides as growth regulators in cereal crops have made use of a strobilurin and a triazole in combination (e.g. Tedford et al 2017) or separately (e.g. Paul et al 2011).

Nelson & Meinhart (2011) suggested that growth regulators may encourage more complete fill of the ear tip in maize. There is also evidence that strobilurin-treated maize has greater stalk strength and less lodging than untreated maize (Paul et al 2011, Kalebich et al 2017). The combination of these trait responses may explain the reported yield benefit of growth regulator application.

The effectiveness of growth regulator application on yield response may depend on cultivar. It is possible that some cultivars would respond more than others, and if such differences were sufficiently strong, this could justify breeding for higher response to growth regulators. To date, studies of growth regulator response involved only small samples of available cultivars adapted to target production environments, and at least one study indicated variability in cultivar response, although it was not consistent over environments and may have been related to disease resistance rather than growth regulator response *per se* (da Costa et al 2012). Relative to the genetic variability available worldwide in maize, the genetic variation within elite commercial Corn Belt Dent hybrids is limited (Goodman 2005). Studies of growth regulator response in a broader array of maize germplasm may lead to discovery of a greater range of potential maize response to growth regulators, and the most highly responsive germplasm might be used in targeted breeding programs to develop commercial-quality cultivars with significant response to growth regulator application, permitting greater overall maize yields.

The objectives of this study were to test a very diverse sample of maize inbred lines and hybrids for response in yield, yield components, and other agronomic traits to strobilurin and triazole growth regulator treatment; and to test for treatment main effects and treatment-by-variety interaction. Treatment-by-variety interactions occur when some varieties are more strongly affected (or affected in a different direction) by the treatment than others, indicating that genotypes vary in their response to growth regulator treatment. The relative importance of these interactions is an indicator of the potential genetic variability for growth regulator response that could be exploited by breeding.

Materials & Methods

To investigate the potential variability and heritability of maize response to strobilurin and triazole application, we planted 22 maize hybrids (Table 2.1) in replicated trials at three locations in North Carolina in 2016 and 2017. These hybrids were drawn from diverse genetic backgrounds, including high-yielding commercial cultivars, crosses between important public inbreds and commercially developed inbreds with expired PVP certificates, and crosses involving temperate and tropical inbreds, popcorn, and sweet corn.

At each location in both 2016 and 2017, the experiment was planted in a split-plot design with three complete replications, where growth regulator treatment comprised the whole-plot factor and hybrid was the sub-plot factor. Experimental units consisted of four 3.6-m rows planted with the same hybrid; row spacing varied from 76cm to 96.5cm depending on location. Seed was sown at a density of 6.8 seeds m⁻¹. Growth regulator treatments were no treatment (control) or application of Syngenta brand Quilt XCel fungicide at growth stage V5/V6, and again at growth stage VT. Quilt XCel is a blend of 13.5% azoxystrobin and 11.7% propiconazole. The growth regulator was applied only to the center two rows of the four-row plots, using a backpack sprayer delivering 241.3 kPa through a flat-fan nozzle, at the labelled application rate of 0.77 L ha⁻¹. Plots were scored for flowering and silking dates, disease pressure, lodging, plant and ear height, number of late-season green leaves, and grain yield and moisture content.

Flowering time was scored on each plot in the Clayton, NC, location. Days to anthesis was the number of days from planting to the day on which at least half of the plants in a plot were shedding pollen. Days to silking was the number of days from planting to the date on which at least half of the plants in a plot had at least one ear with emergent silks. Anthesis-silking interval (ASI) was the difference between days to silking and days to anthesis.

A composite foliar disease rating was taken on each plot using a 0-9 scale. Because each hybrid used in the experiment was not equally susceptible to each disease, the goal of the disease scoring was to generate a rating of overall disease pressure, rather than to note individual diseases in each plot. Thus, a plant that had limited gray leaf spot lesions on leaves below the ear leaf would rate an 8, while another plant with limited rust on lower leaves would also rate an 8. Overall loss of green tissue was the primary rating objective. Rated diseases included northern corn leaf blight, *Exserohilum turcicum* (Leonard & Suggs);

southern corn leaf blight (*Bipolaris maydis* (Nisikado & Miyake); gray leaf spot, *Cercospora zeae-maydis* (Tehon & Daniels); eye spot, *Aureobasidium zeae* (Narita & Hiratsuka); and rust, *Puccinia* sp. (Pers.). A rating of 0 indicated that every plant in the plot was dead from disease, while 9 indicated that no plants showed any signs of disease. Disease rating was accomplished within a week of plot flowering time at each location.

Plant and ear heights were measured approximately two weeks after flowering, with plant height measured from base to flag leaf node, and ear height measured to the insertion point of the primary (highest) ear.

Green leaf counts were made roughly two weeks after maturity, and weekly thereafter until the majority of plants had senesced. Scores are the average number of green leaves (defined as leaves having more than 50% green tissue) per plant in a plot, with six plants per plot being counted.

Lodging was measured at maturity prior to harvest by counting plants leaning more than 30 degrees from vertical, with broken stalks, or with dropped primary ears. Percent lodging was computed as the number of lodged plants divided by the stand count for the plot. For grain yield and moisture content, the center two rows of each plot were mechanically harvested. Machine-harvestable grain yield and moisture of the two center rows of each plot were measured, and yield was adjusted to 15.5% grain moisture.

In 2017, a separate experiment was conducted to measure growth regulator response for 40 diverse maize inbred lines (Table 2.2), using a similar split-plot design with three replications at the same three locations but with a single 3.6-m row as the experimental unit. Growth regulator application was performed in the same manner as in the hybrid experiment. Plots were scored for the same traits as the hybrids except for grain moisture content, as the single-row plots were harvested by hand rather than mechanically. Total ear count per plot was recorded for the inbreds.

Also in 2017, four additional traits were measured for all hybrid and inbred plots: ear length, ear tip fill, 50-kernel weight, and kernel row number. Four random ears were sampled from each plot at maturity to measure ear and kernel traits. Ear length was measured using a Fowler 12" Ultra-Cal IV electronic caliper, from base to tip of ear. The same caliper was then used to measure the unfilled portion of the ear, and ear fill percentage was calculated from this. Kernel row number was counted on the same four ears. These four

ears were then shelled and the kernels bulked, and 50 kernels selected at random and weighed to generate a 50-kernel weight for each plot.

Data were analyzed using Proc MIXED in version 9.4, of the SAS System for Windows (SAS Institute 2018). The following statistical model was used for the hybrid experiment:

$$Y = \mu + \gamma_i + \tau_j + \gamma\tau_{ij} + D_k + S_m + V_n + L_p + B_{q(np)} + VL_{np} + \tau B_{jq(np)} + \gamma T_{in} + \gamma V_{ip} + \gamma TV_{inp} \\ + \gamma\tau V_{ijp} + \gamma\tau TV_{ijnp} + \varepsilon_{ijkmnpq(np)}$$

where Y represents the measured trait value of an experimental unit; μ represents the overall mean; γ represents the fixed effect of hybrid; τ represents the fixed effect of treatment; $\gamma\tau$ represents the interaction between hybrid and treatment; D is a covariate accounting for the effect of disease pressure in each plot; S is a covariate accounting for the effect of differences in stand count; V represents the random effect of year; L represents the random effect of location; B represents the random effect of block within year and location; τB represents the interaction of treatment and block (the whole-plot error effect); additional terms represent interactions among variables; and ε is the residual error.

For the inbred study, the following statistical model was used:

$$Y = \mu + l_i + \tau_j + l\tau_{ij} + D_k + S_m + L_p + B_{q(p)} + \tau B_{jq(p)} + lL_{ip} + l\tau L_{ijp} + \varepsilon_{ijkmpq(p)}$$

where Y represents the measured trait value of an experimental unit; μ represents the overall mean; l represents the fixed effect of inbred line; τ represents the fixed effect of treatment; $l\tau$ represents the interaction between treatment and inbred line; D is a covariate accounting for the effect of disease pressure in each plot; S is a covariate accounting for the effect of differences in stand count; L represents the random effect of location; B represents the random effect of block within location; τB represents the interaction of treatment and block (the whole-plot error effect); additional terms represent meaningful interactions among variables; and ε is the residual error.

Models with and without the disease rating as a covariate were fit to the data from both experiments and results compared to determine if treatment effects or treatment-by-variety interactions depended on disease pressure.

Results

Hybrid experiment

Hybrid main effect was significant at the $p \leq 0.05$ level for all measured traits, and at the $p < 0.001$ level for most traits. The interaction of treatment and hybrid, a measure of variation in response to treatment among hybrids, was significant for only two traits: disease rating (as also reported previously by da Costa et al, 2010) and anthesis-silk interval (ASI; Table 2.3).

Although disease pressure was relatively low in this study (mean rating 7.7 on 9-point scale with 9 reflecting no disease present), treatment main effect was significant for foliar disease ($p < 0.0001$), with application of growth regulator resulting in an average reduction in observable disease level of 1.7 points on the nine-point scale. Hybrid by treatment interaction was also significant ($p = 0.0134$), with disease reduction ranging from a low of 1.2 points for PHB47×LH51 to a high of 2.3 points for B73×LH211; the reduction was also significant for each hybrid individually. Treatment main effects were also significant for grain moisture level at harvest ($p = 0.0135$), with treatment leading to an increase of 0.5% grain moisture on average (increasing from 17.6% to 18.1%). Treatment effect on moisture for individual hybrids was significant in only two cases (Table 2.4). Treatment main effect was also significant on leaf senescence (stay-green), where treatment significantly delayed senescence overall, with treated plants retaining an average of 1.4 more green leaves at the same point late in the season than untreated plants (Figure 2.1). Treatment main effect was not significant for any other traits.

Because grain yield differences may be difficult to identify in small research plots, we measured yield components including ear length, percent cob fill, kernel row number, and kernel weight. If there were any patterns of increased ear length, fill rate, row number, or kernel size, these might be extrapolated to suggest potential yield effects at larger scales. However, none of these yield components were significantly altered by treatment.

Anthesis-silking interval (ASI) is frequently used as a gauge of plant stress during the critical flowering period (Hall et al 1982). If treatment with fungicide reduces plant stress, this might be reflected in reduced ASI for treated plants, which may suggest potential yield benefits if replicated at large scale. ASI varied significantly because of the interaction of material and treatment (Table 2.3), but not in a

consistent direction. ASI increased with growth regulator application for some hybrids but decreased for others (Figure 2.2). ASI was not correlated with yield, nor with any yield component.

Twelve traits were measured in 21 hybrids, resulting in a total of 252 trait-hybrid combinations (although yield and moisture were not measured for one variety; Table 2.1). For 19 of the 250 measured trait-hybrid combinations (7.5%), the p -value associated with the difference between treatment was ≤ 0.05 (Table 4). Multiple testing correction (e.g. Tukey or Bonferroni) would result in no comparisons being significant. Even without correcting for multiple testing, no clear pattern of treatment response was observed: responsive hybrids were not from common germplasm groups, nor were hybrids that showed response to growth regulator in one trait more likely to show response in another.

Although disease rating was itself significantly reduced by treatment, when disease pressure is included as a covariate in the analysis of other traits, it had little effect. The inclusion of the disease pressure covariate had no profound impact on the statistical significance of the treatment or treatment \times environment effects. For example, when the disease covariate was included in the analysis of cob fill, the F-value for treatment was 0.2 ($p = 0.67$); whereas without the covariate, the F-value for treatment was 0.79 ($p = 0.46$). Table 2.3 shows the few exceptions to this rule; however, again no clear patterns emerge with regards to value of disease covariate in significance of measured effect. Table 2.3 additionally shows that disease as a covariate was itself significant at the $p < 0.05$ level for kernel row number.

Inbred experiment

The inbreds used in this study represent an even wider array of maize diversity than the hybrids. However, our findings are consistent with the hybrid experiment in that no pattern of significance emerged among related germplasm. The inbred main effect was strongly significant for every trait, whereas the treatment main effect was significant only for disease rating ($p = 0.04$), days to anthesis, and ASI (Table 2.5). The interaction of inbred with treatment was significant only for lodging and stay-green. The traits for which the treatment-by-genotype interaction was significant in the inbred experiment are entirely distinct from those for which the interaction was significant in the hybrid experiment.

Twelve traits measured on 39 inbreds resulted in a total of 468 comparisons of specific genotype-treatment effects. In 27 of these trait-inbred comparisons (5.8%) the p-value associated with the difference between treatment and control was ≤ 0.05 (Table 2.6); as in the hybrid experiment, multiple-test correction would result in no significant differences. Given the number of tests performed, the proportion of “significant” comparisons at $\alpha = 0.05$ is very close to the 5% expected by random chance. As in the hybrid experiment, no consistent pattern of treatment response with respect to genotypes was observed.

Discussion

The goal of this experiment was to determine if there is heritable variation in maize breeding germplasm for response to treatment with strobilurin and triazole fungicides beyond effects that might be attributable to the effects of protection against disease. The existence of heritable variation for growth regulator response would suggest that specific hybrids could be selected for optimal response to growth regulator, and perhaps that new germplasm sources with greater responses could be identified and incorporated into commercial breeding programs. Our findings suggest limited variability in response to treatment even across a very broad range of genetically diverse maize germplasm. The interaction between hybrid or line and treatment was not significant for yield or any yield components. In the few cases that the interaction was significant, we observed no consistency between treatment and direction of response, which suggests limited or no heritable variation for growth regulator response. The few individual cases of hybrids or inbreds responding for particular traits did not exhibit any clear pattern; the responses were not consistent within germplasm groups, nor were they consistent across traits for a particular hybrid. Commercial hybrids developed by Syngenta were among those with the strongest delayed-senesence effect, suggesting that if this particular response is genetically controlled, it has already been exploited in this commercial breeding program.

This experiment was conducted under natural disease conditions; the observed disease pressure was low in most environments. Despite the low incidence of disease, the one trait for which the growth regulatory response was clearly significant was the disease rating itself. Treated plots were much lower in disease pressure than untreated plots, and this was significant both for all hybrids individually, as well as on average across hybrids. This result demonstrates that the fungicide is effective at reducing disease symptoms even in relatively low-disease environments. Using the disease score as a covariate in the experimental models produced minor and inconsistent changes in results, showing that the reduction in pathogen pressure, although significant, was simply not sufficient to create a clear agronomic benefit in the environments studied. The disease covariate effect itself was significant on one trait, kernel row number, but this had no impact on the significance of treatment main effect, or treatment-by-genotype interaction. The data suggest that treatment effects were too small on most traits to provide a clear or consistent response.

Producers rarely wish to apply pesticides of any kind when the pest pressure is below a certain economic threshold. Other studies have found yield increases in reportedly low-disease conditions with the application of strobilurins and triazoles. Our results suggests that the effects of growth regulator treatment on yield are small and may be dependent on as-yet-undefined environmental conditions.

The results of this study do not suggest that there is any compelling reason to spray these fungicides in the absence of disease purely for their growth regulating effects. Such effects, where present, are small and inconsistent. The potential for development of fungal resistance to these chemicals when routinely spraying pesticides in low disease conditions is well established (Lucas et al 2015, Gisi et al 2012). The potential consequences of promoting the evolution of fungal resistance and the loss of effective fungicidal chemistries by using these products is a contraindication to their use in the absence of substantial disease pressure.

Further study may suggest potential economic benefits of fungicide application in low-disease environments, but the results of this study do not support growth regulator application in the absence of substantial disease pressure. Additionally, future work may illuminate any connection between delayed senescence and potential yield benefits. The only significant correlation with delayed senescence, however, was with increased grain moisture at harvest, which is unfavorable to the grower. Increased grain moisture may result in maize being left in the field beyond the traditional harvest dates to realize a yield increase connected to delayed senescence, which is unlikely to be desirable.

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Table 2.1. Hybrid Germplasm Used In Study		
B73×207	B73×Tx303	
B73×6M502	Hp301×SA24	†
B73×CML247	IL14H×P39	‡
B73×F118	N45P-3122A-EZO	§
B73×Ki3	N58S-3111	§
B73×LH211	N59B-3111A	§
B73×LH252	N60F-3111	§
B73×Mo17	N68B-3111	§
B73×NC350	Oh43×W64A	
B73×PHK46	PHB47×LH51	
B73×PHR58	PHHB4×LH51	
Notes:		
† Yield too low for harvester to measure		
‡ Plots destroyed by wildlife		
§ Seed received from Syngenta		

Table 2.2. Inbred Germplasm Used In Study

§§	207	† §§	F118	‡‡	NC350
‡ §§	6M502	††	Hp301	‡‡	NC358
†	A632	§	IL14H	‡	Oh43
†	B73	‡‡	Ki11	‡	Oh7B
‡	B97	‡‡	Ki3	§	P39
‡‡	CML103		Ky21	§§	PHB47
‡‡	CML228	§§	LH211	§§	PHG35
‡‡	CML247	‡ §§	LH252	§§	PHHB4
‡‡	CML277		M162W	§§	PHK46
‡‡	CML322		M37W	§§	PHR58
‡‡	CML333	‡	Mo17		Tx303
‡‡	CML52		Mo18W		Va35
‡‡	CML69		Ms71		W64A
Notes: † Stiff Stalk ‡ Non Stiff Stalk § Sweet †† Popcorn					
‡‡ Tropical §§ Ex-PVP					

Table 2.3. F-test p values for treatment and hybrid main effects and interaction for each trait measured in hybrid study, from models with and without disease rating as a covariate.

Values with disease covariate												
	Days to Anthesis	Anthesis-Silking Interval (ASI)	Plant Height	Ear Height	Late Green Leaves	Lodging	Grain Moisture	Yield	Ear Length	Cob Fill	Kernel Row Number	50-kernel Weight
Disease covariate	0.8403	0.5753	0.1396	0.7403	0.4067	0.9221	0.877	0.7371	0.4914	0.0756	0.0474*	0.606
Treatment	0.3578	0.8912	0.3669	0.6476	0.0046**	0.3779	0.0135**	0.9764	0.0806	0.6707	0.1986	0.6179
Hybrid	<0.0001***	<0.0001***	0.0352*	<0.0001***	0.0353*	0.0002***	<0.0001***	<0.0001***	<0.0001***	0.0174**	<0.0001***	<0.0001***
Interaction	0.3411	0.0014**	0.8051	0.402	0.7877	0.4403	0.5928	0.5219	0.9247	0.3354	0.5841	0.9854
Values without disease covariate												
Treatment	0.2419	0.7195	0.6044	0.4442	0.0131*	0.303	0.0004***	0.8156	0.0837	0.464	0.7435	0.8001
Hybrid	<0.0001***	<0.0001***	0.0190*	<0.0001***	0.0416*	<0.0001***	<0.0001***	<0.0001***	<0.0001***	0.0174*	<0.0001***	<0.0001***
Interaction	0.3412	0.0921	0.7524	0.388	0.8298	0.2181	0.4204	0.378	0.9949	0.3866	0.6257	0.9856

Table 2.4. Individual hybrid-trait combinations for which treatment effects were significant ($p \leq 0.05$ unadjusted for multiple testing)								
	Lodging	Plant Height	DTA	ASI	Grain Moisture	Cob Fill	Kernel Row #	Green Leaves
B73×207								More late green
B73×6M502								
B73×CML247					More moisture			
B73×F118	Less lodging							
B73×LH211				Shorter interval	More moisture			
B73×LH252								More late green
B73×Mo17								More late green
B73×NC350				Shorter interval				
B73×PHK46								More late green
B73×PHR58				Longer interval			More rows	
B73×Tx303	Less lodging							
N58S-3111							More rows	More late green
N59B-3111A								
N60F-3111								More late green
Oh43×W64A						Less Fill		
PHB47×LH51								More late green

Table 2.5. F-test p values for treatment and hybrid main effects and interaction for each trait measured in inbred study

Values with disease covariate												
	Days to Anthesis	Anthesis-Silking Interval (ASI)	Plant Height	Ear Height	Late Green Leaves	Lodging	Ear Count	Yield	Ear Length	Cob Fill	Kernel Row Number	50-kernel Weight
Disease covariate	0.0691	0.0018**	0.8950	0.1242	0.4760	0.6922	0.4894	0.7565	0.6092	0.2125	0.2799	0.7824
Treatment	0.0391*	0.0013**	0.1669	0.3159	0.0546	0.6875	0.9908	0.8901	0.7268	0.4836	0.9349	0.7790
Inbred Line	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***
Interaction	0.9195	0.7143	0.7757	0.9432	0.0471*	0.0184*	0.6256	0.1806	0.3714	0.9796	0.9412	0.0956
Values without disease covariate												
Treatment	0.4333	0.1801	0.1467	0.5627	0.0700	0.6453	0.6516	0.7484	0.6062	0.9670	0.5950	0.9249
Inbred Line	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***
Interaction	0.8641	0.9058	0.7727	0.9170	0.0413*	0.0514	0.8429	0.2105	0.4068	0.9830	0.9457	0.1023

Table 2.6. Individual inbred-trait combinations for which treatment effects were significant ($p \leq 0.05$ unadjusted for multiple testing)

	Lodging	Plant Height	Ear Count	Yield	Ear Length	50k Weight	Green Leaves	DTA	ASI
207		Shorter							
B97							More green leaves		
CML103	More lodging								
CML228									Shorter interval
CML277								Earlier flowering	
CML52								Earlier flowering	
F118							More green leaves		
IL14H	Less lodging						More green leaves		
Ki11								Earlier flowering	Shorter interval
Ky21									Shorter interval
LH211				More yield		Heavier kernels	More green leaves		
LH252						Lighter kernels			
Mo17									
Ms71							More green leaves		
NC350				More yield					
NC358									Shorter interval
Oh7B									Shorter interval
P39	Less lodging		Fewer ears						
PHHB4						Heavier kernels			
PHK46			Fewer ears						
Tx303			More ears						
Va35				Less yield	Shorter ears				

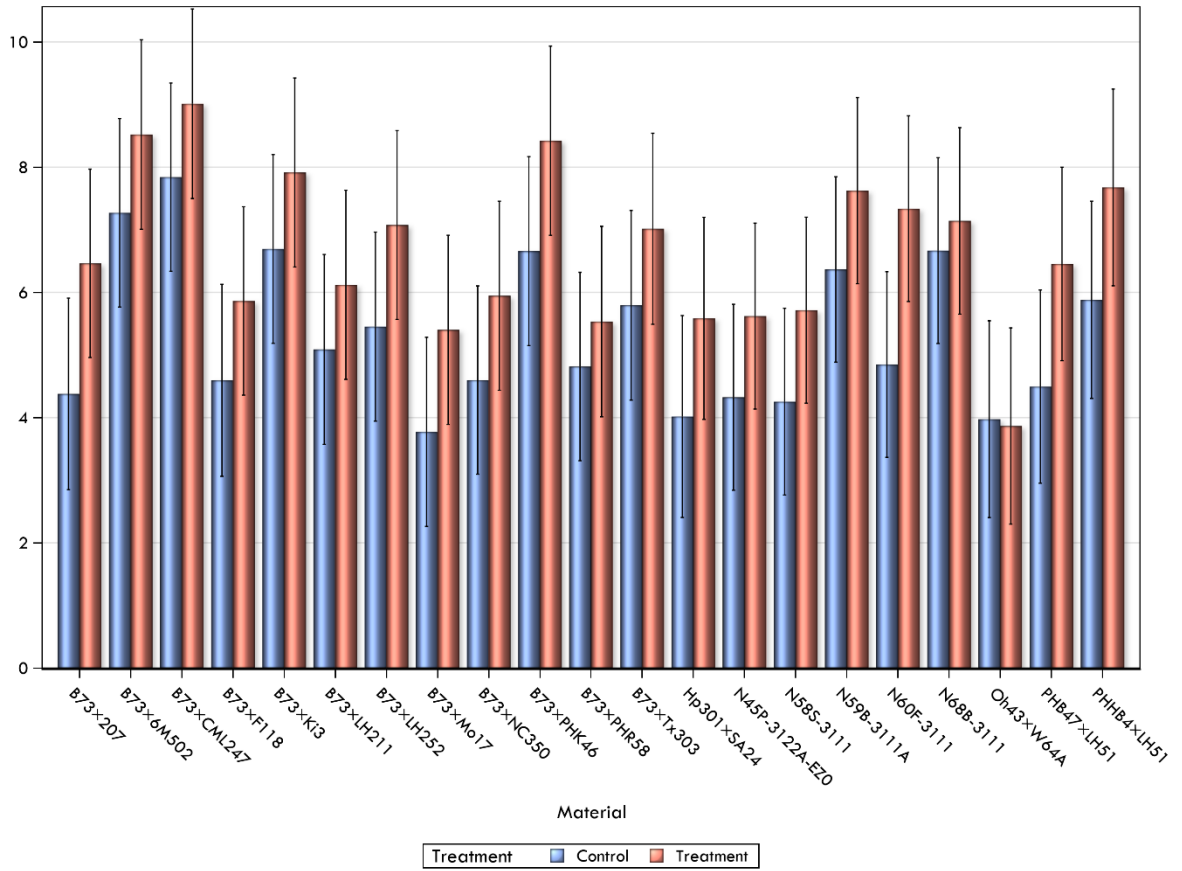


Figure 2.1. End-of-season green leaf count, by treatment, for each hybrid, with standard errors

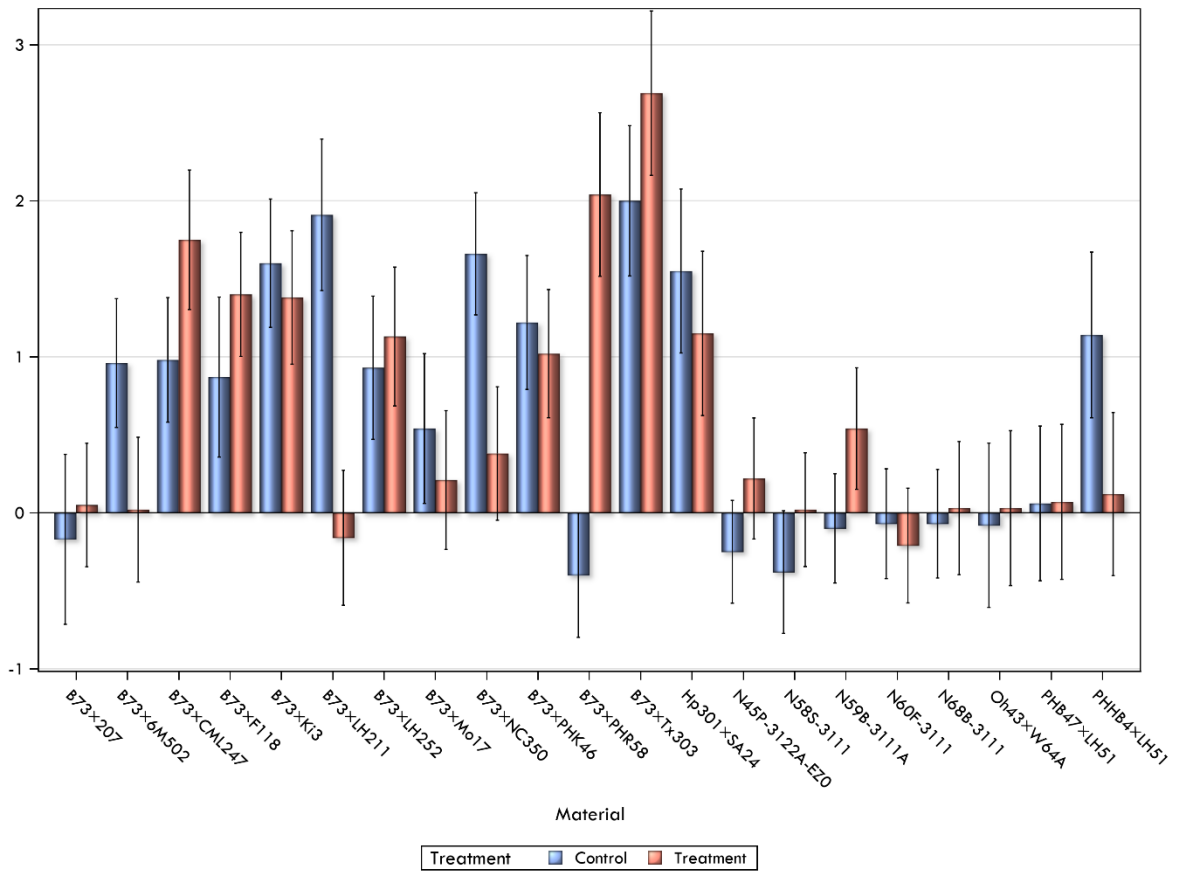


Figure 2.2. Anthesis-silking interval, by treatment, for each hybrid, with standard errors

Chapter 3: Breeding Low-Protein Maize for Protein-Restricted Diets

Abstract

Inborn errors of amino acid metabolism, such as phenylketonuria (PKU), are genetic conditions in humans that result in an inability to metabolize certain amino acids, and require lifelong maintenance of a very restrictive low protein diet. In the case of PKU this diet does not permit consumption of most maize products, yet maize is a major part of the human diet and especially significant in some cultures. Kernel protein content, and to a lesser extent amino acid composition are variable in maize. Breeding maize for low kernel protein content and/or low amounts of specific amino acids such as phenylalanine might expand the restrictive diets and improve quality of life for this subpopulation. To test for the potential of breeding low-protein content maize, an experiment was conducted across two locations in two years to examine a diverse array of hybrid, inbred, and landrace maize varieties for protein content and amino acid composition. Results indicate that there is substantial variability in the kernel protein content across maize varieties and that this trait is highly heritable. Phenylalanine content specifically is also highly heritable although strongly correlated with total kernel protein. Breeding for reduced protein should permit development of maize sufficiently low in phenylalanine to permit broader inclusion in the PKU diet.

Introduction

Phenylalanine is one of the 20 common amino acids that comprise proteins. Phenylalanine is one of the nine “essential” amino acid for humans, which are not produced in the body and must be taken in through the diet. Phenylketonuria (PKU) is a human genetic metabolic disorder in which the body does not produce sufficiently active phenylalanine hydroxylase, the enzyme that converts phenylalanine into tyrosine (Blau et al 2010). Without this enzyme, PKU sufferers cannot eliminate phenylalanine from the body. Over time, excess phenylalanine builds up in the bloodstream and causes irreversible neurological symptoms. PKU can be detected shortly after birth through infant screening, a procedure that is widespread throughout the developed world (de Baulny et al 2007). The treatment for the condition is a diet restriction that sharply limits dietary phenylalanine intake while supplementing with other amino acids. Provided that the diet is strictly maintained, persons with PKU can expect a full lifespan with no symptoms of the condition (Hanley 2004). Maple syrup urine disease is a similar inborn error of amino acid metabolism, second in frequency in humans to PKU, except the enzyme deficiency involves the metabolism of the branched-chain amino acids (BCAA): leucine, isoleucine, and valine (Blackburn et al 2017).

Although small amounts of maize can be included in the PKU diet, maize is not considered a low-phenylalanine food (Dennison 2005). It is unknown whether a maize variety exists that produces unusually low amounts of phenylalanine in the grain, and if so whether that trait is hereditary and can be bred into useful hybrid maize cultivars. A low-phenylalanine maize could be made into tortillas, cornbread, polenta, or other common maize-based foods, expanding the PKU diet while helping patients maintain appropriate blood phenylalanine levels and improving quality of life.

Maize shows significant diversity in both quantity and composition of protein in the kernel, but maize kernels generally contain 12 to 14% protein (Hasjim et al 2009). The major protein of the maize kernel is zein, of which four varieties (α , β , γ , and δ) are identified; in the kernel, β - and γ -zeins form a densely packed vitreous endosperm, while the predominant α -zein forms a soft, starchy endosperm in the center of the kernel (Gibbon & Larkins 2005). Like most grains, maize is deficient in lysine, but the *opaque2* (*o2*) mutation reported by Mertz (1964) results in reduced production of zein proteins and increased production of other lysine-rich proteins. The *o2* mutant phenotype lacks the vitreous endosperm, which leads to greater

susceptibility to kernel breakage, kernel disease, and insect feeding. Further discoveries of modifications to the *o2* mutant led to the development of Quality Protein Maize varieties, which have higher lysine content grain but do not suffer from the problems of the original *o2* mutant varieties (Vasal 2000). Additional kernel composition mutants have also been identified, including *floury1* (*fl1*) and *floury2* (*fl2*), as well as at least 14 other *opaque* mutants (Neuffer et al 1997).

More generally, Flint-Garcia et al (2009a) suggested that “enough variation probably exists in maize inbred lines” to enable breeders to select for specific amino acid contents in addition to total protein in maize grain. Whereas most breeding programs aimed at improving the nutritional quality of grain hope to increase specific components, the development of a specialty maize variety for metabolic disorder diets would aim to reduce concentrations of a specific amino acid in the grain. Wheat grain has recently been targeted for asparagine reduction for the purpose of reducing acrylamide in baked grain products (Rapp et al, 2018), but we are not aware of any ongoing efforts to reduce levels of a particular amino acid in maize.

The range of variation of amino acid composition in maize kernels of different varieties or grown in different conditions is uncertain. Keeney (1970) found that fertilizer regime and genotype affected the overall amino acid composition, with the addition of nitrogen increasing yield and total protein but decreasing the proportion of kernel protein made of important essential amino acids such as lysine (Lys) in certain varieties but not in others. Keeney (1970) and Flint-Garcia et al (2009b) suggested that phenylalanine tended to remain a relatively constant proportion of total protein among varieties and environments.

Baudet et al (1986) analyzed amino acid composition and total kernel nitrogen in a number of maize varieties and concluded that each amino acid varied with total kernel nitrogen according its own unique equation, but that these equations dictated the amino acid breakdown and thus total kernel nitrogen could be used to calculate the amino acid composition of any maize sample regardless of variety or environmental conditions. This disagrees significantly with other findings, but agrees in the suggestion that phenylalanine and total kernel protein are closely correlated. Further work is needed to explore whether

variation in genotype and environment leads to variation in kernel amino acid composition or not, but reducing total protein content should reduce phenylalanine content effectively.

Nitrogen application does increase both total yield and the total protein content of maize kernels, but strongly affects the relative concentrations of only a few amino acids, phenylalanine not among them (Sauberlich et al, 1953; Keeney, 1970). This suggests that simply reducing the nitrogen available to growing maize plants will not be sufficient to depress phenylalanine without incurring undesirable yield losses.

We therefore proposed in the current research to examine a diverse suite of maize genotypes for protein content and amino acid composition. We wished to determine if phenylalanine levels can be reliably inferred from total kernel protein, and if so, how much heritable variation for protein content exists in diverse maize inbreds and hybrids. To do this, we compared protein and amino acid composition of diverse inbreds and some of their hybrids, and also scanned a selection of these diverse genotypes (focusing on those with particularly high or low grain protein content) for any that are unusually low in phenylalanine as a proportion of total protein.

Materials & Methods

To assess the variability in total protein and phenylalanine content in a diverse set of maize germplasm with different kernel types, data from a selection of inbred lines derived from maize landraces reported previously by Flint-Garcia et al (2009b) were re-examined with specific emphasis on the variability of total phenylalanine and its correlation with kernel protein content.

Additionally, two experiments were planted in 2016 and 2017 in Clayton, NC, and Columbia, MO. The first made use of 46 different hybrids and open-pollinated populations (Table 3.1). These included a selection of commercial hybrids, F₁ hybrids of several important public and private inbred lines (including commercial lines with expired PVP certificates), USA and European open-pollinated landrace populations, older open-pollinated varieties, hybrids homozygous for floury or opaque storage protein mutations, and popcorn and sweet corn types.

At each location in each year, varieties were planted in two replications using a randomized complete block design. Experimental units were single row plots 3.7 m in length. In North Carolina, nitrogen was applied at a rate of 53.2 kg ha⁻¹ before planting, then 190.5 kg ha⁻¹ applied as liquid side dress during the growing season, split between two applications. In Missouri, nitrogen was applied at a rate of 134.5 kg ha⁻¹ pre-planting, then 11.2 kg ha⁻¹ applied as side dressing in a single application roughly 4 weeks after planting. Nitrogen regimes at both locations were based on pre-season soil tests and were calibrated to result in similar amounts of available soil N at the two locations. Both locations were irrigated as needed in both years. Up to six plants per plot were self-pollinated, and at maturity, all selfed ears were harvested from each plot and bulk shelled to obtain grain for kernel composition analysis.

The second experiment used 60 different inbred lines, including important public lines, commercial lines with expired PVP certificates, and isogenic inbred pairs differing for a number of kernel composition mutations (Table 3.2). This experiment was also planted in the same locations in Missouri and North Carolina, using the same experimental design and management as the hybrid experiment, except that individual plants in each plot were either self- or sibling-pollinated as needed to ensure adequate grain yield. At maturity, all selfed or sibbed ears were harvested from each plot and bulk shelled to obtain grain for kernel composition analysis.

In both experiments, bulked grain from each individual plot was analyzed with near-infrared spectroscopy (NIRS) equipment, and grain protein, oil and starch contents were estimated using an algorithm developed by Karn et al (2017). A subset of samples from both experiments (Table 3.3) were selected for further analysis via acid hydrolysis by the University of Missouri Agricultural Experiment Station Chemical Laboratories (ESCL), according to AOAC Official Method 982.30 E(a) chp. 35.3.05 (2006). Acid hydrolysis tested each sample for total protein content and individual amino acid composition for 14 individual amino acids and combined glutamine-glutamate and asparagine-aspartate, excluding only cysteine and tryptophan.

Data were analyzed using Proc MIXED in version 9.4 of the SAS System for Windows (SAS Institute 2018). The following statistical model was used for both experiments:

$$y = \mu + G_i + L_j + V_k + B(VL)_{mjk} + GL_{ij} + GV_{ik} + LV_{jk} + GLV_{ijk} + \varepsilon_{ijkm}$$

where y represents the estimated kernel protein content of an experimental unit, μ represents an intercept; G represents the effect of genotype (either inbred or hybrid/population, depending on experiment); L represents the random effect of location; V represents the random effect of year; $B(VL)$ represents the random effect of block within year and location; GL represents the interaction of genotype with location; GV represents the interaction of genotype with year; GLV represents the interaction of all three elements together; and ε represents residual error.

Heritabilities on a plot basis for grain protein content were estimated for hybrids and inbreds separately, using the following equation:

$$\hat{H} = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \hat{\sigma}_{GL}^2 + \hat{\sigma}_{GV}^2 + \hat{\sigma}_{GLV}^2 + \hat{\sigma}_\varepsilon^2}$$

Line mean-basis heritabilities were also estimated for hybrids and inbreds separately, using the following equation, where n_L , n_V , and n_B represent the number of locations, years, and blocks, respectively:

$$\hat{H} = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \frac{\hat{\sigma}_{GL}^2}{n_L} + \frac{\hat{\sigma}_{GV}^2}{n_V} + \frac{\hat{\sigma}_{GLV}^2}{n_L n_B} + \frac{\hat{\sigma}_\varepsilon^2}{n_L n_V n_B}}$$

Results

Variability and heritability of maize kernel protein content

Our results demonstrate a substantial range in maize total kernel protein content and significant variation for amino acid composition within both hybrids and inbreds. Among hybrids and landraces, average estimated protein content from NIRS ranged from a low of 8.5% dry weight for Illinois Low Protein Synthetic Cycle 62 (ILP), to a high of 16.5% for Taos Blue (Table 3.4). Of note, both these are open-pollinated populations; the most extreme F₁ hybrids were Tx807 × Mo17 o2 (10.9%) and Oh43 fl1 × W64A fl1 (15.1%); furthermore, both of these hybrids are homozygous for well-characterized kernel composition mutants (*opaque2* and *floury1*). The kernel protein content range for non-mutant F₁s between common public and/or private sector inbreds was a somewhat narrower 11.3% (B73 × LH252) to 13.5% (B73 × PHR58).

Among inbred lines, average estimated protein content ranged from a low of 11.0% dry weight for PHK46 to a high of 16.0% of dry weight for W64A fl1 (Table 3.5); this last was also a parent of the highest-protein F₁ hybrid in the other study. Overall, the range of kernel protein content across all hybrids/landraces was wider than the range among inbreds, although non-mutant F₁ hybrids showed a smaller range.

A subset of samples from both experiments was sent to the Missouri ESCL for amino acid quantification via acid hydrolysis to test amino acid composition, compare total amino acid content to the NIRS reported protein, and to look for any unusual low-phenylalanine genotypes. The subset was selected from the lowest-protein and highest-protein hybrids and inbreds that had sufficient seed samples. The correlation between total grain protein content estimated by NIRS and by acid hydrolysis was $r=0.73$ for the whole subset (Figure 3.1a). The relationship between protein content estimated by acid hydrolysis vs. by NIRS was curvilinear, with NIRS tending to overestimate kernel protein in both the lowest and highest protein content materials, although estimates tended to be quite good in the midrange (Figure 3.1).

Estimating the correlation between protein contents estimated by NIRS and acid hydrolysis separately for outbred and inbred material revealed a much closer relationship for the hybrids and open-pollinated populations ($r=0.85$, $N = 67$) than found in the inbreds ($r=0.51$, $N = 75$).

However, these subgroups are quite small; larger conclusions regarding the suitability of NIRS for general protein estimation in maize cannot be inferred from these results.

Much of the observed variation in maize protein content was heritable: the entry mean-basis heritability of kernel protein content, as estimated by NIRS, was 0.80 for hybrids and landraces, and 0.89 for inbreds (Table 3.6).

Phenylalanine and other amino acid contents in maize kernels

Our analysis of the data presented by Flint-Garcia et al (2009b) suggests that phenylalanine content in maize grain is strongly correlated ($r=0.98$) with total protein content of the grain (Table 3.7). The correlation between phenylalanine and total protein contents is unusually high; apart from tyrosine no other amino acid levels are so highly correlated to total kernel protein. The correlation between phenylalanine content and total protein content in our samples (as analyzed by acid hydrolysis) was $r=0.97$, consistent with our analysis of the data of Flint-Garcia et al. (2009). Phe content had a lower correlation with total protein content estimated with NIRS, $r=0.55$ for inbreds and $r=0.88$ for hybrids and populations (Figure 3.2).

In our results total kernel protein estimates were not useful predictors for either lysine or methionine contents (Figure 3.3), but they were effective ($r=0.75$) at predicting total content for the branched chain amino acids (BCAAs: leucine, isoleucine, and valine) (Figure 3.4).

Kernel phenylalanine content ranged from a low of 2.2 g kg⁻¹ (or 0.22% dry weight) to a high of 8.5 g kg⁻¹. The nine lowest phenylalanine content varieties (mostly hybrids) were also the nine lowest-protein content varieties overall (although their rankings changed). Phenylalanine content changed in a highly correlated manner with total analyzed protein content (Figure 3.2d).

Kernel lysine content ranged from 2.4 g kg⁻¹ to 5.7 g kg⁻¹ (Table 3.8). Both hybrids and inbreds carrying the *opaque 2* mutant were consistently among the varieties with highest kernel lysine content, along with the Illinois High Oil Synthetic population. Kernel BCAA content ranged from 10.3 g kg⁻¹ to 36.3 g kg⁻¹, all higher than the permissible range for a BCAA-restricted diet, primarily due to the very high leucine content.

Our sample also included the public inbred Oh43, and three near-isogenic variants carrying the *opaque2*, *floury1*, or *floury2* mutations. The *opaque2* mutant had significantly improved lysine content, as expected (Figure 3.5). Both the *opaque2* and *floury2* mutants showed reduced levels of both phenylalanine and BCAAs (Figure 3.5), but these reductions were not of practical significance. None of the mutants altered total protein content appreciably.

Heterosis for protein content

Several of the F₁ crosses in the hybrid experiment represent the progeny of two parents in the inbred experiment, which allows estimation of mid-parent heterosis as the difference between hybrids and the means of their inbred parents. In 23 of 26 such comparisons, the NIRS estimates of hybrid protein contents were significantly lower than the average of the two parental inbreds (Figure 3.6); in 17 of these comparisons the hybrid was lower than the lower-protein inbred parent. Eight such parental line-F₁ trios were also submitted for amino acid analysis, and these results demonstrated that hybrid kernels are typically lower in phenylalanine and other tested amino acid contents, in roughly equivalent amounts, compared to the mean of the inbred parents' kernels (Figure 3.7). For example, in 7 of 8 trios the hybrid kernels were lower in phenylalanine than the lower-protein parent. These results indicate that heterosis is generally negative for protein content and Phe content in maize.

Discussion

The results of this experiment suggest that although no mutation or large effect variant that suppresses relative phenylalanine composition in maize kernels was identified, kernel protein content is highly heritable and highly correlated with grain Phe content, suggesting its utility as an economically efficient indirect selection criterion for Phe content. The ease and low cost of determining protein content with NIRS makes the latter preferable to amino acid hydrolysis for large scale germplasm or breeding population evaluations. Whether there might exist a mutation that strongly and specifically affects Phe content remains an open question that will not be easily answered. Given this, the most appropriate way to breed for low-phenylalanine content maize grain is by selecting for low protein content maize.

Given this it seems likely to be worthwhile to calibrate a new NIRS algorithm for low protein maize. Our current algorithm was developed for a broad population of maize and overestimates total protein in low-protein samples (Figure 3.1); with a sample set taken primarily from lower-protein materials this bias could be substantial. While even with the bias our algorithm was able to accurately rank low-protein samples, it would be valuable for future research in this area to train a new algorithm for more accurate estimation. Our results indicate that the algorithm also struggles as protein levels approach the high end, which suggests that multiple algorithms may be necessary when working with extreme populations.

Hybrid plants typically produce larger kernels and larger ears than their inbred parents. Our data support the observation going back to at least Hayes & Garber (1919) that this extra seed size is primarily due to increased starch, rather than additional protein. In general, the open-pollinated populations tested here had higher protein content than the F1 hybrids, which may also reflect the negative correlation between yield and protein content, since open-pollinated populations have lower yield potential than F1 hybrids. Hybrid cultivars specifically developed for a very niche market such as metabolic disorder diets may be difficult to produce due to the limited commercial incentive, however, so an open-pollinated population with low protein content may be more easily disseminated and reproduced.

It further remains to be seen if the trend noted among F₁s to have lower overall protein and lower Phe than the midpoint of the inbred parents, and often lower than either parent, will continue to be the case when both parents are at the low end of the kernel protein range. B73 was a parent in 14 of our F₁s and

has near-average kernel protein in this population; all but three of the hybrids produced from B73 had less kernel protein than the line itself. Would the same be true of a substantially lower-protein tester such as PHK46?

Finally there is the question of where the lowest bound for maize kernel protein is, and whether that is low enough to meet the requirements of a restricted-protein diet. In the Illinois Long-Term Selection Experiment from which our ILP line was drawn, kernel protein stabilized after about cycle 60 (our seed source was cycle 62) and the ILP line was discontinued after cycle 93 due to lack of continued response to selection and poor germination, although the high protein line (as well as both high and low oil lines) have continued to respond to selection through over 100 cycles (Lucas et al 2013). This implies that at least for Burr White (for which we were unable to find any original seed stock), the lower biological limit for kernel protein is around four to five percent. We cannot conclude that this is the floor for all maize, however, and selection in unrelated lines may determine a lower biological limit for kernel protein. A seed requires some nitrogen to germinate, and foods considered “free” on the PKU diet have 1% or less protein and are not seed-based. A truly “free” maize is not likely to be possible, but even 4-6% substantially alters the protein exchange calculation and allows more freedom for dieters.

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Table 3.1. Hybrid and landrace germplasm planted in 2016-2017.

215E fl1 Oh43×215E fl1 W64A	Oh43 o2×W64A o2	
406C fl2 Oh43×406C fl2 W64A	Oh43×W64A	
AS-DK(S)C3 (Cuzco × MN lines)	P1498	
B73×207	PHB47×LH51	
B73×6M502	PHHB4×LH51	
B73×CML247	PHT60×Cacahuacintle Food Corn C4	
B73×F118	PHT60×Hickory King	
B73×Ki3	PHT60×Maiz Ancho Blanco C3	
B73×L163	R78×R84	
B73×LH211	R84×R78	
B73×LH252	T177×T179	
B73×Mo17	Tx807×Mo17 o2	‡
B73×NC350	Tx807×W64A o2	‡
B73×PHK46	Illinois High Oil Synthetic Cycle 62	
B73×PHR58	Illinois Low Protein Synthetic Cycle 62	†
B73×Shoe Peg	Mohawk Round Nose	
B73×Tx303	Oaxacan green dent	†
Cacahuacintle Food Corn C4×Hickory King	Pueblo blue	
Hickory King×Maiz Ancho Blanco C3	Righetta Bianca ottofile	
Hp301×SA24	Rostrato o dente di cane piemontese	
IL14H×P39	Taos blue	
N58S-3111	Zapalote Chico 2451F	

Notes: † - Not planted in 2016 ‡ - Fixed for the o2 mutation because Tx807 is an o2 line

Table 3.2. Inbred germplasm planted in 2016-2017.

207	Hickory King	Oh43 o2	
215E <i>f1</i> Oh43	Hp301	Oh7B	
215E <i>f1</i> W64A	IL14H	P39	
406C <i>f2</i> Oh43	Ki11	PHB47	
6M502	Ki3	PHHB4	
A632	Ky21	PHK46	
B104	L163	PHR58	
B73	LH211	PHT60	
B97	LH252	PHT60 <i>f2</i>	‡
CML103	M162W	R78	
CML228	M37W	R84	
CML247	Mo17	SA24	
CML277	Mo17 o2	† Shoe Peg	
CML322	Mo18W	Tx303	
CML333	Ms71	Tx807	§
CML52	NC262	W64A	
CML69	NC350	W64A o2	
F118	NC358		
GT112	Oh43		
Cacahuacintle Food Corn C4			

† - 2016 only ‡ - 2017 only § - line fixed for o2 mutation

Table 3.3 Germplasm samples subjected to acid hydrolysis.			
Germplasm	No. of Samples	Germplasm	No. of Samples
215E fl1 Oh43 × 215E fl1 W64A	5	406C fl2 Oh43	4
B73 × 6M502	5	6M502	4
B73 × LH252	5	B104	4
B73 × NC350	4	B73	4
B73 × PHK46	5	Cacahuacintle Food Corn C4	4
Cacahuacintle × Hickory King	4	CML228	2
Hp301 × SA24	4	CML277	4
IL14H × P39	5	CML322	1
Illinois High Oil Synthetic C62	5	GT112	4
Illinois Low Protein Synthetic C62	4	Hickory King	3
Mohawk Round Nose	1	IL14H	5
P1498	4	LH211	1
PHHB4 × LH51	1	LH252	5
PHT60 × Cacahuacintle Food Corn C4	4	Mo18W	1
PHT60 × Hickory King	4	NC262	4
Pueblo blue	1	Oh43	4
Taos blue	1	Oh43 o2	3
Tx807 × Mo17 o2	5	P39	1
215E fl1 Oh43	4	PHK46	5
215E fl1 W64A	4	PHT60	4
207	1		

Table 3.4. Mean kernel protein contents for 43 maize hybrids and populations measured with near-infrared spectrophotometry.

Hybrid or Population	Kernel protein, % by weight	Hybrid or Population	Kernel protein, % by weight
† Illinois Low Protein Synthetic Cycle 62	8.2	Oh43 o2 × W64A o2	13.0
P1498	10.8	B73 × 207	13.1
Tx807 × Mo17 o2	10.9	Righetta bianca ottofile	13.2
IL14H × P39	11.2	B73 × L163	13.2
B73 × LH252	11.3	B73 × CML247	13.2
B73 × 6M502	11.4	R84 × R78	13.2
B73 × PHK46	11.6	R78 × R84	13.3
PHHB4 × LH51	11.9	Cacahuacintle FC × Hickory King	13.4
B73 × Shoe Peg	11.9	B73 × LH211	13.5
T177 × T179	12.0	Hp301 × SA24	13.5
B73 × NC350	12.0	B73 × PHR58	13.5
PHB47 × LH51	12.2	Zapalote Chico 2451F	13.9
Tx807 × W64A o2	12.5	Rostrato o dente di cane piemontese	14.2
N58S-3111	12.6	Hickory King × Maiz Ancho Blanco C3	14.2
PHT60 × Hickory King	12.6	† Oaxacan Green Dent	14.5
B73 × Mo17	12.7	AS-DK(S)C3 (Cuzco × MN lines)	14.5
B73 × F118	12.7	406C fl2 Oh43 × 406C fl2 W64A	14.5
PHT60 × Cacahuacintle Food Corn C4	12.8	215E fl1 Oh43 × 215E fl1 W64A	15.2
PHT60 × Maiz Ancho Blanco C3	12.8	Illinois High Oil Synthetic Cycle 62	16.0
B73 × Ki3	12.9	Mohawk Round Nose	16.3
Oh43 × W64A	12.9	Pueblo Blue	16.4
B73 × Tx303	13.0	Taos Blue	16.5

Notes: Standard error 0.8, except † = 0.9

Table 3.5. Mean kernel protein contents for 58 maize inbred lines measured with near-infrared spectrophotometry.

Inbred Line	Kernel protein % by weight	Inbred Line	Kernel protein % by weight
PHK46	11.0	W64A o2	13.4
IL14H	11.4	Ki3	13.5
B104	11.6	Hp301	13.6
LH252	11.7	F118	13.7
Oh43 o2	11.9	Oh43	13.8
† CML228	11.7	Tx303	13.8
B97	12.0	Ms71	14.1
6M502	12.3	R84	14.2
GT112	12.4	CML247	14.2
PHT60	12.6	2O7	14.2
Mo18W	12.6	CML333	14.2
† PHT60 fl2	12.8	Ki11	14.3
NC262	12.7	R78	14.3
† Mo17 o2	12.5	PHB47	14.3
W64A	12.7	406C fl2 Oh43	14.4
NC358	12.8	Hickory King	14.4
CML103	12.9	215E fl1 Oh43	14.4
Mo17	13.0	L163	14.4
CML277	13.0	A632	14.5
M162W	13.1	NC350	14.5
Tx807	13.1	M37W	14.6
† P39	13.1	Oh7B	14.7
CML69	13.2	PHR58	14.8
B73	13.2	CML52	14.8
PHHB4	13.3	CML322	14.9
Shoe Peg	13.3	Cacahuacintle Food Corn C4	15.4
SA24	13.3	LH211	15.9
Ky21	13.3	215E fl1 W64A	16.0

Notes: Standard error 0.6, except † = 0.7

Table 3.6. Heritability estimates (and their standard errors) on a line mean-basis and on a plot-basis for grain protein content		
	Line mean-basis Heritability	Plot-basis Heritability
Hybrids/Open-pollinated populations	0.80 (0.07)	0.26 (0.07)
Inbreds	0.89 (0.03)	0.38 (0.07)

Table 3.7. Correlation between total protein content and single amino acid content of maize grain in a sample of 44 inbred lines and landraces grown in 2005-06 (based on data from Flint-Garcia et al, 2009b)

Tyr 0.94	Phe 0.98	Lys 0.72	Val 0.12	Ile 0.12	Leu 0.11	Trp 0.13	Thr 0.11	Met 0.06	His 0.17
-------------	-------------	-------------	-------------	-------------	-------------	-------------	-------------	-------------	-------------

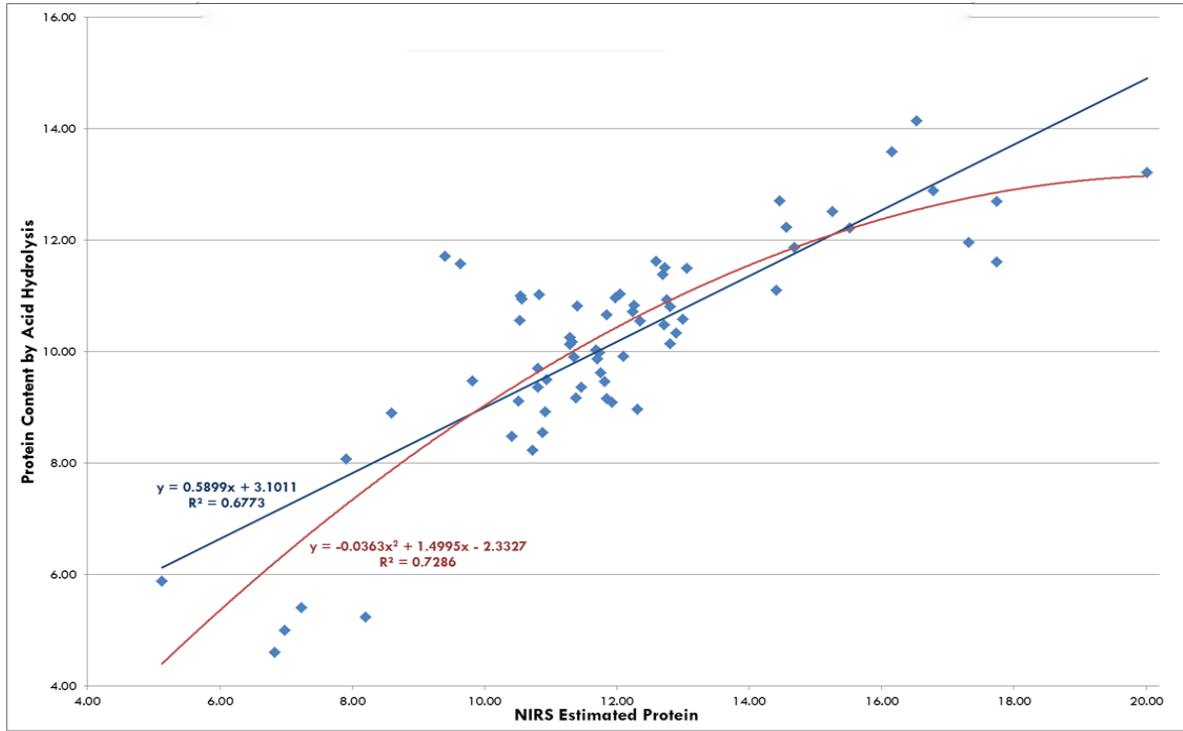


Figure 3.1a. NIRS Estimate vs Protein Content for All Samples

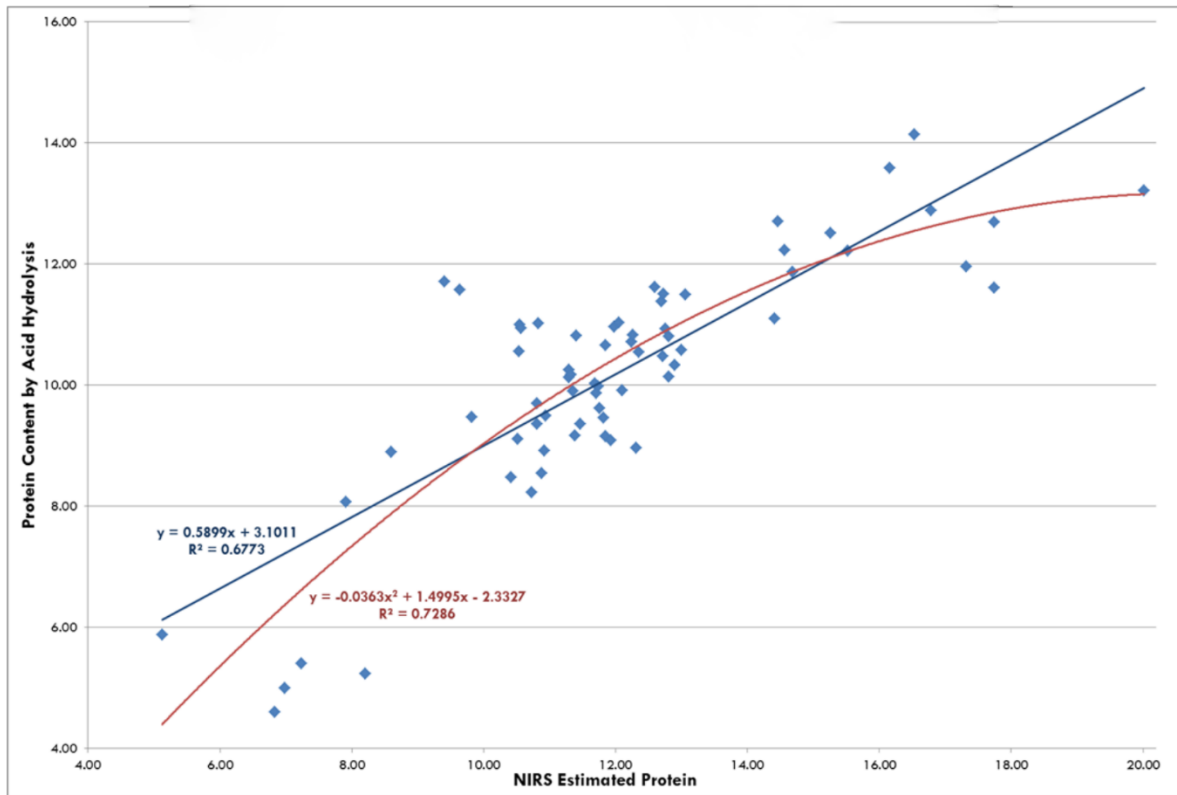


Figure 3.1b. NIRS Estimate vs Protein Content for Hybrids and Landraces

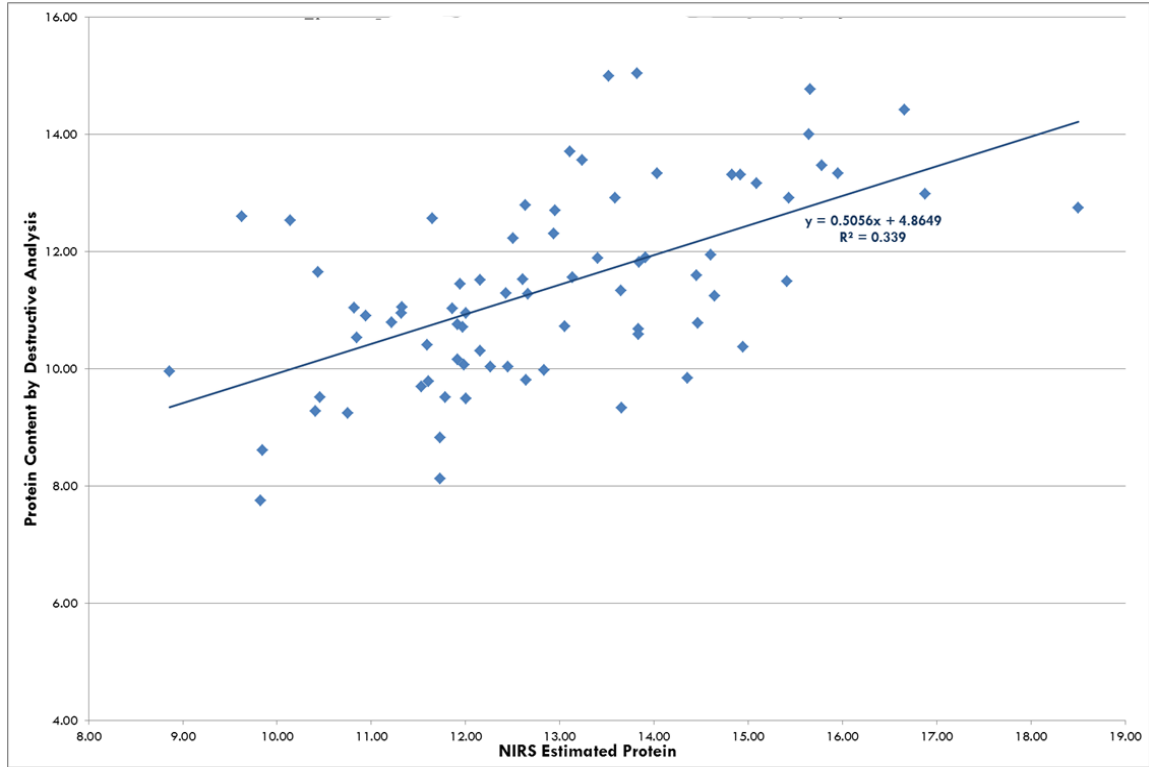


Figure 3.1c. NIRS Estimate vs Protein Content for Inbred Lines

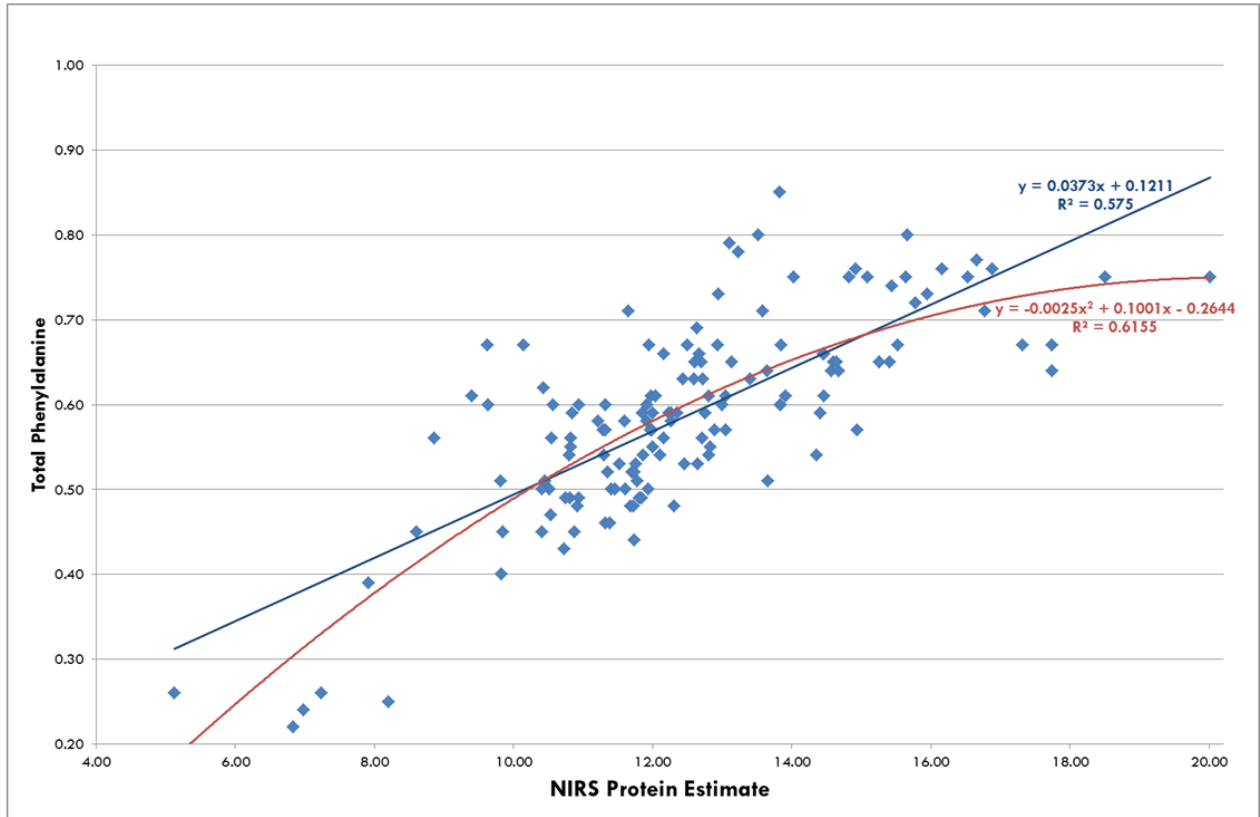


Figure 3.2a. NIRS Protein Estimate vs Phe for All Samples

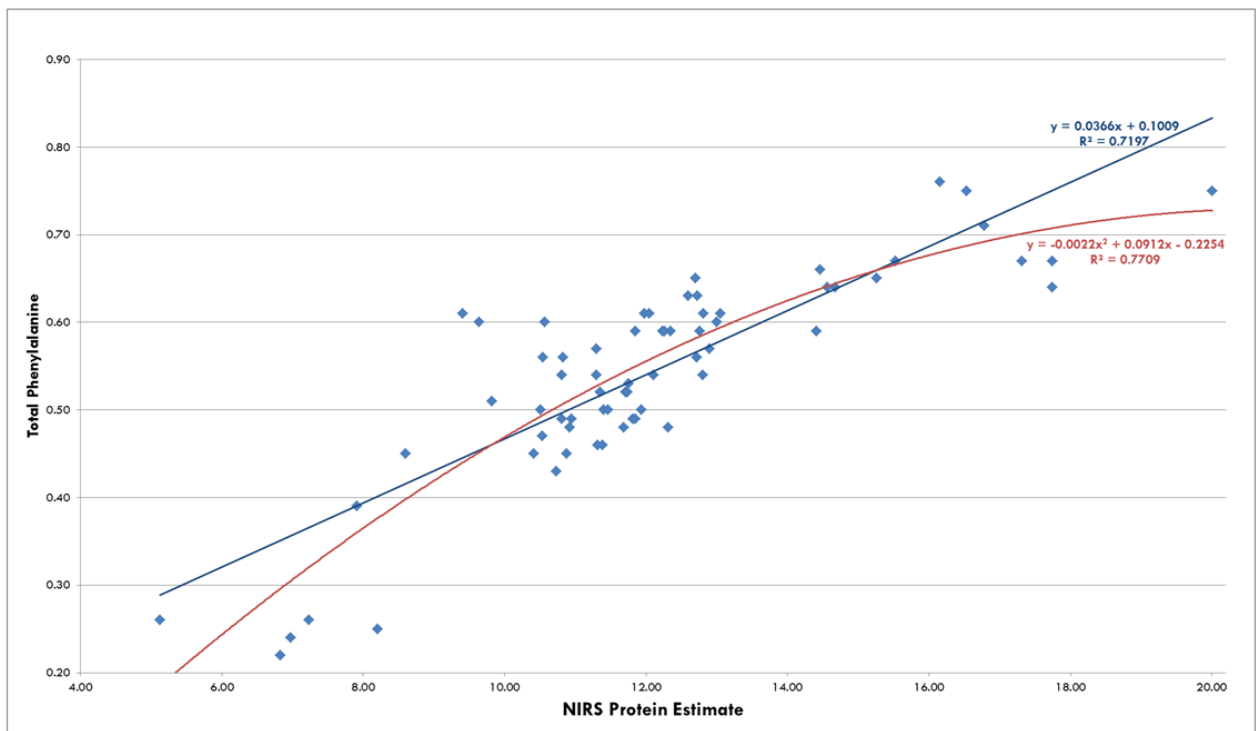


Figure 3.2b NIRS Protein Estimate vs Phe for Hybrids and Landraces

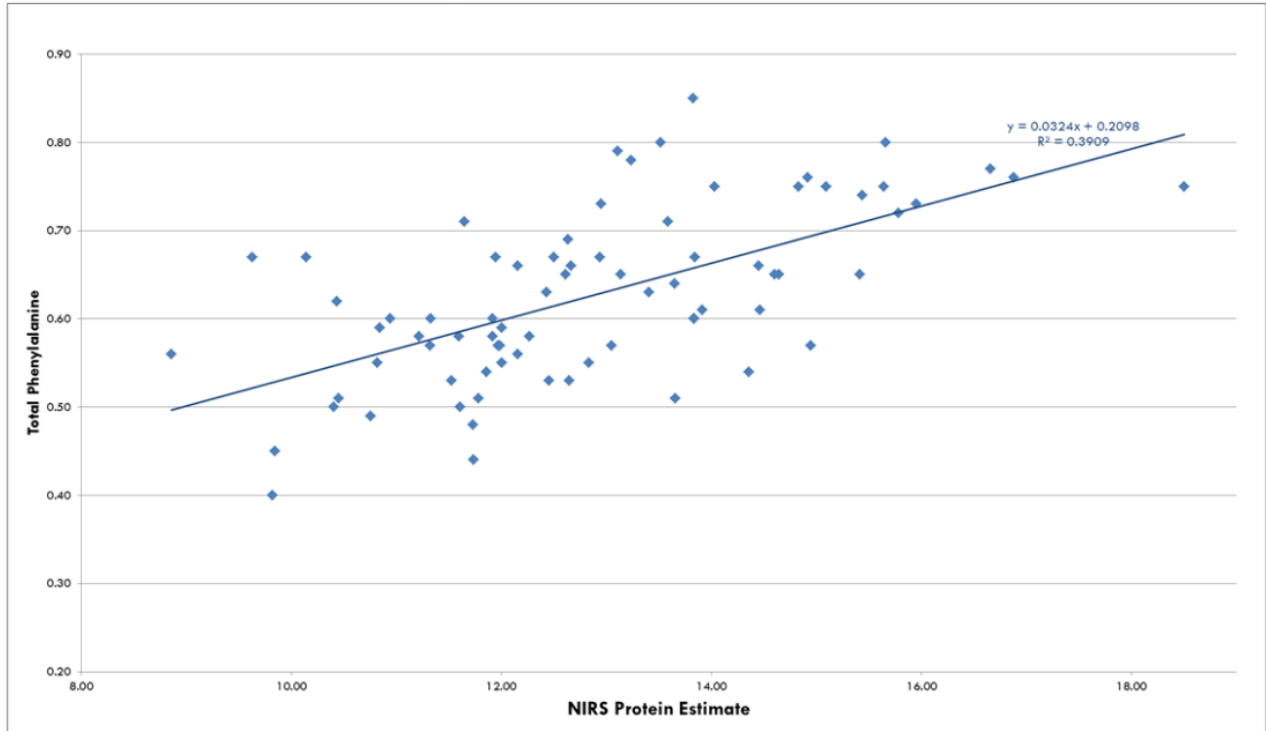


Figure 3.2c. NIRS Protein Estimate vs Phe for Inbred Lines

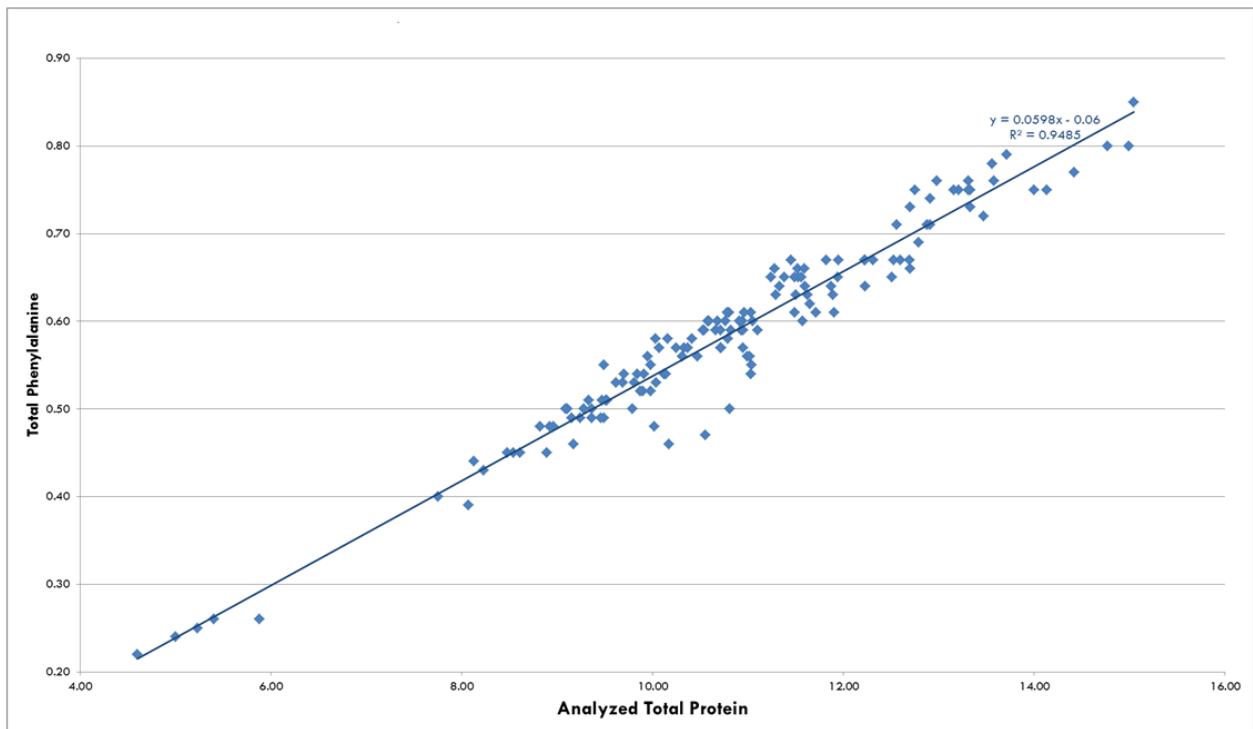


Figure 3.2d. Measured protein vs Phe for All Samples

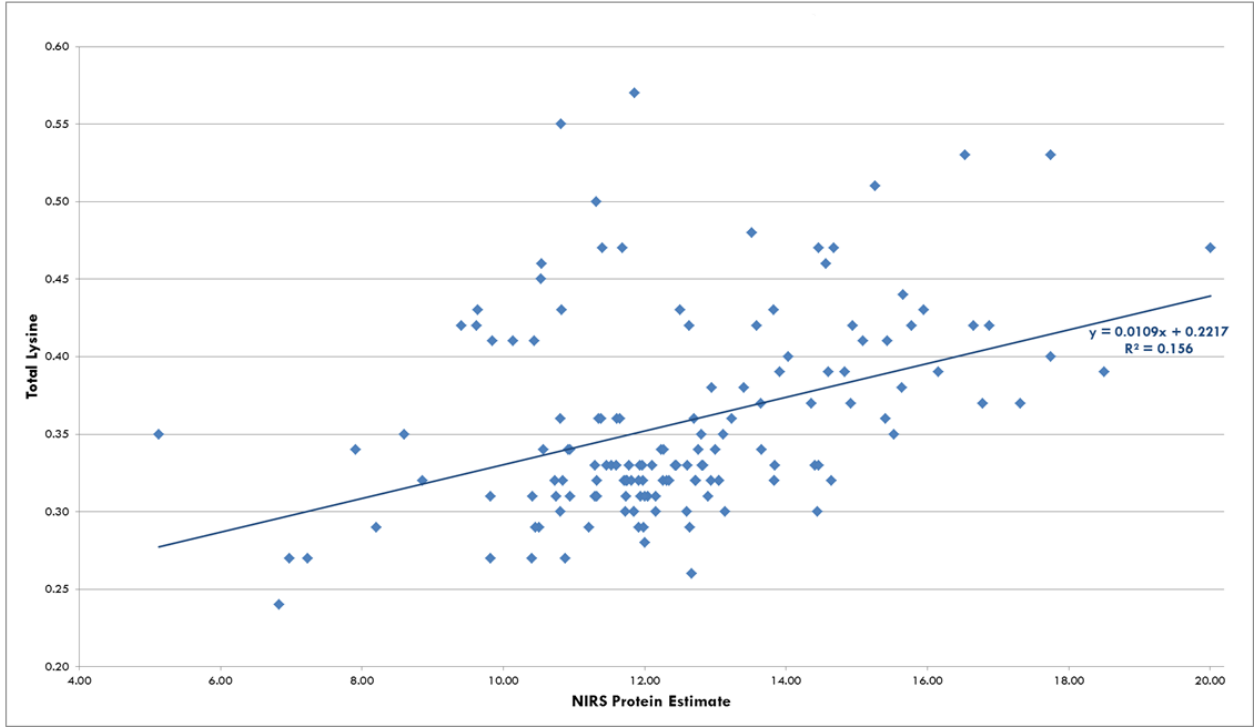


Figure 3.3a. NIRS Protein Estimate vs Lys for All Samples

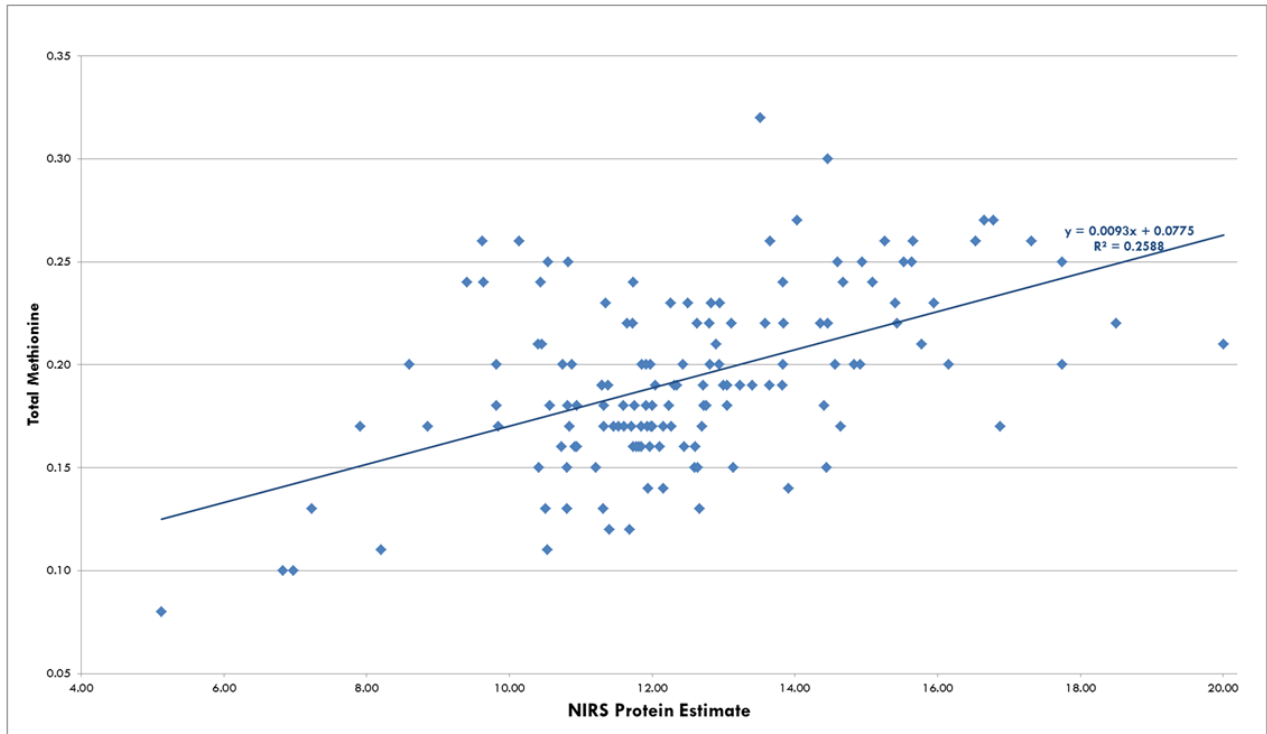


Figure 3.3b. NIRS Protein Estimate vs Met for All Samples

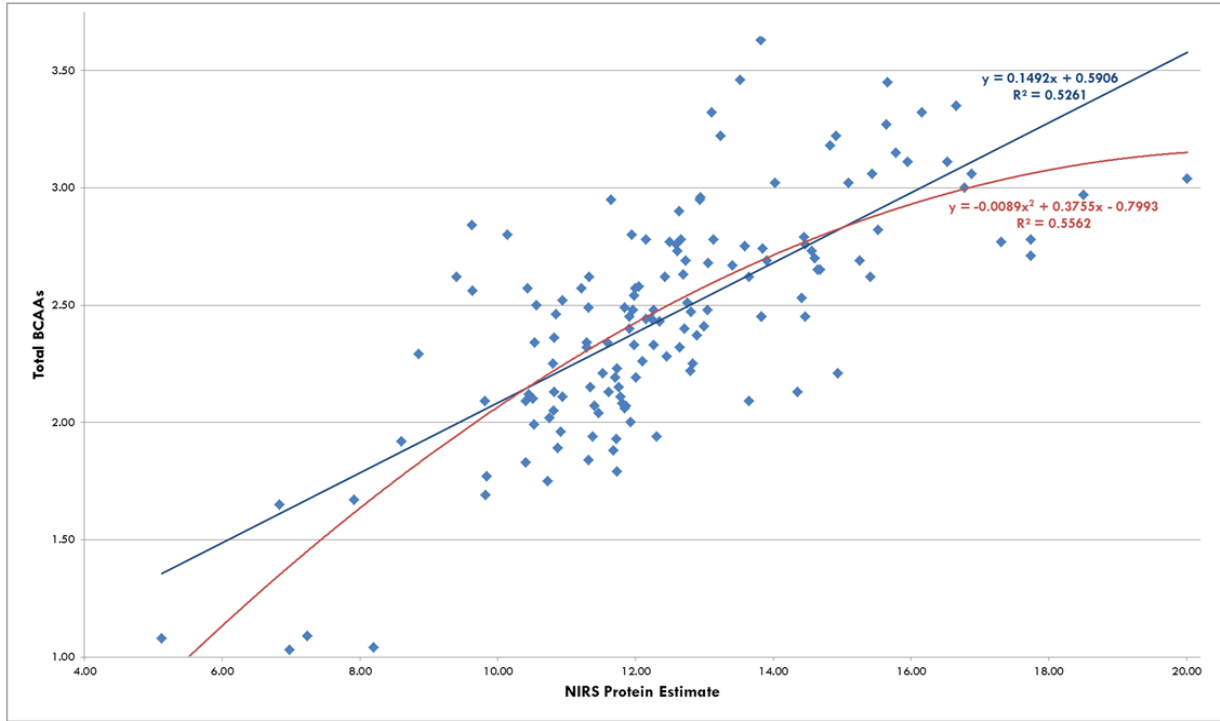
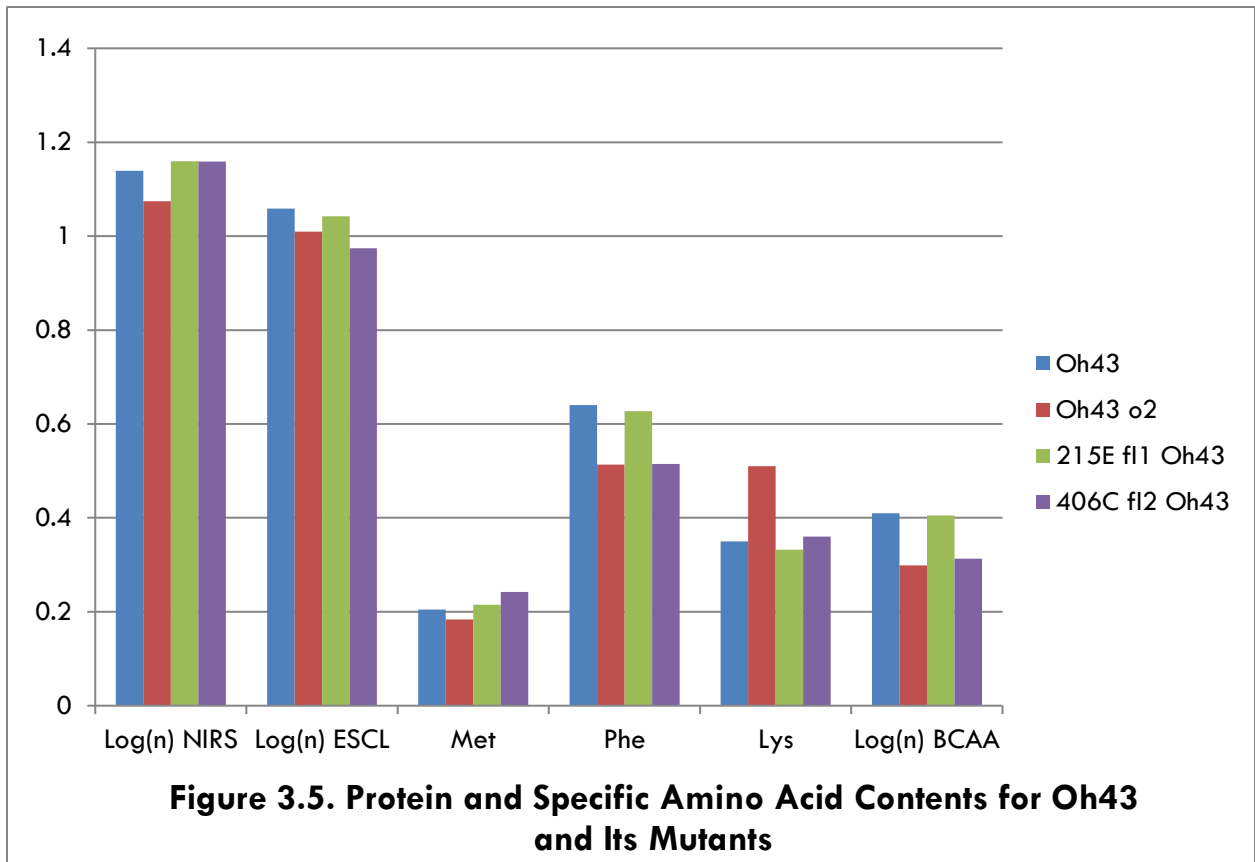


Figure 3.4. NIRS Protein Estimate vs BCAAs for All Samples



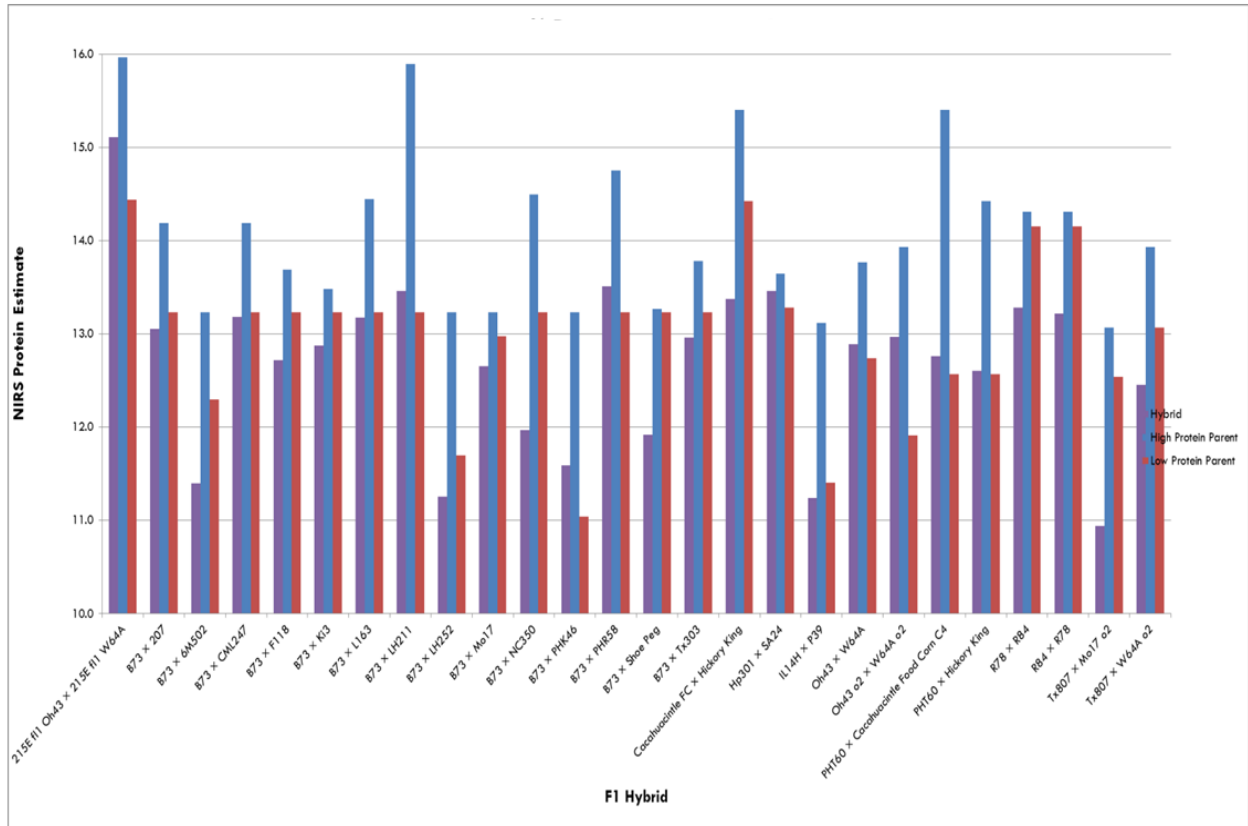


Figure 3.6. Estimated Protein (% Dry Weight) for F₁ Hybrids and Parents

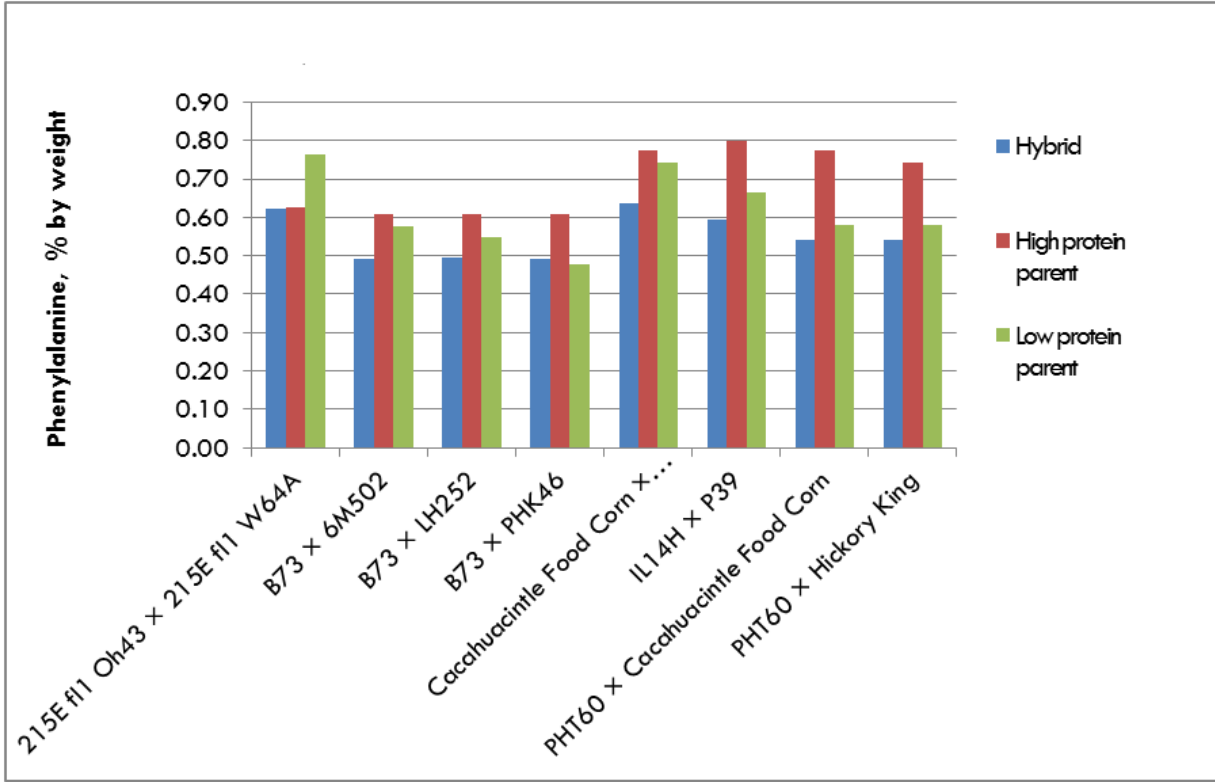


Figure 3.7a. Phe for F₁ Hybrids and Parents

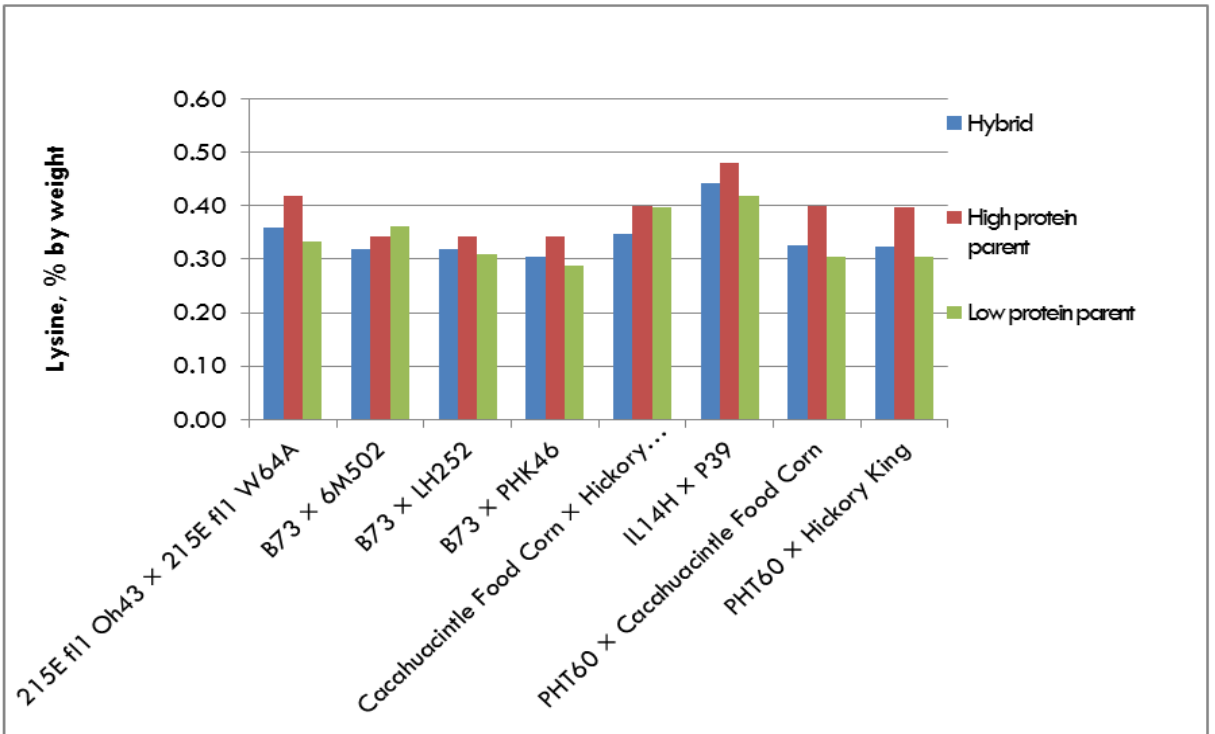


Figure 3.7b. Lys for F₁ Hybrids and Parents

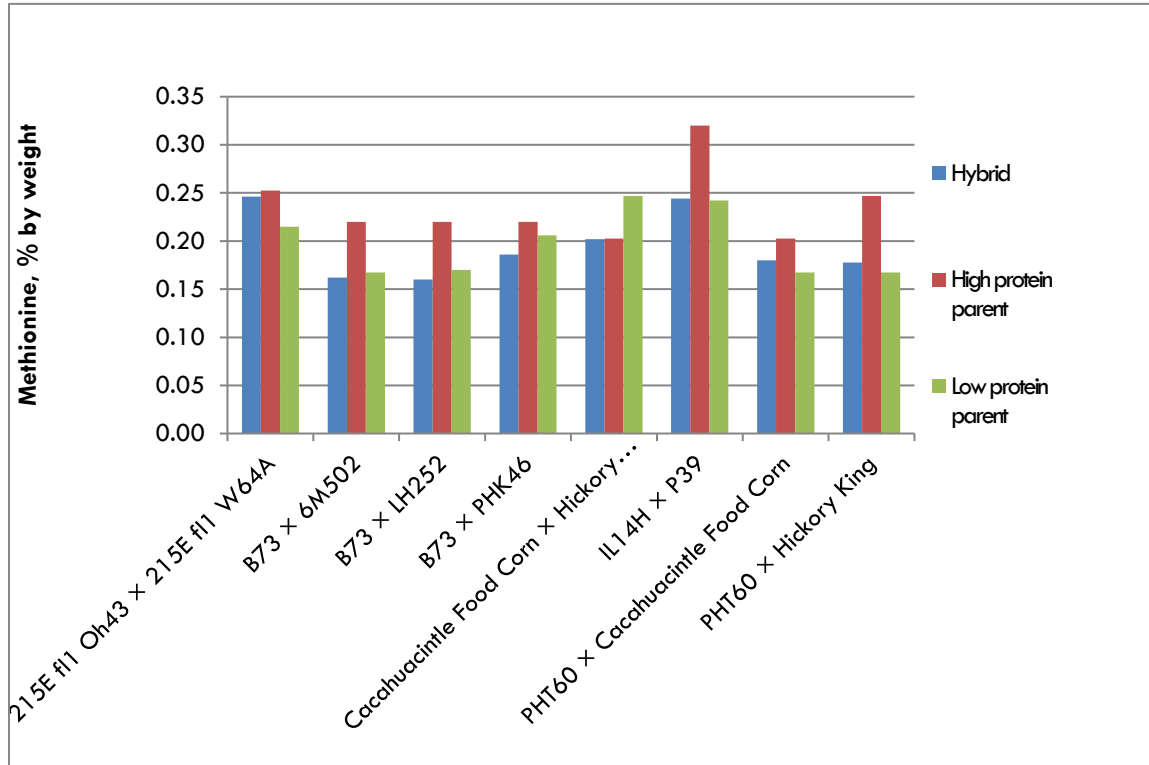


Figure 3.7c. Met for F₁ Hybrids and Parents

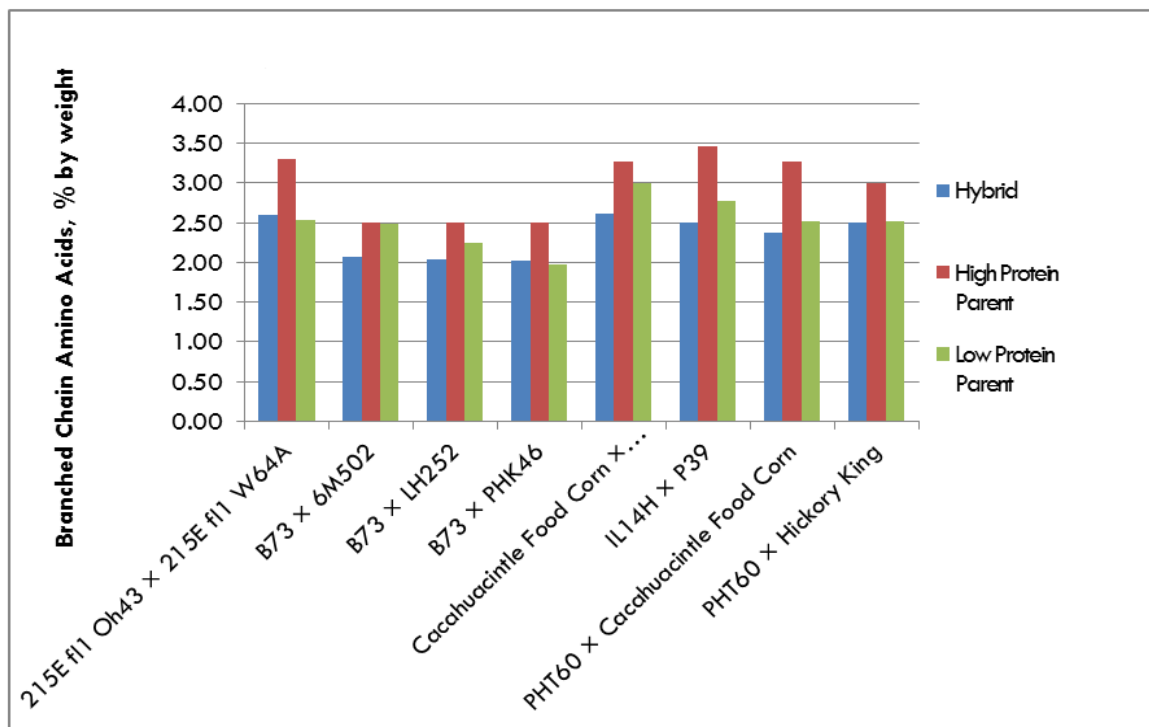


Figure 3.7d. BCAAs for F₁ Hybrids and Parents