TOTAL YELLOW PIGMENT CONTENT AND OXIDATIVE STRESS LEVEL DURING KERNEL DEVELOPMENT OF DURUM WHEAT

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment
for the Degree of
MASTER OF SCIENCE

Major Department:
Plant Sciences

April 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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MASTER OF SCIENCE

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ABSTRACT

The focus of this research was to determine the effects of cultivar and environment on levels of total yellow pigment content and oxidative stress during grain development and in semolina and pasta. Lipoxygenase activity increased just before and declined after physiological maturity, while change in malondialdehyde content in the kernel mirrored that of yellow pigment and declined with maturity. After grain reached physiological maturity, levels of lipoxygenase activity, malondialdehyde content and yellow pigment content per kernel were lowest in 2011, while polyphenol oxidase activity was lowest in 2010. Lipoxygenase activity and yellow pigment content decreased after milling and processing, whereas malondialdehyde content decreased after milling but increased with processing. No lipoxygenase activity was detected after processing. Stereomicroscopy and scanning electron microscopy were used to observe anatomical and morphological changes that occurred as durum wheat caryopsis progressed from anthesis to final desiccation.

ACKNOWLEDGEMENTS

I am so grateful my path crossed with Dr. Deckard during the winter of 2009, as he explained to me what graduate school was all about, and what my opportunities might be while encouraging me to apply. I am grateful for his support and friendship which have been critical throughout my studies. I am so thankful that my wise advisor, Dr Frank Manthey, took a chance on the potential that he saw in me, and offered me a project to work with under his supervision. I am grateful for his patience in teaching and mentoring me throughout the research project and thesis writing. Thank you, Dr. Manthey, for all your hard work, which is very much appreciated! My committee members, Dr. Clifford Hall III, Dr. Ed Deckard, and Dr. Elias Elias provided valuable council, comments, and recommendations during my thesis defense, and I am very grateful for their involvement. I am grateful to Dr. Elias Elias and his wonderful durum wheat team for planting my field experiments. My sincere appreciation goes to a very kind and knowledgeable person, Reena Dash, for assisting me with performing the lab tests. I am grateful to Dr. Horsley, Dr. Hammond, and Jyoti for helping with the statistical part of my thesis.

Very special thanks go to my parents, Nikolai and Nina, for always believing in me and for being there for me. As lucky as I am, my special thanks also goes to my second set of parents, Arlen and Ronna Gilbertson, for their love and care, for all their involvement in my school life, and great support throughout my studies. I am so grateful for the patience and understanding that came from both of my sets of parents when it was most needed. Special love and thanks go to Eric and Evelyn Butcher, who took on the task of being my American grandparents, and their faithful example of living uprightly and setting the right priorities in life have given me something for which to aspire. I am grateful for my all my friends and fellow students, for their encouragement, and for making the grad school life fun and memorable.

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FORMAT OF THESIS

This thesis has an overall ABSTRACT, GENERAL INTRODUCTION, and LITERATURE REVIEW. The Literature Cited in the GENERAL INTRODUCTION and LITERATURE REVIEW are given at the end of each section. The thesis is written as three separate papers. Each paper has an Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusions followed by Literature Cited. At the end of three papers, there is an OVERALL CONCLUSION and a brief discussion on FUTURE RESEARCH AND COMMERCIAL APPLICATIONS. The last part of the dissertation is the APPENIDIX. Redundancy may be expected in some places due to the format of the thesis.

GENERAL INTRODUCTION

Carotenoids are a group of pigments that are found in many biological systems (Trocolli et al 2000). Functions of carotenoid pigments differ depending on their chemistry and location in plants. Carotenoids are classified as carotenes or xanthophylls. Carotenes are unsaturated hydrocarbons while xanthophylls are oxygenated derivatives of carotenes. Xanthophylls are the predominant carotenoid pigment class in wheat kernel. Lutein and zeaxanthin are the main xanthophylls found in wheat kernel. Carotenoid pigments are located mainly in the outer layers (pericarp/embryo) of the wheat kernel, with the embryo, pericarp, and endosperm containing decreasing levels, respectively (Hentschel et al 2002). While carotenoid pigments might not be uniformly distributed in a wheat kernel, lutein might be. It has been reported that α-, β-carotene, and zeaxanthin are located mainly in the embryo while lutein, the most abundant pigment, is equally distributed across the kernel (Panfili et al 2004). β-Carotene, xanthophyll, particularly lutein, and flavones, found in the amyloplasts of endosperm cells, contribute to the yellow appearance of the cereal endosperm, a valued quality trait in durum wheat (*Triticum turgidum* L. var. durum) (Gelinas et al 1998; Abdel-Aal et al 2007). The degree of yellowness in durum wheat endosperm depends on the presence and concentration of carotenoid pigment, which final concentration is influenced by genotype and environment (Borrelli et al 2003).

Abiotic stress includes any environmental conditions or combination of them that negatively affect the expression of genetic potential for growth, development and reproduction (Jones et al 1984). Abiotic stress often triggers the formation of reactive oxygen species (ROS) inside plant cells including cells found in grain. Reactive oxygen species include hydroperoxides and peroxyradicals. At low concentrations, ROS serve as signals in cells that activate specific transcription factors needed for gene expression. At high concentrations, ROS can oxidize

macro-molecules such as lipids, DNA, and proteins (Dat et al 2000). Malondialdehyde (MDA) is a byproduct of polyunsaturated fatty acid peroxidation. Malondialdehyde content has been used as an indicator of oxidative stress in plants (Al-Ghamadi 2009).

To counteract the toxicity of ROS, a highly efficient antioxidant defense system, including both nonenzymatic and enzymatic constituents, is present in plant cells (Logginni et al 1999). Carotenoids contribute to the nonenzymatic antioxidant system found in seeds by scavenging peroxyradicals, thus limiting free radical-induced membrane deterioration and seed ageing (Mittler 2002).

Oxidative enzymes, such as polyphenol oxidase (PPO) and lipoxygenase (LOX), can lead to the initiation of deterioration reactions, such as undesirable color, flavor and nutritional changes (Goncalves et al 2010). Polyphenol oxidase is the enzyme associated with the conversion of phenolic compounds to quinones and their products' polymerization. Two kinds of reactions generated by PPO are the hydroxylation of monophenols to *o*-diphenol and oxidation of *o*-diphenol to *o*-quinone (Tomas-Barberan and Espin 2001). Polyphenol oxidase oxidizes diphenols in the presence of molecular oxygen that results in enzymatic browning (Schweiggert et al 2005). Lipoxygenase catalyzes the hydroperoxidation of polyunsaturated fatty acids, esters and glycerides containing a 1-*cis*, 4-*cis*-pentadiene structure, which can oxidize carotenoid pigment (MacDonald 1979; Schweiggert et al 2005). According to Borrelli et al (1999), a decrease of 16.3% in semolina β-carotene content was observed during pasta processing. This loss was attributed to LOX activity. Polyphenol oxidase and LOX have been shown to vary in activity among genotypes and seem to be affected by environment. However, none of these enzymes have been assessed for their variation in the durum germplasm currently used in breeding program at North Dakota State University.

Published research has mainly focused on amount and distribution of color components in grain (Borrelli et al 2008), but little is known about the accumulation of carotenoid or yellow pigments and their relationship to oxidative stress levels in the grain during kernel development. Understanding the nature and behavior of carotenoid pigment accumulation and LOX and PPO activities during grain filling will benefit the attempts to reduce the negative effects of oxidative stress on durum product quality.

The present study was conducted to determine:

1. the effect of cultivar and environment on yellow (carotenoid) pigment accumulation and enzymatic activity during grain development;
2. the relationship between oxidative stress levels and yellow pigment content during the grain development;
3. stability of yellow pigments and changes in enzymatic activity after milling and pasta processing; and
4. the relationship between enzymatic activity and carotenoid defense system after milling and processing.

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LITERATURE REVIEW

Background

Durum wheat, 2n=28, genome AABB, is in the family Poaceae, tribe Triticeae. It evolved in the Middle East via interspecific hybridization and chromosome doubling followed by domestication (Bozzini 1988). The parent species, *T. monococcum* and *Aegilops speltoides*, contributed the A and B genomes, respectively. It is more drought tolerant than hexaploid bread wheat (*T. aestivum*), and has the hardest kernel of all wheat types (Elias and Manthey 2005).

Durum wheat is milled primarily to produce semolina. Pasta is made from semolina and water that is formed into a dough, and extruded under vacuum through a die. Although durum is also made into bread in some parts of the world, this is a relatively small proportion of worldwide durum usage (Trocolli et al 2000).

Kernel Development

Pollination and Fertilization

Wheat development is divided into vegetative and reproductive stages. Growth stages up until jointing are vegetative. Growth stages from jointing to heading are both vegetative and reproductive. The reproductive stage starts with heading (Poehlman 1959). Wheat is predominantly a self-pollinating crop. The cross-fertilization rate may be as high as 1 to 2%, although it can be less than 1% (Sleeper and Poehlman 2006).

Wheat flower consists of three stamens and a pistil. Pollen is formed in the anther. Pollination is consummated by the transfer of pollen grains from the anther onto the stigma. Mature pollen grain germinates on stigma and a slender pollen tube grows through the style and enters the ovary. The pericarp layer develops from integuments that surround the ovary. Hence, the pericarp is maternal tissue. Two sperm nuclei are released from the pollen tube into the

embryo sac. One sperm nuclei (1N) fuses with the egg nuclei (1N) to form a zygote (2N). The zygote develops into the embryo. The second sperm fuses with the two polar nuclei (1N each) to form primary endosperm nucleus (3N). Hence, double fertilization occurs. The first fertilization results in the zygote (embryo) and the second fertilization results in the endosperm.

The primary endosperm nucleus divides numerous times to form the nuclear endosperm. The initial endosperm is termed a nuclear endosperm since there is nuclear division without cell wall formation (Olsen 2004). Next, the multinucleated endosperm undergoes cellularization; whereby cell walls are deposited around each nucleus. Cellularization is complete in about four days after pollination. All subsequent cell division involves both nuclear division and cell wall deposition.

Cell Differentiation and Kernel Development

The fully developed cereal endosperm consists of four main cell types: starchy endosperm cells, aleurone cells, transfer cells, and cells that surround the embryo. Transfer cells develop in the basal region of the endosperm that covers the main vascular tissue of the maternal plant. They facilitate solute transfer (mainly amino acids, sucrose, and monosaccharides) between the symplast and apoplast. Starchy endosperm cells accumulate starch and prolamin storage proteins as coded for by the nuclear DNA in each cell. Starchy endosperm cells originate from the inner cells that are formed by cellularization. They are oriented randomly. The other source of starchy endosperm cells are derived from daughter cells of the aleurone cells. In wheat, the aleurone is a single layer of cells that cover the perimeter of the endosperm except for the transfer cell region. The aleurone cells are active during seed germination. They contain enzymes required to degrade the storage starch and proteins into glucose and amino acids, respectively.

During grain filling, the grain undergoes six developmental stages: watery stage, milk stage, soft dough stage, hard dough stage, kernel hard stage, and harvest ripe. Physiological maturity occurs during the end of the hard dough stage when the kernel moisture declines below 35% (Sofield et al 1977). Physiological maturity concludes the assimilation of dry matter into the kernel stages of kernel development. At physiological maturity, wheat kernels dehydrate and reduce in size. The reduction in the kernel size is a property of the connective protein network of the endosperm (Anjum and Walker 1991). The protein network in the endosperm contracts eliminating open spaces and results in a vitreous dense endosperm.

Kernel Storage Material

Starch Deposition and Quality

In nearly all types of plants, starch occurs in two forms, the transitory starch in photosynthetically active leaves, and the storage starch in heterotrophic tissues such as endosperm (Ugalde and Jenner 1990). Transitory starch is produced by photosynthesis in green leaves. Starch in leaves is converted to hexose sugars that are used to form sucrose. Sucrose is translocated via phloem to the amyloplast of endosperm cells during kernel development. Sucrose is converted to ADP-glucose, which is the primary substrate involved in formation of two types of α-glucans, amylose and amylopectin (Hawker and Jenner 1993; Tester et al 2004). Durum wheat starch granules typically contain 73 to 74% amylopectin (Leloup et al 1991).

Both amylose and amylopectin consist of homoglucans with α-1,4 bond forming the main chain and α-1,6 bond at branch points (Smith 2001). Amylose is a relatively linear molecule consisting of long chain of α-1,4 linked glucose units with an occasional α-1,6 bond at branch points. Amylopectin has a high degree of structural organization, the linear chain of α-1,4-linked glucoses with high frequency (relative to amylose) of α-1,6 branches (Myers et al 2000). Large,

disk-shaped A-starch granules (20-45 μm) appear first and continue to increase in size throughout the grain-filling period, whereas spherical B-starch granules (<10 μm) are initiated approximately two week after anthesis and remain considerably smaller (Ao and Jane 2007). At maturity, the ratio of A- and B-starch granules is 1:10 (Jenner et al 1991).

The functional properties of starch, particularly, the ability of starch or flour to absorb water and form a paste, are affected by variations in the proportions of amylose and amylopectin, the size distribution of starch granules, as well as the composition and chemical structures of the A- and B-granules (Ao and Jane 2007; Liu et al 2011). Soh et al (2006) reported that an increased B-granule content increased farinograph water absorption, and that cooked spaghetti firmness was highest with B-type granules at 32–44% (volume percentage basis), which is approximately 10–15% higher than normally found in durum starch. Also increasing the amylose content in the starch caused the dough to be more extensible, increased spaghetti firmness, and decreased water absorption.

Protein Deposition and Quality

The quality of wheat flour for end-use depends on the visco-elastic properties of dough, which are influenced by quantity and quality of the gluten-forming storage proteins of wheat endosperm (Sissons et al 2007). Storage protein is located within membrane-bound spherical bodies. These bodies, typically 0.5-1.5 μm in diameter, are derived from the endoplasmic reticulum and Golgi apparatus and appear closely associated with rough endoplasmic reticulum (Kim et al 1988). Protein deposition begins at about 10 days after anthesis, and continues until physiological maturity (Jenner et al 1991). During the final stages of grain filling, many protein bodies fuse, forming a continuous, tight protein matrix in which the starch granules are embedded.

Durum wheat storage protein is composed of gliadin and glutenin proteins, the group of proteins which exert the most influence on the strength and elastic properties of dough. Gliadins are monomeric proteins with virtually all disulfide bonds existing within the same molecule; whereas glutenins are polymeric proteins with intermolecular disulfide bonds that create networks (Miflin et al 1983). Thus, protein structure defines their functional properties in gluten and dough.

Variation in gliadin to glutenin ratio affects gluten and dough characteristics. Monomeric gliadins are responsible for dough extensibility; whereas dough strength is determined by the capacity of glutenin subunits to form large polymers with different sizes (Liu et al 1996). Dough strength is enhanced by increasing the glutenin-to-gliadin ratio by addition of glutenin enriched fraction to base flour (Uthayakumaran et al 1999; Edwards et al 2003). The low correlation between the glutenin-to-gliadin ratio and measures of dough strength observed by Edwards et al (2007) may be related to a variation in glutenin subunits ratio. Glutenins are subdivided into low molecular (LMW-GS) and high molecular weight (HMW-GS) subunits (Shewry 1999).

There is limited information about how variation in HMW-GS-to-LMW-GS ratio influences pasta quality. A study conducted by Sissions et al (2007) showed that increased HMW-GS-to-LMW-GS ratio in semolina dough greatly increased dough strength, but did not increase cooked pasta firmness. This study was contradicted by Edwards et al (2007) who found a significant negative correlation between HMW-GS-to-LMW-GS ratio and semolina dough strength, measured by means of alveograph and mixograph.

Growing environment can negatively influence the glutenin-to-gliadin ratio by shortening the period of grain development (Alternbach et al 2003). The initial formation and accumulation of glutenin polymers starts later than the synthesis of gliadins monomers during grain

development (Ng et al 1991; Abonyi et al 2007). Gao et al (2010) observed a difference in the accumulation dynamics of glutenin subunits and glutenin polymer in wheat cultivars with different qualities during grain development. Formation and accumulation of HMW-GS and LMW-GS were expressed earlier in the grain development stage in strong-gluten cultivars than in weak-gluten cultivars.

Yellow Pigment

Bright yellow color of semolina end-products is a desirable characteristic in pasta industry as dictated by consumer demand (Singh et al 2009). Yellow pigment in durum wheat endosperm is caused primarily by carotenoids, whose content can be affected by genotype and growing environment. Taghouti et al (2010) reported that the component of variation due to genotype was larger than that due to the environment, indicating the greater influence of genotypes on the yellow pigment index. These results are similar to other studies in which yellow pigment content were shown to be mainly influenced by genotypic effects (Rharrabti et al 2003; Pozniak et al 2007).

The genetics of yellow pigment concentration in durum wheat has been studied extensively, and is controlled largely by genetic factors with additive effects (Elouafi et al 2001; Singh et al 2009). Major quantitative trait loci (QTL) for yellow pigment have been found on the group 2 chromosomes 6B and 7B (Pozniak et al 2007) and 3B (Patil et al 2008). Two linked QTL for yellow pigment were reported on 7A (Singh et al 2009).

A high level of carotenoid pigments in semolina does not, however, solely guarantee a high color of pasta itself. The degree of yellowness of pasta color is additionally affected by semolina extraction rate (Dexter and Matsuo 1978; Borrelli et al 1999), oxidative degradation by lipoxygenase enzyme during processing, and processing conditions (Borrelli et al 2003).

Carotenoid Synthesis

Carotenoids are a diverse group of yellow-orange pigments found in many biological systems (Panfili et al 2004). They can be divided into two general classes: carotenes and xanthophylls. Carotenes, such as α-carotene, β-carotene, and lycopene, are hydrocarbons; whereas xanthophylls are oxygenated derivatives of carotenes, and include the compounds β-cryptoxanthin, lutein, and zeaxanthin.

Carotenoids usually are synthesized *de novo* in differentiated plastids of roots, flowers, fruits, and seeds, accumulating mostly in chloroplasts (green photosynthetic plastids) and chromoplasts (colored plastids), but also in amyloplasts (starch-storing plastids), leucoplasts (colourless plastids), etioplasts (dark-grown precursors of the chloroplast), and elaioplasts (lipid-storing plastids) (Cazzonelli and Pogson 2010). Carotenoids in plant cells are synthesized in plastids via nonmevalonate (non-MVA) methylerythol (MEP) pathway (Hsieh and Goodman 2005).

The MEP pathway utilizes pyruvate (generated from the Calvin cycle or glycolysis) and glyceraldehyde-3-phosphate (GA3P) to produce isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP) separately via a branching of MEP pathway (Figure 1; Cunningham 2002). Gradual condensation of four IPP units yields geranylgeranyl pyrophosphate (GGPP). Two molecules of GGDP (C_{20}PP) are condensed and are further catalyzed by phytoene synthase (PSY) to produce the first colorless carotenoid 15-*cis*-phytoene. Phytoene synthase being a rate-limiting enzyme of carotenoid biosynthesis is considered to be a key regulator of the carotenoid biosynthetic pathway (Giuliano et al 2008). The production of all-*trans*-lycopene from 15-*cis*-phytoene involves four enzymes: phytoene desaturase synthase

(PDS), ζ-carotene isomerase (ZISO), ζ-carotene desaturase synthase (ZDS), and carotenoid isomerase (CrtISO) as well as a light-mediated photoisomerization (Chen et al 2010).

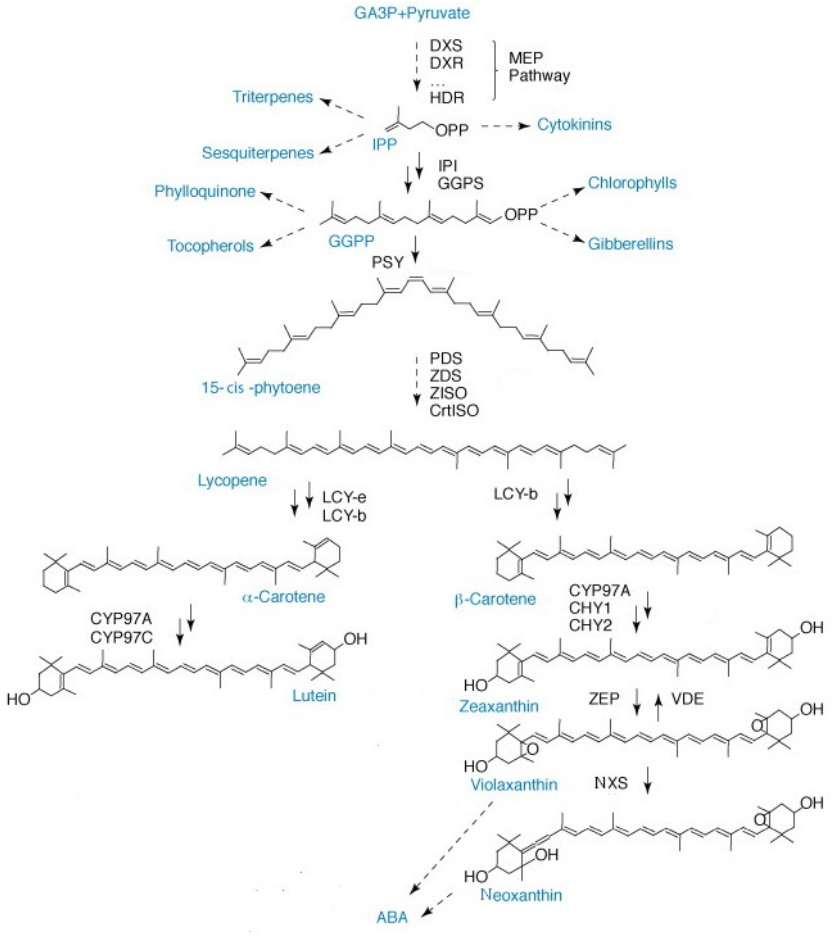

Figure 1. The carotenoid biosynthetic pathway. Names of compounds are in blue, and names of enzymes are in black.

Source: Giuliano (2008) with some modifications

Lycopene is the substrate of two competing cyclases: ε-cyclase (LCY- ε) and β-cyclase (LCY-β). These two cyclases act together on the two ends of the molecule, which leads to the formation of α-carotene; whereas the action of LCY-β alone forms β-carotene (Howitt et al

2009). The carotenes serve as substrates for the production of xanthophylls including lutein, violaxanthin and neoxanthin.

Beta- and α-carotene are redundantly hydroxylated by non-heme (CHY1, CHY2) as well as cytochrome P450 (CYP97A and CYP97C) hydroxylases. Enzyme β-hydroxylase acts on the β-ring and ε-hydroxylase acts on the ε-ring of the α-carotene to form lutein (Pfundel and Bilger 1994). Lutein is the end-product of the α-carotene branch. β-Hydroxylase acts in two steps to produce β-cryptoxanthin and zeaxanthin in the β-carotene branch. Zeaxanthin can be epoxidized to produce violaxanthin by zeaxanthin epoxidase (ZEP). Violaxanthin is converted to neoxanthin by neoxanthin synthase (NXS) (Cazzonelli 2011). Neoxanthin is the final carotenoid of the β-β branch of the classical biosynthetic pathway (Al-Babili et al 2000). Cleavage of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin by 9-*cis*-epoxycarotenoid dioxygenase leads to the products that are further modified to form abscisic acid (Cazzonelli 2011).

Carotenoid Function

The role of carotenoid pigments in plants is determined primarily by whether the tissue they are found in is photosynthetic or non-photosynthetic. In non-photosynthetic tissues, carotenoids determine or contribute to the color of flowers and fruits. Chromoplasts are carotenoid-containing plastids responsible for the yellow, orange, and red colors of many flower petals and fruits. In the absence of chlorophyll, the main function of chromoplast carotenoids is the attraction of pollinating insects and animals (Bartley and Scolnic 1995). Carotenoid colors, that range from pale yellow to a reddish brown depend upon the number of conjugated double bonds along the C_{40} backbone, as well as other cyclic and oxygenic modifications (Cazzonelli 2011).

Carotenoids participate in the light harvesting process in photosynthetic tissue. They absorb light in the blue region of the spectrum (400 to 600 nm), and the energy absorbed can be transferred to chlorophylls. Therefore, carotenoids serve as accessory pigments by harvesting radiant light in a region of the spectrum not covered by the chlorophylls (Demming-Adams et al 1996).

The most important function of carotenoids in photosynthetic plant tissue is photoprotection against harmful oxygen species. Due to the presence of carotenoids in photosynthetic tissue plants are able to balance between absorbing sufficient light for photosynthetic processes, while avoiding photo-oxidative damage to membranes and proteins caused by excessive light. Carotenoids scavenge ROS, like singlet oxygen ($^1O_2$), formed during photooxidative stress, to inhibit oxidative damage and quench triplet sensitizer ($3Chl^*$) and excited chlorophyll (Chl^*) to prevent the formation of $^1O_2$ and protect the photosynthetic apparatus (Sharma et al 2012).

In seeds, carotenoids contribute to the nonenzymatic antioxidant system, which functions to limit free radical-induced membrane deterioration and seed ageing (Calucci et al 2004). During seed germination, higher antioxidant contents were correlated with an inhibition of peroxidase activity and promotion of seed germination (Rogozhin et al 2001). Lutein and zeaxanthin are the major carotenoids, included in the group of xanthophylls, that function as antioxidants and protect from photo-induced free radical damage in wheat kernels (Demmig-Adams et al 1996). Comparison of the free radical accumulation with the antioxidant (lutein) decline showed a loss of protection and, thus, an increase in the free radical formation (Galleschi et al 2002). Seeds that were 'acceleratory aged' at 40°C and 100% relative humidity gradually lost lutein. The decrease in lutein after three days of acceleratory ageing conditions was

subsequently accompanied by an increase in ROS after 10 days. By day 14, lutein content was reduced by more than 50%; ROS levels in flour, derived from the aged seeds, increased 2.5 fold (Galleschi et al 2002). A rapid decrease in the lutein content of wheat seeds during storage has been observed by Pinzino et al (1999).

Carotenoid production in the seed is important for abscisic acid production and seed dormancy (Maluf et al 1997). The carotenoid, zeaxanthin, serves as a substrate for the production of phytohormones such as abscisic acid and strigolactone as well as other signaling molecules. In some situations, carotenoid biosynthetic genes, such as β-carotene hydroxylase, were shown to be rate-limiting for abscisic acid biosynthesis and can alter the plants resistance to drought and oxidative stress by modulating the levels of xanthophylls and abscisic acid synthesis (Du et al 2010).

Oxidative Stress

Plant oxidative stress is a complex physiological phenomenon. It occurs as a result of overproduction of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), and accompanies virtually all biotic and abiotic stresses (Al-Ghami 2009). Oxidative stress may arise due to (1) the development of an imbalance between production and scavenging of ROS due to severe disturbance of cell physiology (Prasad et al 1994), or (2) generating of ROS by activating various enzymes, such as, NADPH-oxidase, cell wall-bound peroxidases, and amine oxidases in the apoplast, as a part of stress signaling and immunity response system in plants (Grant and Loake 2000).

The role of ROS during abiotic stress appears to be opposite to the role of ROS during pathogen defense. When abiotic stresses occur, ROS scavenging enzymes (e.g. superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, and catalase) are induced to decrease

the concentration of toxic intercellular ROS levels (Apel and Hirt 2004). Nonenzymatic antioxidants include ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids, and carotenoids. However, during the pathogen defense ROS, particularly hydrogen peroxide (H_2O_2) is produced to diffuse into cells and activate programmed cell death, which potentially limits the spread of disease from the infection point. During plant pathogen reaction, the activity and levels of the ROS detoxifying enzymes ascorbate peroxidase and catalase are suppressed by salicylic acid and nitrous oxide. Thus, during the plant pathogen defense, the plant produces more ROS while decreasing ROS scavenging capacities (Dangle and Jones 2001).

When ROS concentration exceeds a threshold, lipid peroxidation occurs in cellular membranes. Lipid peroxidation aggravates the oxidative stress by production of lipid-derived radicals, which react with and damages protein and DNA. Thus, the level of peroxidation is often use as an indicator of ROS damage to the cell membrane. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell damage and is one main parameter for evaluating membrane oxidation extent (Al-Ghamadi 2009).

Lipoxygenase

Role of lipoxygenase (LOX) in conferring resistance against pathogens have been established (Slusarenko 1996), partially due to its involvement in pathway of jasmonic acid and traumatic acid biosynthesis upon wounding (Figure 2; Siedow 1991). Traumatic acid has been reported to mimic the physiological effects seen upon wounding of plant tissue, inducing cell division and subsequent callus formation (Bonner and English 1938), while jasmonic acid was suggested to inhibit growth and to act as a promoter of plant senescence (Creelman and Mullet 1995).

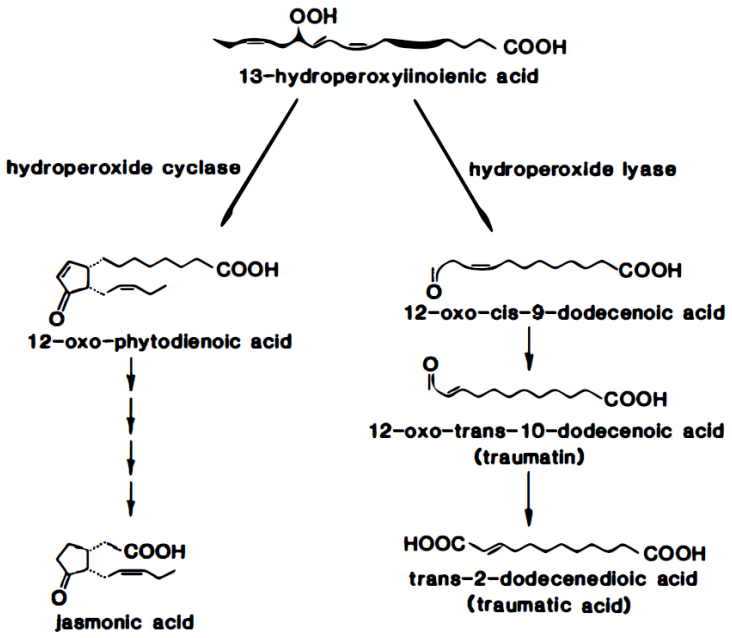

Figure 2. The "lipoxygenase pathway" for the biosynthesis of jasmonic acid, traumatin, and traumatic acid from the lipoxygenase product, 13-hydroperoxylinolenic acid.

Source: Siedow (1991)

Lipoxygenase refers to a group of enzymes that catalyze the oxidation of polyunsaturated fatty acids containing a *cis*-*cis*-1,4-pentadiene system, producing firstly free radicals and, subsequently after oxygenation, conjugated hydroperoxidiene derivatives (Troccoli et al 2000). Fatty acid radicals produced during the intermediate steps of polyunsaturated fatty acid peroxidation by LOX are responsible for the oxidative degradation of α-tocopherol and carotenoid pigments, such as β-carotene, xanthophylls and chlorophylls (Trono et al 1999; Sissons 2008). Lipoxygenase activity is greatest in embryo, followed by pericarp and endosperm (Rani et al 2001); thus a higher amount of tocopherol and carotenoids in semolina may prevent the bleaching activity of LOX and improve semolina and pasta color. Unfortunately, the content of tocopherol and carotenoids in semolina is decreased significantly because the embryo and wheat outer layers, where the large amounts of them are located, are removed during milling (Lintas 1988).

β-Carotene acts as an inhibitor of LOX activity, preventing semolina bleaching and improving pasta quality (Trono et al 1999). A decrease of 16.3% in semolina β-carotene content during pasta processing versus a 7.9% loss during milling was observed by Borrelli et al (1999). Simple correlations and the linear multiple regression confirm that among other factors, LOX activity is the main factor involved in loss of color (Borrelli et al 1999). In fact, it has been demonstrated that high pigment content in pasta products is not always derived from high β-carotene content in whole grain, and that LOX activity levels are more important than for retaining yellow pigment content than the β-carotene content in whole grain or semolina (Borrelli et al 1999). It had been reported that LOX activity can be inactivated by temperatures above 65°C (Yemenicioglu and Ercan 1999).

Lipoxygenase levels in durum wheat are cultivar-related and depend on the environment (Troccoli et al 2000). In fact, molecular analysis of LOX expression has demonstrated that genotypic variation is due to different transcriptional levels of the relative gene(s) (Manna et al 1998; Carrera et al 2007). Research by De Simone et al (2010) showed that activity of LOX in semolina was determined by transcript level, isoforms (isozyme) of LOX present, and amount of LOX enzyme.

Polyphenol Oxidase

Polyphenol oxidase (PPO) is an enzyme that has wide distribution in plants (Yoruk and Marshall 2003). Polyphenol oxidases catalyze the oxidation of phenolic compounds into quinones in the presence of molecular oxygen. Generated *o*-quinones further react with amines and thiol groups or undergo self-polymerization to produce the dark/brown colored polyphenols (Anderson and Morris 2003). Polyphenol oxidase normally functions as a phenol oxidase *in vivo* only in senescent or damaged cells. In plants, inactive PPOs are localized in plastids while their

phenolic substrates are located mainly in the vacuole. So the enzymatic browning occurs only when sub-cellular compartment is ruptured in the presence of oxygen (Vaughn et al 1988). Although high PPO activity can be beneficial in variety of products, such as prunes, dark raisins, tea, and coffee, brown color tends to mask the yellow color in semolina end-products during processing, when it reaches substantial levels (Sissons 2008).

Polyphenol oxidase activity occurs early in grain development and decreases with kernel maturation. A large portion of PPO activity is located in the endosperm of immature seeds. As the kernel matures, the level of PPO activity in the endosperm decreases, whereas the level of PPO activity in the outer layers and embryo increases (Kruger 1976). In mature grain after wheat fractioning, it was observed that wheat pericarp contained the highest PPO activity, there was little PPO activity found in wheat white flour, and no PPO activity was observed in the embryo (Marsh and Galliard 1986). Milling of wheat grain at a higher flour extraction rate raises darkening effect in end-products, suggesting that PPO enzymes reside mainly in pericarp and grain layers (Baik et al 1994). Polyphenol oxidase activity varies with genotype, and durum wheat cultivars have lower PPO activity than common wheat cultivars (Lamkin et al 1981).

Polyphenol oxidase has been frequently implicated in resistance to diverse pathogens and insects. Quinones, the primary products of PPO, are highly reactive and can undergo complex secondary reaction pathways that may lead to generation ROS. Pathogen and insect defense role of PPO was demonstrated on a tomato plant, where the reduction in PPO activity levels resulted in enhanced susceptibility to the pathogens, while PPO overexpression increased resistance of tomato plants to *Pseudomonas syringae* pathogen (Thipyapong et al 2007).

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PAPER 1. EFFECT OF CULTIVAR AND ENVIRONMENT ON GRAIN FILLING OF *TRITICUM TURGIDUM* VAR. DURUM

Abstract

Research was conducted to determine the effect of cultivar and environment on the total yellow pigment content and oxidative stress levels during grain development. Five durum cultivars were grown in field experiments established in 2009 - 2011 near Prosper, ND. Spikes were collected every 3 to 4 days from each plot beginning 7 days after anthesis. Lipoxygenase activity, polyphenol oxidase activity, and malondialdehyde content were measured as determinants of oxidative stress. Compared to the 30 year average, the average air temperature during grain filling was low in 2009, average in 2010, and high in 2011. Cultivars did not differ in their deposition patterns for total yellow pigment content, protein content, ash content or kernel weight. Environmental stress (high temperature) was high in 2011, which was reflected in low kernel weights. Total yellow pigment and malondialdehyde concentrations per kernel declined with maturity and after grain reach its physiological maturity were lower in 2011 than in 2009 and 2010. Total yellow pigment content correlated positively with MDA content, with correlation coefficient of $r=0.85$ ($P \leq 0.01$) in 2009, $r=0.86$ ($P \leq 0.01$) in 2010, and $r=0.71$ ($P \leq 0.01$) in 2011 (Tables A-1, A-2, and A-3). Lipoxygenase activity peaked just before and declined after physiological maturity, and was generally greater in 2010, followed by 2011 and 2009. Polyphenol oxidase activity was higher in 2009 initially and declined with maturity. Polyphenol activity was lowest in 2010 during grain filling suggesting effect of environmental conditions during grain filling.

Introduction

In cereal crops, carotenoid pigments, natural yellowish pigments that include β-carotene, xanthophyll, and flavones are found in the amyloplasts of endosperm cells (Gelinas et al 1998). They contribute to the yellow color of endosperm and protect the integrity of cellular membranes from lipid peroxidation by acting as free radical scavengers. Carotenoid content in durum wheat has been reported to vary with cultivar and environment (Clarke et al 2006; Singh 2008). Cultivar main effect has a larger influence on carotenoid pigment content than environment main effect (Borrelli et al 1999; Rharrabti et al 2003) as total yellow pigment content is a highly heritable trait and is controlled by additive gene effects (Clarke et al 2006).

A period of rapid cell division occurs after floret fertilization, which results in the formation of endosperm and embryo. Deposition of starch in endosperm cells begins towards the end of cell division and continues until physiological maturity (Jones et al 1985). Starch found in the endosperm is derived from carbon dioxide fixed by photosynthesis that occurred during the period of the kernel development and remobilization of the stored carbohydrates to growing kernels (Jenner et al 1991). Protein deposition begins at a later stage than starch deposition, with gliadin synthesis greatest early in grain filling and glutenin synthesis greatest mid to end of grain fill (Abonyi et al 2007). Environment affects rate and duration of grain filling. However, genotypes vary significantly in grain filling rate rather than in grain filling duration (Dias and Lidon 2009). The optimum temperature during grain growth to achieve maximum yields of wheat generally is considered to be between 15 and 20°C (Al-Khatib and Paulsen 1984; Dupont and Altenbach 2003). Elevated temperatures (Johnson et al 1981; Plaut et al 2004) and water deficit (Lawlor 2002; Martinez et al 2003; Tambussi et al 2005) after anthesis decrease the rate and duration of photosynthesis (Harding et al 1990). Elevated temperatures,

however, increased the rate of protein deposition but decreased duration of grain fill which resulted in reduction of total amount of protein per kernel, and altered glutenin to gliadin ratio (Hurkman and Wood 2011).

The mineral accumulation in the grain primarily depends on the translocation from the leaves and the minerals removed from the lower parts of the plant after the onset of senescence (Wardlaw 1990). Under drought stress the re-translocation of minerals from vegetative organs is much higher than that in well watered condition, which leads to a decrease in ash content in the flag leaf at maturity and an increase in ash content in the grain at maturity (Merah et al 1999).

Unfavorable growing environment such as drought, salinity, and extreme temperatures during grain fill cause morphological changes and cellular damage to the mature grain (Sancho et al 2008). Oxidative stress is manifested by the presence of reactive oxygen species (ROS) which serve as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction pathway at low concentration (Mittler 2002). However, at high concentrations, ROS can be extremely reactive, and can oxidize multiple cellular components like proteins, lipids, DNA, and RNA. Reactive oxygen species react with polyunsaturated lipids, forming malondialdehyde (MDA), an advanced lipoxidation end-product. The production of this aldehyde is often used as a biomarker to measure the level of oxidative stress in cereal crops (Fercha 2011).

Lipase and lipoxygenase (LOX) enzymes found in the cell membrane are activated when tissue is disrupted or injured. Lipase, which is found predominantly in the pericarp of the kernel, hydrolyzes triacylglycerides to produce free fatty acids (Galliard 1986; Steele 2004), while LOX peroxidizes polyunsaturated fatty acids and their monoacylglycerides to produce f hydroperoxides (Siedow 1991; Bhirud and Sosulski 1993). Lipoxygenase activity is greatest in

the embryo, followed by pericarp and endosperm (Rani et al 2001). In durum wheat, LOX activity is cultivar-related and depends on the environment (Troccoli et al 2000). Borrelli et al (1999) reported that LOX activity varied with cultivar and environment, but that cultivar had a much greater effect on LOX activity than did growing environment. In regard to LOX, the availability of substrates and the presence of natural inhibitors could modify the enzymatic activity. Lipoxygenase activity is inhibited by α-tocopherol and carotenoids (Abdel-Aal et al 2007). α-Tocopherol is concentrated in the embryo, and thus removed during the milling.

Polyphenol oxidase (PPO), a bicopper metalloenzyme, is involved in enzymatic browning using oxygen and phenolic compounds as the substrates (Nicolas et al 2003). In food products derived from durum and common wheat (*Triticum aestivum* L.), PPO is a major contributor to time-dependent discoloration (Baik et al 1994). Generally, PPO activity is found mostly in the pericarp and more specifically in the aleurone layer of wheat kernels (Sullivan 1946). The phenolic compounds are oxidized to *o*-quinones, which then react with themselves, other phenolics or nucleophilic amino acids to form brown colored melanoidins (Amiot et al 1992). Durum wheat cultivars have lower levels of PPO activity than common wheat cultivars (Lamkin et al 1981). Levels of PPO activity in wheat grain vary among genotypes, as it is a highly heritable trait (Nilthong et al 2012), and PPO activity is also influenced by environment (Baik et al 1994; Park et al 1997).

Relatively limited assessment has been made on changes in carotenoid pigment content in relation to oxidative stress, as measured by LOX activity, MDA content, and PPO activity in kernel during grain development. Thus, the main objective of the present study was to determine the effect of cultivar and environment on the relationship between oxidative stress levels and carotenoid (yellow) pigment content during the grain development.

Materials and Methods

Plant Material

A field experiment was established near Prosper, ND in 2009, 2010 and 2011. The experiment consisted of five durum wheat cultivars ('AC-Commander', 'Alkabo', 'Divide', 'Mountrail', and 'Rugby'), which were selected based on their differences in pigment content (preliminary unpublished results). Durum cultivars were sown in four blocks (replicates). Each cultivar was seeded at 80 g/plot in eight-3 m rows that were spaced 0.3 m apart. The soil type at Prosper was a Parilla loam. Nitrogen was applied at 23 kg N/ha. In 2009 and 2010, nitrogen was applied in the form of anhydrous ammonia and in 2011 it was applied as urea. Weather data was obtained from the weather station located at the Prosper experiment station (www.ndawn.ndsu.nodak.edu).

In 2009, plots were sprayed for weed control twice. The first application consisted of Prowl® (2.3L/ha) and DiscoverNG® (1.12 kg/ha) and was applied when the durum was in the 3-leaf stage. The second application consisted of Puma® (0.7 kg/ha), Widematch® (0.56 kg/ha), and Harmony Extra® (0.028 kg/ha) applied at the 5-leaf stage. In 2010, the herbicide Wolverine® (0.95L/ha) was applied at 5-leaf stage. In 2011, Wolverine® (0.95 L/ha) and Prowl® (1.12 L/ha) were applied for weed control at the 4-leaf stage. Herbicides were applied with a tractor-mounted sprayer delivering 94 L/ha at 276 kPa through 8002 nozzles. Field plots were not treated with fungicide or insecticide.

Durum wheat spikes (50) were collected randomly from each plot twice a week beginning about seven days after anthesis and ending at harvest. An effort was made to collect spikes from the main tiller. General kernel development categories (watery, milk, soft dough, hard dough, and mature) were determined by squeezing a couple of kernels that were removed

from the center of the spike. Moisture content of spikes was determined by drying three spikes from each plot in a forced-air dryer at 135°C for 2 hr. The remaining spike samples were air-dried at room temperature and threshed using a threshing machine (Agriculex SPT-1, Guelph, Ont., Canada) belt thresher. Grain was placed in plastic bags and stored at 4°C until grain quality tests were conducted.

Physical Kernel Characteristics

1000-Kernel weight (1000-KWT) was determined by counting the number of kernels in 10 g of clean grain and adjusting weight to 1000 kernels. To determine 1000 kernel weight for a sample with less than 10 g of kernels, the weight of 200 kernels were determined and multiplied by five.

Chemical Kernel Characteristics

Wheat kernels were ground using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ) fitted with a 0.4 mm screen and tested for ash content, moisture content, protein content, yellow pigment content, MDA content, and LOX activity. Approved methods (AACC International 2010) were used to determine ash content (Ash – Basic Method, method 08-01.01), moisture content (Moisture – Air Oven Method, method 44-15.02), and protein content (Crude Protein - Combustion Method, method 46-30.01).

Total yellow pigment content was determined using the water-saturated n-butanol Method 14-50.01 (AACC International 2010) as modified by use of 2 g of ground material. Water-saturated n-butanol (10 mL) was added to 2 g of whole meal and shaken 2 min. After resting 30 min, the extracts were filtered through Whatman number 1 filter paper, and absorbance was measured using a spectrophotometer (Beckman Coulter DU 720 General Purpose UV/Vis Spectrophotometer) at a wavelength of 435.8 nm. Measurements per extracted sample were

recorded and values averaged and converted to yellow pigment concentration (μg/g) using the extinction coefficient (1.6632) for β-carotene (Sims and Lepage 1968).

Malondialehyde (MDA) content was determined according to the method described by Al-Ghamdi (2009). Ground samples (1 g) were transferred to screw-capped plastic tubes and homogenized following addition of 5 mL of trichloroacetic acid (TCA)–2-thiobarbituric acid (TBA)–HCl reagent [15% (w/v) TCA, 0.37% (w/v) TBA, 0.25 M HCl and 0.01% butylated hydroxytoluene (BHT)]. After homogenization, samples were incubated at 90°C for 30 min in a hot water bath, chilled in crushed ice for 10 min and centrifuged at 12,000 x *g* for 10 min. Absorbance of the aliquot was measured at 535 nm and 600 nm against TCA-HCL-BHT reagent. Malondialdehyde reacts with TBA and the resulting chromophore absorbs at 535 nm. Malondialdehyde concentration was calculated directly using the extinction coefficient of 1.55×10^5 mM cm^{-1} (Kwon et al 1965).

Lipoxygenase activity was measured using the method described by Li and Schwarz (2012). The assay is based on the determination of lipid hydroperoxides by a ferrous oxidation-xylenol orange (FOX) assay. Lipoxygenase extracts were prepared daily. Each sample was run in duplicates. Ground samples (1g) were extracted at 4^oC with 10 mL of 0.1 *M* potassium phosphate buffer, pH=6.6. The mixture was vortex mixed every 15 min for 1 hr. Samples were filtered into a new set of test tubes using Whatman GF/A 90 mm glass microfiber filters. Linoleic acid, substrate for the assay, was prepared by adding 4 mL of 1% Tween 20 and 72 uL of linoleic acid to 25.2 mL of 0.1 M ice-cold phosphate buffer. To eliminate the cloudiness, 0.8 mL of ice-cold 0.1 M NaOH was added to the substrate. Preparation of the ferrous oxidation-xylenol orange (FOX) reagent include mixing 67.3 mg xylenol orange, 98 mg ammonium ferrous sulfate, and 1.39 mL sulfuric acid in 98.6 mL of distilled water for 1 hr. One volume of

this concentrated reagent was mixed with nine volumes of 100% methanol solution containing 0.97g/L BHT.

The enzymatic reaction mixture consisted of 110 µL of 0.1 M phosphate buffer (pH=6.6) and 10 µL of whole-wheat extract was mixed in a glass tube, and then 30 µL of freshly prepared linoleic acid substrate was added. The enzymatic reaction ran at 25^{o}C for 5 min. Then, FOX reagent (2.85 mL) was added to the glass tube and immediately mixed. The resultant solution was allowed to stand at room temperature for 10 min, after which, the absorbance at 560 nm was read against the reaction blank. The reaction blank consisted of whole wheat extract (10 uL) mixed with 110 µL of 0.1 M phosphate buffer (pH 6.6) and 2.85 mL of FOX reagent.

Lipoxygenase activity (U/g)= (Δ560nm/Incubation time) × (Total Volume in Cell/Aliquot Assayed) x 1/EmM x (Extraction Volume/Sample Weight) x Dilution, where EmM (Extinction Coefficient)=47 (Li and Schwartz 2012).

Polyphenol oxidase activity was determined using intact kernels as described by Anderson and Morris (2001) given in Approved Method 22-85.01 (AACC International 2010). A 1.5-mL aliquot of 10 m*M* of L-DOPA (L-3,4-dihydroxyphenylalanine) containing 0.02%, v/v, Tween-20 as substrate in 50 m*M* MOPS [3-(*N*-morpholino) propane sulfonic acid] buffer, pH 6.5, was added to five undamaged seeds in a 2-mL microcentrifuge tube. The tubes were placed on an orbital shaker (Glas-Col, Terre Haute, IN, USA) and rotated for 1 hr at room temperature to allow the reaction to occur. Polyphenol oxidase activity was measured as the change in absorbance at 475 nm using a Beckman Coulter spectrophotometer (Beckman Coulter DU 720 General Purpose UV/Vis Spectrophotometer). Each sample was run in duplicate. The L-DOPA solution was made fresh daily.

Statistical Analysis

The collected data were analyzed using the Statistical Analysis System (SAS) computer packages version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA). Experimental design was a randomized complete block design (RCBD) with split plot in time arrangement. All treatments had four replications and data collected were subjected to analysis of variance (ANOVA). Cultivar and spike collection times (days after anthesis, DAA) were fixed effects, and year (environment) was a random effect. Means were separated by using Fisher's-protected LSD at P=0.05. F-tests were significant at $P \leq 0.05$.

For each dependent variable, the error mean squares from 2009, 2010, and 2011 were tested for homogeneity using Bartlett's Chi-square test (Steele and Torrie 1980).
To assess the relationship between the variables involved in oxidation and defense system in grain, a Pearson correlation analysis were performed with the SAS 9.3 software package (SAS Institute Inc., Cary, NC, USA). Correlation coefficients were tested for homogeneity as described by Gomez and Gomez (1984) and combined when appropriate.

Results and Discussion

Dates of planting, anthesis, physiological maturity, and harvest in 2009, 2010 and 2011 are shown in Table 1. Differences in growing conditions and accumulated growing degree days (AGDD) before anthesis and during grain development in 2009, 2010, and 2011 are shown in Table 2. In 2009, a cool wet spring led to water-saturated fields that delayed planting. Harvest in 2009 was delayed until September 4 due to late maturity and rainfall (Tables 1 and 2). Grain reached its physiological maturity 32 days after anthesis (DAA). Average minimum air temperature was generally lower than normal with a lower than normal amount of precipitation. Top soil and subsoil moisture was rated adequate (NASS 2009).

Table 1. Dates of Planting, Anthesis, Physiological Maturity, and Harvest in 2009, 2010 and 2011

Growing year	Planting date	Estimated anthesis	Estimated physiological maturity	Harvesting date
2009	May 28	July 28	August 29	September 4
2010	April 19	July 1	July 22	August 10
2011	May 20	July 21	August 09	August 22

In 2010, durum was planted and harvested within the normal time frame, and grain reached its physiological maturity 21DAA. Average minimum air temperatures and rainfall was within normal limits. Top soil and subsoil moisture was rated adequate (NASS 2010).

Saturated fields and low soil temperature delayed planting in 2011 until the end of May (Table 1). Grain was harvested on August 22. Due to high temperatures during grain filling in 2011, grain reached its physiological maturity on approximately 18DAA. Average minimum air temperature in 2011 was generally higher than normal. Rainfall was higher than normal (Table 2). Top soil and subsoil moisture was rated surplus (NASS 2011).

Averaged daily maximum and minimum air temperatures after anthesis were generally higher in 2011 than in 2009 or 2010 (Table 2). During the first two weeks after anthesis, the difference in averaged minimum air temperatures in 2009, 2010, and 2011 was even more pronounced and was 10.5°C, 13.3°C, and 16.7°C, respectively (Figure 3). Rainfall was greatest in 2011 and least in 2009 (Figure 4). Thus, 2009 was cool and dry during grain fill while 2011 was warm and wet during grain fill.

1000-Kernel Weight

Error variances for 1000-KWT, kernel ash and kernel protein (both as percentage and weight per kernel), were not found to be homogeneous within each variable among years; therefore, data for each year was analyzed separately. Cultivar x DAA interaction was

significant for 1000-KWT, percent kernel protein and protein weight per kernel for 2009, 2010, and 2011, and ash weight per kernel in 2009 and 2011 (Tables 3, 5-7). However, plotted line graphs of these variables indicate that their respective interactions were due to differences in magnitude as their deposition patterns were similar for all cultivars (Figures A-1, A-2, A-3, and A-4). Cultivar main effect was significant for 1000-KWT in 2009 and 2011 (Table 3). In 2009, 1000-KWT, averaged across DAA, was highest in Alkabo (37.3 g), and lowest in Divide (32.9 g) (Table 8). In 2011, the highest 1000-KWT was in Divide (21.1 g); and lowest in AC-Commander (17.1 g) when averaged across DAA and compared with other cultivars (Table 8).

Days After Anthesis main effect was significant for mean 1000-KWT for 2009, 2010, and 2011 (Table 3). Rapid increase in 1000-KWT occurred from 10 to 25DAA in 2009, and from 10 to 20DAA in 2010 (Figure 3). In 2011, kernel weight showed increase from 10DAA to 15DAA. Mature kernels averaged across cultivars weighed 42.7 g in 2009 and 39.4 g in 2010. In 2011, 1000-KWT was the lowest (22.1 g), reflecting the short grain fill period (Table 2) due to the effect of high night temperatures (Austin et al 1986).

Table 2. Growing Conditions in 2009, 2010, and 2011, and the 30-Year Averages

	2009			2010			2011		
	Planting to anthesis	Anthesis to physiol. maturity	Physiological maturity to harvest	Planting to anthesis	Anthesis to physiol. maturity	Physiological maturity to harvest	Planting to anthesis	Anthesis to physiol. maturity	Physiological maturity to harvest
Averaged Daily max temperature, °C	25.0	24.6	22.2	22.0	27.8	27.7	25.0	28.1	27.8
[1]30-Year average maximum air temperature, °C	26.3	27.9	25.4	21.8	27.8	28.4	25.6	28.4	27.9
Averaged Daily min air temperature, °C	11.1	11.6	8.0	9.4	14.3	15.3	13.9	16.7	13.8
30-Year average min air temperature, °C	12.9	13.5	10.8	7.9	14.4	14.4	12.2	14.4	13.4
Rainfall, cm	8.4	6.1	0.3	17.2	6.0	5.0	29.1	12.7	0.3
30-Year average rainfall, cm	19.2	6.4	1.8	20.0	6.0	4.3	19.7	3.7	3.0
Accumulated growing degree days (AGDD), °C	1052	552	77	1137	422	371	1126	404	253
30-Year AGDD, °C	1171	644	90	1088	429	392	1109	393	251

Source: NDAWN 2009, 2010, and 2011.

[1]Normal values give for the specified time frame/period represent the 30 years average for the same time frame/period.

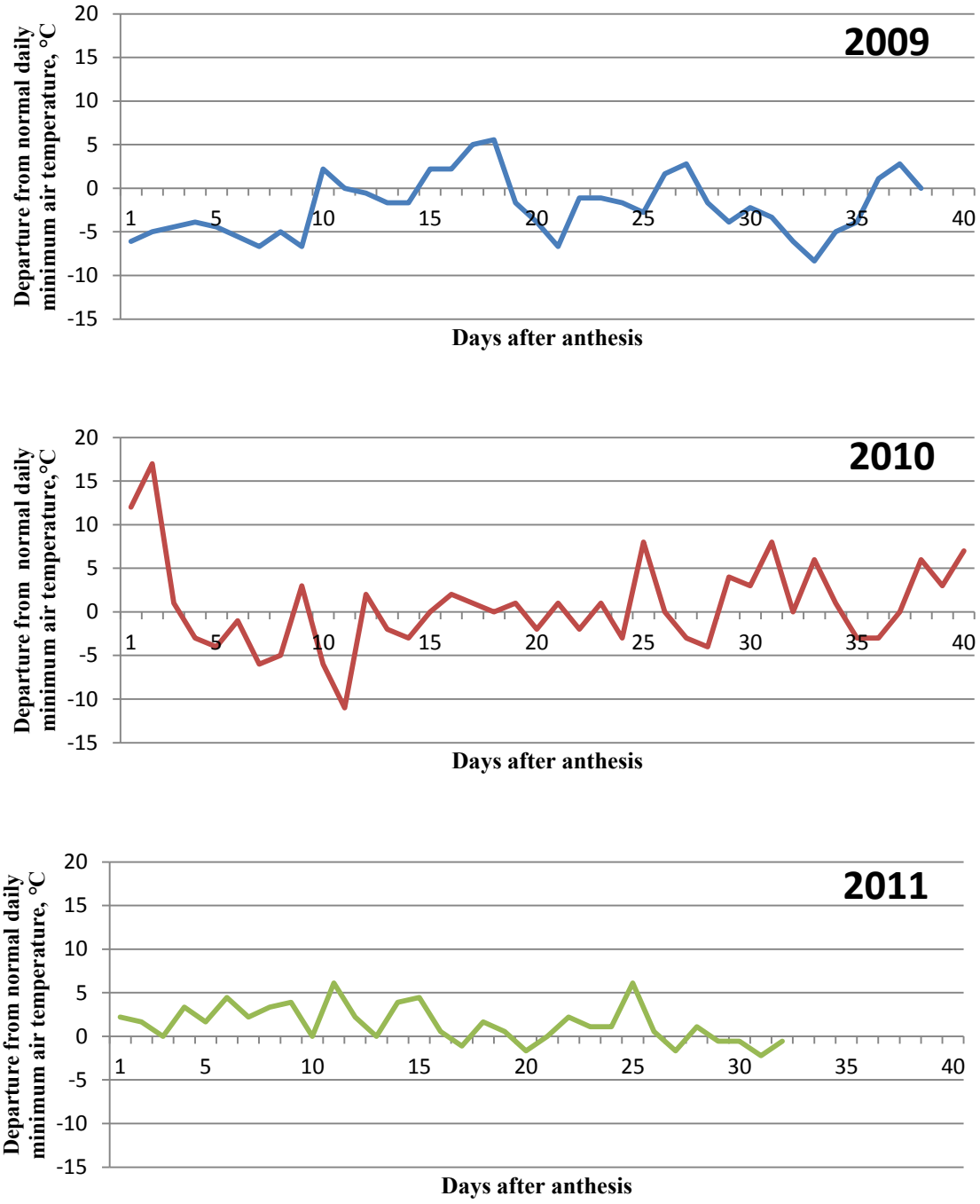

Figure 3. Departure from normal daily minimum air temperature in 2009, 2010, and 2011.

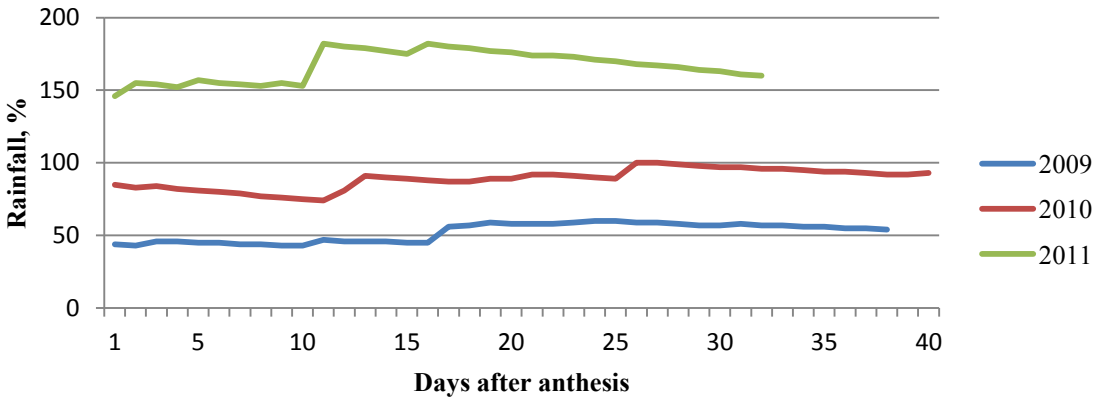

Figure 4. Percent of normal rainfall in 2009, 2010, and 2011.

Grain filling period is more susceptible to stresses and has a lower optimum temperature (15°C) when compared with vegetative growth and development (20°C) for wheat (Wardlaw et al 1989). The cool temperatures enhance carbohydrate supply to the grain by prolonged green leaf area duration and an extended total growth cycle (Spiertz et al 2006). High minimum air temperature and excessive soil moisture during vegetative growth in 2011 could have elevated the relative humidity in the crop canopy. High humidity favores the onset of plant diseases, such as tan spot, which is caused by the fungus *Pyrenophora tritici-repentis* and stem and leaf rust, which are caused by fungi *Puccini graminis* and *P. recondita* (personal observation). Leaf diseases would reduce the functional leaf area and hastened the decline in photosynthesis.

Feng et al (2000) reported that during grain filling high temperatures resulted in reduced supply, translocation and deposition of assimilates in the developing grain; thus limiting grain dry mass. The negative effect of high maximum air temperature was more pronounced when followed by the high minimum air temperatures, as was observed during grain filling in 2011 (Table 2). Altenbach et al (2003) observed a significant reduction in the duration of grain fill in wheat at moderate and high temperature regimes. In their experiment, the period between

anthesis and kernel maturity spanned 44, 35 and 26 days under 24/17°C, 37/17°C and 37/28°C day/night temperature regimes, respectively.

Table 3. Analysis of Variance for 1000-Kernel Weight (g) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	8.30	3.48	
	Cultivar	4	99.65	3.79	*
	Error (a)	12	26.32	11.02	
	Days After Anthesis (DAA)	6	1991.87	881.06	**
	Error (b)	18	2.26	0.95	
	Cultivar*DAA	24	4.42	1.85	*
	Error (c)	72	3.4		
2010	Rep	3	8.86	13.56	
	Cultivar	4	70.10	20.19	ns
	Error (a)	12	3.47	5.31	
	Days After Anthesis (DAA)	6	358.27	350.57	**
	Error (b)	18	1.02	1.56	
	Cultivar*DAA	24	7.71	11.8	**
	Error (c)	72	0.65		
2011	Rep	3	41.01	39.76	
	Cultivar	4	3.03	0.62	**
	Error (a)	12	4.90	4.76	
	Days After Anthesis (DAA)	5	1893.05	1225.35	**
	Error (b)	15	1.55	1.50	
	Cultivar*DAA	20	3.40	3.30	**
	Error (c)	57	1.03		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant;
Df = degree of freedom; and MS=mean square.

Table 4. Analysis of Variance for Kernel Ash (%) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.0479	9.59	
	Cultivar	4	0.0019	0.06	ns
	Error (a)	12	0.0356	7.13	
	Days After Anthesis (DAA)	6	0.6126	62.93	**
	Error (b)	18	0.0097	1.95	
	Cultivar*DAA	24	0.0068	1.36	ns
	Error (c)	72	0.0049		
2010	Rep	3	0.0679	35.49	
	Cultivar	4	0.0947	15.33	**
	Error (a)	12	0.0062	3.22	
	Days After Anthesis (DAA)	6	0.5634	43.06	**
	Error (b)	18	0.0131	6.83	
	Cultivar*DAA	24	0.0031	1.62	ns
	Error (c)	72	0.0019		
2011	Rep	3	0.0235	3.44	
	Cultivar	4	0.4124	35.52	**
	Error (a)	12	0.0116	1.70	
	Days After Anthesis (DAA)	5	0.5867	75.16	**
	Error (b)	15	0.0078	1.14	
	Cultivar*DAA	20	0.0204	2.98	ns
	Error (c)	60	0.0069		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 5. Analysis of Variance for Kernel Ash (mg/kernel) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.004	1.43	
	Cultivar	4	0.001	0.06	*
	Error (a)	12	0.004	1.57	
	Days After Anthesis (DAA)	6	0.366	114.7	**
	Error (b)	18	0.003	1.24	
	Cultivar*DAA	24	0.005	1.78	*
	Error (c)	72	0.003		
2010	Rep	3	0.007	4.61	
	Cultivar	4	0.017	15.06	**
	Error (a)	12	0.001	0.75	
	Days After Anthesis (DAA)	6	0.517	375.07	**
	Error (b)	18	0.001	0.90	
	Cultivar*DAA	24	0.002	1.48	ns
	Error (c)	72	0.002		
2011	Rep	3	0.002	1.02	
	Cultivar	4	0.003	2.42	ns
	Error (a)	12	0.001	0.52	
	Days After Anthesis (DAA)	5	0.173	76.56	**
	Error (b)	15	0.002	0.93	
	Cultivar*DAA	20	0.007	2.78	*
	Error (c)	60	0.002		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant;
Df = degree of freedom; and MS=mean square.

Table 6. Analysis of Variance for Kernel Protein (%) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.404	2.81	
	Cultivar	4	3.458	4.29	*
	Error (a)	12	0.806	5.61	
	Days After Anthesis (DAA)	6	1.225	10.64	**
	Error (b)	18	0.115	0.80	
	Cultivar*Time	24	0.265	1.85	*
	Error (c)	72	0.143		
2010	Rep	3	1.603	15.32	
	Cultivar	4	3.627	16.04	**
	Error (a)	12	0.226	2.16	
	Days After Anthesis (DAA)	6	12.912	32.93	**
	Error (b)	18	0.392	3.75	
	Cultivar*DAA	24	0.444	4.25	**
	Error (c)	72	0.104		
2011	Rep	3	0.218	0.36	
	Cultivar	4	9.427	4.50	*
	Error (a)	12	2.092	3.42	
	Days After Anthesis (DAA)	5	1.001	3.55	*
	Error (b)	15	0.282	0.46	
	Cultivar*DAA	20	1.123	1.84	*
	Error (c)	60	0.611		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 7. Analysis of Variance for Kernel Protein (mg/kernel) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.239	4.20	
	Cultivar	4	1.205	2.18	ns
	Error (a)	12	0.553	9.74	
	Days After Anthesis (DAA)	6	41.063	635.62	**
	Error (b)	18	0.065	1.14	
	Cultivar*DAA	24	0.128	2.24	*
	Error (c)	72	0.057		
2010	Rep	3	1.080	47.96	
	Cultivar	4	0.328	4.88	*
	Error (a)	12	0.067	2.98	
	Days After Anthesis (DAA)	6	35.802	422.66	**
	Error (b)	18	0.085	3.76	
	Cultivar*DAA	24	0.059	2.62	*
	Error (c)	72	0.023		
2011	Rep	3	0.212	7.04	
	Cultivar	4	0.526	13.62	**
	Error (a)	12	0.039	0.254	
	Days After Anthesis (DAA)	5	10.406	267.78	**
	Error (b)	15	0.389	1.29	
	Cultivar*DAA	20	0.193	6.40	**
	Error (c)	57	0.030		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 8. Means of 1000-Kernel Weight (g), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	36.5ab	31.5a	17.1b
Alkabo	37.3a	31.7a	20.4a
Divide	32.9b	31.1a	21.1a
Mountrail	33.4ab	31.2a	18.1b
Rugby	34.8ab	30.9a	20.3a

Means within columns followed by the same letter are not significantly different at P<0.05 level, n=28 in 2009 and 2010, n=24 in 2011.

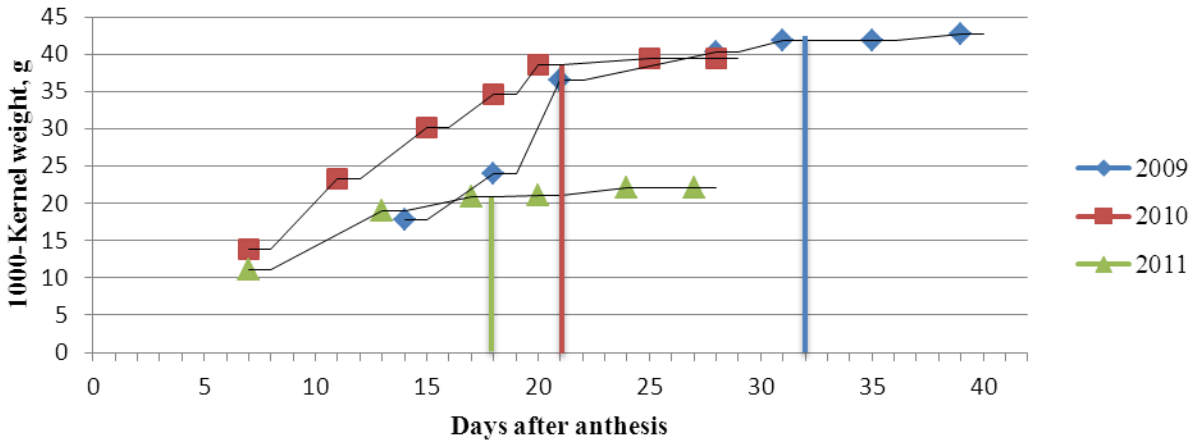

Figure 5. Effect of days after anthesis on mean 1000-kernel weight averaged across cultivars. Vertical bars indicate time when grain reached its physiological maturity. $LSD_{0.05}$=1.0 in 2009, $LSD_{0.05}$=0.8 in 2010, and $LSD_{0.05}$=0.7 in 2011.

Kernel Ash

Cultivar main effect was significant for ash content per kernel in 2009 and 2010 (Table 5). In 2009, the ash content per kernel was highest in Alkabo and AC-Commander (0.65 mg/kernel) and lowest in Divide and Mountrail (0.58 mg/kernel). In 2010 the lowest ash content per kernel was in Mountrail (0.57 mg/kernel) (Table 9). Ash content per kernel was similar for all cultivars in 2011 (Table 9).

Table 9. Means of Kernel Ash (mg/kernel), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	0.65a	0.63a	0.5ab0
Alkabo	0.65a	0.62a	0.50ab
Divide	0.58b	0.61a	0.52a
Mountrail	0.58b	0.57b	0.49b
Rugby	0.61ab	0.63a	0.51ab

Means within columns followed by the same letter are not significantly different at P<0.05 level, n=28 in 2009 and 2010, n=24 in 2011.

Ash content per kernel averaged across cultivars and DAA in 2011 (0.51 mg/kernel) was lower than in 2009 and 2010 (0.61 mg/kernel). Low ash content per kernel in 2011 as well as low 1000-KWT (Figures 5 and 6) can be attributed to elevated temperatures and higher than normal amount of precipitation after anthesis in 2011. This combination of temperature and precipitation reduced grain maturation period, thus limiting supply of assimilates and minerals in the grain.

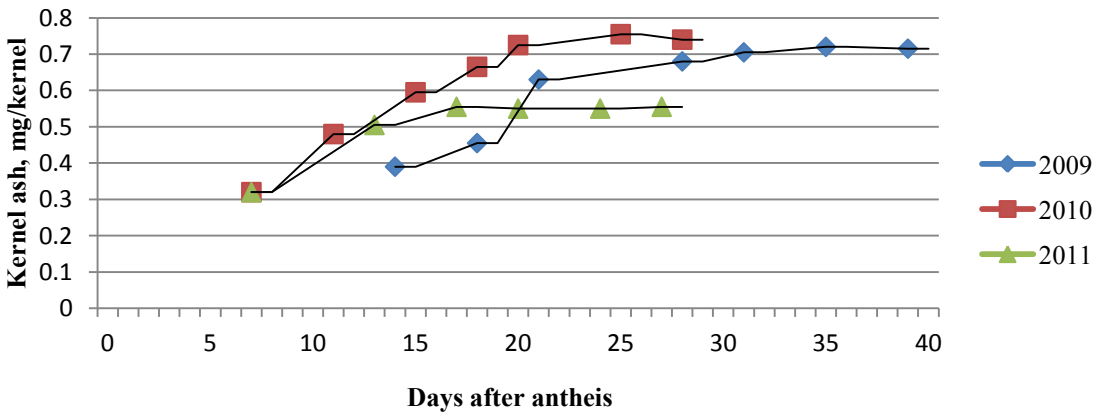

Figure 6. Effect of days after anthesis on mean kernel ash per kernel averaged across cultivars. $LSD_{0.05}$=0.04 in 2009, $LSD_{0.05}$=0.02 in 2010, and $LSD_{0.05}$=0.03 in 2011.

Days after anthesis main effect for ash content per kernel was significant in 2009, 2010, and 2011 (Table 5). Ash content per kernel increased gradually in all three years as grain filling progressed (Figure 6). In 2009, ash content per kernel showed an increase from 0.39 mg/kernel on the first sampling date until 0.71 mg/kernel at estimated physiological maturity (32 DAA). In 2010, kernel ash content increased from 7DAA (0.32 mg/kernel) until 21DAA (0.73 mg/kernel), the date of estimated physiological maturity. In 2011, ash content was 0.32 mg/kernel at 7DAA and increased to 0.55 mg/kernel at the time of physiological maturity (18DAA) (Figure 6).

On the wt/wt (%) basis, overall ash content of mature grain was highest in 2011 (2.55%) followed by 2010 (1.88%) and 2009 (1.68%), suggesting that the effect of growing environmental conditions were more pronounced on a kernel dry weight than mineral accumulation in grain (Figure 7). In 2011, the greater number of kernels per weight made up for the lower ash content per kernel, thus resulting in higher wt/wt (%) ash content.

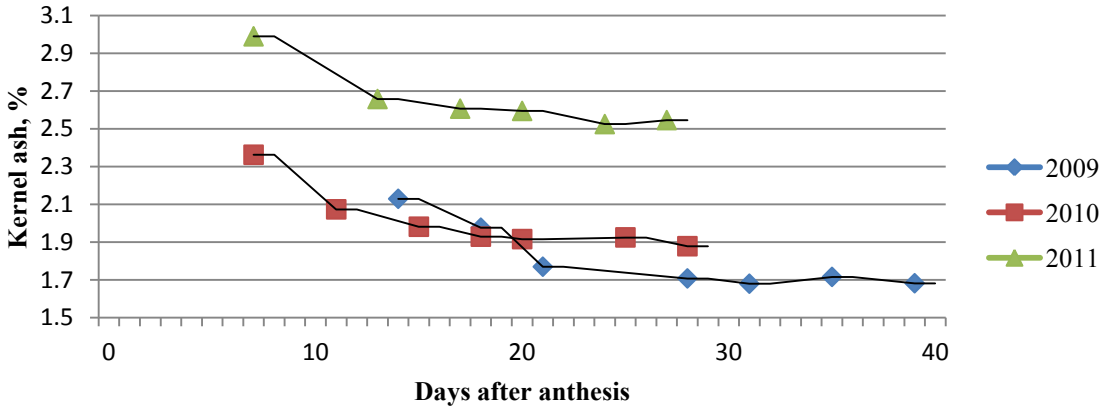

Figure 7. Effect of days after anthesis on mean kernel ash averaged across cultivars. $LSD_{0.05}$=0.07 in 2009, $LSD_{0.05}$=0.08 in 2010, and $LSD_{0.05}$=0.06 in 2011.

Kernel Protein

Cultivar main effect was significant for kernel protein content (%) in 2009, 2010, and 2011 (Table 6). When averaged across the DAA, Divide had the highest total protein content among cultivars in 2009 (14.8%) and 2010 (13.9%), whereas AC-Commander and Rugby had the lowest kernel protein content in 2009 (13.9%). Mountrail and Alkabo had the lowest kernel protein content in 2010 (12.7%). Kernel protein content averaged across DAA in 2011 was the highest in AC-Commander (18.0%) and lowest in Rugby (16.3%) (Table 10).

Table 10. Means of Kernel Protein (%), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	13.9c	13.0b	18.0a
Alkabo	14.1bc	12.7c	16.7bc
Divide	14.8a	13.6a	17.0bc
Mountrail	14.2b	12.7c	17.3ab
Rugby	13.9bc	13.1b	16.3c

Means within columns followed by the same letter are not significantly different at $P<0.05$ level, n=28 in 2009 and 2010, n=24 in 2011.

Days after anthesis main effect was significant for kernel protein content (%) in 2009, 2010, and 2011 (Figure 6). In 2009, kernel protein accumulation was fairly uniform, protein content averaged across cultivars was 14.5% at 14DAA and declined to 13.9% at 18DAA, it fluctuated up to 14.5% at 28DAA and declines slightly to 14.1% by 32DAA, an estimated date for physiological maturity, and ceased. In 2010, kernel protein content decreased slightly from 13.8% at 7DAA to 12.5% at 14DAA and remained similar until physiological maturity (21DAA), when it increased slightly to 14.3% and declined to 13.4% at 28DAA. In 2011, protein content averaged across cultivars was 17.2% at 7DAA and maintain similar throughout

the grain filling period with an slight increase to 17.4% after grain reached its physiological maturity at and decrease to 17.0 afterwards (Figure 6). An increase in kernel protein would occur when the rate of deposition of protein was greater than the rate of deposition of starch. The converse is also true. The decrease in kernel protein (%) would occur when deposition of protein was less than the deposition of starch; this occurred during early part of grain filling (Figure 8).

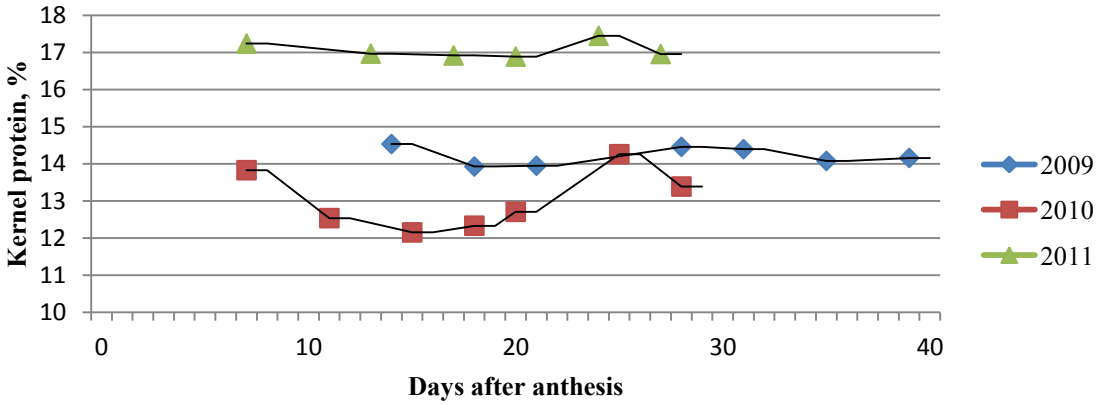

Figure 8. Effect of days after anthesis on mean kernel protein averaged across cultivars. $LSD_{0.05}$=0.2 in 2009, $LSD_{0.05}$=0.4 in 2010, and $LSD_{0.05}$ =0.01 in 2011.

Cultivar main effect was significant for protein content per kernel in 2010 and 2011 (Table 7). When averaged across the DAA, Alkabo had the highest protein per kernel (5.25 mg/kernel) among cultivars in 2009. Divide had the highest protein per kernel content among cultivars in 2010 (4.19 mg/kernel) and 2011 (3.55 mg/kernel), whereas Mountrail had the lowest protein per kernel in 2009 (4.73 mg/kernel), 2010 (3.91 mg/kernel) and 2011 (3.15 mg/kernel) (Table 11).

Table 11. Means of Kernel Protein (mg/kernel), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	5.11ab	4.13ab	3.24bc
Alkabo	5.25a	4.03cb	3.31b
Divide	4.87ab	4.19a	3.55a
Mountrail	4.73b	3.91c	3.15c
Rugby	4.87ab	4.13ab	3.24cb

Means within columns followed by the same letter are not significantly different at $P<0.05$ level, n=28 in 2009 and 2010, n=24 in 2011.

Days after anthesis main effect was significant for protein content per kernel in 2009, 2010 and 2011 (Table 7). In 2009 protein content averaged across cultivars was 2.57 mg/kernel at 14DAA and nearly doubled by 21DAA (5.09 mg/kernel). Protein content per kernel in 2009 reached 6.03 mg/kernel by 32DAA, an estimated date for physiological maturity, and ceased. In 2010, protein content per kernel averaged across cultivars was 1.90 mg/kernel at 7DAA and doubled by 15DAA (3.67 mg/kernel). Protein content continued to increase until grain reached its estimated physiological maturity and was 5.63 mg/kernel at 25DAA. In 2011, protein content averaged across cultivars was 1.89 mg/kernel at 7DAA and nearly doubled to 3.24 mg/kernel six days later (13DAA), it increased slowly to 3.54 mg/kernel by 18DAA where it reached estimated physiological maturity (Figure 7).

Wheat is a cool season crop. The optimum temperatures during grain development vary between 18-25°C during the day, and between 15-17°C during the night (Austin et al 1986). Temperatures in this range give the longest duration of grain fill and the greatest accumulation of starch and protein per kernel (Dupont and Altenbach, 2003). The greater protein accumulation in 2009 can be attributed to cooler night time temperatures after anthesis that prolonged time until

physiological maturity and thus accumulation of protein in the kernel. Grain filling was condensed in 2011 due to high night temperatures during grain developing stage.

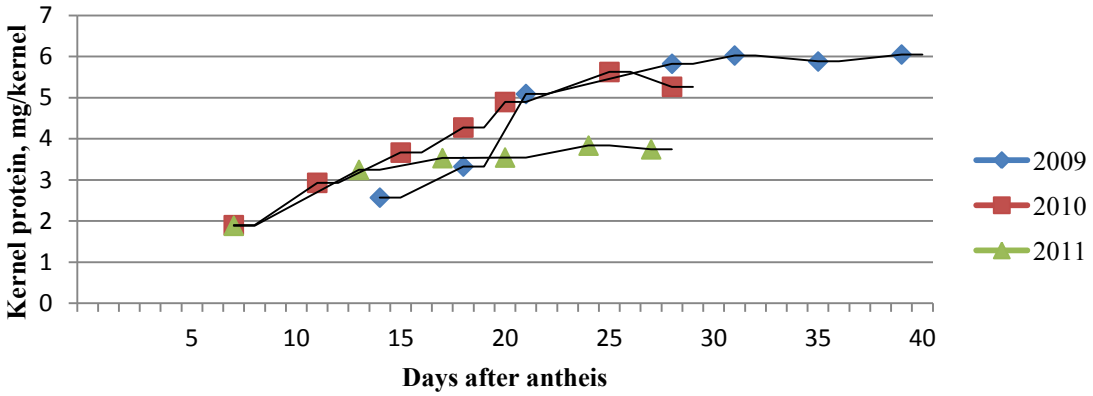

Figure 9. Effect of days after anthesis on mean kernel protein per kernel averaged across cultivars.
$LSD_{0.05}$=0.02 in 2009, $LSD_{0.05}$=0.02 in 2010, and $LSD_{0.05}$=0.01 in 2011.

Total Yellow Pigment Content

Cultivar by DAA interaction was significant for total yellow pigment (TYP) content (ppm) in 2009 and 2010 (Table 12). Cultivar by DAA interaction was determined to be due to differences in magnitude as the shape of the curves appeared to be similar for all cultivars (Figure A-5). Cultivar main effect was significant for TYP content (ppm) in 2009, 2010, and 2011 (Table 12). In 2009, AC-Commander (28.3 ppm) had the highest TYP while Mountrail (22.3 ppm) had the lowest. In 2010, Alkabo (19.1 ppm) had the highest TYP content while Mountrail (13.0 ppm) had the lowest (Table 13). In 2011, AC-Commander (17.3 ppm) had the highest TYP content while Rugby (13.8 ppm) the lowest among cultivars (Table 13).

Table 12. Analysis of Variance for Total Yellow Pigment Content (ppm) in 2009, 2010 and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	24.99	2.71	
	Cultivar	4	167.39	5.08	*
	Error (a)	12	32.93	3.57	
	Days After Anthesis (DAA)	6	9927.74	1007.26	**
	Error (b)	18	9.86	1.07	
	Cultivar*DAA	24	34.26	3.72	**
	Error (c)	72	9.21		
2010	Rep	3	4.41	1.35	
	Cultivar	4	36.24	9.85	**
	Error (a)	12	3.68	1.12	
	Days After Anthesis (DAA)	6	1824.59	251.81	**
	Error (b)	18	7.24	2.21	
	Cultivar*DAA	24	33.26	10.14	**
	Error (c)	72	3.28		
2011	Rep	3	46.01	10.99	
	Cultivar	4	38.26	6.86	**
	Error (a)	12	5.57	1.33	
	Days After Anthesis (DAA)	5	537.75	34.79	**
	Error (b)	15	15.45	3.69	
	Cultivar*DAA	20	6.78	1.62	ns
	Error (c)	60	4.18		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 13. Means of Total Yellow Pigment Content (ppm), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010 and 2011

Cultivar	2009	2010	2011
AC-Commander	28.3a	15.5b	17.3a
Alkabo	23.0b	19.1a	15.6b
Divide	22.7b	15.3b	16.2ab
Mountrail	22.6b	13.0b	15.2bc
Rugby	23.2b	15.4b	13.8c

Means within columns followed by the same letter are not significantly different at $P<0.05$ level, n=28 in 2009 and 2010, and n=24 in 2011.

Days after anthesis main effect was significant for TYP content when averaged across cultivars in 2009, 2010, and 2011 (Tables 12). In 2009, TYP content decreased by 24% from 62.9 ppm at 14DAA to 47.6 at 18DAA, there was a further 49% decrease to 24.4 ppm at 21DAA (Figure 10). At estimated physiological maturity (32DAA) TYP content (ppm) was 8.8 ppm and showed no further significant decrease. In 2010, TYP content declined by 60% from 35.4 ppm at 7DAA to 14.2 at 11DAA. TYP content continued to decline slightly until day of estimated physiological maturity, it was 9.8 ppm at 21DAA and then ceased changing. In 2011, carotenoid pigment content decreased by 22% from 24.0 ppm at 7DAA to 18.8 ppm at 13DAA. In 2011, carotenoid pigment content decreased steady as grain filling progressed and was 15.7 ppm at 17DAA, it continue to decrease after the date of estimated physiological maturity and was 10.1 ppm at harvest time (27DAA). In early stages of grain development, TYP content was greatest in 2009 (62.2 ppm at 14DAA) and lowest in 2011 (24.0 ppm at 7DAA). At the time grain reached its physiological maturity, TYP pigment content was slightly higher in 2011 than 2009 and 2010 (Figure 10).

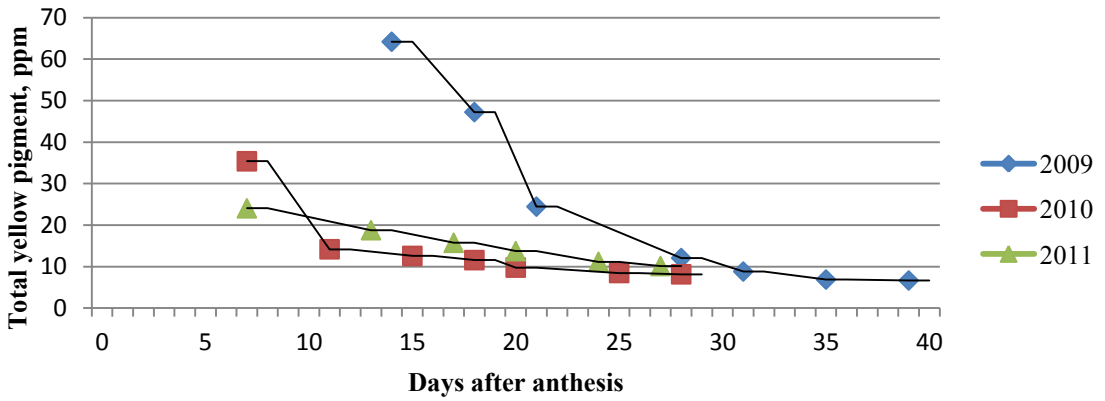

Figure 10. Effect of days after anthesis on mean total yellow pigment content averaged across cultivars.
$LSD_{0.05}$=2.4 in 2009, $LSD_{0.05}$=1.7 in 2010, and $LSD_{0.05}$=2.6 in 2011.

Cultivar x DAA interaction for TYP per kernel, averaged across cultivars, was significant in 2010 and 2011 (Table 14); however, the significance was due to the magnitude (Figure A-6). Cultivar main effect was significant for TYP content per kernel in 2010 and 2011 (Table 14). Alkabo (0.34 µg/kernel) had the highest TYP content per kernel in 2010 and 0.21 µg/kernel in 2011, while Mountrail had the lowest TYP concentration per kernel at 0.21 µg/kernel (2010) and 0.18 µg/kernel (2011) (Table 15).

Days after anthesis main effect averaged across cultivars was significant for TYP content per kernel in 2009, 2010, and 2011 (Table 14). In 2009, the pigment per kernel content decreased by 51% from 1.11 µg/kernel (14DAA) to 0.55 µg/kernel (18DAA), with no significant decrease after 21DAA (Figure 9). There was 53% decrease from 21DAA (0.51 µg/kernel) until 28DAA (0.24 µg/kernel), and 25% decrease at 31DAA (0.18 µg/kernel). In 2011, TYP concentration per kernel increased from 0.27 µg/kernel at 7DAA to 0.36 µg/kernel at 13DAA. Total yellow pigment content per kernel then decreased by about 50% and was 0.16 µg/kernel at 18DAA, the date of estimated physiological maturity, with little non-significant decrease afterwards.

Table 14. Analysis of Variance for Total Yellow Pigment Content per Kernel (µg/kernel) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.0106	1.15	
	Cultivar	4	0.0376	1.27	ns
	Error (a)	12	0.0295	3.20	
	Days After Anthesis (DAA)	6	2.4690	251.33	**
	Error (b)	18	0.0098	1.07	
	Cultivar*DAA	24	0.0070	0.76	ns
	Error (c)	72	0.0092		
2010	Rep	3	0.1676	27.20	
	Cultivar	4	0.0565	5.28	**
	Error (a)	12	0.0107	1.74	
	Days After Anthesis (DAA)	6	0.5178	4.36	*
	Error (b)	18	0.1186	19.26	
	Cultivar*DAA	24	0.0130	2.12	**
	Error (c)	72	0.0061		
2011	Rep	3	0.0023	2.28	
	Cultivar	4	0.0031	3.31	**
	Error (a)	12	0.0009	0.90	
	Days After Anthesis (DAA)	5	0.1872	101.81	**
	Error (b)	15	0.0018	1.76	
	Cultivar*DAA	20	0.0026	2.53	**
	Error (c)	60	0.0010		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 15. Means of Total Yellow Pigment Content per Kernel (µg/kernel), Averaged Across DAA as Affected by Cultivar in 2009, 2010 and 2011

Cultivar	2009	2010	2011
AC-Commander	0.45a	0.28ab	0.19abc
Alkabo	0.44a	0.34a	0.21a
Divide	0.42a	0.25bc	0.20ab
Mountrail	0.37a	0.21c	0.18c
Rugby	0.38a	0.28ab	0.19bc

Means within columns followed by the same letter are not significantly different at P<0.05 level, n=28 in 2009 and 2010, n=24 in 2011.

Initial concentration of carotenoid pigment per kernel was highest in 2009, it was 1.11 µg/kernel at 14DAA, whereas in 2010 it was 0.58 µg/kernel at 7DAA and 0.19 µg/kernel at 15DAA, in 2011 TYP concentration per kernel was 0.27 µg/kernel at 7DAA and 0.36 µg/kernel at 13DAA. After estimated physiological maturity there was no significant decrease observed in all three years, and the levels of TYP content per kernel was slightly lower in 2011, followed by 2009, and 2010.

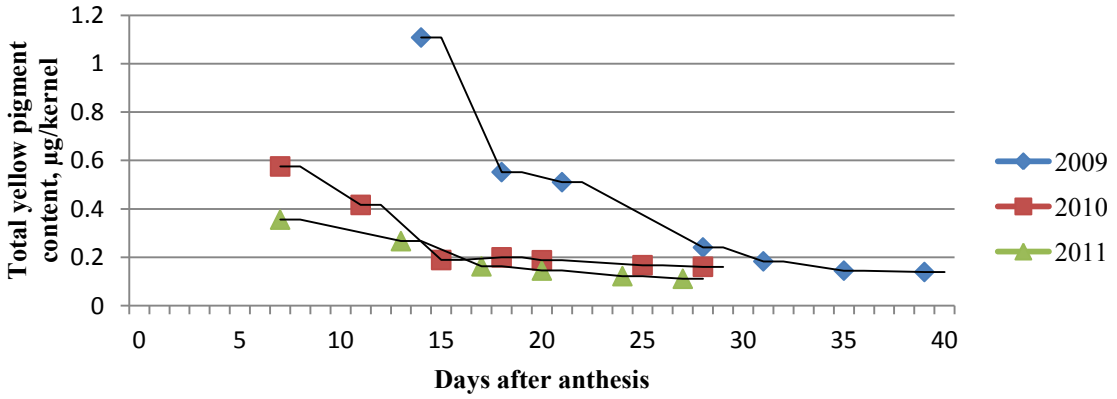

Figure 11. Effect of days after anthesis on mean total yellow pigment content per kernel averaged across cultivars.
$LSD_{0.05}$=0.07 in 2009, $LSD_{0.05}$=0.23 in 2010, and $LSD_{0.05}$=0.03 in 2011.

Total yellow pigment (TYP) in durum endosperm is composed primarily of the carotenoid xanthophylls: lutein, zeaxanthin, and β-cryptoxanthin (Singh 2008). The decline of

carotenoid compounds, primarily *trans*- lutein, during grain fill period were reported by Ramachandran et al (2010) suggesting termination of the flux of substrate for its biosynthesis early in a pathway. Kean et al (2007) suggested that decline in lutein content in sorghum was due to conversion of lutein to apocarotenoids or aromatic compounds. The decreased amount of carotenoid pigment per kernel might be associated with their antioxidant activity in the cell. Carotenoids act both as singlet oxygen quenchers and as quenchers of triplet excited states of molecules (Howitt and Pagson 2006). There were two possible mechanism of carotenoid action proposed by Mortensen and Skibsted (1997). Firstly, carotenoids can react with radicals to form an adduct; in the second mechanism, they can transfer on electron to the radical, giving rise to a stable carotenoidic radical cation and regenerating the original molecule (Mortensen and Skibsted 1997). Carotenoids dissipate excess energy via xanthophyll-mediated non-photochemical quenching (e.g. xanthophyll carotenoids include zeaxanthin, antheraxanthin and lutein). The physiological relevance of xanthophylls to photosynthesis and plant biology in general is displayed by bleaching (Cazzonelli 2011).

Oxidative Stress

Cultivar x DAA interaction was significant for lipoxygenase (LOX) activity in 2009, 2010 and 2011 (Table 16). Cultivar x DAA was significant for malondialdehyde (MDA) content per kernel in 2011 (Table 18). The interactions, however, were due to magnitude, as the behavior of all five cultivars in each year was similar for LOX and MDA content (Figures A-7 and A-8).

Lipoxygenase Activity

Cultivar main effect was significant for LOX activity in all three years (Table 16). The greatest LOX activity was observed in Rugby and was 0.54 U/g, 1.18 U/g, and 0.93 U/g in 2009, 2010, and 2011 year, respectively (Table 20). The least LOX activity was observed in AC-

Commander and was 0.04 U/g, 0.10 U/g and 0.07 U/g in 2009, 2010, and 2011 years, respectively (Table 20).

Days after anthesis main effect was significant for LOX activity in 2009, 2010, and 2011 (Tables 16). In 2009, LOX activity averaged across cultivars was 0.14 U/g at 14DAA and there was a slight increase to 0.33 U/g by the 28DAA; however, the increase was not significantly different. Lipoxygenase activity had a rapid increase to 0.81U/g at physiological maturity (32DAA) with a rapid decrease to 0.48 U/g afterwards (35DAA) (Figure 12). In 2010, LOX activity averaged across cultivars was 0.39 U/g at 7DAA, it increased steadily and peaked at 21DAA (1.19 U/g), estimated physiological maturity, with a slight decrease afterwards to 0.93 U/g at 28DAA (Figure 12). In 2011, LOX activity increased from 0.50 U/g at 7DAA to 1.12 U/g at 18DAA, with a sharp decrease afterwards.

Lipoxygenase catalyzes the oxidation of polyunsaturated fatty acids to hydroperoxides, in which singlet oxygen and superoxide anions can be formed (Lynch and Thompson 1994). Sofo et al (2004) reported that LOX activity increased under drought environment indicating that water deficit is associated with lipid peroxidation mechanisms. The increase in lipoxygenase activity at the physiological maturity in 2009, 2010, and 2011 might reflect the programmed cell death that occurs in endosperm as it approaches physiological maturity. In the early stages of grain development, LOX activity averaged across cultivars was higher in 2011 as compared to 2009 and 2010. After the physiological maturity, lipoxygenase activity in 2011 was slightly lower than in 2010. Lipoxygenase activity averaged across cultivars was at similar levels in 2009 and 2011 after the physiological maturity. The reduction in LOX activity in 2011 may be attributed to the reduced amount of free radicals due to antioxidant activity of carotenoid pigments.

Table 16. Analysis of Variance for Lipoxygenase Activity (U/g) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.06	2.80	
	Cultivar	4	1.13	49.81	**
	Error (a)	12	0.02	1.06	
	Days After Anthesis (DAA)	6	1.16	12.37	**
	Error (b)	18	0.094	4.37	
	Cultivar*DAA	24	0.08	3.17	**
	Error (c)	72	0.02		
2010	Rep	3	0.056	2.74	
	Cultivar	4	5.093	175.47	**
	Error (a)	12	0.029	1.40	
	Days After Anthesis (DAA)	6	1.681	16.52	**
	Error (b)	18	0.101	4.90	
	Cultivar*DAA	24	0.193	9.32	**
	Error (c)	72	0.021		
2011	Rep	3	0.09	2.40	
	Cultivar	4	3.07	87.08	**
	Error (a)	12	0.03	0.95	
	Days After Anthesis (DAA)	5	1.13	10.90	**
	Error (b)	15	0.10	2.79	
	Cultivar*DAA	20	0.10	2.79	**
	Error (c)	60	0.03		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 17. Analysis of Variance for Malondialdehyde Content (nmol/g) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	1.17	2.54	
	Cultivar	4	0.36	0.56	ns
	Error (a)	12	0.64	1.39	
	Days After Anthesis (DAA)	6	68.16	148.80	**
	Error (b)	18	0.46	0.99	
	Cultivar*DAA	24	0.43	0.93	ns
	Error (c)	72	0.46		
2010	Rep	3	5.83	8.40	
	Cultivar	4	0.45	0.64	ns
	Error (a)	12	0.70	1.01	
	Days After Anthesis (DAA)	6	49.49	11.37	**
	Error (b)	18	4.35	6.26	
	Cultivar*DAA	24	0.17	0.25	ns
	Error (c)	72	0.69		
2011	Rep	3	0.44	4.43	
	Cultivar	4	0.41	11.36	**
	Error (a)	12	0.03	0.36	
	Days After Anthesis (DAA)	5	17.30	31.39	**
	Error (b)	15	0.55	5.47	
	Cultivar*DAA	20	0.15	1.51	ns
	Error (c)	60	0.10		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 18. Analysis of Variance for Malondialdehyde Content per Kernel (nmol/kernel) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.00213030	7.22	
	Cultivar	4	0.00265305	2.36	ns
	Error (a)	12	0.00112369	3.81	
	Days After Anthesis (DAA)	6	0.00143636	3.55	*
	Error (b)	18	0.00040495	1.37	
	Cultivar*DAA	24	0.00047701	1.62	ns
	Error (c)	72	0.00029508		
2010	Rep	3	0.00362145	18.53	
	Cultivar	4	0.00486714	5.01	**
	Error (a)	12	0.00097135	4.97	
	Days After Anthesis (DAA)	6	0.00063408	2.05	ns
	Error (b)	18	0.00030923	1.58	
	Cultivar*DAA	24	0.00012200	0.62	ns
	Error (c)	72	0.00019541		
2011	Rep	3	0.00002627	0.39	
	Cultivar	4	0.00045708	7.78	**
	Error (a)	12	0.00005878	0.87	
	Days After Anthesis (DAA)	5	0.00100438	9.45	**
	Error (b)	15	0.00010623	1.58	
	Cultivar*DAA	20	0.00017825	2.65	**
	Error (c)	60	0.00006730		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 19. Analysis of Variance for Polyphenol Oxidase Activity (ΔOD_{475}/hr/g) in 2009, 2010, and 2011

Year	Sources of variation	Df	MS	*F* value	
2009	Rep	3	0.0001	0.06	
	Cultivar	4	0.0031	10.35	**
	Error (a)	12	0.0003	0.25	
	Days After Anthesis (DAA)	6	0.0271	57.70	**
	Error (b)	18	0.0004	0.39	
	Cultivar*DAA	24	0.0012	0.98	ns
	Error (c)	72	0.0012		
2010	Rep	3	0.0007	4.40	
	Cultivar	4	0.0004	3.02	ns
	Error (a)	12	0.0001	0.87	
	Days After Anthesis (DAA)	6	0.0069	26.30	**
	Error (b)	18	0.0003	1.78	
	Cultivar*DAA	24	0.0002	1.07	ns
	Error (c)	72	0.0001		
2011	Rep	3	0.0008	1.53	
	Cultivar	4	0.0004	0.76	ns
	Error (a)	12	0.0006	1.15	
	Days After Anthesis (DAA)	5	0.0011	1.29	ns
	Error (b)	15	0.0008	1.46	
	Cultivar*DAA	20	0.0006	1.07	ns
	Error (c)	60	0.0005		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Table 20. Means of Lipoxygenase Activity (U/g), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	0.04c	0.10c	0.07c
Alkabo	0.43b	0.92b	0.80b
Divide	0.50ab	1.08a	0.89ab
Mountrail	0.42b	0.86b	0.83ab
Rugby	0.54a	1.18a	0.93a

Means within columns followed by the same letter are not significantly different at P<0.05 level, n=28 in 2009 and 2010, n=24 in 2011.

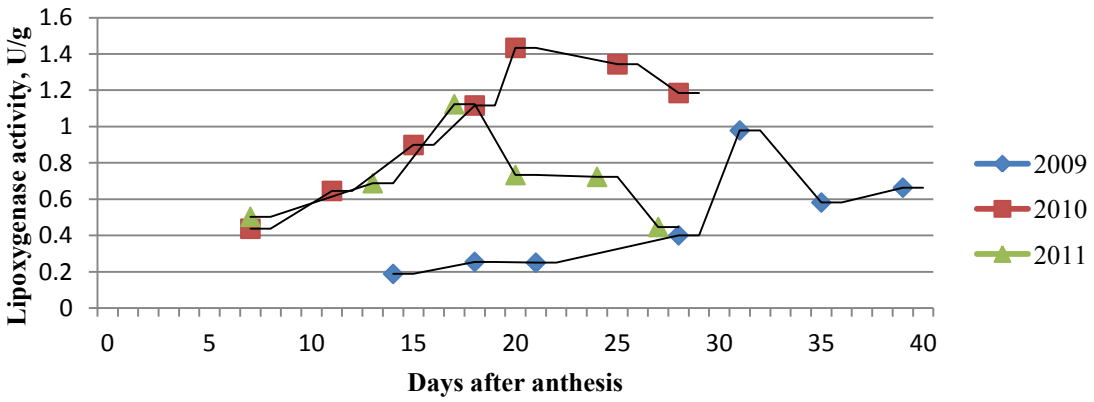

Figure 12. Effect of days after anthesis on mean lipoxygenase activity averaged across cultivars. $LSD_{0.05}$=0.20 in 2009, $LSD_{0.05}$=0.21 in 2010, and $LSD_{0.05}$=0.21 in 2011.

Malondialdehyde Content

Cultivar main effect was significant for MDA content in 2011 (Tables 17). In 2011, Divide had the highest MDA content (4.7 nmol/g), whereas Alkabo had the lowest MDA content (3.9 nmol/g).

Table 21. Means of Malondialdehyde Content (nmol/g), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	4.9a	4.2a	4.1c
Alkabo	4.7a	4.4a	3.9d
Divide	4.7a	4.1a	4.3a
Mountrail	4.9a	4.3a	4.2ab
Rugby	4.8a	4.2a	4.1bc

Means within columns followed by the same letter are not significantly different at P<0.05 level, n=28 in 2009 and 2010, n=24 in 2011.

Days after anthesis main effect was significant for MDA content in 2009, 2010, and 2011 (Table 17). In 2009, the highest MDA contents averaged across cultivars were expressed at 14DAA (8.7 nmol/g) in 2009, at 7DAA (7.7 nmol/g) in 2010, and at 7DAA (5.9 nmol/g) in 2011 with significant rapid decrease at 11DAA to 4.4 nmol/g in 2010, and at 13DAA to 4.2 nmol/g in 2011, then leveled off (Figure 13). Malondialdehyde content averaged across cultivars in 2009 leveled off at 28DAA (3.7 nmol/g). Malondialdehyde content averaged across cultivars were similar in 2009, 2010, and 2011 (Figure 13).

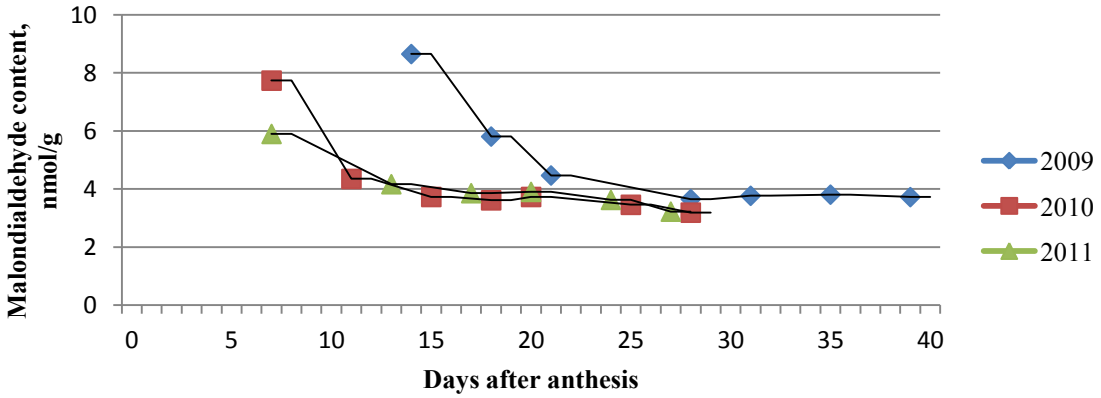

Figure 13. Effect of days after anthesis on mean malondialdehyde content averaged across cultivars.
$LSD_{0.05}$=0.4 in 2009, $LSD_{0.05}$=1.4 in 2010, and $LSD_{0.05}$=0.5 in 2011.

The cultivar main effect was significant for MDA content per kernel in 2010 and 2011 (Table 18). In 2010, MDA content per kernel averaged across years was highest in Alkabo (0.13 nmol/kernel) and remained similar in other cultivars (0.12 nmol). In 2011, MDA content per kernel averaged across years was lowest in AC-Commander (0.07 nmol/kernel) and remained the same in the other cultivars (0.08 nmol/kernel) (Table 22).

Days after anthesis main effect was significant for MDA content per kernel in 2009 and 2011 (Table 18). Malondialdehyde content per kernel fluxuated during grain filling. Malondialdehyde content per kernel declined slightly from 0.16 nmol/kernel at 14DAA to 0.14 nmol/kernel at 18DAA, followed by an increase to 0.16 nmol/kernel at 21DAA and a decrease to 0.15 nmol/kernel at 28DAA.

After physiological maturity (32DAA) MDA levels per kernel leveled out and remain 0.16 nmol/kernel. Malondialdehyde content per kernel in 2011 increased from 0.06 nmol/kernel at 7DAA to 0.08 nmol/kernel at 14DAA where it leveled out and remain similar until 24DAA, with slight decrease to 0.07 nmol/kernel at harvest time (Figure 14).

Table 22. Means of Malondialdehyde Content per Kernel (nmol/kernel), Averaged Across Days After Anthesis as Affected by Cultivar in 2009, 2010, and 2011

Cultivar	2009	2010	2011
AC-Commander	0.16a	0.12ab	0.07b
Alkabo	0.16a	0.13a	0.08a
Divide	0.14b	0.11b	0.08a
Mountrail	0.15ab	0.12ab	0.08a
Rugby	0.15ab	0.12ab	0.08a

Means within columns followed by the same letter are not significantly different at $P<0.05$ level, n=28 in 2009 and 2010, n=24 in 2011.

Malondialdehyde, a decomposition product of polyunsaturated fatty acids, has been utilized as a suitable biomarker/landmark for lipid peroxidation (Smirnoff 1993, Mittler 2002). In 2011, MDA content averaged across cultivars was lower than in 2009 and 2010. Low MDA content in 2011 can be due to activation of enzymatic and non-enzymatic antioxidant activity as a response to unfavorable environmental conditions.

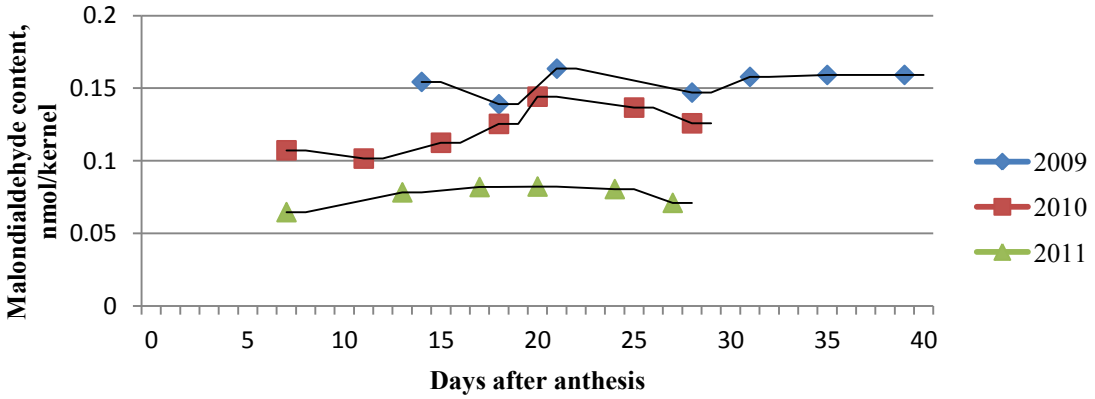

Figure 14. Effect of days after anthesis on mean malondialdehyde content per kernel averaged across cultivars.
$LSD_{0.05}$=0.01 in 2009, $LSD_{0.05}$=0.02 in 2010, and $LSD_{0.05}$=0.01 in 2011.

Polyphenol Oxidase Activity

Cultivar main effect was significant for polyphenol oxidase (PPO) activity in 2009 (Table 19). Rugby and AC-Commander cultivars had the highest PPO activity of 0.16 ΔOD_{475}/hr/g, whereas Alkabo and Divide had the lowest (0.14 ΔOD_{475}/hr/g). Days after anthesis main effect was significant for PPO activity in 2009 and 2010 (Table 19).

In 2009, a decrease of PPO activity was observed as grain development progressed, whereas in 2010 a decrease from the 7DAA (0.073ΔOD_{475}/hr/g) until 15DAA (0.051 ΔOD_{475}/hr/g) was observed, followed by an increase from 18DAA (0.066 ΔOD_{475}/hr/g) to 28DAA (0.099 ΔOD_{475}/hr/g). Mean PPO activity across cultivars in 2010 was lower than in 2009 and 2011, which might be related to the weather conditions during grain development (Figure 15).

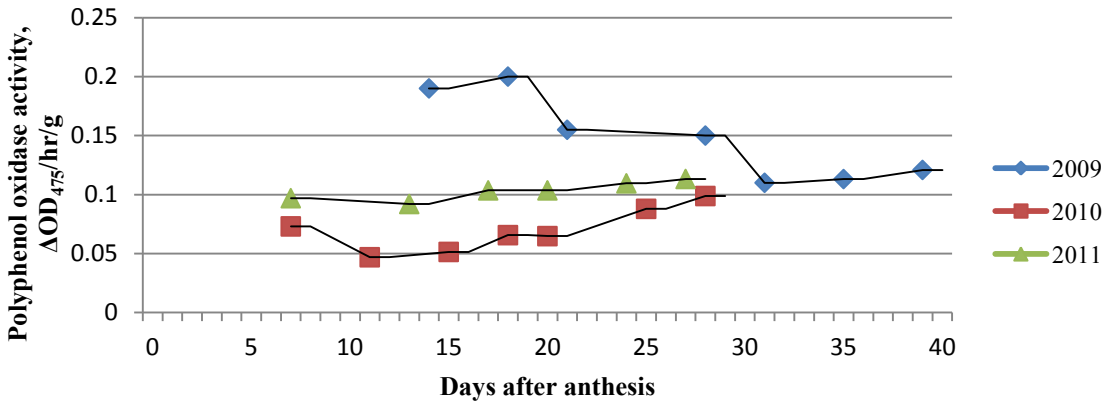

Figure 15. Effect of days after anthesis on mean polyphenol oxidase activity averaged across cultivars.
$LSD_{0.05}$=0.01 in 2009, $LSD_{0.05}$=0.01 in 2010, and $LSD_{0.05}$=0.02 in 2011.

The temperatures and rainfall in 2010 were similar to normal minimum and maximum air temperatures, and normal total rainfall during the same period of time, whereas lack of rainfall in 2009, and high daily minimum air temperature along with the excess of rainfall in 2011, might account for an increase in PPO activity.

Conclusions

Both cultivar and environment affect grain characteristics, however, environmental factor appear to affect grain characteristics more than cultivar. Elevated temperatures, excess soil moisture, and disease pressure in 2011 reduced the length of grain filling, which resulted in low kernel weight, increased kernel ash content, and significantly reduced protein accumulation per kernel. Deposition of yellow pigment was similar for all years and declined with maturity. Lower TYP content per kernel along with lower content of MDA in 2011 suggests that the yellow pigments participated in antioxidant mechanism in plants and were involved in maintaining membrane integrity from lipid peroxidation.

Lipoxygenase activity was affected by cultivar and environment. Lipoxygenase activity peaked just before and declined after physiological maturity in all years, which might reflect its

role in ABA production. Abscisic acid promotes development processes such as seed maturation and dormancy. Generally LOX levels were greater in 2010, followed by 2009 and 2011, which indicate their possible suppression by enzymatic and non-enzymatic antioxidant systems. Lipoxygenase activity was minimal in AC-Commander and maximum and Rugby, however TYP content and malondialdehyde content, and levels of PPO activity did not vary with cultivars to a great extent, suggesting that membrane peroxidation was caused by factors additional to lipoxygenase activity.

Mean polyphenol activity across cultivars in 2010 was lower than in 2009 and 2011. Less favorable weather conditions during grain development in 2009 and 2011 due to the less than normal precipitation in 2009, and elevated temperature in 2011 may account for higher PPO activity.

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PAPER 2. EFFECT OF CULTIVAR AND ENVIRONMENT ON TOTAL YELLOW PIGMENT CONTENT, MALONDIALDEHYDE CONTENT AND LIPOXYGENASE ACTIVITY IN SEMOLINA AND PASTA

Abstract

Research was conducted to determine the effect of cultivar and environment on total yellow pigment (TYP) content and oxidative stress levels in semolina and pasta. Malondialdehyde (MDA) content and lipoxygenase (LOX) activity were measured as determinants of oxidative stress. Five durum cultivars were grown in a field experiment established in 2009 - 2011 near Prosper, ND. Compared to the 30 year average, the mean air temperature during grain filling was low in 2009, average in 2010, and high in 2011. Removal of embryo and pericarp during milling reduced TYP content an average of 18%; LOX activity 81% and MDA content 44%. Effect of milling on TYP content and LOX activity varied with cultivar. The least and greatest loss of TYP content, averaged over the years, was observed in AC-Commander (12%) and Mountrail (29%), respectively. The lowest loss of LOX activity after milling was observed in Rugby (65%). Total yellow pigment loss was greatest in 2009 (22%) and least in 2011 (15%). Conversely, MDA content loss was greatest in 2011 (47%) and least in 2009 and 2010 (42%). Pasta processing resulted in a 12% decline in TYP content and a 23% increase in MDA content. There was no detectable LOX activity in dried pasta.

Introduction

Pasta is the most utilized durum wheat product, particularly in European and North American countries (Trocolli et al 2000). Durum wheat has a hard, vitreous kernel, a yellow endosperm, and a grain protein content ranging from about 12.0 to 14.5% (Matsuo and Dexter 1980). The quality of end-products is related to the quality of the durum grain. Pasta made from

durum wheat cultivars of superior quality results in a bright, translucent, yellow color, and it retains, after cooking, firmness and absence of stickiness.

The yellow color derived from the endosperm is valued in the milling and pasta industries for its consumer appeal. Yellow color of durum endosperm is highly correlated with *trans*-lutein, the major carotenoid in durum wheat (Panfili et al 2004; Ramachandran et al 2010). Although environmental factors have important roles in the carotenoid concentrations in cereals, the genetic component is predominant (Borrelli et al 1999; Taghouti et al 2010).

High concentration of carotenoid pigments in semolina does not guarantee a bright yellow color of pasta, because this parameter is also affected by levels of lipoxygenase and polyphenol oxidase activity (Borrelli et al 1999; McDonald 1979). Lipoxygenase catalyzes the addition of molecular oxygen to polyunsaturated fatty acids containing *cis*-*cis*-1,4 pentadiene system, producing free radicals and then after oxygenation, conjugate *cis*-, *trans*- diene hydroperoxydes (Borrelli et al 1999). The level of peroxidation often is use as an indicator of damage to the cell membrane. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is one main parameter for evaluating the extent of membrane oxidation (Al-Ghamadi 2009).

Lipoxygenase activity in durum wheat is cultivar-related and depends on the environment (Borrelli et al 1999). Lipoxygenase is localized primarily in embryo and pericarp (aleurone layer), thus it is removed during milling to some extend (Borrelli et al 1999). Additionally, pasta processing results in a large decrease in lipoxygenase activity (Yemenicioglu and Ercan 1999). During pasta processing, pigment destruction starts with the hydration of semolina and can increase with the increased water absorption, temperature, or oxygen concentration in the mixing environment (Matsuo et al 1970). Lipoxygenase catalyzes the formation of fatty acid radicals

are responsible for oxidative degradation of the carotenoid pigments (Siedow 1991). Lipoxygenase activity was stable during drying at 50°C but was rapidly inactivated at 65°C and 75°C (Yemenicioglu and Ercan 1999).

The main objective of this study was to determine the effect of cultivar and growing environment on grain and semolina quality characteristics, as well as the effects of milling and processing on TYP content, MDA content, and lipoxygenase activity.

Materials and Methods

Plant Material

A field experiment was established near Prosper, North Dakota in 2009, 2010 and 2011. The experiment consisted of five durum wheat cultivars ('AC-Commander', 'Alkabo', 'Divide', 'Mountrail', and 'Rugby'), which were selected based on their differences in TYP content (preliminary unpublished results). Durum cultivars were sown in four blocks (replicates). Each cultivar was seeded at 80 g/plot in eight-3 m rows that were spaced 0.3 m apart. The soil type at Prosper was a Parilla loam. Nitrogen was applied at 23 kg/ha as anhydrous ammonia in 2009 and 2010 and as urea in 2011. Weather data was obtained from the weather station located at the Prosper experiment station (www.ndawn.ndsu.nodak.edu).

In 2009, plots were sprayed for weed control twice. The first application consisted of Prowl® (2.3L/ha) and DiscoverNG® (1.12 kg/ha) and was applied when the durum was in the 3-leaf stage. The second application consisted of Puma® (0.7 kg/ha), Widematch® (0.56 kg/ha), and Harmony Extra® (0.028 kg/ha) applied at the 5-leaf stage. In 2010, the herbicide Wolverine® (0.95L/ha) was applied at 5-leaf stage. In 2011, Wolverine® (0.95 L/ha) and Prowl® (1.12 L/ha) were applied for weed control at the 4-leaf stage. Herbicides were applied

with a tractor-mounted sprayer delivering 94 L/ha at 276 kPa through 8002 nozzles. Field plots were not treated with fungicide or insecticide.

Each plot was harvested using HEGE 140 series plot combine (Hans-Ulrich HEGE GmbH, Waldenburg, Germany) when grain moisture reached approximately 14%. Harvested grain samples were cleaned using a Carter-Day dockage tester (Simon-Carter Company, Minneapolis, MN) that was configured with a number 25 riddle and a number 8 top sieve and a number 2 bottom sieve. Grain was stored at 4°C until time for analyses.

Whole Grain Quality

1000-Kernel weight was determined by counting the number of kernels in 10 g of clean grain, using electronic seed counter (Seedburo Equipment Co., Chicago, IL). Grain was ground on a laboratory hammer mill (model 3100, Perten Instruments, Springfield, IL, USA). Approved methods (AACC International 2010) were used to determine ash content (Method 08-01.01), moisture content (Method 44-15.02), and falling number (Method 56-81.03). Protein content was determined using an Infratec 1226 Whole Grain Analyzer (FOSS Tecator, Sweden).

Semolina Sample Preparation

Durum wheat was milled into semolina using a Quadramat Jr. Mill (C.W. Brabender Instruments, Inc. South Hackensack, NJ, USA) according to Method 26-50.01 (AACC International 2010). Before milling, all samples were tempered in two stages based on grain moisture: (1) tempered to 12.5% moisture for at least 72 hr; and (2) tempered to 15%, 24 hr before milling. Semolina samples were kept at 4°C until time for analysis.

Hydrated/dried (further referred to as 'processed') semolina was prepared by hydrating semolina to 32% and mixed in 10 g bowl mixograph (National Manufacturing, TMCO Division, Lincoln, NE) for 8 min. Hydrated samples were dried on trays in laboratory pasta dryer

(Standard Industries, Fargo, ND, USA) using a high (70°C) temperature drying cycle as described by Yue et al (1999). Once dried, semolina was ground using a Udy cyclone mill (UDY Corp., Boulder, CO, USA) fitted with a 60 mesh sieve and stored in plastic bags at 4°C.

Semolina Quality

Approved methods (AACC International 2010) were used to determine ash content (Method 08-01.01), moisture content (Method 44-15.02), and protein content (Method 39-25.01, adapted for FOSS Infratec 1241 Grain Analzyer (Foss North America, Eden Prairie, MN, USA). Wet gluten and gluten index were determined with the glutomatic instrument (Perten Instruments, Springfield, IL, USA) according to AACC Approved Method 38-12.02 (2010).

Total yellow pigment content was determined using a modified water-saturated n-butanol Method 14-50.01 (AACC International 2010). Water-saturated n-butanol (10 mL) was added to 2 g of whole meal or semolina and shaken 2 min. After resting 30 min, the extracts were filtered through Whatman number 1 filter paper, and absorbance was measured using a spectrophotometer (Beckman Coulter DU 720 General Purpose UV/Vis Spectrophotometer) at a wavelength of 435.8 nm. Measurements per extracted sample were recorded and values averaged and converted to yellow pigment concentration (μg/g) using the extinction coefficient (1.6632) for β-carotene.

Malondialdehyde (MDA) content was determined according to the method described by Al-Ghamdi (2009). Ground samples (1 g) were transferred to a screw-capped plastic tubes and homogenized following addition of 5 mL of trichloroacetic acid-2-thiobarbituric acid-HCl (TCA–TBA–HCl) reagent [15% (w/v) TCA, 0.37% (w/v) TBA, 0.25 M HCl and 0.01% butylated hydroxytoluene (BHT)]. After homogenization, samples were incubated at 90°C for 30 min in a hot water bath, chilled in crushed ice for 10 min and centrifuged at 12,000 x *g* for 10

min. Absorbance of the aliquot was measured at 535 nm and 600 nm against TCA-HCL-BHT reagent. Malondialdehyde reacts with thiobarbituric acid and the resulting chromophore absorbs at 535 nm. Malondialdehyde concentration was calculated directly using the extinction coefficient of 1.55×10^5 mM cm^{-1}.

Lipoxygenase activity in the grain was determined using the method developed by Li and Schwarz (2012). The assay is based on the determination of lipid hydroperoxides by a ferrous oxidation-xylenol orange (FOX) assay. Each analysis was performed in duplicate.

Lipoxygenase extracts were prepared daily. Ground samples (1 g) were extracted at 4°C with 10 mL of 0.1 *M* potassium phosphate buffer, pH=6.6. The mixture was vortex mixed every 15 min for 1 hr. Samples were filtered into a new set of test tubes using Whatman GF/A 90 mm glass microfiber filters. Linoleic acid, substrate for the assay, was prepared by adding 4 mL of 1% Tween 20 and 72 uL of linoleic acid to 25.2 mL of 0.1 M ice-cold phosphate buffer. To eliminate the cloudiness, 0.8 mL of ice-cold 0.1 M NaOH was added to the substrate. Preparation of the ferrous oxidation-xylenol orange (FOX) reagent include mixing 67.3 mg xylenol orange, 98 mg ammonium ferrous sulfate, and 1.39 mL sulfuric acid in 98.6 mL of distilled water for 1 hr. One volume of this concentrated reagent was mixed with nine volumes of 100% methanol solution containing 0.97g/L BHT.

The enzymatic reaction mixture consisted of 110 µL of 0.1 M phosphate buffer (pH=6.6) and 10 µL of whole-wheat extract was mixed in a glass tube, and then 30 µL of freshly prepared linoleic acid substrate was added. The enzymatic reaction ran at 25°C for 5 min. Then, FOX reagent (2.85 mL) was added to the glass tube and immediately mixed. The resultant solution was allowed to stand at room temperature for 10 min, after which, the absorbance at 560 nm was

read against the reaction blank. The reaction blank consisted of whole wheat extract (10 uL) mixed with 110 μL of 0.1 M phosphate buffer (pH 6.6) and 2.85 mL of FOX reagent.

Lipoxygenase activity (U/g)= (Δ560nm/Incubation time) × (Total Volume in Cell/Aliquot Assayed) x 1/EmM x (Extraction Volume/Sample Weight) x Dilution, where EmM (Extinction Coefficient)=47.

Statistical Analysis

The collected data was analyzed using the Statistical Analysis System (SAS) computer packages version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA). Experimental design was a randomized complete block design (RCBD). All treatments had four replications and data collected were subjected to analysis of variance (ANOVA). Cultivar was a fixed effects and year (environment) was a random effect. Means were separated by using Fisher's-protected LSD at P=0.05. F-tests were significant at $P \leq 0.05$.

For each dependent variable, the error mean squares from 2009, 2010, and 2011 were tested for homogeneity of error variance using Bartlett's Chi-square test (Steele and Torrie 1980). Data were pooled together when appropriate. Year main effect could not be statistically evaluated, because years cannot be replicated.

Results and Discussion

In 2009, a cool wet spring led to water-saturated fields that delayed planting (Table 1, page 36). Grain harvest in 2009 was delayed until September 4. In 2010, durum was planted on April 19 and harvested on August 10, which is within the normal time frame for ND. Average minimum air temperatures and rainfall were within normal limits. Saturated fields and low soil temperature delayed planting in 2011 until the end of May. Grain was harvested on August 22.

Average minimum air temperature in 2009 was generally lower than the 30 year average, with a lower than normal amount of precipitation (Table 2, page 38). Average minimum air temperature in 2011 was generally higher than the 30 year average. Rainfall was higher than normal.

Averaged daily maximum and minimum air temperatures after anthesis were generally higher in 2011 than in 2009 or 2010 (Table 2, page 38). Rainfall was greatest in 2011 and least in 2009 (Figure 4, page 41). Thus, 2009 was cool and dry during grain fill; 2010 had average temperature and rainfall during grain fill; while 2011 was hot and wet during grain fill. Surface and subsoil moisture was adequate during grain fill period in 2009 and 2010 and was adequate to surplus in 2011 (NASS 2009, 2010, 2011). Heavy disease pressure was noted due to the wet, hot conditions in 2011 (personal observations).

Whole Grain Quality

The year x cultivar effect was significant for 1000-KWT, total protein content, total ash content, and falling number (FN) (Table 23). However, the interactions were significant due to magnitude as all five cultivars showed similar pattern over the years (Figures A-9).

The lowest 1000-KWT was in 2011 (18.9 g) as compared to 36.2 g in 2010 and 35.6 g in 2009. Means for 1000-KWT in 2009 and 2010 were typical of those commonly reported for durum wheat. However, means for 1000-KWT in 2011 were lower than normal (North Dakota Wheat Commission 2009, 2010, and 2011).

These data reflect the environmental conditions during grain filling, where the maximum and minimum air temperature and rainfall were below average in 2009, were average in 2010, and were above average in 2011.

Table 23. Analysis of Variance for Whole Grain Characteristics Averaged Over Years

Parameter	Sources of variation	Df	MS	*F* value	
1000-KWT, g	Year	2	1922.62	630.89	
	Rep (Year)	9	4.48	1.47	
	Cultivar	4	17.92	1.63	ns
	Year*Cultivar	8	10.96	3.60	**
	Error	36	3.04		
Ash content, mg/kernel	Year	2	0.10731	45.14	
	Rep (Year)	9	0.00238	1.81	
	Cultivar	4	0.00284	1.32	ns
	Year*Cultivar	8	0.00214	1.64	ns
	Error	36	0.00131		
Total ash content, %	Year	2	5.29358	378.10	
	Rep (Year)	9	0.01400	1.93	
	Cultivar	4	0.05302	1.83	ns
	Year*Cultivar	8	0.02891	3.98	**
	Error	36	0.00727		
Protein content, mg/kernel	Year	2	26.32	536.31	
	Rep (Year)	9	0.08	1.65	
	Cultivar	4	0.67	10.55	**
	Year*Cultivar	8	0.06	1.29	ns
	Error	36	0.05		
Total protein content, %	Year	2	71.12	367.12	
	Rep (Year)	9	0.19	1.64	
	Cultivar	4	4.21	2.14	ns
	Year*Cultivar	8	1.96	16.56	**
	Error	36	0.12		
Falling number, sec	Year	2	43062.71	74.18	
	Rep (Year)	9	2243.90	3.87	
	Cultivar	4	14281.82	4.71	*
	Year*Cultivar	8	3030.65	5.22	**
	Error	36	580.55		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

Duration of grain filling (time from anthesis to physiological mature) was 32 days in 2009, 21 days in 2010, and 18 days in 2011. Lower 1000-KWT in 2011 can be attributed to the higher than normal night temperatures during grain development that shortened the duration of grain filling (Spiertz et al 2006).

The amount of ash per kernel was greatest in 2010 (0.62 mg/kernel), followed by 2009 (0.54 mg/kernel) and 2011 (0.47 mg/kernel), reflecting a shortened period for grain filling in 2011, thus limited mineral accumulation. Conversely, total ash content averaged across cultivars was highest in 2011 (2.50%), followed by 2010 (1.71%) and 2009 (1.53%), reflecting negative effect of hot and wet growing conditions in 2011 on grain weight and size. The same weight of grain contains a higher number of kernels when they are small and shriveled, thus greater number of kernels per weight in our case overcame the lower ash content per kernel resulting in higher wt/wt (%) ash content in 2011.

The cultivar main effect was significant for protein per kernel (Table 23). Divide (4.9 mg/kernel) had the highest protein content per kernel while Mountrail had the lowest protein content per kernel (4.3 mg/kernel) (Figure 16).

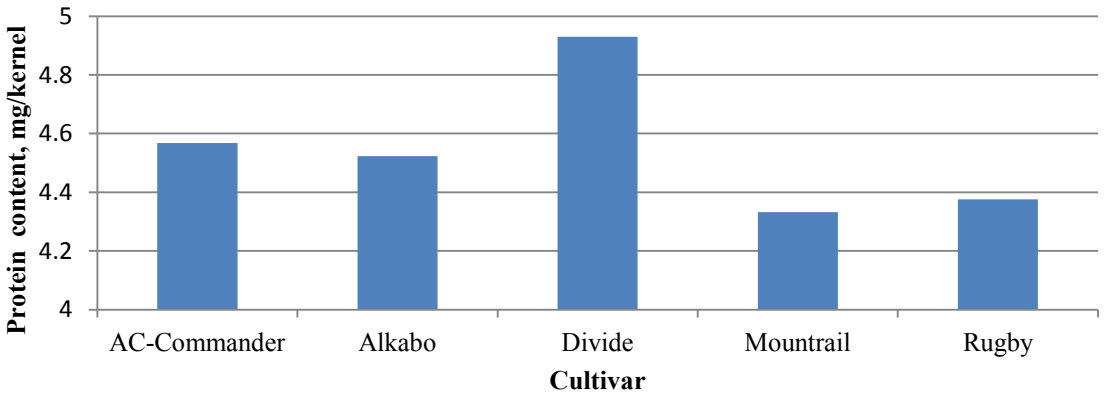

Figure 16. Cultivar main effect on protein content per kernel averaged over years. $LSD_{0.05}$=0.2.

The amount of protein per kernel was greatest in 2009 (5.5 mg/kernel), followed by 2010 (4.9 mg/kernel) and 2011 (3.3 mg/kernel). However, total protein content averaged across cultivars was highest in 2011 (17.4%) followed by 2009 (15.4%) and 2010 (13.6%). The percentage of kernel that was protein was greater in 2011 because the kernel weight was low. High temperature and disease pressure reduced grain filling period and photosynthesis, resulting in lower per kernel protein. The weight of protein per kernel was greatest in 2009 which reflects the relatively long period of grain filling (Table 2, page 38).

The cultivar main effect was significant for FN (Table 23). AC-Commander (428 sec) had the greatest mean FN, while Rugby (353 sec) had the lowest mean FN (Figure 17). Falling number was greater in 2011 (420 sec) and 2009 (411 sec) than in 2010 (335 sec; $LSD_{0.05}$=34). These FN are within the acceptable range for durum wheat used to make pasta.

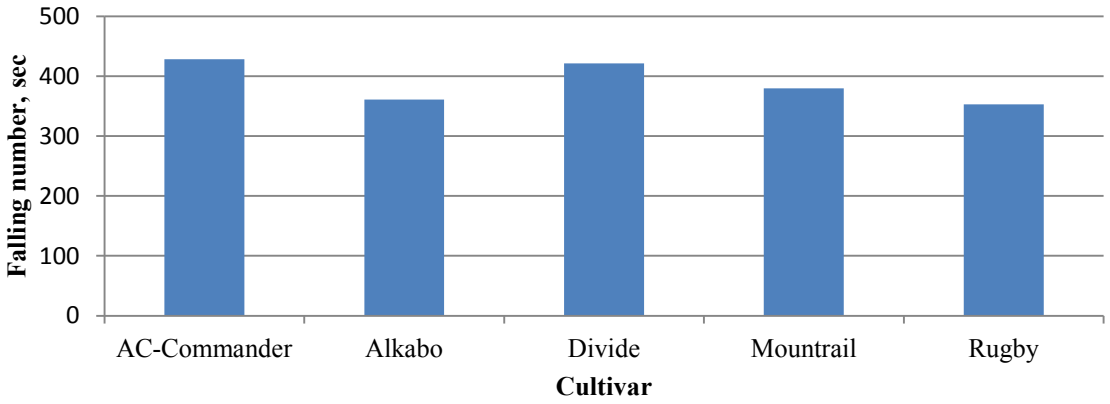

Figure 17. Cultivar main effect on falling number averaged over years. $LSD_{0.05}$=52.

Falling number is an indicator of sprouting damage to the semolina due to elevated levels of α-amylase activity. According to Matsuo et al (1982), high amylotic activity, determined by FN test (FN below 100 sec), in spaghetti increases the amount of residue in the cooking water and tends to give slightly softer cooked pasta. Donnelly (1980) reported that severe checking

and cracking of spaghetti samples processed from severely sprouted wheat (FN below 120 sec) occurred after one month of storage.

Semolina Quality

The year x cultivar interaction was significant for semolina quality parameters, such as total semolina protein, total semolina ash content, gluten index, and wet gluten (Table 24). However, the interactions for total semolina protein content, total semolina ash content, and wet gluten were due to magnitude as the response of all five cultivars were similar (Figure A-10).

Protein content averaged across cultivars was highest in 2011 (15.1%), followed by 2009 (13.1%) and was lowest in 2010 (12.0%). Ash content averaged across cultivars was highest in 2011 (1.40%), followed by 2010 (0.92%) and was lowest in 2009 (0.77%).

The wet gluten (WG) content is directly correlated to the grain protein. Any increase in total protein content of the flour will result in a gluten content increase (Perten et al 1992). Ratio between WG content and grain/semolina protein (P) content showed that WG/P ratio is considered as an indicator of wet gluten production per protein unit. Wet gluten averaged across cultivars was highest in 2011 (56.9%), followed by 2010 (34.5%) and 2009 (32.1/%)

Year x cultivar interaction was significant for Gluten index. Gluten index did not significantly change over the years in Rugby and was 3.3, 3.5, and 3.9 in 2009, 2010, and 2011, respectively. There was no significant difference in gluten index values for AC-Commander from 2009 (97.5) and 2010 (95.2) and Divide from 2009 (92.1) and 2010 (92.9). However, both cultivars showed significant reduction in gluten index in 2011. In 2011, gluten index for AC-Commander was 44.4, and for Divide it was 39.5. Alkabo and Mountrail showed significant reduction in gluten index values from 2009 to 2010, and further reduction in 2011 (Figure 18).

Table 24. Analysis of Variance for Semolina Quality Parameters Averaged Over Years

Parameter	Sources of variation	Df	MS	*F* value	
Protein content, %	Year	2	49.38	505.35	
	Rep (Year)	9	0.15	1.51	
	Cultivar	4	3.09	1.90	ns
	Year*Cultivar	8	1.63	16.65	**
	Error	36	0.10		
Ash content, %	Year	2	2.157	914.54	
	Rep (Year)	9	0.004	1.59	
	Cultivar	4	0.038	1.37	ns
	Year*Cultivar	8	0.027	11.73	**
	Error	36	0.002		
Wet gluten, %	Year	2	3727.39	435.03	
	Rep (Year)	9	10.41	1.22	
	Cultivar	4	483.36	0.98	ns
	Year*Cultivar	8	492.38	57.47	**
	Error	36	8.56		
Gluten index	Year	2	6775.66	381.84	
	Rep (Year)	9	30.48	1.72	
	Cultivar	4	11721.00	15.19	**
	Year*Cultivar	8	771.49	43.48	**
	Error	36	17.74		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

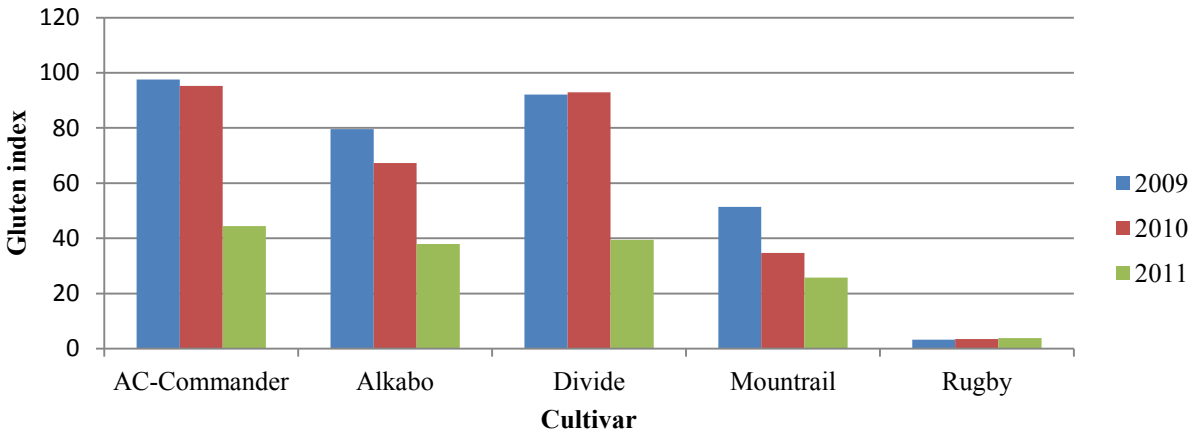

Figure 18. Effect of year x cultivar interaction on gluten index in 2009, 2010, and 2011.

Elevated temperatures in 2011 during grain development increased total grain protein content, and thus wet gluten content, through reduction in starch deposition (Stone and Nicolas 1998; Gooding et al 2003). Several investigations have documented that short periods of heat stress (daily mean temperatures above 35°C) during grain filling caused reduced size of glutenin polymers and weakened gluten strength. Exposure to heat stress decreases synthesis of glutenin, while synthesis of gliadins remains stable or increases (Majoul et al 2003).

Effect of Milling and Processing

The cultivar main effect was significant for initial total yellow pigment (TYP) content (ppm) in wheat (Table 25). Highest TYP content, averaged over the years, was in AC-Commander (10.0 ppm) and Alkabo (9.5 ppm) while lowest in Mountrail (7.3 ppm) and Rugby (6.8 ppm, $LSD_{0.05}$=1.1). Total yellow pigment content (ppm) averaged across cultivars was highest in 2011 (11.2 ppm), followed by 2010 (7.7 ppm) and 2009.

Total yellow pigment content was lower in semolina than in the kernel. The year x cultivar interaction was significant for the loss of TYP after milling (Table 25).

Table 25. Analysis of Variance for the Effect of Milling and Processing on Total Yellow Pigment Content (ppm) Averaged Over Years

Parameter	Sources of variation	Df	MS	*F* value	
TYP content, ppm	Year	2	125.20	120.41	
	Rep (Year)	9	0.50	0.63	
	Cultivar	4	23.37	17.45	**
	Year*Cultivar	8	1.34	1.67	ns
	Error	36	0.80		
Loss of TYP with milling, %	Year	2	238.44	14.75	
	Rep (Year)	9	66.01	4.08	
	Cultivar	4	560.57	8.49	**
	Year*Cultivar	8	66.05	4.09	**
	Error	36	16.17		
Loss of TYP, with processing, %	Year	2	243.78	62.63	
	Rep (Year)	9	10.89	2.80	
	Cultivar	4	189.29	8.26	**
	Year*Cultivar	8	22.91	5.89	**
	Error	36	3.89		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

In Alkabo, AC-Commander, Mountrail, and Rugby, the loss of TYP content after milling was lowest in 2011 and highest in 2009. However, in Divide, the loss of TYP was highest in 2011 (25.1%) as compared to 2009 (20.9%) and 2010 (15.3%) (Figure 19). The greatest loss of TYP content was in Mountrail (29%) while the smallest loss occurred with AC-Commander (12%)

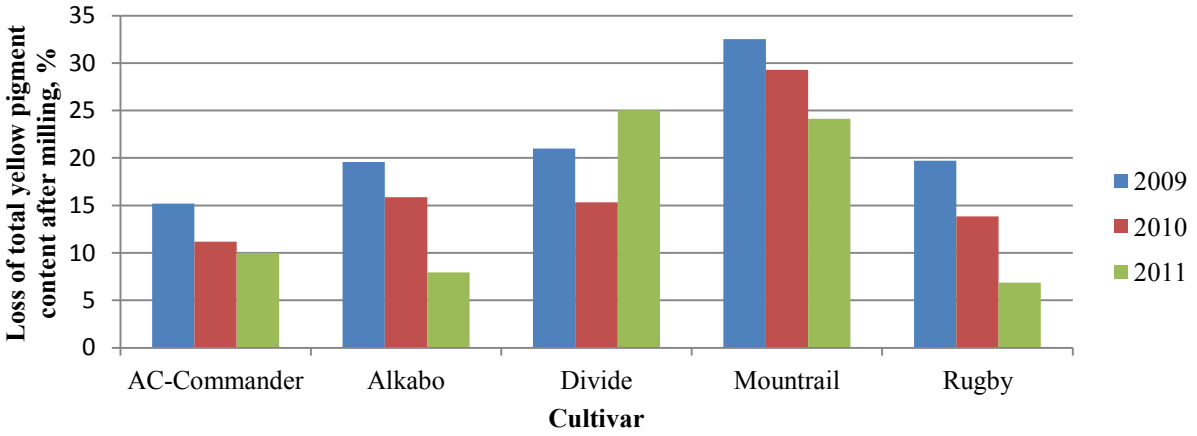

Figure 19. Effect of year x cultivar interaction on loss of total yellow pigment content after milling in 2009, 2010, and 2011.

The loss of total yellow pigment (TYP) in whole grain after milling reported by Singh (2008) varied from 2.3 to 20% depending on cultivar, year, and location. The mean values of β-carotene content loss in grain after milling varied from 3.3 to 6.6% for the group of durum cultivars with high β-carotene content and 4.0 to 16.2% for the group of durum cultivars with low β-carotene content (Borrelli et al 1999).

The year x cultivar interaction was significant for the loss of TYP content with processing (Table 25). The loss of TYP content with processing in Divide, Mountrail, and Rugby did not differ significantely in 2009 as compared to 2010 (Figure 20). The loss of TYP content in Alkabo was greater in 2010 (15.7%) than in 2009 (10.2%), while in AC-Commander the loss of TYP content with processing was greater in 2009 (9.7%) than in 2010 (4.3%). Loss of TYP content with processing was generally less in all cultivars in 2011 as compared to 2009 and 2010 (Figure 20).

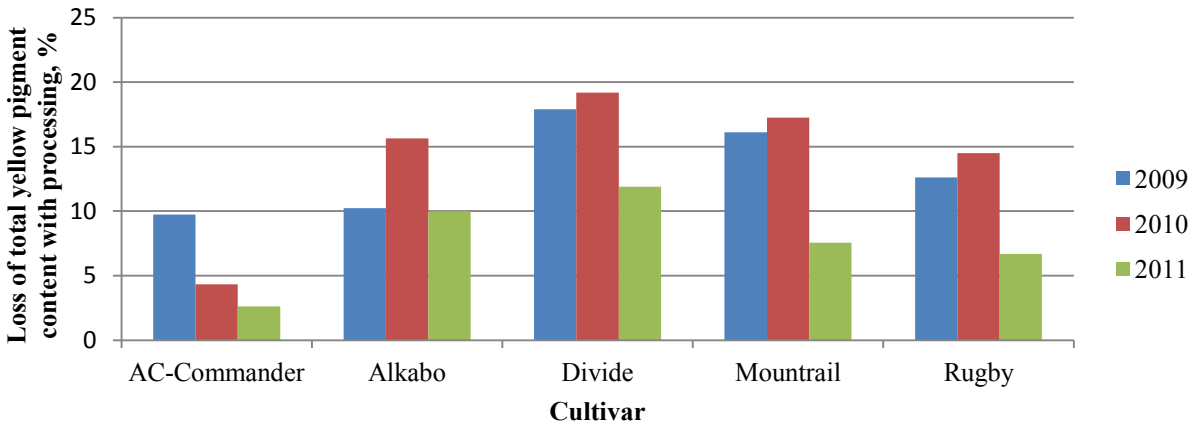

Figure 20. Effect of year x cultivar interaction on loss of total yellow pigment content with processing in 2009, 2010, and 2011.

The carotenoid pigments in durum wheat kernel are not distributed homogeneously. Embryo contains high levels of pigment, followed by pericarp and endosperm (Panfili et al 2004). Thus during milling, a variable amount of yellow (carotenoid) pigments are removed with the pericarp and embryo (Borrelli et al 1999). In addition, a significant amount of carotenoid pigments are bleached during hydration step due to LOX activity (Borrelli et al 2003) and thermal stress during high temperature drying. Destruction of carotenoid pigment occurs after semolina is hydrated and is enhanced by increased temperature and oxygen concentration in the mixing environment (Matsuo et al 1970). Unsaturated fatty acids react with LOX to generate hydroperoxide, which in turn oxidizes the pigment.

Year x cultivar interaction was significant for LOX activity in whole grain (Table 26); however, the interaction was due to magnitude (Figure A-7). The cultivar main effect was significant for LOX activity. Lipoxygenase activity, averaged over the years was highest in Divide (0.71 U/g) and lowest in Mountrail (0.48 U/g; $LSD_{0.05}$=0.24). Little or no LOX activity detected in AC-Commander in whole grain or semolina. Lipoxygenase activity averaged across cultivars was highest in 2010 (0.57 U/g) and 2009 (0.55 U/g) while lowest in 2011 (0.25 U/g).

The loss of LOX activity with milling was highest in Alkabo (82.6 %), followed by Mountrail (81.3 %), and Divide (79.6 %), while the least loss of LOX activity with milling was observed in Rugby (65.4%; $LSD_{0.05}$=4.9). Because AC-Commander had little or no detectable LOX activity, data for LOX activity in AC-Commander was not analyzed.

Experiments with wheat flour showed that LOX activity is relatively high in flours where the embryo and pericarp are maintained together with the endosperm (Rani et al 2001). This is due to the fact that both the pericarp and the embryo are rich in LOX. Wheat embryo and wheat pericarp were 17 and four times higher in lipoxygenase activity respectively as compared to endosperm (Rani et al 2001). There was no LOX activity determined in semolina after processing. Lipoxygenase activity was reported to be stable at 50°C; however it was inactivated at 65°C to 75°C (Yemencioglu and Ercan 1999).

Year x cultivar interaction was significant for MDA content in whole grain (Table 26). However, the interaction was due to magnitude (A-1). Malondialdehyde content was highest in 2009 and 2011 (3.1 nmol/g) and lowest in 2010 (2.9 nmol/g). Year x cultivar interaction was significant for the loss of MDA content with milling (Table 26). The MDA content loss was greatest in Divide in 2010 and 2011 as compared to 2009, whereas in Mountrail and Alkabo the greatest loss of MDA content during milling was observed in 2011 as compared to 2009 or 2010 (Figure 21). MDA content loss with milling of AC-Commander did not vary with year.

The year x cultivar interaction and cultivar main effect were not significant for the increase of MDA content after processing (Table 26). However, MDA content was 22.7% higher after processing. These data indicate a high level of lipid oxidation occurred during processing. These samples were processed without a vacuum. Typically, pasta processing is

done under vacuum so as to reduce the oxygen available for LOX activity and subsequent membrane damage as detected by MDA content.

During pasta processing, pigment destruction starts with the hydration of semolina and increases with the increased water absorption and oxygen concentration. Lipoxygenase catalyzes the oxidation of polyunsaturated fatty acids by molecular oxygen, producing, firstly, free radicals and, subsequently, after oxygenation, conjugate *cis*, *trans*- diene hydroperoxides. Lipid hydroperoxides are converted to MDA under the conditions of the thiobarbituric acid (TBA) test. Thus, the TBA test measures both endogenous MDA and MDA derived from lipid hydroperoxides. Radicals, however, are responsible for degradation of pigments, such as β-carotene, xanthophylls and chlorophylls. β-Carotene also serves as inhibitor of LOX activity, preventing semolina bleaching and improving pasta quality. Thus, the loss of TYP content due to the bleaching of carotenoid pigments during processing as well as lipid peroxidation manifested by increased MDA content is likely due to increased activity of LOX during mixing. Lipoxygenase activity was not detected after drying because high temperature during drying probably denatured the LOX.

Table 26. Analysis of Variance for the Effect of Milling and Processing on Lipoxygenase Activity (U/g) and Malondialdehyde Content (nmol/g) Averaged Over Years

Parameter	Sources of variation	Df	MS	*F* value	
LOX activity in grain, U/g	Year	2	0.675	11.72	
	Rep (Year)	9	0.013	0.62	
	Cultivar	4	0.939	14.36	**
	Year*Cultivar	8	0.065	3.21	**
	Error	36	0.020		
Loss of LOX activity with milling, %	Year	2	1.16	0.03	
	Rep (Year)	9	36.47	0.60	
	Cultivar	3	768.11	31.41	**
	Year*Cultivar	6	24.46	0.40	ns
	Error	27	65.01		
MDA content in grain, nmol/g	Year	2	0.251	7.24	
	Rep (Year)	9	0.035	2.76	
	Cultivar	4	0.045	0.95	ns
	Year*Cultivar	8	0.048	3.81	**
	Error	36	0.012		
Loss of MDA content with milling, %	Year	2	155.10	7.90	
	Rep (Year)	9	88.29	4.50	
	Cultivar	4	13.92	0.18	ns
	Year*Cultivar	8	77.75	3.96	**
	Error	36	19.64		
Increase of MDA content with processing, %	Year	2	418.67	2.83	
	Rep (Year)	9	308.31	2.08	
	Cultivar	4	212.25	1.77	ns
	Year*Cultivar	8	119.73	0.81	ns
	Error	36	148.04		

* and ** Significant at P=0.05 and P=0.01, respectively; ns = non-significant; Df = degree of freedom; and MS=mean square.

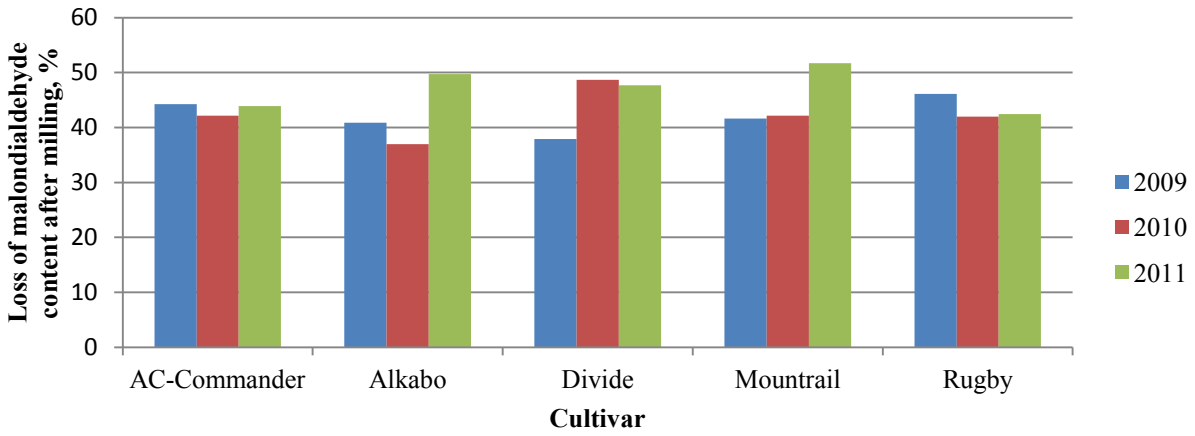

Figure 21. Effect of year x cultivar interaction on loss of malondialdehyde content after milling in 2009, 2010, and 2011.

Conclusions

Grain and semolina quality characteristics varied greatly with cultivar and environment. Environment had a large effect on grain weight, protein and ash content. Unfavorable growing conditions of 2011 resulted in a low grain weight, high total protein content but weak gluten in semolina. Cultivar and environment had an effect on TYP content and oxidative stress in semolina and pasta. During milling, TYP content, LOX activity and MDA content were reduced as a result of the removal of embryo, pericarp, and aleurone layer, where the main levels of carotenoid pigment and LOX activity are located. After processing TYP content declined, while MDA content increased and there was no LOX activity detected in the samples.

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PAPER 3. MORPHOLOGICAL CHANGES IN DURUM WHEAT CARYOPSIS DURING GRAIN DEVELOPMENT

Abstract

Stereomicroscopy and scanning electron microscopy (SEM) were used to observe anatomical and morphological changes that occurred as durum wheat kernel/caryopsis progressed from anthesis to physiological maturity to final desiccation. Changes were observed via SEM in vascular tissue, aleurone layer, endosperm, and embryo of durum caryopsis collected from field grown plants at 7, 11, 15, 18, 20, 25, and 28 days after anthesis (DAA). Stereo-microscopy showed images of fresh kernels collected from greenhouse grown plants at 7, 11, 14, 21, 25, 32, and 39DAA with emphasis on cross section and longitudinal section of kernel. The purpose of this work was to relate the existing studies on wheat seed formation and development processes and the images taken with scanning electron microscope and light stereomicroscope.

Introduction

Meiosis generates the production of pollen in the anthers and the embryo sac in the carpel. It occurs during the boot stage, when the head is fully developed and can be easily seen in the swollen section of the leaf sheath below the flag-leaf. In wheat, meiosis starts in the middle of the spike, continuing later above and below this zone (Zadocks et al 1974). Depending on the environment, anthesis (flowering/pollen shed) occurs within four to seven days after the spike emerges from the flag-leaf sheath. Flowering begins in the central part of the spike and continues towards the basal and apical ends (Kirby 1974).

The development of endosperm results from double fertilization, a fertilization of two polar nuclei in the central cell of the embryo sac by one sperm cell nucleus, which generates a triploid nucleus (3n) (Nadaud et al 2010). Whereas the diploid (2n) embryo or zygote originates

from fertilization of the egg cell by the second sperm cell nucleus (Sabelli and Larkins 2009). The primary endosperm cell then exhibits syncytial growth (nuclear division without cell wall formation), followed by cellularization (formation of cell wall resulting in single nucleus cells), and cell differentiation.

The differentiation stage includes the formation of four major cell types: transfer cells, aleurone, starchy endosperm, and cells surrounding the embryo. After mitosis and endo-reduplication, the endosperm starts accumulating storage compounds: proteins and starch (Olsen 2001). The transfer cells differentiate very early and are observed over the nucellar projection, bordering the endosperm cavity (Thompson et al 2001). The aleurone cells form a peripheral layer around the starchy endosperm, except in the transfer cell region. The differentiation of the aleurone cells is independent from that of the transfer cells (Becraft 2001). The maturation phase of wheat grain includes programed cell death, dormancy, and desiccation. At harvest, only the embryo and aleurone layer contain living cells. The pericarp layer and endosperm, excluding aleurone layer, are not living.

This paper will pictorially show morphological changes that wheat grain undergoes during early development and maturation in the light of recent advances in our understanding of physiological processes during seed formation.

Materials and Methods

Kernels for scanning electron microscopy were selected from the spikes of 'Alkabo' durum wheat that was grown in field plots in 2010. Spikes were collected at 7, 11, 15, 18, 20, 25, and 28 days after anthesis (DAA) and were air-dried at room temperature, threshed and stored at 4°C until used.

For stereomicroscopy, fresh kernels of Alkabo durum wheat were obtained from the spikes of plants grown in the greenhouse. The spikes were cut 15 minutes before use. Spikes were collected at 7, 11, 14, 21, 25, 32, and 39DAA.

Scanning Electron Microscopy

Scanning electron microscopy was conducted at the Electron Microscopy Center located at North Dakota State University, Fargo, ND. Selected kernels were divided into two equal halves with a razor blade. Halves of the kernels with the embryo attached were fixed to the aluminum mounts and sputter coated with the palladium using a Balzer SCD030 sputter coater (Anatech Ltd, Virginia). Images were obtained using JEOL JSM-6300 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Stereomicroscopy

Stereomicroscopy was conducted at the Electron Microscopy Center located at North Dakota State University, Fargo, ND. Each kernel was cross and longitudinal sectioned using razor blade. Images were obtained using Olympus SZH stereomicroscope (Olypmus Optical Co. Ltd. Tokyo, Japan) with Nikon Coolpix 5700 digital camera (Nikon Corporation, Nikon, Japan).

Results and Discussion

In cereals, the development of coenocytic endosperm results from the triple fusion of one sperm nuclei and two polar nuclei, and, within 0-1 day after anthesis leads to syncytium (cellular) formation (Bennet et al 1975). Cells in the endosperm exhibit three stages of development: syncytial growth, cellularization, and cell differentiation (Olsen 2001). During syncytial growth (1-2 days), nuclear division occurs without cell wall formation. Syncytial growth results in peripheral multinucleated cells. Grain cellularization stage occurs within 3-6DAA is associated with cell wall formation and intense mitotic activity, which lasts about 10

days and proceeds centripetally until the central cell cavity is completely filled with cells (Figures 23 and 24). During mitotic activity in durum wheat caryopsis, the enlargement of the structure results mainly from influx of water. Grain mass is correlated with the number of endosperm cells at the end of cell division, that ceased at 15-20DAA (Jenner et al 1991; Nadaud et al 2010). Lastly, is the cell differentiation stage, which begins 7-8DAA and lasts for 10-12 days (Olsen 2001; Sabelli 2009).

The cell differentiation stage includes the formation of four major cell types: transfer cells, aleurone, starchy endosperm, and cells surrounding the embryo. There is rapid accumulation of storage material in starchy endosperm. Kernel maturation follows differentiation stage. Accumulation of material in endosperm slows and finally reaches physiological maturity followed by desiccation (Olsen 2001).

Transfer Cells

Transfer cells develop over the main vascular tissue of the maternal plant (Figures 22-24). Transfer cells are involved in transfer of photosynthate (sucrose and amino acids) from the maternal tissues to the developing endosperm (Robert et al 2011). In wheat transfer cells are located over the nucellar projection (Thomson et al 2001).

Aleurone Cells

In wheat, aleurone layer is composed of single layer of living cells that cover the entire perimeter of the outer starchy endosperm except for the transfer cell region (Figures 24, 25A,B) (Brouns et al 2012). Botanically aleurone layer is a part of endosperm; however, millers consider aleurone layer as a part of the pericarp and remove it from the endosperm with the grain outer layers.

Olsen (2001) stated that aleurone cell differentiation from the outer layer of endosperm begins after completion of the cellularization process, which is between 6 and 10 days after anthesis (Figure 24). During grain development, aleurone layer accumulates high levels of phytic acid, which chelated several minerals. Intercellular medium of aleurone cells is characterized by high amounts of protein, minerals, B vitamins and lipid compounds (Buri et al 2004). Mature aleurone cells appear cuboidal (Figures 24 and 25). The function of aleurone cells in germinating seed is to produce cell wall degrading, proteolytic, and hydrolytic enzymes to convert the storage protein and starch granules of the endosperm into sugars and amino acids to uptake by the growing embryo (Hoecker et al 1999).

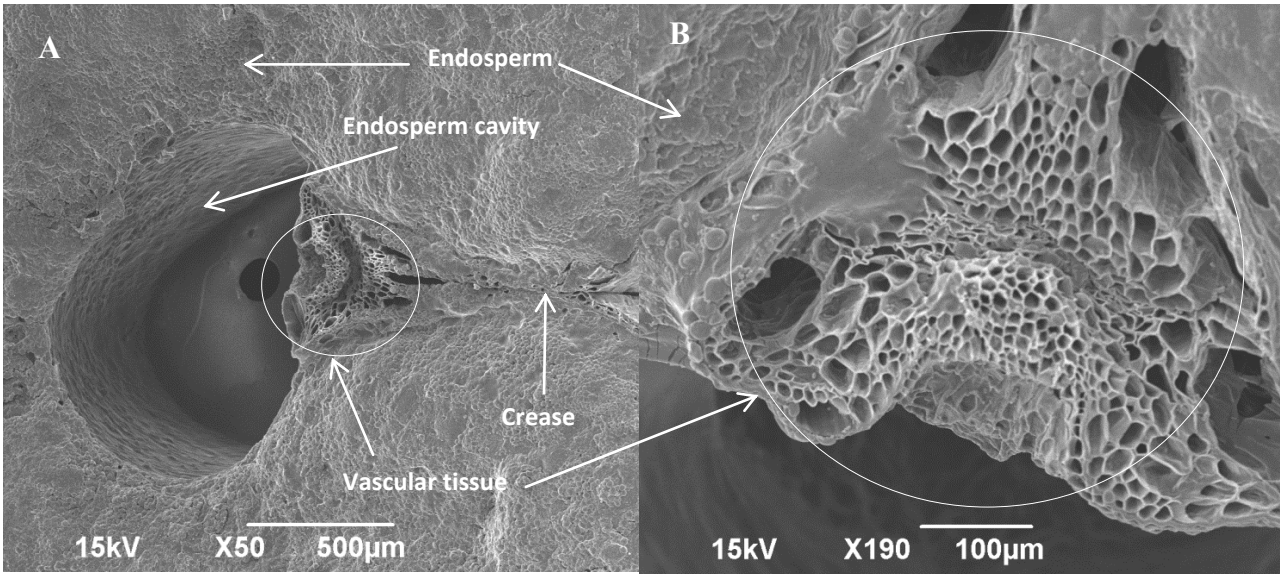
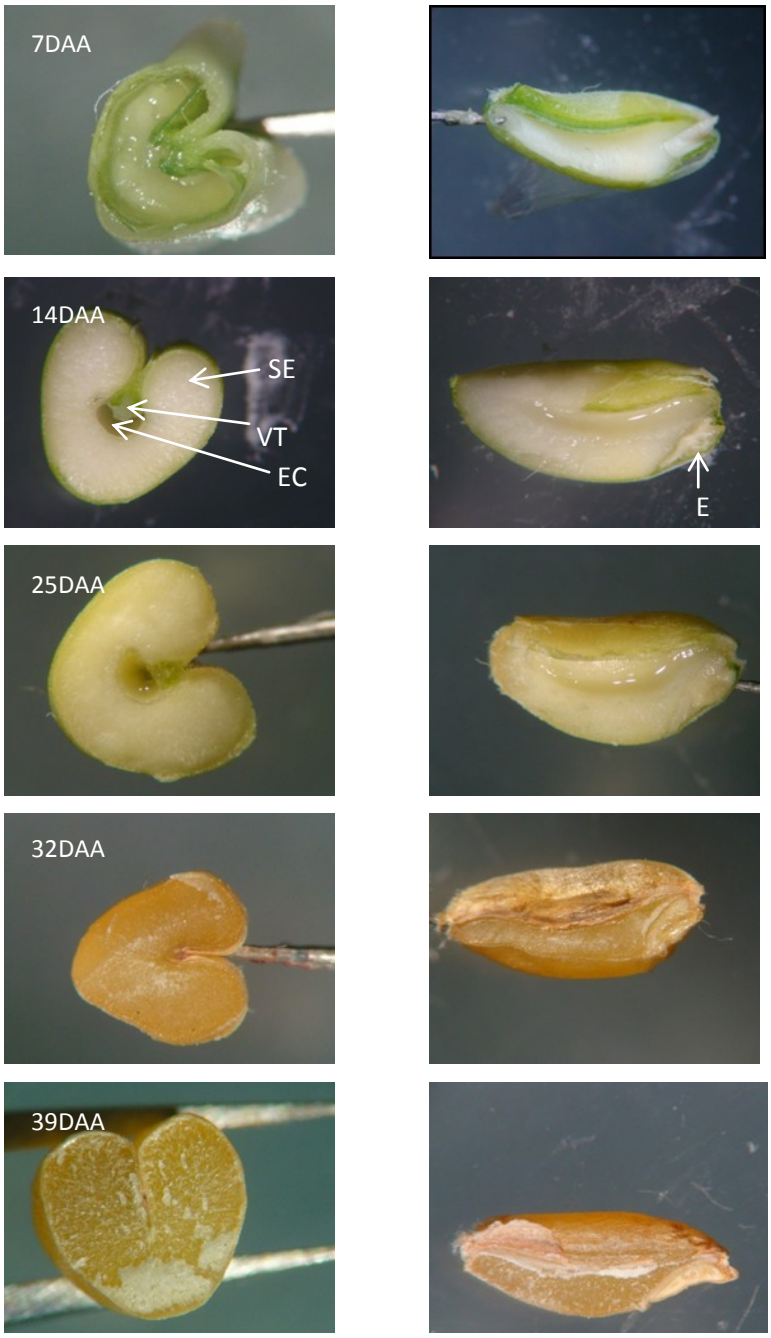


Figure 22. Scanning electron microscopy images of vascular tissue by the crease at 50x (A) and 190x (B).



Watery stage: Grain enlargement is mainly due to the influx of water.

Milk stage: the endosperm has started development and has a milky liquid consistency. The embryo is visible.

Soft dough stage: the endosperm has a floury paste consistency. The embryo is 1-3 mm long.

Hard dough: The endosperm appears as thik bread dough.

Mature: the kernel attained maximum dry matter accumulation and cannot be easliy mashed between the fingers. Kernels generally have 30-35% moisture when mature but must dry to 12-14% before storage.

Figure 23. Light microscopy images of the cross (left), and longitudinal (right) sections of fresh durum wheat seed at different periods of time after anthesis (down). The images show little cell differentiation at 7DAA. Grain filling begins at approximately 11DAA until about 25DAA with maturation and dissection afterwards (32 and 39DAA). Abbreviation: SE= starchy endosperm, VT=vascular tissue, EC=endosperm cavity, E=embryo.

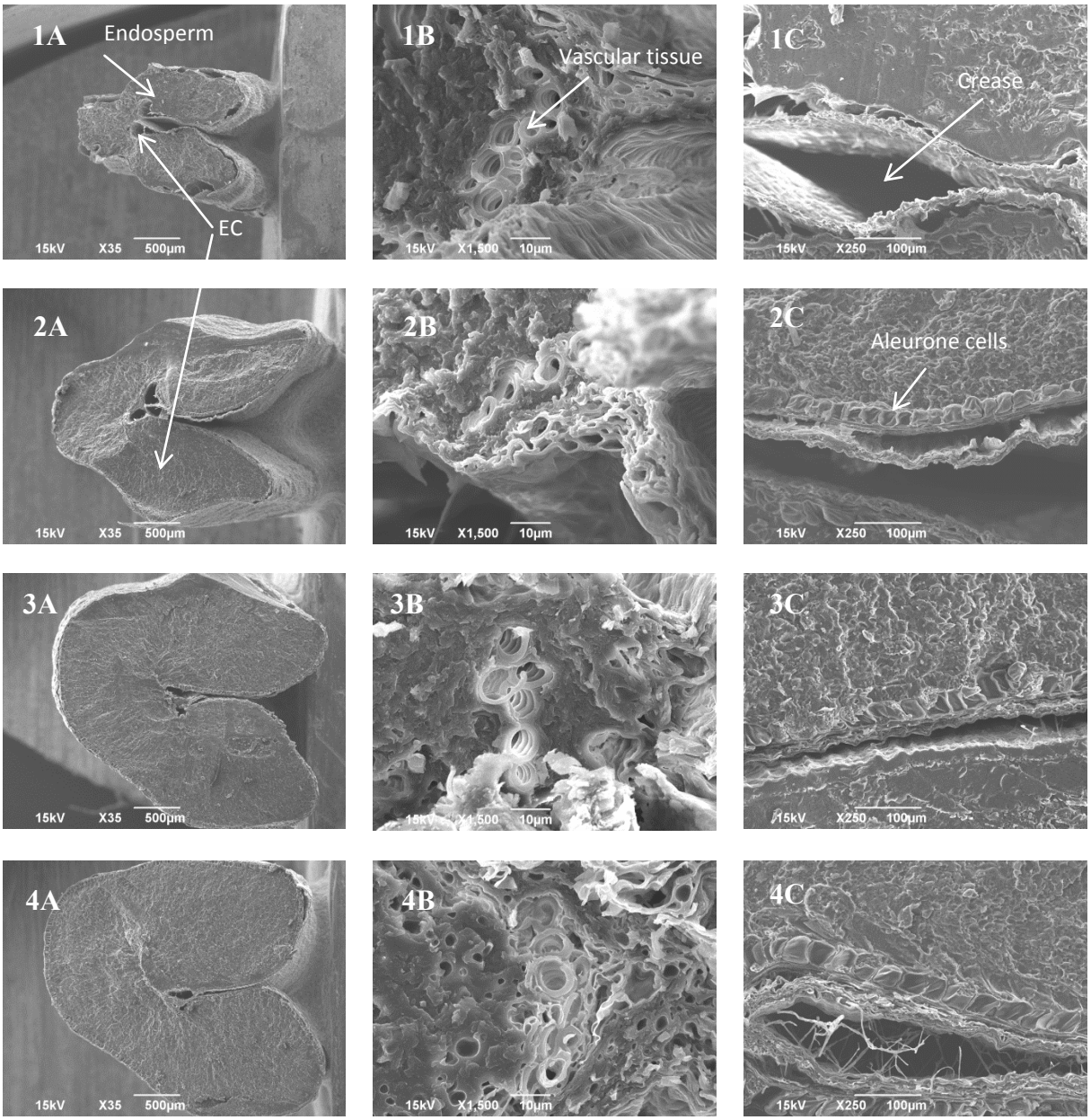


Figure 24. Scanning electron microscopy images of Alkabo kernel during development. Cross section at 35x (left), vascular tissue right above the crease at 1,500x (middle), aleurone layer along the crease at 250x (right) at different sampling times (down), that are 7, 15, 20, 28 days after anthesis (DAA), respectively. Grain cellularization stage with cell wall formation and intense mitotic activity proceeds centripetally until the central cell cavity is completely filled with cells (1A-4A). Endosperm cavity (EC) disappears as grain filling progresses (1A-4A). Column B images show formation of vascular tissue. Column C shows development of aleurone layer. Aleurone layer is not visible on the image 1C (7DAA) but become more pronounced with time (1C-4C). Olsen (2001) stated that aleurone cell differentiation from the outer layer of endosperm begins after completion of the cellularization process, which is between 6 and 10DAA.

Starchy Endosperm

Grain filling involves the deposition of starch and protein in the cells formed during the grain enlargement phase. For a time grain filling stage overlap with grain enlargement stage as grain filling begins 10-15 days after anthesis and lasts 20-30 days, depending on the environmental conditions. Starch is deposited in amyloplasts in the form of granules and

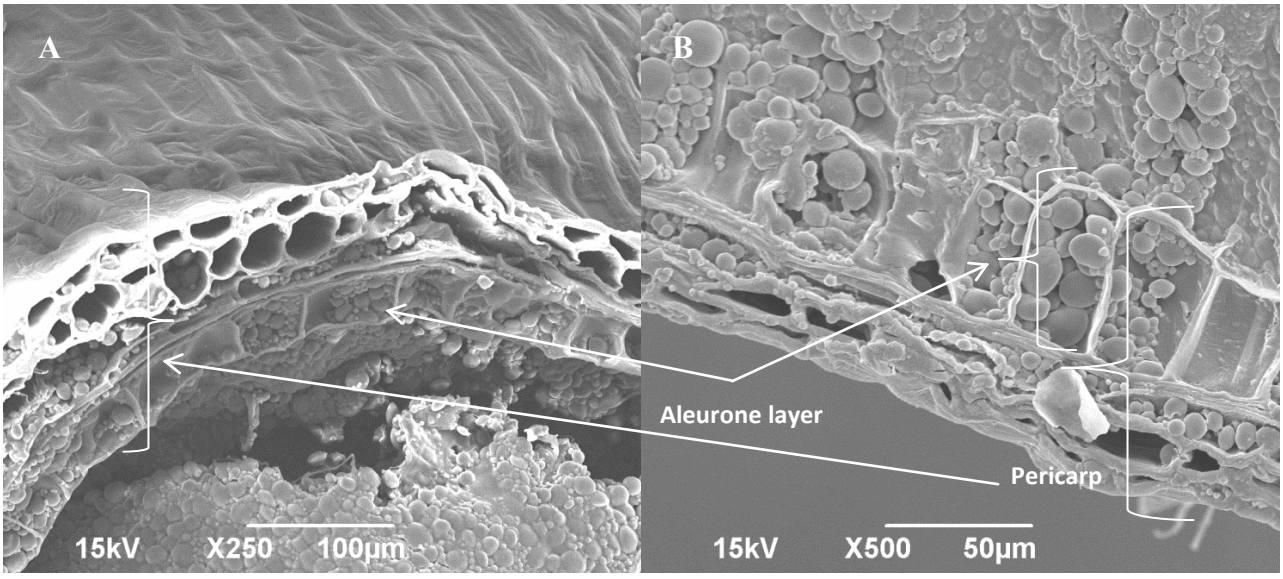


Figure 25. Scanning electron microscopy images of pericarp tissue at 250x (A) and 500x (B). Wheat pericarp consists of epidermis, hypodermis, cross cells, tubes cells, seed coat (testa), nucellar tissue, and aleurone layer. At maturity pericarp layers become fused and indistinguishable. However, aleurone layer can be well identified. In wheat the diameter of aleurone cells is 20-75 µm (Brouns et al 2012).

comprises approximately 70% w/w of the endosperm in mature wheat (Sissons 2008). Depending on the type of amyloplasts, there are three types of starch granules formed in endosperm (Figure 26). The A-type granules formed during the first week after anthesis, followed by smaller B-type granules that are synthesized during the second week after anthesis, while the C-type granules developed during the third week after anthesis (Bechtel and Wilson 2003).

Storage protein is located within membrane-bound spherical bodies. These bodies, typically 0.5-1.5 μm in diameter, derived from rough endoplasmic reticulum and the Golgi apparatus (Kim et al 1988). Protein deposition begins at about 10 days after anthesis and continues until physiological maturity (Jenner et al 1991). During the final stages of grain filling, many protein bodies fuse, forming a continuous, tight protein matrix in which the starch granules are embedded (Figure 27).

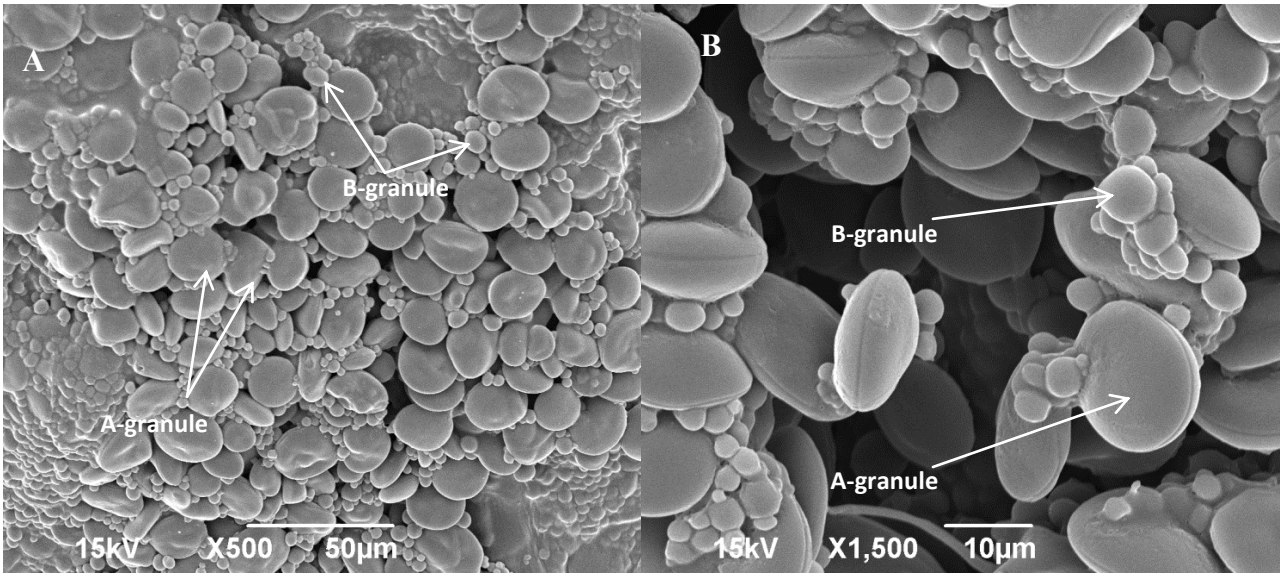


Figure 26. Scanning electron microscopy images of starch granules at 500x (A) and at 1,500x (B). Two types of starch granules in matured air-dried 'Alkabo' durum wheat kernel are clearly distinguish: A-type starch granules are disk-like or lenticular in shape with an average diameter of 10–35 μm and contribute >70% of the total weight and approximately 3% of the total granule number of endosperm starch. Whereas B-type starch granules are shaped spherically and range from 1 to10 μm in diameter. B-granules account for >90% of the total granule number but <30% of the total weight of starch in wheat endosperm.

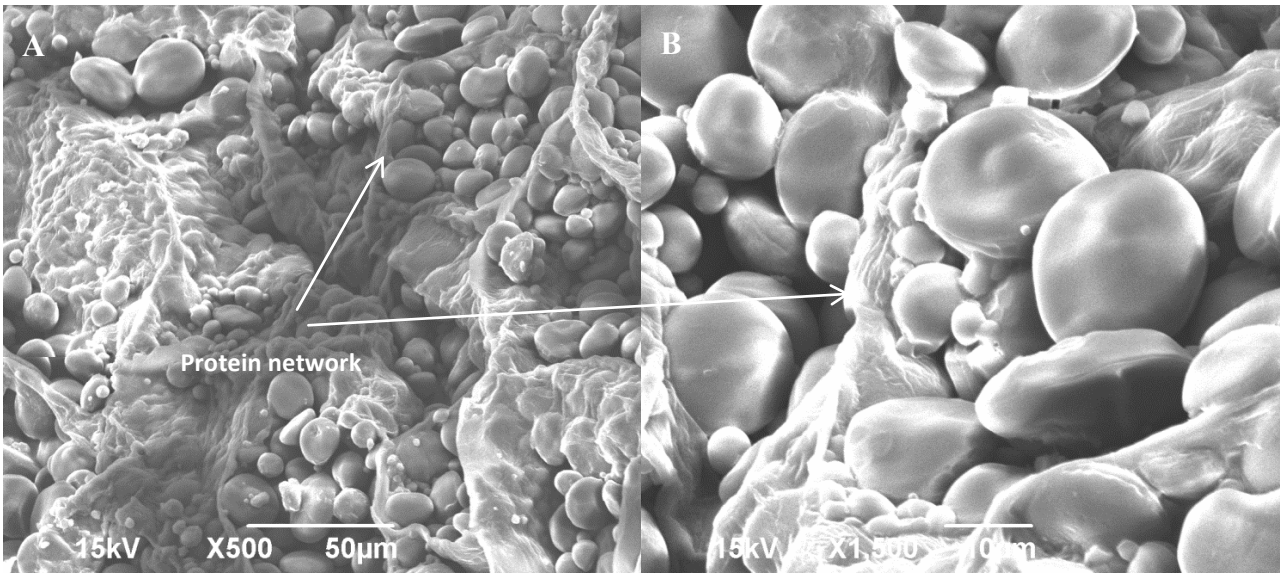


Figure 27. Scanning electron microscopy images of starch granules embedded in protein network at 500x (A) and 1,500x (B). At physiological maturity of grain, the protein network in the endosperm contracts eliminating open spaces and results in a vitreous dense endosperm (Olsen 2001).

Embryo-Surrounding Region

The embryo-surrounding region (ESR) represents the cells that line the cavity of the endosperm in which the embryo develops (Olsen 2001). The definitive information on the function of ESR in wheat is lacking but may include a role of in embryo nutrition or establishing a physical barrier between the embryo and the endosperm during seed development (Figures 28 and 29).

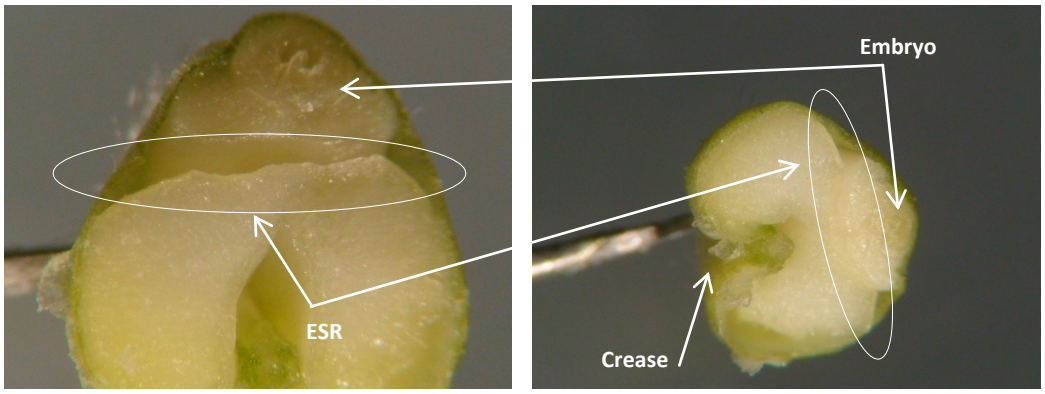


Figure 28. Light microscopy image of wheat kernel cross cut through embryo. The embryo-surrounding region (ESR) represents the cells that line the cavity of the endosperm in which the embryo develops.

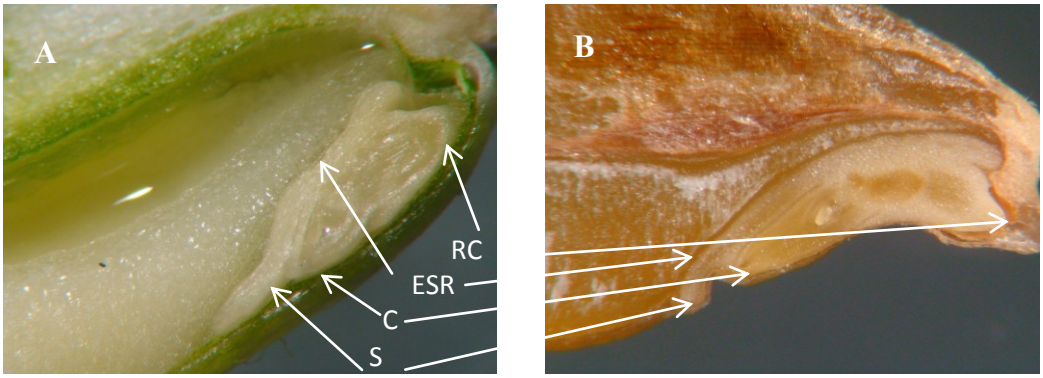


Figure 29. Light microscopy images of wheat embryo at 15DAA (A) and 25DAA (B). S=scutellum. Scutellum is the most obvious part of the embryo and is modified leaf attached to the first node. C=coleoptile. The shoot portion of the main axis consist of the growing point, nodes, and several rudimentary leaves called the coleoptile, which is attached to the stem at the second node. RC=root cap. Root portion of the main axis consist of rudimentary radicle surrounded by coleorhiza cells. Thus, the embryo consists of leaf, stem, and root and is surrounded by embryo surrounding region (ESR).

Conclusions

Progression of grain filling was monitored using light microscope and scanning electron microscope. This research focused on grain filling which begins about 7 to 10 days after anthesis. Much metabolic activity occurs in the first seven days after anthesis. Future microscope work should concentrate on morphological and anatomical developments that occur

in the first 15 days after anthesis and how stress, particularly air temperature and soil moisture affect these developments.

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OVERALL CONCLUSION

All five cultivars showed similar patterns in total yellow pigment (TYP) content during grain development. Total yellow pigment content per kernel declined as grain development progressed. Total yellow pigment content varied with environment and cultivar from 0.18 to 0.45 μg/kernel. In 2011, TYP per kernel was slightly lower than in 2009 and 2010. Lipoxygenase (LOX) activity increased just before and decreased after physiological maturity for all five cultivars. Little or no LOX activity was detected in AC-Commander. Lipoxygenase activity during grain filling period varied greatly among cultivars and over environments from 0.45 to 1.19 U/g. Levels of LOX activity and MDA content were lower in 2011, suggesting involvement of enzymatic and nonenzymatic (including carotenoids) defense system to suppress the stress levels. Levels of PPO activity were lowest in 2010, the year with temperature and rainfall in the range of normal.

After milling, there was 12-29% loss of TYP content, depending on cultivar and environment. The greatest loss of TYP content after milling was observed in 2009 while the least occurred in 2011. Processing resulted in additional loss of TYP content from 6 to 16% depending on cultivar and environment. The range of the loss of lipoxygenase activity (65-83%) after milling was mainly cultivar related, while decrease in malondialdehyde content was mainly affected by environment. The decrease in MDA content after milling was greatest in 2011; however, it increased significantly and was the highest after processing, suggesting higher activity of LOX during mixing period. There was no lipoxygenase activity detected in processed semolina samples due to their denaturation at high drying temperatures. Increased MDA content after processing in AC-Commander suggest that there are additional factors besides LOX activity or different LOX isozymes that affect lipid peroxidation.

FUTURE RESEARCH AND COMMERCIAL APPLICATIONS

In both natural and agricultural conditions, plants are frequently exposed to environmental stresses, which negatively affect their growth and development. Understanding the physiological processes that underlie stress injury and the adaption mechanism of plants to environmental stress is important to identify means by which plant growth and metabolism can be regulated under acute stress conditions for maintaining crop productivity and quality of crops grown under unfavorable weather conditions.

To precisely measure oxidative damage through membrane peroxidation, the environmental conditions should be under control, thus done in the greenhouse. Malondialdehyde content, along with primary products of lipid peroxidation, and two or more types of isozymes of lipoxygenase could be a give a more precise measurement of oxidation in grain during development. Measuring polyphenol oxidase (PPO) activity in whole wheat kernel instead of the pericarp only will give a better understanding of the enzyme behavior under stressed conditions. Total antioxidant capacity, along with enzymatic defense system (superoxide dismutase, ascorbate peroxidase, and catalase) and nonenzymatic defense system (tocopherols along with TYP content) in developing kernel can be measured.

The information will be useful for plant breeders in their effort to screen lines for the traits that define optimum balance between enzymatic and nonenzymatic defense systems and oxidative activity.

APPENDIX

Table A-1. Pearson's Correlation Coefficient of Grain Parameters During Kernel Development in 2009

	Protein content, mg/kernel	Ash content, mg/kernel	TYP content, ppm	TYP, µgkernel	MDA content, nmol/g
1000-KWT, g	0.94 ***	0.87 ***	-0.90 ***	-0.84 ***	-0.84 ***
Protein content, mg/kernel		0.94 ***	-0.63 ***	-0.85 ***	-0.57 ***
Ash content, %		-0.70 ***	0.75 ***	0.68 ***	0.74 ***
Ash content, mg/kernel			-0.80 ***	-0.77 ***	-0.81 ***
TYP content, ppm				0.91***	0.85 ***
TYP content, µg/kernel					0.71 ***

*, **, and *** = significantly different at 0.05, 0.01, and 0.001 levels of probability, respectively; ns=not significant at P=0.05 (n=140).

Table A-2. Pearson's Correlation Coefficient of Grain Parameters During Kernel Development in 2010

	Protein content, mg/kernel	Ash content, mg/kernel	TYP content, ppm	TYP, µg/kernel	MDA content, nmol/g
1000-KWT, g	0.93 ***	0.94 ***	-0.64 ***	-0.33 ***	-0.69 ***
Protein content, mg/kernel		0.95 ***	-0.57 ***	-0.33 ***	-0.66 ***
Ash content, %		-0.75 ***	0.46 ***	0.20 *	0.58 ***
Ash content, mg/kernel			-0.59 ***	-0.31 ***	-0.68 ***
TYP content, ppm				0.89 ***	0.86 ***
TYP content, µg/kernel					0.67 ***

*, **, and *** = significantly different at 0.05, 0.01, and 0.001 levels of probability, respectively; ns=not significant at P=0.05 (n=140).

Table A-3. Pearson's Correlation Coefficient of Grain Parameters During Kernel Development in 2011

	Protein content, mg/kernel	Ash content, mg/kernel	TYP content, ppm	TYP, µg/kernel	MDA content, nmol/g
1000-KWT, g	0.96 ***	0.92 ***	-0.74 ***	-0.41 ***	-0.80 ***
Protein content, mg/kernel		0.90 ***	-0.74 ***	-0.45 ***	-0.83 ***
Ash content, %		-0.65 ***	0.65 ***	0.30 ***	0.67 ***
Ash content, mg/kernel			-0.66 ***	-0.38 ***	-0.74 ***
TYP content, ppm				0.78 ***	0.81 ***
TYP content, µg/kernel					0.52 ***

*, **, and *** = significantly different at 0.05, 0.01, and 0.001 levels of probability, respectively; ns=not significant at P=0.05 (n=120).

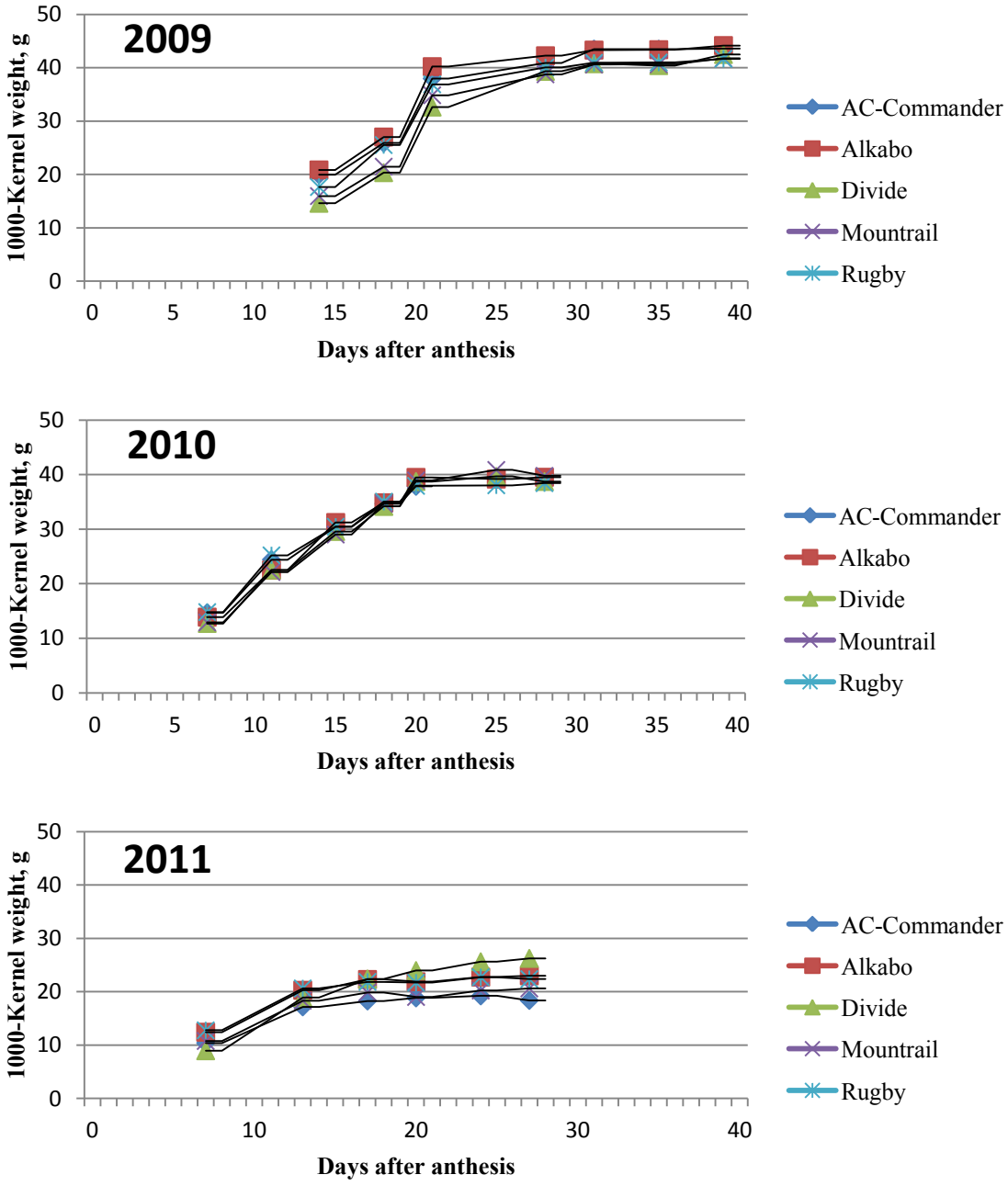


Figure A-1. Effect of cultivars x days after anthesis interaction on a 1000-kernel weight in 2009, 2010, and 2011.

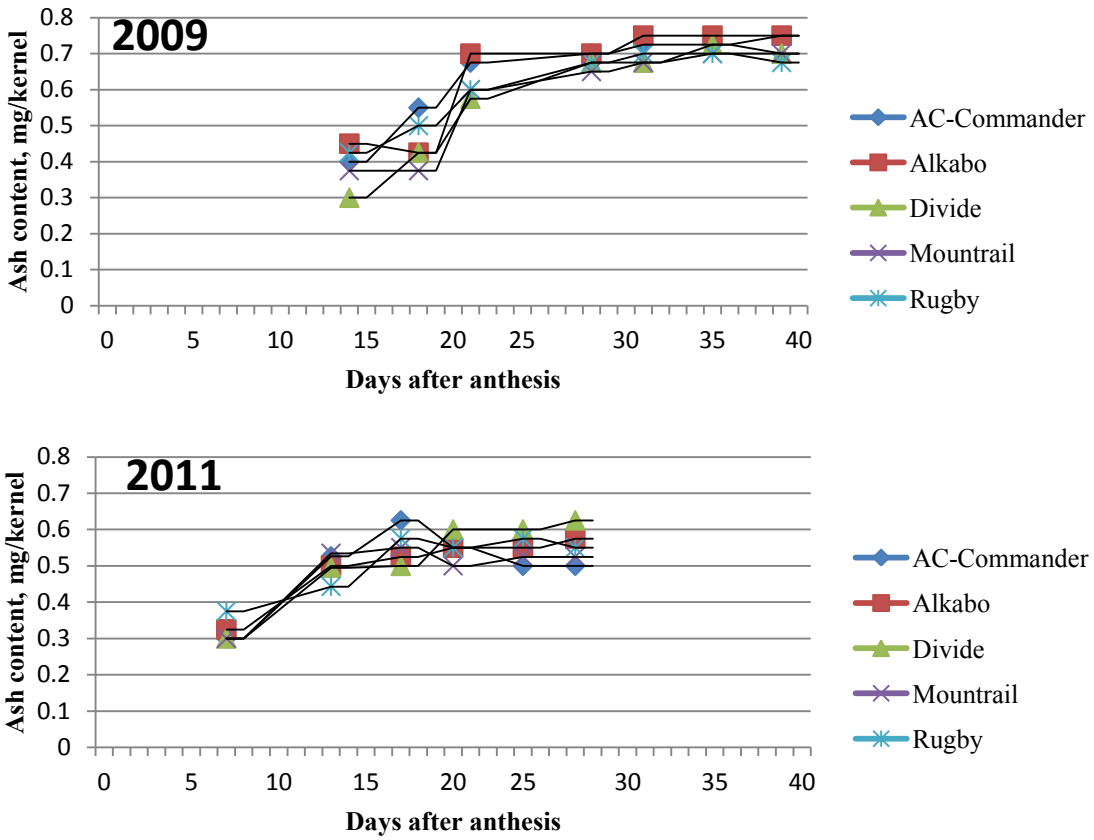


Figure A-2. Effect of cultivars x days after anthesis interaction on ash content per kernel in 2009 and 2011.

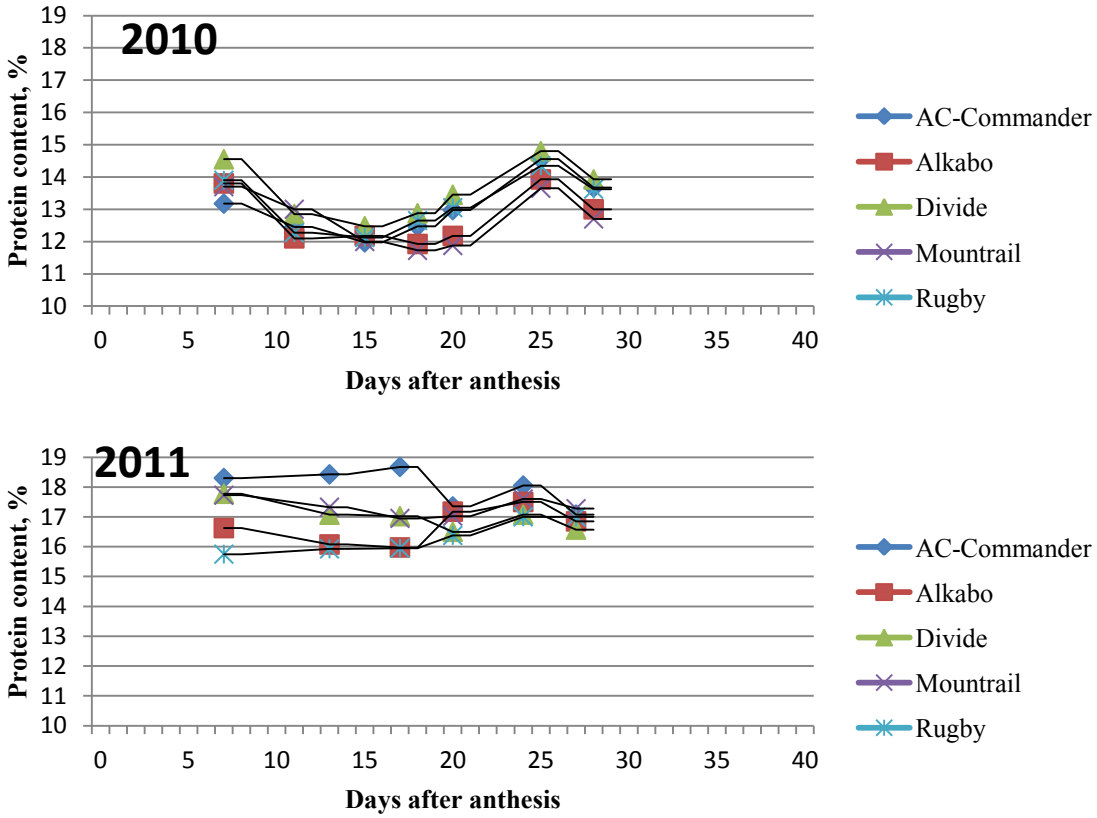


Figure A-3. Effect of cultivars x days after anthesis interaction on total kernel protein content in 2009, 2010, and 2011.

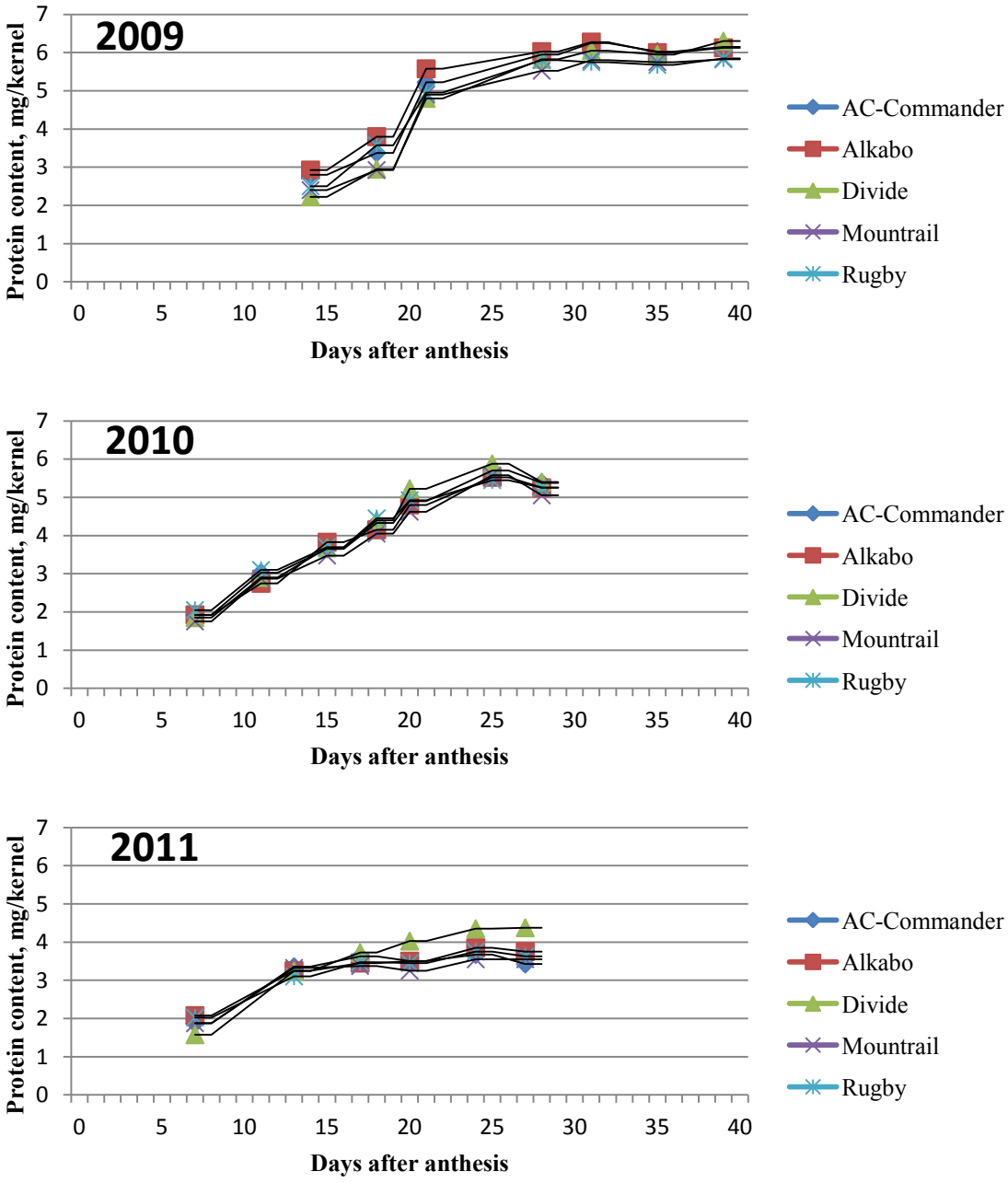


Figure A-4. Effect of cultivars x days after anthesis interaction on protein content per kernel in 2009, 2010, and 2011.

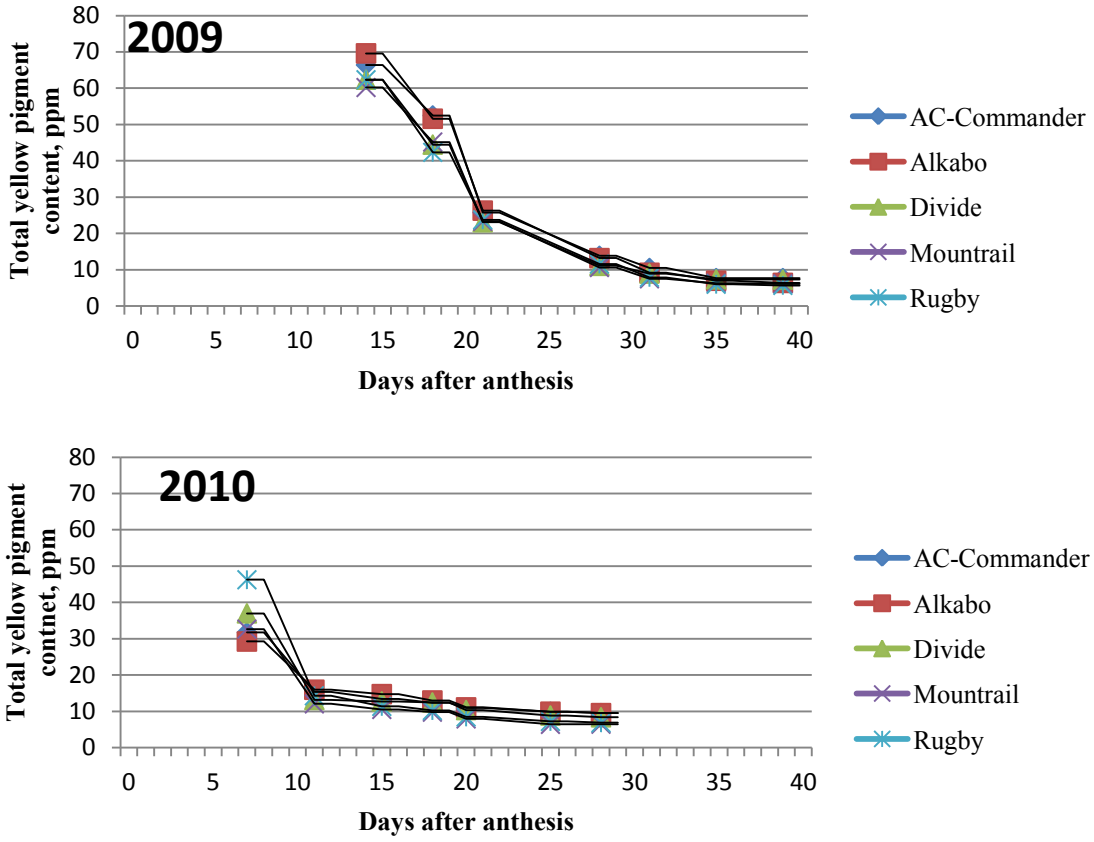


Figure A-5. Effect of cultivars x days after anthesis interaction on total yellow pigment content in 2009 and 2010.

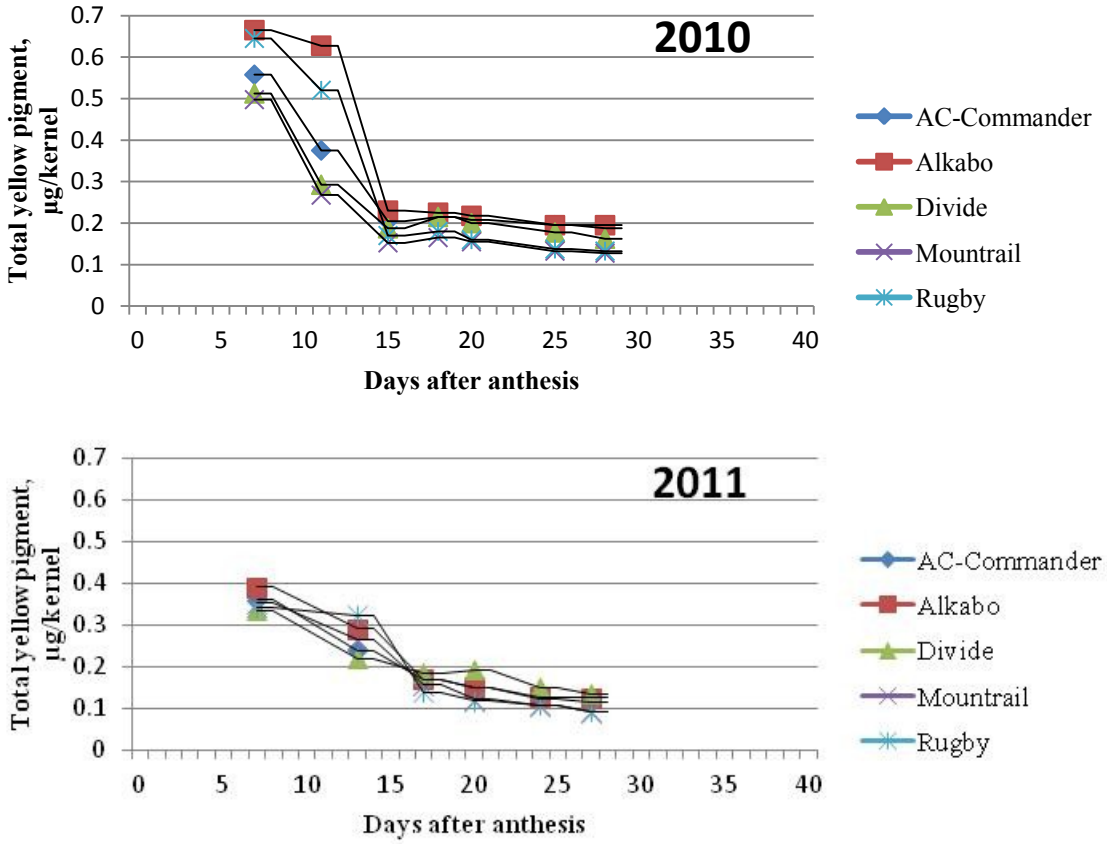


Figure A-6. Effect of cultivars x days after anthesis interaction on TYP content per kernel (µg/kernel) in 2010 and 2011.

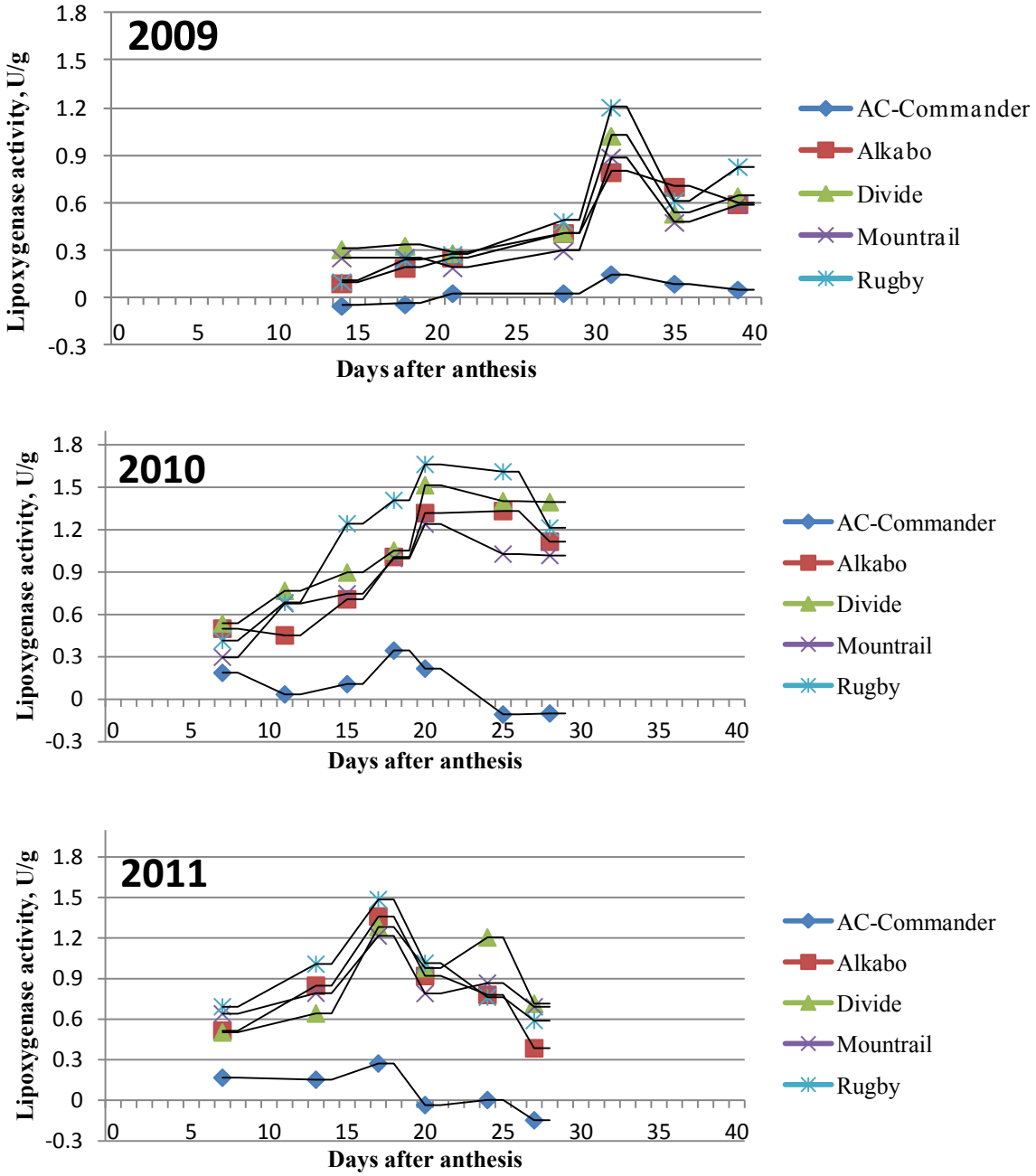


Figure A-7. Effect of cultivars x days after anthesis interaction on lipoxygenase activity in 2009, 2010, and 2011.

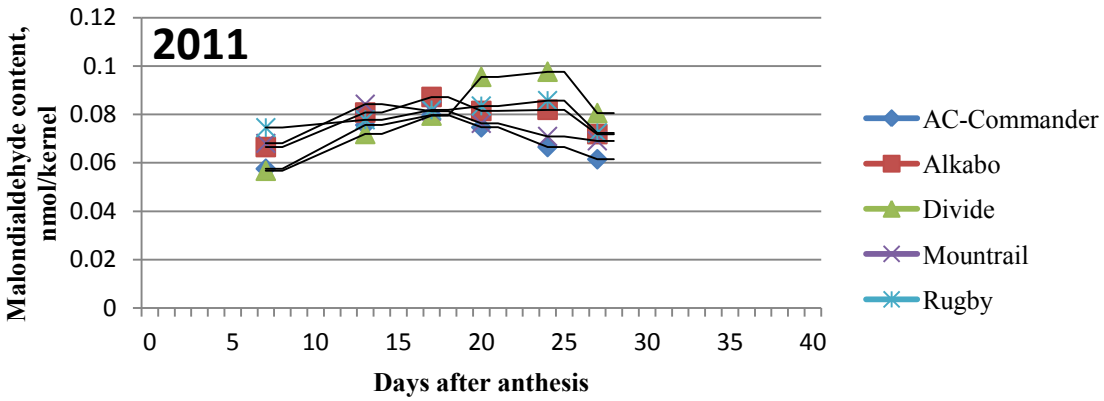


Figure A-8. Effect of cultivars x days after anthesis interaction on malondialdehyde content per kernel in 2011.

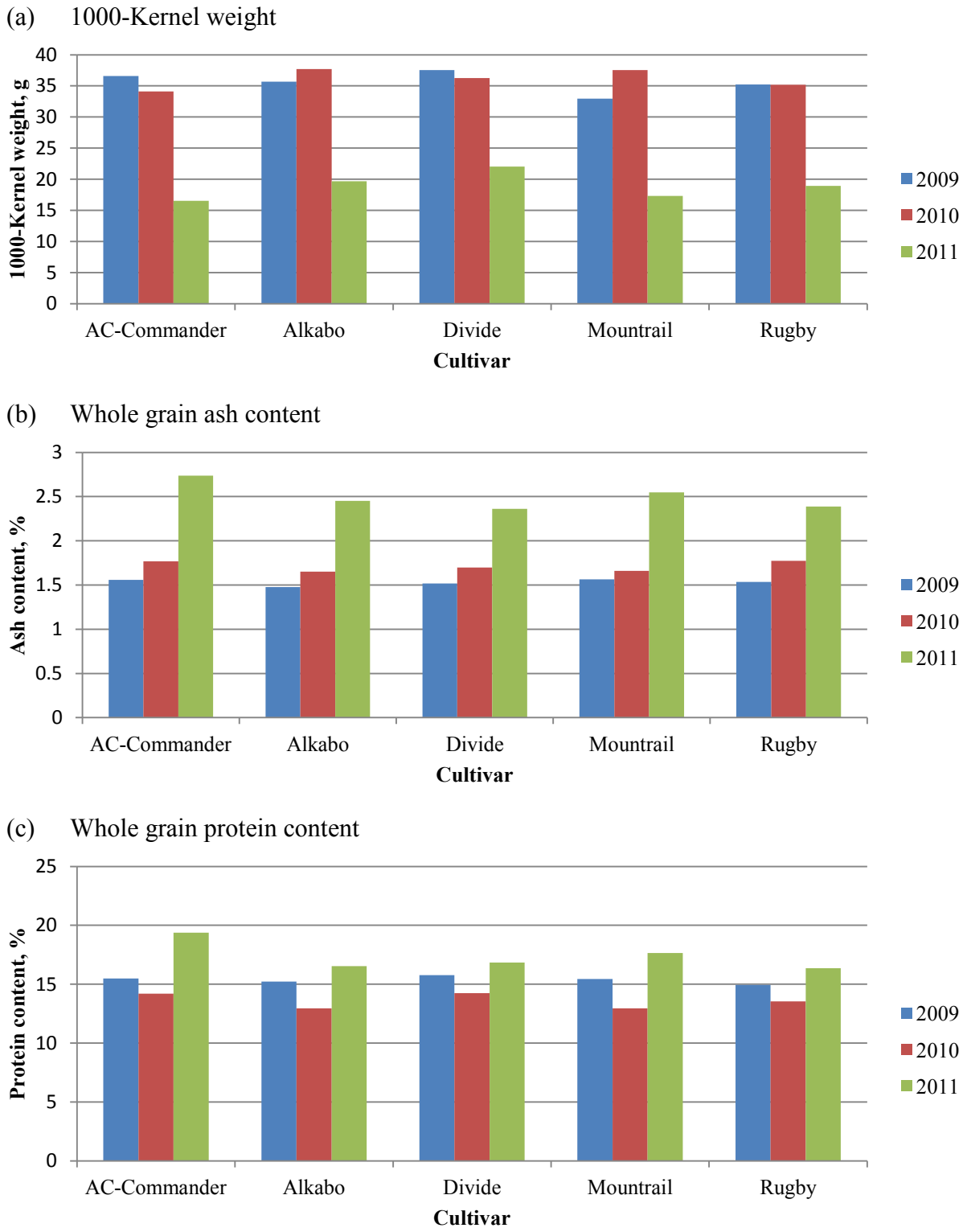
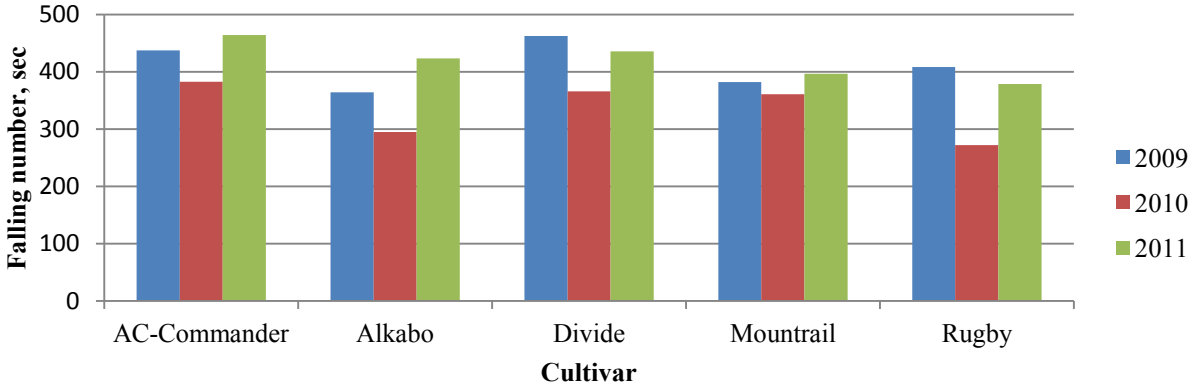
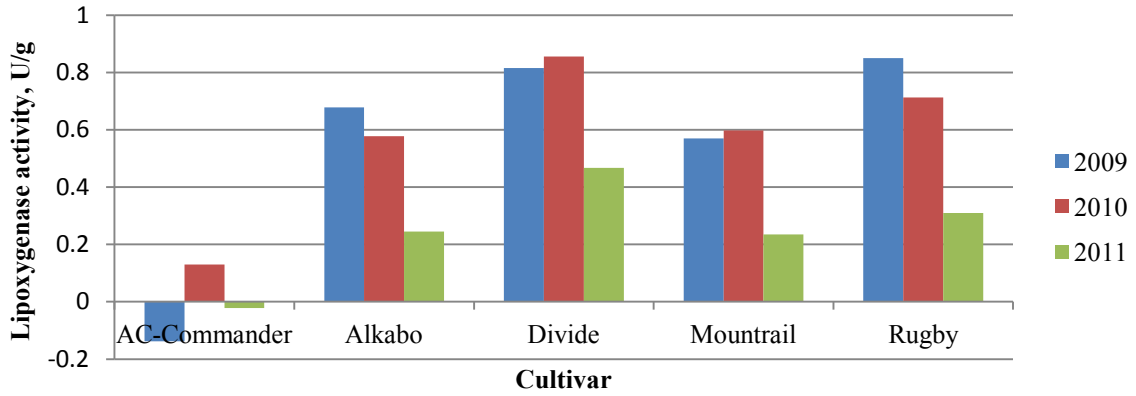


Figure A-9. Effect of year x cultivar interaction on 1000-kernel weight (a), whole grain ash content (b), whole grain protein content (c), falling number (d), whole grain lipoxygenase activity (e), and whole grain malondialdehyde content (f) in 2009, 2010, and 2011.

(d) Falling number



(e) Whole grain lipoxygenase activity



(f) Whole grain malondialdehyde content

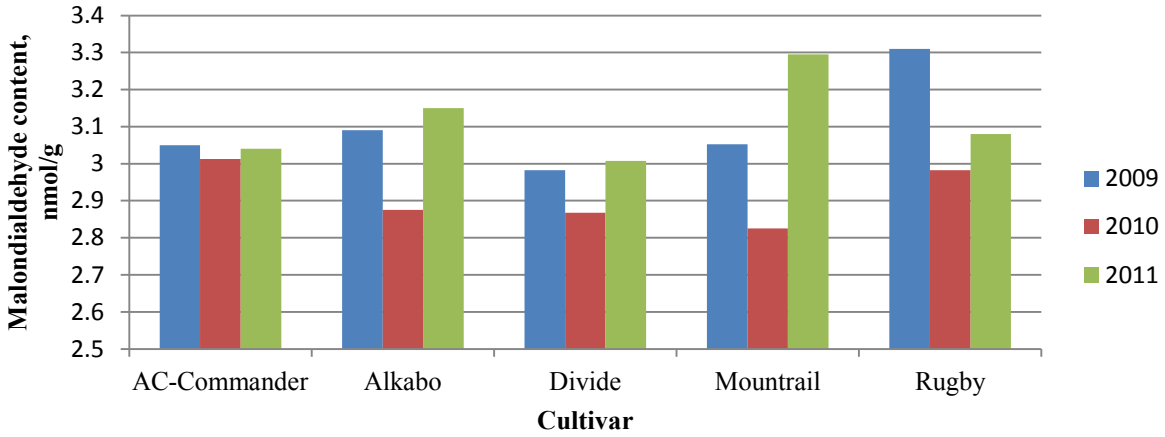


Figure A-9. Effect of year x cultivar interaction on 1000-kernel weight (a), whole grain ash content (b), whole grain protein content (c), falling number (d), whole grain lipoxygenase activity (e), and whole grain malondialdehyde content (f) in 2009, 2010, and 2011 (continued).

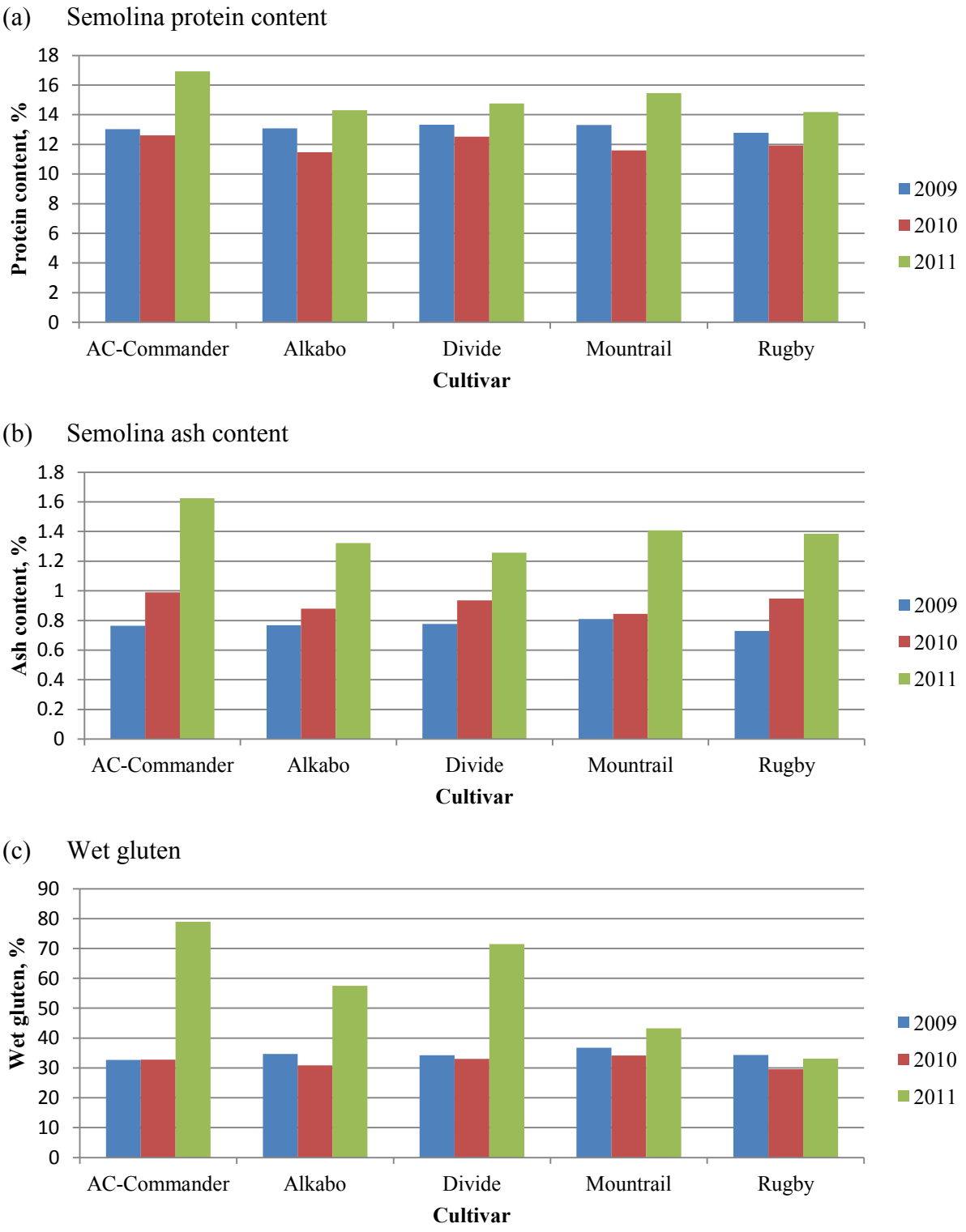


Figure A-10. Effect of year x cultivar interaction on semolina protein content (a), semolina ash content (b), and Wet gluten (c) in 2009, 2010, and 2011.