Nitrogen and sulfur effects on hard winter wheat quality and asparagine concentration and survey of Kansas soil sulfur conditions

by

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Abstract

Soil sulfur (S) deficiency is becoming increasingly common throughout the U.S. due to The Clean Air Act of 1990; S deficiency is typically found in high sand and low organic matter soils and looks very similar to nitrogen deficiency with stunted growth and chlorosis. Over application of N when there is a S deficiency has shown to be detrimental to wheat quality. Sulfur deficiency is detrimental to baking quality due to its effects on formation of disulfide bonds. These bonds are formed from the sulfhydryl groups of cysteine, which influence viscoelasticity of dough. Soil S deficiency can also lead to an increase of free asparagine concentration which indicates acrylamide forming potential of baked products. Acrylamide is a potential carcinogen; S fertilization has been shown to decrease acrylamide forming potential in wheat flour. Therefore, the objectives of this study were to: i) determine the effect of genotype, N, and S fertility on overall wheat quality and rheological characteristics; ii) determine the typical range of asparagine in wheat grain in Kansas: and iii) determine the influence of S availability in soils on asparagine in wheat grain. In the first study we found that in a soil with low S availability, S fertility significantly increased overall grain quality and rheological characteristics as well as increasing yield. Protein composition was evaluated by the ratio of total polymeric/ total monomeric protein using high performance liquid chromatography. In both years, the ratio of polymeric to monomeric protein was increased by sulfur fertilization. Solvent retention capacity (SRC) was evaluated using the whole grain lactic acid-sodium dodecyl sulfate solvent retention test (lactic acid- SDS SRC). In 2018, S application increased the SRC from 217% to 308%. Sulfur application increased average farinograph stability from 9.2 min to 14.6 min. Farinograph stability was effectively predicted by the SRC test (R^2=0.78). Free asparagine concentration decreased significantly in both years across all genotypes when S was applied.

Although, in Chapter 2 we found that poor wheat quality in a S deficient soil was increased with S fertilizer application, we did not find a strong correlation between available S in the soil and wheat quality or free asparagine concentration in Chapter 3. In 2018 a S sufficient location, Ashland had the highest free asparagine concentration (37 μmol/g) which was likely due to hot dry weather during grain fill. Thus, further investigation of factors influencing free asparagine in winter wheat is necessary for us to have a better understanding of how to decrease the risk of elevated levels. Information from this study will help winter wheat producers in the Great Plains diagnose sulfur deficiency and determine best practices for increasing grain quality and decreasing free asparagine concentration.

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Dedication

This thesis is dedicated to my grandfather Dr. Marvin Margolis. Thank you for always inspiring me to follow in your footsteps and being one of my biggest role models.

Chapter 1 - Literature Review

Importance and Origin of Wheat

Wheat (*Triticum aestivum L.*) is an allohexaploid species originating from two successive rounds of hybridization. The second hybridization event is thought to have occurred in the fertile crescent during the Neolithic period, estimated to be about 8,000 to 10,000 years ago (Heun et al., 1997). This event lead to an increase in food security and played a large role in the development of permanent settlements of humans. Since this time, what we commonly have known as bread wheat has traveled many miles and evolved through human migration (Bonjean et al. 2001). Hard winter wheat called 'Turkey Red' was brought to central Kansas in 1873, this genotype was unlike any other wheat at the time and was much better suited for the soil and weather conditions in Kansas compared to the soft and semi-hard wheat genotypes that were originally brought by settlers (Gwirtz et al., 2006). Wheat improvement had its formal beginning in 1897 when the U.S. Department of Agriculture set up a program of wheat research and development. This was the start of grading wheat, not only for yield performance and disease resistance, but also developing quality requirements of millers and bakers (U.S Wheat Association (USWheat.org)). Wheat is one of the most important crops across the globe for food production because it is a primary source of calories for the world population and contains significant amounts of essential nutrients. Wheat provides many essential nutrients such as proteins, fiber, minerals, vitamins and lipids that help contribute to a healthy diet (Shewry et al., 2015). Annual global production of wheat is estimated to be around 765 million metric tons in 2019 (FAOSTAT, 2019), with China being the world's leading wheat producing country with 134 million metric tons, followed by India (98 million), Russia (85 million), United States (47 million), and France (36 million) (FAOSTAT, 2019). The Great Plains is the leading wheat

producing region in the U.S and in 2019, Kansas was the leading wheat producing state with 51.3 million metric tons (Kansas Wheat Alliance, 2019).

Sulfur-Deficient Soil

The 1990 US Federal Clean Air Act amendments have successfully improved air quality through regulation of power plant air emissions (USEPA, 2015). Sulfur emissions have decreased by 80%, resulting in measured decreases in sulfur deposition (NADP, 2015). This 80% reduction has also been shown, not only by the Acid Rain Program (ARP) efforts, but also by the Cross-State Air Pollution Rule (CSAPR). Through these two programs, the United States has been able to reduce the amount of air pollution and SO_2 emissions from commercial plants (Figure 1) (USEPA, 2019). It is estimated that S removal based on average Kansas wheat yields is 0.04 kg S per 27 kg of grain harvested (KSU Wheat Production Handbook, 1997).

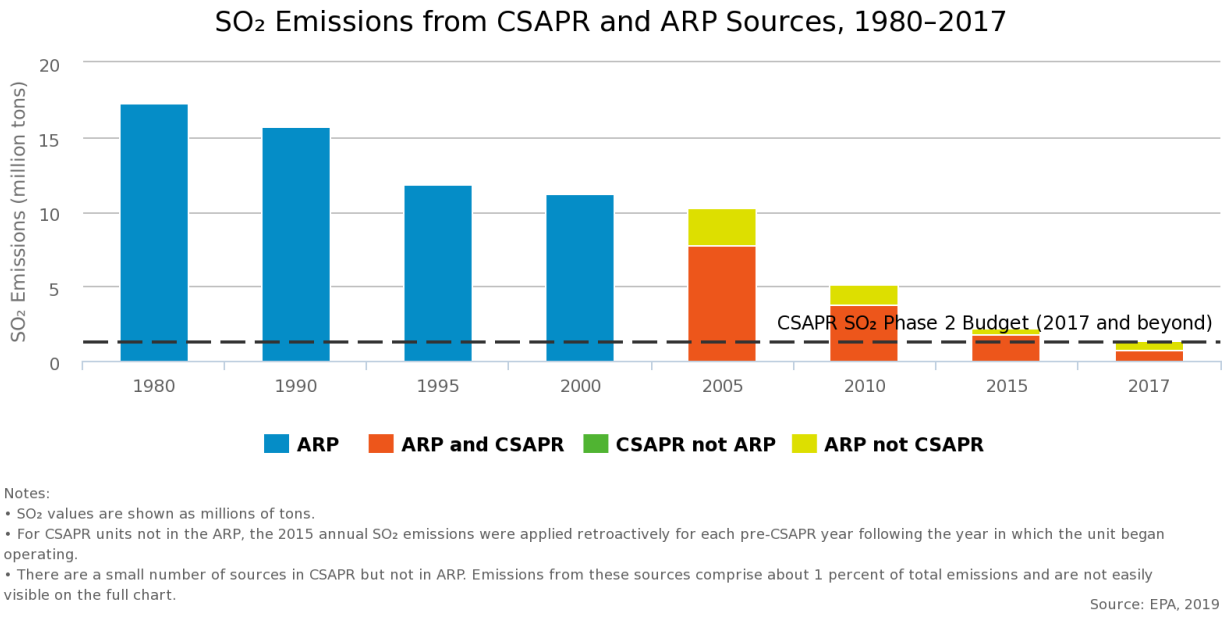

Figure 1. SO_2 emissions in the U.S. from CSAPR and ARP sources from the dates of 1980-2017. SO2 values are shown as millions of tons

Along with lower S deposition, soil S deficiency is becoming increasingly common throughout the world due to lack of S fertilization (David et al., 2016). Sulfur deficiencies are common in low organic matter soils, sandy soils and intensely farmed soils that do not receive S fertilizer. Higher performing wheat genotypes are likely a significant cause of increased S depletion because they remove larger amounts of S from the soil along with intensive cropping systems such as double cropping, less crop residue left behind and higher tillage (Lamond,1997). Although S deficiency is not extremely common throughout the Great Plains where most winter wheat is produced, there is a trend for sandy soils to become S-deficient as they are expected to have lower organic matter. Typically, S is found in organic matter so when organic matter is limited we would expect for there to be a decrease in available S. Sandy soil is also prone to having S deficiency due to increased leaching in the soil since S is a mobile nutrient (Rhue and Kamprath, 1973).

Sulfur Soil Fertility and Agronomics

Sulfur (S) is one of 17 elements that are essential for crop growth and development. Sulfur is considered a secondary macronutrient (Lamond, 2004), is sometimes considered to be the fourth most important nutrient in terms of plant growth and quantity taken up by plants (Jeschke et al., 2017). Cereal crop grain yield and protein concentration are dependent on adequate soil fertility. Adequate S also is required for wheat growth and development, as S is essential for the biosynthesis of amino acids, proteins, and chlorophyll (Jamal et al., 2010). Sulfur is a key constituent of enzymes involved in nitrogen (N) metabolism (Campbell, 1999; Mendel, 2003; Swamy et al., 2005), and S deficiency can decrease N assimilation (Salvagiotti et al., 2009) leading to a less productive plant. Without an adequate supply of S, wheat is unable to reach its full yield potential and cannot efficiently utilize N for protein biosynthesis (Yu et al.,

2018). Glutathione (GSH) is the main transport and storage form of reduced sulfur in plants and helps plants with stress management by scavenging reactive oxygen species and metal sequestration (Hameed et al., 2014), limited GSH levels can significantly decrease the crop yield, protein and quality potential. (Hossain et al., 2017).

A study that took place in Australia investigating the effects of mid-season S deficiency over multiple S rates found that at the rate of 30 kg S/ha increased yield, nitrogen use efficiency (NUE), harvest index and grain protein. This study found that protein yield, harvest index and NEU all peaked when 30 kg/ha S was applied and if there was an overabundance of S over 30 kg/ha (Yu et al., 2018), then the positive effects would tail off due to excess of nutrients. Producers are especially interested in increasing NUE so that plants are utilizing the N that is applied to perform better, thus making the producers investment more profitable.

Sources of Sulfur

Sulfur is critical for proper plant growth and development (Salvagiotti and Miralles, 2007). In well-drained agricultural soils, organic sulfur typically accounts for over 95% of the total sulfur, Organic sulfur is converted to inorganic sulfate through mineralization, making it available for plant uptake. Organic matter content of the soil greatly affects the amount of sulfur available to the crop through mineralization. On average, one percent of organic matter will supply about 1-1.5 kg of available sulfur annually (Jeschke et al., 2017). Where organic matter is limited, it may be critical for producers to apply S containing fertilizers. Sulfate S containing sulfur fertilizers have been a popular choice for producers. Ammonium sulfate is a popular and effective S source as it contains 21% N and 24% S and is readily available. Ammonium thiosulfate provides a portion of its S in a plant available form and can be an effective S source (Grant et al., 2004). Ammonium thiosulfate is 50% elemental S and 50% sulfate S, thus only

50% is plant available at time of fertilization, although elemental S is slowly converted to sulfate over time through microbial processes requiring both water and oxygen this oxidation rapidly occurs in warm moist soils (Germida et al. 1993) . Ammonium thiosulfate is a liquid fertilizer and is the most popular choice for those using liquid synthetic fertilizers. Potassium magnesium (Mg) sulfate is 22% sulfur and is often applied to soils that are deficient in both S and Mg. Elemental S is a common type of S fertilizer containing 90-95% S. Although elemental S contains high amounts of S there is a concern about the availability for immediate plant uptake. Elemental S must first be oxidized by soil microorganisms to plant available sulfate-S before it becomes available to crops. Soil temperature and moisture are very critical for oxidization. Elemental S is not recommended for cool season grasses such as winter wheat due to lack of oxidation. Gypsum (calcium sulfate dihydrate -- $CaSO_4 \cdot 2H_2O$) is also used as an S source. Gypsum is slightly soluble and may offer advantages in reducing losses in high leaching environments and for long season crops (Aulakh and Malhi, 2004). In a field trial in Saskatchewan, gypsum was shown to be a successful S source (Wen et al., 2003). Less common products used to apply S to crops are potassium thiosulfate (TKS). Potassium thiosulfate is a liquid fertilizer and has 17% S, TKS is harmful to the seed, for this reason, it is not a very commonly used product. Ammonium polysulfide has 40-45 % S, and it is not very common due to a strong S smell. Potassium sulfate has 17% S and is a good source of S but is not readily available in the marketplace. In past times, many different commonly used fertilizers would have a high concentration of S due to contamination and impurities within the solution/granular product, but that is no longer a problem (Lamond, 1997). This is another factor that is leading to decreased S in soil in the Great Plains; producers in the past had been inadvertently applying S and now are not due to cleaner fertilizer sources.

Sulfur Deficiency and Quality

Sulfur plays an important role in two critically important amino acids that contain S, methionine and cysteine. Methionine is the initiating amino acid in the synthesis of all proteins, and cysteine, by virtue of its ability to form disulfide bonds, plays a crucial role in protein folding (Brosnan and Brosnan, 2006 and Györi, 2005). Sulfur deficiency is detrimental to baking quality due to its effects on formation of disulfide bond if S is not available plants are not able to form disulfide bonds. These bonds are formed from the sulfhydryl groups of cysteine, which influence viscoelasticity of dough. Bread making quality is determined by the gluten proteins that form a viscoelastic dough with a gas retention ability necessary to produce loaves with large volumes and desirable texture (Schofield, 1994). These viscoelastic properties are made up of the elastic properties, when dough is mixed the elasticity gives dough the ability to return to original form when mixing is stopped where the viscosity allows dough to remain deformed while mixing is maintained. Viscoelastic properties improve dough strength, softness and shelf-life thus it is important for wheat to have balanced elasticity and viscosity. The baking quality of wheat grown on a S deficient soil is increased with S fertilization showing high correlation between loaf volume and sulfur content of grain, thus improving rheological properties by increasing extensibility of dough (Salih, 2003). Sulfur deficit may result in harder grain; the dough made from such grain is usually stiff and is not elastic (Ryant & Hřivna, 2004).

Zhao et al. (1997, 1999) found that when using the Chorleywood bread process grain S status was a better indicator of loaf volume than grain N content. A study that took place in Australia and New Zealand found that there is a need for adequate flour SO_4-S to lower work input (WI) in industrial bakeries (Wooding et al., 2000). This study found that when they fertilized N to S with a 3:1 ratio that it prevented undesirable increases in WI and increasing S

fertilization decreased maximum resistance; although loaf volume, water absorption and crumb gain were not significantly affected by S fertilization (Wooding et al., 2000). Researchers have made conclusions that N and S fertilizers may be used to manipulate the concatenation between the mixing requirements and the dough strength, but this effect was genotype dependent. A study by (Tea et al., 2007) found that flour protein levels increased by 3% when S was applied alone, but when S and N were applied together, they found a protein increase of 7%. The same study found that S treatments improved dough swelling and extensibility, but the tenacity to extensibility ratio decreased after S treatment. Most studies looking at the effects of S fertilization also looked at the interaction with N as they work together as protein building blocks. A greenhouse experiment in Norway found that S fertilization increased sodium dodecyl sulfate sedimentation values and affected the N/S ratio with minimal effect on yield. The sodium dodecyl sulfate sedimentation test is important due to its ability to predict gluten strength and baking quality, higher values imply that there is stronger gluten strength and baking quality is higher (Carter et al. 1999). Balanced fertilization with N and S is very important to secure high yield and high wheat quality. It is important to carefully consider the S fertilization regime, particularly at high N fertilization rates. Variations in wheat quality have been recorded where S is limited even though the yield responses were relatively small (Flæte et al., 2005). It is very important to focus on fertility from a quality point of view not just a yield standpoint.

Asparagine and Acrylamide

Asparagine accumulates under conditions of stress as a biological response to the restriction of protein synthesis. Such stress conditions can be caused by drought, pathogen attack, toxicity or nutrient deficiency (Lea et al., 2007). Asparagine often plays a key role in the active site of enzymes (Mansfield et al., 2006). Biosynthesis of all essential amino acids can be

achieved from asparagine as a donor of fixed N. High free asparagine concentrations in grain indicate poor N use efficiency, making the plant less efficient overall. This leads to lower quality grain. Although there is limited information on asparagine concentration for wheat cultivars produced in the United States, a previous study in Nebraska found that asparagine concentration in grain was influenced both by genotype and production environment, with production environment having a larger effect on asparagine concentration than genotype (Navrotskyi et al., 2018).

Researchers in the United Kingdom (UK) found in a greenhouse experiment that free asparagine concentration was correlated with acrylamide concentration in heated flour with a R^2-value of 0.9945 (Figure 2A). Total grain S also correlated strongly with acrylamide concentration with a R^2 value of 0.9462 (Figure 2B), suggesting that grain S and/or free asparagine concentration could be used for quality control checks (Curtis et al., 2009). The same research team found that free asparagine concentration was significantly lessened when S was applied to many different cultivars of wheat growing in S deficient soils (Curtis and Halford, 2016) (Figure 3). Sulfur deficiency leads to the plant being less resilient to stress, therefore leading to increased free asparagine concentration. Where there is extreme S starvation in the soil, the concentration of free asparagine can increase by 30-fold (Muttuchumaru et al., 2006). Typically, when plants are S stressed, asparagine will make up about 50% of the free amino acid pool (Halford et al., 2012)

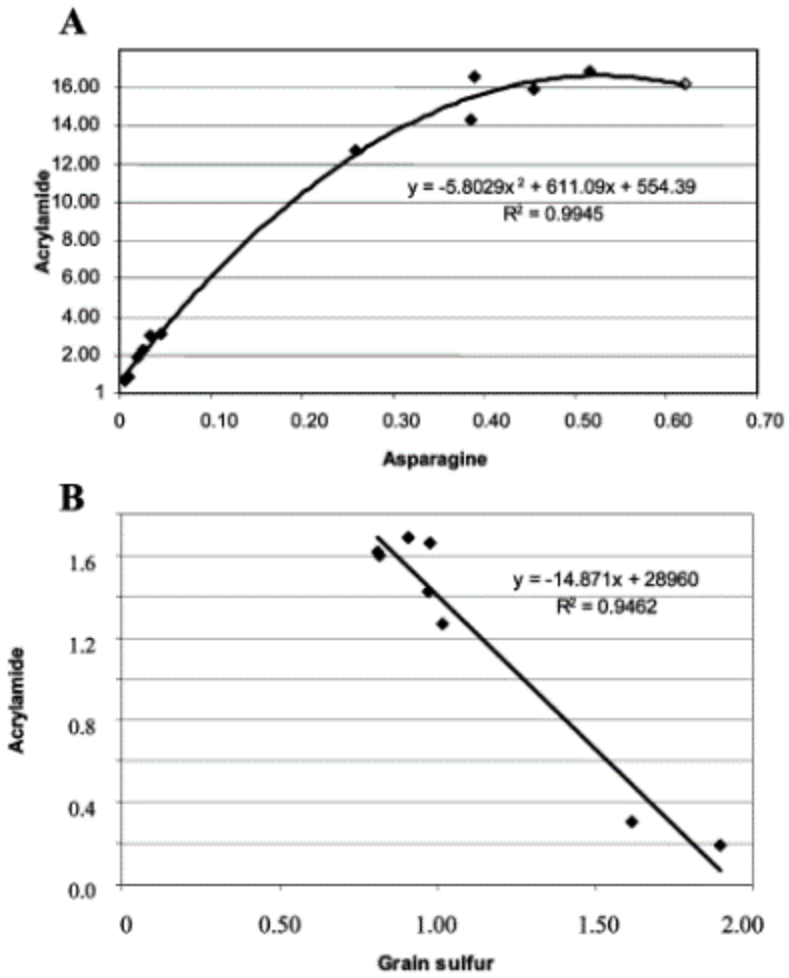

Figure 2. (A) Free asparagine concentration (mmol/kg) in wheat grain plotted against acrylamide formed in heated flour (mg/kg). (B) Total grain sulfur(mg/g) plotted against acrylamide formed in heated flour (mg/kg) (Curtis et al. 2009).

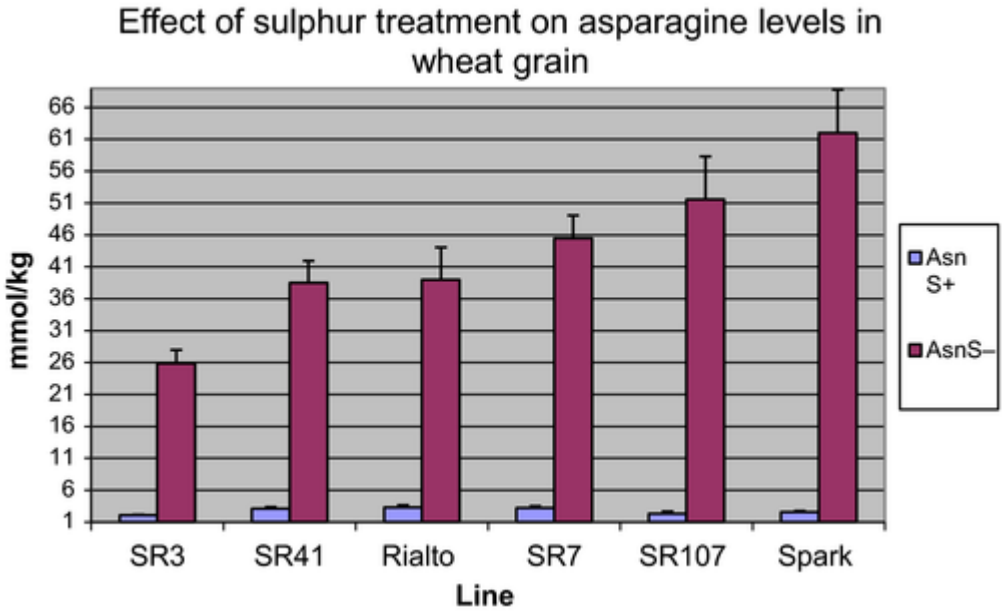

Figure 3. Asparagine concentration (mmol/kg) under sulfur sufficient (blue) and sulfur deficient (red) treatments (Curtis et al. 2009)

Free asparagine concentration, therefore, reflects the general acrylamide-forming potential of grain. Acrylamide has been described as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC, 1994). Acrylamide has also been described as mutagenic, reprotoxic and neurotoxic (Tepe, 2016). Unfortunately the mechanism of acrylamide synthesis is still not fully understood, so it is very important for food processors to monitor, detect and avoid acrylamide production in food products (Stobiecka et al., 2007) Although there is not a set threshold of acrylamide that is known to be toxic to humans, The European Food Safety Authority (EFSA) described the risk characterization using the reference point of the bench mark dose level (BMDL) of 0.17 mg/kg bw/day of acrylamide in food, the developmental effects of acrylamide are not currently a concern at current dietary levels (EFSA, 2015). Although the current dietary levels are not a concern, The Food and Agriculture

Organization and the World Health Organization (FAO/WHO) have concluded that the presence of acrylamide in the human diet is a concern (World Health Organization, 2006).

Shortly after acrylamide had been characterized as a thermally generated constituent of many processed foods (Tareke et al., 2002), it has become a challenge for the food industry to determine how to reduce the concentration of this potential carcinogen, particularly in bread. There are many pathways for formation of acrylamide in foods although the most prevalent and important pathway of acrylamide formation seems to be through the reaction between asparagine and a reducing sugar via the Maillard reaction (Soares et al., 2015) (Figure 2). Acrylamide formation from asparagine is dependent on numerous food processing parameters including temperature, formulation and cooking time (Council for Agricultural Science and Technology, 2006). The reaction between amino acids, typically asparagine, and reducing sugars is by the Maillard reaction, generates desirable flavor and color compounds in heated foods (Jackson and Al-Taher, 2010).

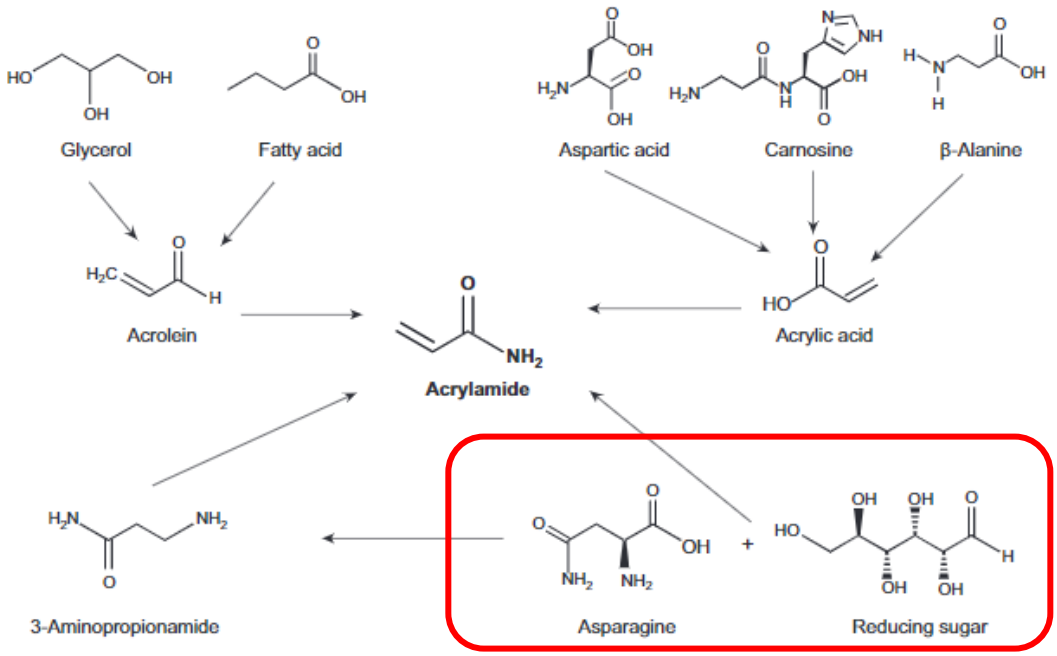

Figure 4. Acrylamide formation pathways. The bottom left shows the reaction between asparagine and a reducing sugar (Soares et al. 2015).

Free asparagine concentration in wheat flour is a significant determinant of acrylamide forming potential in end use products (Muttuchmaru et al., 2006). Therefore, by decreasing the asparagine concentration in grain, adequate S and N fertilization may decrease the potential health concern of acrylamide in food products (Curtis et al., 2018). It is highly unlikely for any agricultural production ground to have no S, but as we deplete our soils without adding S amendments, it is going to be more likely to find grain that has high free asparagine and this acrylamide forming potential.

Conclusions

A key conclusion from the scientific literature indicates that S fertilization can increase flour quality in many ways. Sulfur tends to increase maximum dough resistance and increase protein content. Sulfur fertilization can also increase dough swelling, making for a more profitable loaf due to higher water absorption. Sulfur fertilization was also found to decrease free

asparagine concentration in flour, thus, decreasing acrylamide forming potential. It may be very important for producers to apply S fertilizer, not only because it can create a better quality and safer flour, but it can also help increase yields for most crops that have a high S requirement. In the future, it may be increasingly important for producers to apply S fertilizer due to decreasing S concentrations in soil and limited soil S amendments. Although there has been some research on S fertilization and the connection to wheat quality and free asparagine concentration, there is still quite a lot of work and research to be performed to better our understanding of the impact and connection.

Rational and Objectives of Study

Wheat producers in the Great Plains of the United States may be seeing large increases of soil S deficiencies in the near future with increasing yields and vigorous crops pulling out more S than is being deposited into the soil. Although there are not many, studies have shown that S fertility, where S is deficient, can increase yield protein and quality (Yu. et al., 2018; Curtis et al., 2009). Researching and understanding S, and its impact on both agronomic and quality parameters, will help producers be able to make better decisions going forward with S fertility to achieve the full crop potential in all aspects.

These studies were conducted to: 1) determine agronomic effects of S fertility; 2) determine the effect of S deficiency on wheat rheological characteristics; 3) identify adequate S fertility rates to correct deficiency; 4) determine the effect of S deficiency on free asparagine accumulation in grain; 5) survey the state of Kansas for available S levels; 6) help producers determine the value of S fertilization.

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Chapter 2 - Nitrogen and Sulfur Effects on Hard Winter Wheat Quality and Asparagine Concentration

Abstract

Grain protein concentration and composition are key factors affecting winter wheat quality and are influenced by wheat genotype, available fertility, and growing conditions. These same parameters can affect free asparagine concentration in grain, and elevated asparagine can lead to acrylamide production in baked food products, which can be a health concern. The objectives of this study were to determine the effect of genotype, nitrogen (N), and sulfur (S) fertility on protein concentration, protein quality, dough rheology, and asparagine concentration in winter wheat grown on S-deficient soils. Treatments were arranged in a 3x2x4 factorial design in 2017 and 3x2x5 factorial design in 2018. There were three levels of N (56, 101 and 146 kg ha^{-1}), two levels of S (0 and 22 kg ha^{-1}), four levels of genotype in 2017, and five levels of genotype in 2018. Protein composition was evaluated as the percent polymeric protein using size exclusion high performance liquid chromatography. In both years, the ratio of polymeric to monomeric protein was increased by sulfur fertilization. Solvent retention capacity (SRC) was evaluated using the whole grain lactic acid-sodium dodecyl sulfate test. In 2018, S application increased the SRC from 217% to 308%. However, in 2017, SRC improvement was limited to two genotypes and was modest, likely a consequence of the reduced protein concentration in S-treated plots. Free asparagine concentration averaged 9.8 µmol/g and 20.9 µmol/g in 2017 and 2018, respectively. Asparagine concentration in grain was affected by N, S, genotype, and their interactions. Sulfur application substantially reduced asparagine concentrations in both years. Dough rheology was evaluated in the 2018 trial using the farinograph test. Sulfur application increased average farinograph stability from 9.2 min to 14.6 min. Farinograph stability was

effectively predicted by the SRC test (R^2=0.78). These results demonstrate the importance of ensuring adequate S fertility in winter wheat production.

Introduction

Wheat (*Triticum aestivum* L.) grain yield and protein concentration are dependent on adequate soil fertility. Sulfur (S) and nitrogen (N) fertilizer application on hard red winter wheat can have many benefits. Nitrogen is an essential nutrient important for wheat growth and protein biosynthesis. Low N availability can limit crop growth, yield, and quality (Sinclair and Horie, 1989). Adequate S also is required for wheat growth and development, as S is essential for the biosynthesis of amino acids, proteins, and chlorophyll (Jamal et al., 2010). Sulfur is a key constituent of enzymes involved in N metabolism (Campbell, 1999; Mendel, 2003; Swamy et al., 2005), and S deficiency can decrease N assimilation (Salvagiotti et al., 2009). When N supply restricts crop growth, grain yield will increase when N fertilizer is applied, but yield may plateau if a critical factor other than N is limiting. Without an adequate supply of S, wheat is unable to reach its full yield potential and cannot efficiently utilize N for protein biosynthesis (Yu et al., 2018). Therefore, N use efficiency and crop yields are increased when N and S are applied together (Aulakh and Malhi, 2004; Salvagiotti et al., 2009).

While all amino acids contain N, two critically important amino acids contain S, methionine and cysteine. Methionine is the initiating amino acid in the synthesis of all proteins, and cysteine, by virtue of its ability to form disulfide bonds, plays a crucial role in protein folding (Brosnan and Brosnan, 2006). Sulfur and N deficiency are detrimental to baking quality due to their effects on formation of disulfide bonds formed from the sulfhydryl groups of cysteine, which influence viscoelasticity of dough (Györi, 2005). Dough viscoelasticity is related to many factors, including the intrinsic nature of the flour, dough ingredients, temperature, water

absorption, air incorporation and type of mixing (Mirsaeedghazi et al. 2008). Dough rheological tests predict manufacturing quality, such as the bakery performance of bread (Dobraszczyk B.J. et al., 2003).

Asparagine accumulates under conditions of stress as a biological response to the restriction of protein synthesis. Such stress conditions can be caused by drought, pathogen attack, toxicity or nutrient deficiencies (Lea et al., 2007). Asparagine often plays a key role in the active site of enzymes (Mansfield et al., 2006), and biosynthesis of all essential amino acids can be achieved from asparagine as a donor of fixed N. High free asparagine concentrations in grain indicate poor N utilization efficiency, defined as the conversion of assimilated N to grain protein, leading to lower quality grain. Although there is limited information on asparagine concentration for wheat cultivars produced in the United States, a previous study in Nebraska found that asparagine concentration in grain was influenced both by genotype and production environment, with production environment having a larger effect on asparagine concentration than genotype (Navrotskyi et al., 2018).

Acrylamide is formed from asparagine in the presence of reducing sugars in baked foods via the Maillard reaction (Council for Agricultural Science and Technology., 2006) Acrylamide formation from asparagine is dependent on numerous food processing parameters including temperature, formulation and cooking time. Free asparagine concentration, therefore, reflects the general acrylamide-forming potential of grain. Acrylamide is classified as a neurotoxin and has been described as “probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC, 1994). By decreasing asparagine concentration in grain, optimized S and N fertilization may decrease the potential health concern of acrylamide in wheat food products (Curtis et al., 2018).

The 1990 US Federal Clean Air Act amendments have successfully improved air quality through regulation of power plant air emissions (USEPA, 2015). Sulfur emissions have decreased by 80%, resulting in measured decreases in sulfur deposition (NADP, 2015). Along with lower S deposition, soil S deficiency is becoming increasingly common throughout the world due to lack of S fertilization (David et al., 2016). Sulfur deficiencies are common in low organic matter soils, sandy soils and intensely farmed soils that do not receive S fertilizer.

Hard winter wheat is produced on 4.8 million hectares in the central Great Plains of the United States (Kansas, Oklahoma, Colorado), and 2018 production totaled 11.4 million metric tons (USDA, 2018). There is limited research on the interaction of genotype, sulfur, and nitrogen management in this major grain producing region. The goal of this study was to evaluate the interaction effects of cultivar, S, and N application on crop quality and asparagine concentration in hard red winter wheat grown on S-deficient soil under the low rainfall conditions of the central Great Plains of the United States.

Materials and Methods

Field Experiment

This study was conducted as a small-plot field experiment at the Natural Resource Conservation Service Plant Materials Center near Manhattan, Kansas in 2017 and 2018. The soil selected for the study is mapped as Belvue Silt Loam with soil properties typically associated with S deficiency. Different experimental areas on the same experimental farm were used in the two years of the trial. In each year, baseline soil conditions were recorded from three composite soil samples collected from the experimental area at 0-15 cm and 15 to 46 cm prior to the experiment, and analyzed for organic matter by loss on ignition (Combs and Nathan, 1998),

SO_4-S by monocalcium phosphate extraction (Combs et al., 1998),and pH in water (Watson and Brown, 1998) conducted by the Kansas State University Soil Testing Laboratory.

The experiment was established in a randomized arrangement of a split-split-plot design with four replications. Treatments were arranged in a factorial design with three levels of N (56, 100 and 145 kg/ha applied as urea), two levels of S (0 and 22 kg/ha as ammonium sulfate), and four levels of genotype in 2017 (cvs. 'Everest', 'Fuller', 'Jagger', and 2137), and five levels of genotype in 2018 (Everest, Fuller, Jagger, 2137 and 'SY Monument'). Nitrogen treatments were applied as the whole plot, S treatments were applied as the subplot, and genotypes were the sub-subplot. Urea rates were adjusted for all S treatments to balance the N application. Wheat was planted on October 31, 2016 and October 21, 2017 with a no-till drill into soybean residue. Urea was broadcast by hand on February 16, 2017 and ammonium sulfate was broadcast by hand on April 6, 2017. In 2018, urea and ammonium sulfate were broadcast by hand on February 19. Plots were harvested with a small plot combine on June 20, 2017 and June 18, 2018. Harvested grain was cleaned with an air blast cleaner (Almaco, Nevada, Iowa). Test weight was measured gravimetrically according to American Association of Cereal Chemists (AACC) Method 55-10.01.

Genotypes were selected based on their nitrogen use efficiency, measured as grain yield per unit of applied N, and based on their nitrogen uptake efficiency, measured as N assimilated per unit of applied N, as identified in a previous trial (Dorsey, 2014). Jagger had low N use efficiency (low yielding) and high N uptake, Fuller had low N use efficiency (low yielding) and low N uptake, Everest had high N use efficiency (high yielding) and high N uptake, and 2137 had high N use efficiency (high yielding) and low N uptake. Agronomic experience with SY Monument, not included in Dorsey (2014), suggests that it is a high nitrogen use efficiency

genotype because it is high yielding with high grain protein concentration across a large number of trials (unpublished). The cultivar Everest, included in both years of the study, was the most widely grown wheat cultivar in Kansas in each year from 2013-2018. SY Monument was added in 2018 due to its rapid rise in importance in Kansas production. SY Monument was the second most widely grown wheat in Kansas in 2018 and became the most widely grown wheat in Kansas in 2019 (https://www.nass.usda.gov/Statistics_by_State/Kansas/Publications/Cooperative_Projects/Wheat_Varieities/, accessed December 31, 2019).

Grain Compositional Analysis

Whole grain protein concentration and moisture content were analyzed using near infrared reflectance (DA7250, Perten Instruments North America, Springfield, IL, USA) according to AACC Method 39-25.01. Kernel hardness, size and weight were measured using a single kernel characterization system (SKCS 4100, Perten Instruments North America) according to AACC Method 55-31.01. Protein composition was evaluated as the ratio of total polymeric/ total monomeric protein; whole grain samples were ground with a cyclone mill (2010-030 cyclone sample mill, UDY, Ft. Collins, CO, USA) with a 1 mm stainless steel screen. Protein composition was evaluated using size exclusion chromatography. Protein was extracted from 20 mg flour with bran in 1 mL 50 mM sodium phosphate pH 6.9 containing 0.5% sodium lauryl sulfate. Samples were vortex mixed for 5 sec, then vortexed for 5 min at room temp. Samples in 2 ml microcentrifuge tubes were then sonicated for 20 sec at input of 6W (Q-Sonica XL 2000 series, Newton, CT, USA) in cold metallic thermal beads. Sonicated samples were centrifuged at 12,000 x *g* for 5 min and supernatants were transferred to 2 ml Spin X centrifuge tube filters containing a 0.45 μm nylon membrane (Corning, Costar, Corning, NY, USA) and centrifuged at

12,000 x *g* for 5 min. The filtrate was analyzed using an Agilent 1200 series high performance liquid chromatograph (HPLC), (Agilent, Santa Clara, CA, USA) equipped with variable wavelength UV detector operating at 214 nm. The column used for SE-HPLC was a 300 x 7.8mm BioSepSEC-s4000 Pore size 500 Å (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a 50/50 ratio of deionized water + 0.1% trifluoroacetic acid (TFA) and acetonitrile + 0.1% TFA (Sigma Aldrich, St. Louis, MO). The run time was 30 min at a flow rate of 0.5 ml/min with pressure of 28 bar at 30ºC.

Free amino acids were extracted from 0.5 g of whole grain flour in 10 mL 0.001 N hydrochloric acid. Extracts were vortexed and then shaken for 10 min. Samples were allowed to rest for 15 minutes, then centrifuged at 6,000 x *g* for 15 min. An aliquot of the supernatant was transferred to a clean microcentrifuge tube and stored at -20°C until analysis. Samples were thawed, centrifuged at 12,000 x g for 2 min, then derivatized using the EZ: Faast kit (Phenomenx, Torrance, CA, USA) and analyzed by gas chromatography mass spectrometry (GC-MS) (Agilent 7890B GC and 5977B MS Santa Clara, CA, USA) equipped with a multipurpose auto sampler robot (Gerstel MPS, Mülheim , Germany). The GC-column was a Zebron ZB-AAA (Phenomenex) 10m x 0.25 mm x 0.15 mm. The oven temperature was initially held at 110°C for 1 min, raised at 25°C/min to 320°C, and held for 1 min. The column flow was 22.154 mL/min. The injection volume was 1µL with a run time of 10 min 40 sec. Calibration standards as recommended by the manufacturer and provided by the EZ: Faast kit were supplemented with additional asparagine standards to encompass the range of concentrations observed in the experimental samples.

Total grain N and SO_4S were analyzed by the Kansas State University Soil Testing Lab. Whole grain samples were ground with a cyclone mill (2010-030 cyclone sample mill, UDY, Ft.

Collins, CO, USA) with a 1 mm stainless steel screen. Analysis of SO_4S was analyzed by inductively coupled plasma (ICP) spectrometry on Nitric-Perchloric digested whole ground meal. The Nitric-Perchloric digest was conducted as follows; prepared whole ground meal (0.2500g) was weighed into a 50 ml Kimax digestion tube. Boiling chips and 5 ml of Nitric acid was then added in a fume hood. The tubes were then covered with saran wrap overnight. 5ml of Perchloric acid was added to predigested plant material and tubes were placed on a cold Techator digestion block. Heat was set at 200°C and digests were heated for 3 hours or until white fumes appear and the acid is clear (and colorless when cooled to room temperature). Tubes were then diluted to 25 or 50 ml with ddH2O and mixed by inverting twice. Total N was calculated by a LECO TruSpec CN combustion analyzer on a 0.15-g sample of prepared whole ground meal. This test reports total levels (inorganic and organic) of N on a weight percent basis.

Functional Quality Analysis

Grain from the 2018 trial was milled and flour was characterized by Grain Craft (Wichita, KS). Flour protein concentration was measured by NIR spectrometry. Ash concentration was measured per AACC Method 08-01. Farinograph (AACC Method 54 -21 02) stability, absorption, arrival, mixing tolerance index (MTI) and peak were recorded.

Lactic-acid sodium dodecyl sulfate (SDS) solvent retention capacity (SRC) was measured on whole wheat meal from all plots in both the 2017 and 2018 trials. The method was generally as described by Seabourn et al. (2012). Wheat meal samples (1 g) were accurately weighed into a 50 mL centrifuge tube to which 5 mL of 0.47% lactic acid was then added. The meal was suspended in the lactic acid solution by mixing on a vortex mixer (Fisher Scientific Fisherbrand digital vortex, Waltham, MA) for 6 sec. Then 20 mL of 1.25% (w/v) SDS solution was added to

the tube and mixed on vortex mixer for an additional 6 sec. Tubes were then shaken on an orbital shaker for 4 min at 300 rpm (Scientific Industries, Mini- 300 orbital genie with adhering mat, Bohemia, NY) then centrifuged (Beckman Coulter Avanti J-E, Indianapolis, IN, USA) for 2 min at 3200 x *g*. Supernatant was removed by decanting the tubes then placing them upside down on a disposable lab mat for 5 min to drain any remaining liquid. Solvent retention capacity was calculated from the weight of the wheat meal and the weight of the final pellet as described in Seabourn et al. (2012). Flour moisture was estimated as 10%.

Statistical Analysis

Data were analyzed using a mixed model analysis of variance. Each trial year was analyzed separately because of the additional genotype included in the second year of the study. Data analysis was carried out in R software version 3.5.1 (R Core Team, 2017) using the Agricolae package (Mendiburu, 2015). Main plot error (rep × N) was used to test the effect of N rate. Subplot error (S × N (rep)) was used to test the effects of S and the N × S interaction. Residual error was used to test the effects of genotype and all interactions of fertility with genotype.

Results and Discussion

Agronomic Traits

The soils of the two experimental areas in which trials were grown were considered deficient (Leikam et al. 2003) due to the low organic matter (< 1.8%) and low SO_4-S concentrations in the soil (Table 1) averaging around 2 mg/kg. In 2018, the soil had slightly higher organic matter therefore having higher available S, although the S content of the soil was still considered deficient. The weather was more favorable for wheat production in 2017 than in

2018, with cooler temperatures and greater available moisture in 2017 (Figure 5 A&B). Mean grain yield in 2017 was 2783 kg/ha and in 2018 was 1580 kg/ha. Although N rates affected some parameters, most notably grain protein concentration, genotypes generally responded similarly to increased N, and the three-way interactions of N, S, and genotype were generally non-significant (Tables 2-4). Therefore, our analysis will focus principally on the effects of S application and the range of responses of genotypes to S application. Because the three-way interactions of N, S, and genotype were generally non-significant, conclusions regarding the responses of genotypes to S application are valid across the range of N rates included in the study. In 2017, S application increased yield by 1201 kg/ha (55%), and in 2018, S increased yield by 626 kg/ha (49%) (Table 5). Yield of wheat genotypes responded to S in a genotype-dependent manner in 2017, while in 2018, S was the primary determinant of grain yield (Table 2). In 2017, increasing N rates increased grain yield, but only when S was applied (Figure 6). Nitrogen rate did not affect grain yield in 2018.

Table 1. Soil analysis prior to conducting the experiment.

				SO_4-S (kg/ha)		
Year	Organic matter	pH	S from OM† (kg/ha)	0-15 cm	15-46 cm	Avail. S (kg/ha)
2017	0.9%	6.1	2.6	2.5	5.4	10.4
2018	1.7%	6.1	4.8	3.8	6.7	15.4

† Estimated as 2.5 times % organic matter (Leikam et al., 2003).

Table 2. Test for significance of main effects and interactions based on analysis of variance for grain yield, test weight and kernel weight.

	F-statistic					
	Grain Yield		Test Weight		Kernel Weight	
Effect	2017	2018	2017	2018	2017	2018
Nitrogen rate (N)	1.7 ns	2.2 ns	2.7 ns	11.9**	11.7**	1.3
Sulfur rate (S)	191.3***	118.0***	2.7 ns	119.9***	99.0***	14.7**
Genotype	89.0***	1.0 ns	8.8***	18.9***	1.9 ns	6.2***
N × S	8.5*	0.4 ns	0.4 ns	0.8 ns	11.5**	0.3 ns
N × Genotype	0.8 ns	1.8 ns	0.10 ns	2.5*	1.4 ns	1.4 ns
S × Genotype	4.7***	2.3 ns	1.3 ns	3.4*	0.4 ns	0.5 ns
N × S × Genotype	1.4 ns	0.2 ns	1.6 ns	1.6 ns	1.0 ns	1.3 ns

*, **, *** indicate significance at $p < 0.05$, 0.01, 0.001, respectively. [†]ns, non-significant

Table 3. Test for significance of main effects and interactions based on analysis of variance for grain protein concentration, ratio of total polymeric protein to total monomeric protein (TPP/TMP), free asparagine concentration and whole grain lactic acid-sodium

	F-statistic									
	Grain protein concentration		TPP/TMP		Asparagine		Lactic Acid – SDS SRC		N:S Ratio	
Effect	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Nitrogen rate (N)	83.4***	44.4***	4.6 ns	3.8 ns	6.8*	19.6**	4.0 ns	0.2 ns	12.5 **	6.4 *
Sulfur rate (S)	119.0***	65.0***	347.5***	55.1***	80.3***	418***	5.2*	362.2***	212.5 ***	2123.9 ***
Genotype	104.4***	93.7***	58.7***	7.0***	6.8***	8.8***	200.5***	42.5***	3.3 *	30.1***
N × S	4.2 ns	16.9***	5.8*	1.6 ns	5.4*	3.6 ns	18.6***	8.5**	5.6 *	17.9 ***
N × Genotype	0.30 ns	1.1 ns	0.4 ns	0.4 ns	0.4 ns	1.5 ns	3.1*	1.0 ns	0.57 ns	0.99 ns
S × Genotype	12.1***	9.3***	8.7***	3.9**	7.2***	5.3***	7.7***	3.9**	1.3 ns	6.6 ***
N × S × Genotype	1.8 ns	0.8	1.2 ns	1.0 ns	0.4 ns	2.0 ns	0.2 ns	2.6**	1.2 ns	0.90 ns

*, **, *** indicate significance at $p < 0.05$, 0.01, 0.001, respectively. †ns, non-significant

Table 4. Test for significance of main effects and interactions based on analysis of variance for flour yield, flour ash concentration, and farinograph absorption, arrival, MTI, peak and stability determined on grain grown in the 2018 trial.

	F-Statistic						
Effect	Flour yield	Flour ash concentration	Farinograph absorption	Farinograph arrival	Farinograph MTI	Farinograph peak	Farinograph stability
Nitrogen	9.0*	3.1 ns	12.5**	29.7***	3.6 ns	9.0*	5.5*
Sulfur	0.3 ns	27.4***	7.8*	95.4***	18.4**	149.3***	94.6***
Genotype	2.0 ns	0.9 ns	12.9***	23.6***	14.0***	12.6***	50.5***
N × S	0.6 ns	2.9 ns	5.7*	6.0*	0.9 ns	2.8 ns	0.3 ns
N × Genotype	1.0 ns	2.9**	1.0 ns	0.8 ns	1.0 ns	0.7 ns	1.4 ns
S × Genotype	4.6**	6.2***	7.1***	9.8***	5.6***	6.0***	1.2 ns
N × S × Genotype	1.5 ns	2.2*	2.0 ns	3.8***	1.8 ns	0.3 ns	1.3 ns

*,**,*** indicate significance at $p < 0.05$, 0.01, 0.001, respectively. †ns, non-significant

Table 5. Effect of S on grain yield, test weight, and kernel weight.

	Grain yield (kg/ha)		Test weight (g/l)		Kernel weight (mg)		Protein Yield (kg/ha)	
Sulfur treatment	2017	2018	2017	2018	2017	2018	2017	2018
0 kg/ha S	2182	1268	773	756	30.3	26.3	343	192
22 kg/ha S	3383	1894	766	714	28.0	23.3	487	316
F-statistic	191.3***	118.0***	2.7 ns	119.9***	99.0***	14.7**	622.4***	322.4***

***, **, * indicate significance at $p < 0.001$, 0.01, 0.05, respectively.

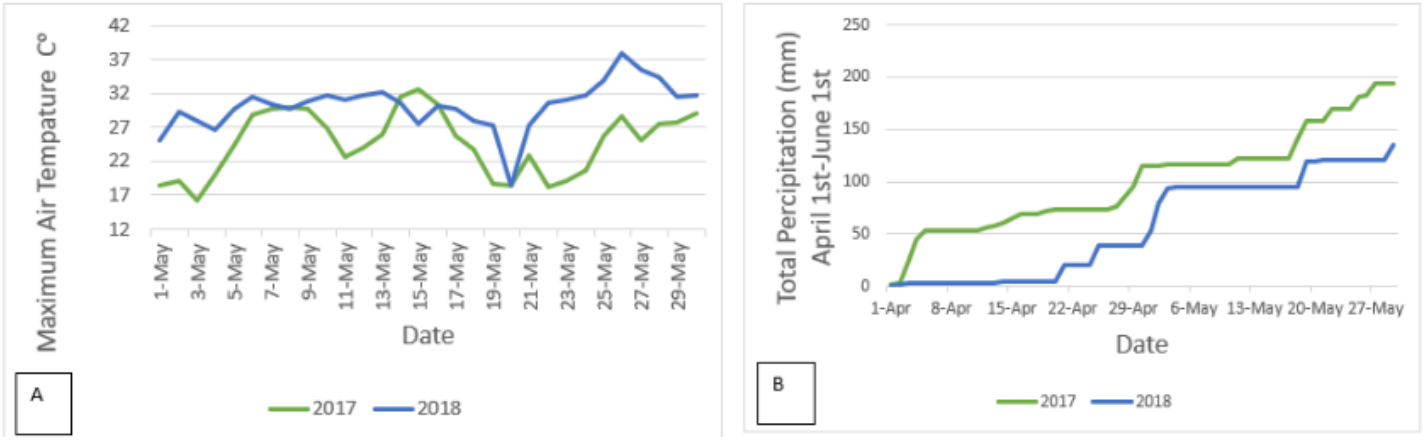

Figure 5. (A) Maximum air temperature (Cº) for May 2017 and 2018 during grain fill. (B) Cumulative precipitation from April 1 – Jun 1. Data collected from Kansas Mesonet (http://mesonet.k-state.edu/).

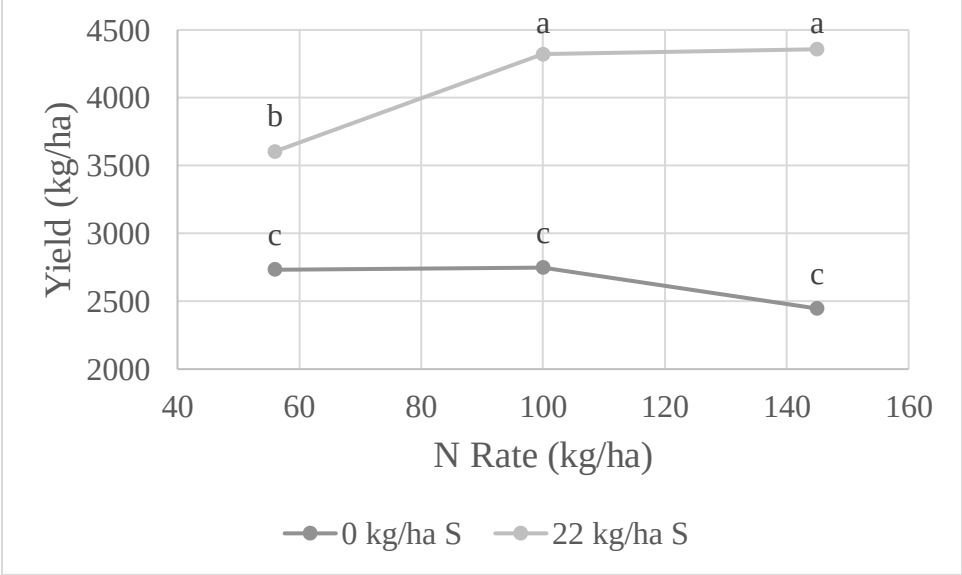

Figure 6. Nitrogen by sulfur interaction effect on grain yield in 2017 rates. (Different letters above bars indicate means are significantly different at p<0.05)

In 2017, genotypes differed in test weight, but fertility treatments had no effect on test weight. In 2018, however, test weight of genotypes responded differently to S and N applications, and S application significantly decreased test weight (Tables 2&5). In 2018, cool

early spring temperatures favored tiller production in plants with adequate available fertility. Then, at flowering, high temperature and low rainfall limited the ability of well-tillered plants to fill grain.

Fertility treatments affected kernel weight in 2017, and genotypes had similar kernel weights and responded similarly to fertility treatments in 2017. However, in 2018, both genotype and S application affected kernel weight, but kernel weight of the five genotypes again responded similarly to fertility treatment (Table 2). Sulfur application decreased kernel weight in both years (Table 5). Our hypothesis is that under higher fertility conditions, plants initiate additional seeds, but under moisture-limited conditions, plants are less able to translocate photosynthate to the developing seeds, thereby decreasing seed weight. Kernel weight is important because small kernels decrease the amount of flour produced and increase ash content (Gaines et al. 1997), producing a less desirable flour and dough quality.

Grain Composition

In both 2017 and 2018, genotype and fertility treatments affected grain protein concentration, and the effect of S application on protein concentration varied among genotypes (Table 3). Protein concentration was much higher in 2018 (average = 16.1 %) than in 2017 (average = 12.4 %). In 2017, S application tended to decrease protein concentration in all genotypes, but the effect was greater in some genotypes than others (Fig. 7 A&B). The decrease in protein concentration was likely due to dilution due to increased yield. Grain protein yield was increased significantly by S application both years (Table 5). The net yield of protein in 2017 averaged 343 kg ha^{-1} in the absence of S and averaged 487 kg ha^{-1} when S was applied. In 2018, S application tended to increase protein concentration, although the effect was larger in some

genotypes than others (Figure 8 A&B). The net yield of protein in 2018 averaged 195 kg ha^{-1} in the absence of S and averaged 298 kg ha^{-1} when S was applied. In 2018, increasing N rates increased protein concentration only when S was applied (Figure 9). Increased protein concentration in 2018 may be a consequence of hot, dry weather late in the growing season that reduced kernel weights (Table 5). Nitrogen to S ratios are a relatively inexpensive test and are helpful to producers in differentiating between nitrogen and sulfur deficiency as well as give information on S availability for the next growing season. If S is adequate the ratio will be less than 15 (Goos 2014). Nitrogen to S ratios were significantly improved both years with S application (Table 3), in 2017 ratios decreased from 23.6 to 15.0 and in 2018 32.3 to 18.4 (Figure 7). Nitrogen to S ratio was very predictive or many quality and agronomic parameters. There was a strong positive correlation with free asparagine concentration and N:S ratio in both 2017 and 2018 as well as test weight in 2018 and total N in grain in 2017. There was a strong negative correlation between N:S ratio and grain yield, protein yield, and SO_4S in grain in both years as well as lactic acid- SDS SRC in 2018 (Table 6). Thus, N:S ratio may be a good tool to predict quality and agronomic parameters of wheat grain.

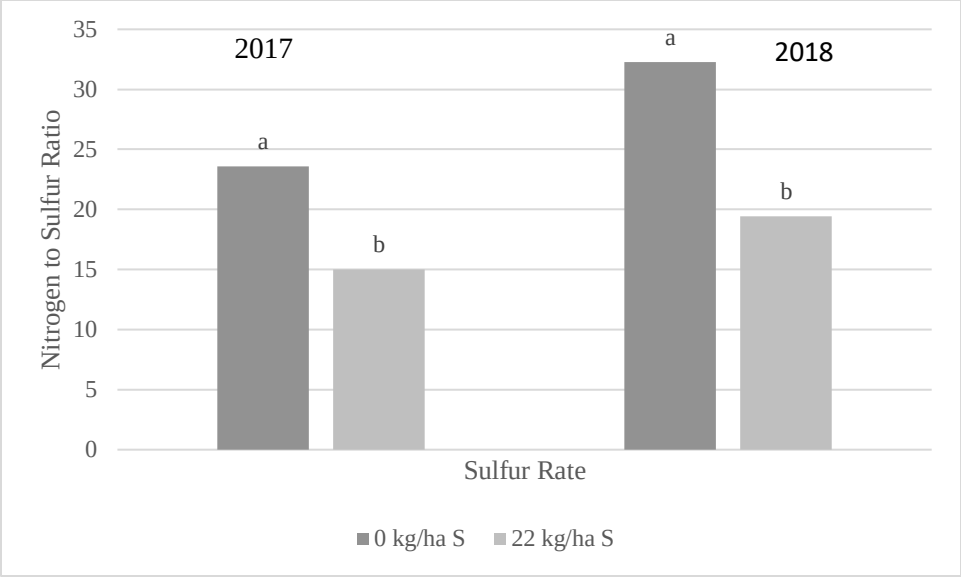

Figure 7. Effect of S application on the ratio of Nitrogen to Sulfur in 2017 and 2018. Means followed by the same letter are not significantly different according to an LSD test (α = 0.05).

Table 6. Correlation between N:S ratio in meal and various quality parameters. (Strong and moderate correlations highlighted in yellow > ±0.50)

CORRELATION TO N:S RATIO

	r	
	2017	**2018**
TOTAL POLYMERIC/TOTAL MONOMERIC PROTEIN	-0.72	-.52
LACTIC ACID- SDS SRC	-0.23	-0.88
TEST WEIGHT	0.04	0.63
FREE ASPARAGINE CONCENTRATION	0.81	0.93
THOUSAND KERNEL WEIGHT	0.38	0.40
GRAIN PROTEIN	-0.74	-0.42
GRAIN YIELD	-0.81	-0.81
PROTEIN YIELD	-0.65	-0.81
TOTAL N IN GRAIN	0.71	0.14
SO_4S IN GRAIN	-0.90	-0.95
FLOUR ASH	NA	-0.41
FLOUR PROTEIN	NA	-0.26
FARINOGRAPH ABSORBTION	NA	-0.27
FARINOGRAPH MTI	NA	0.35
FARINOGRAPH PEAK	NA	-0.63
FARINOGRAPH STABILITY	NA	-0.60

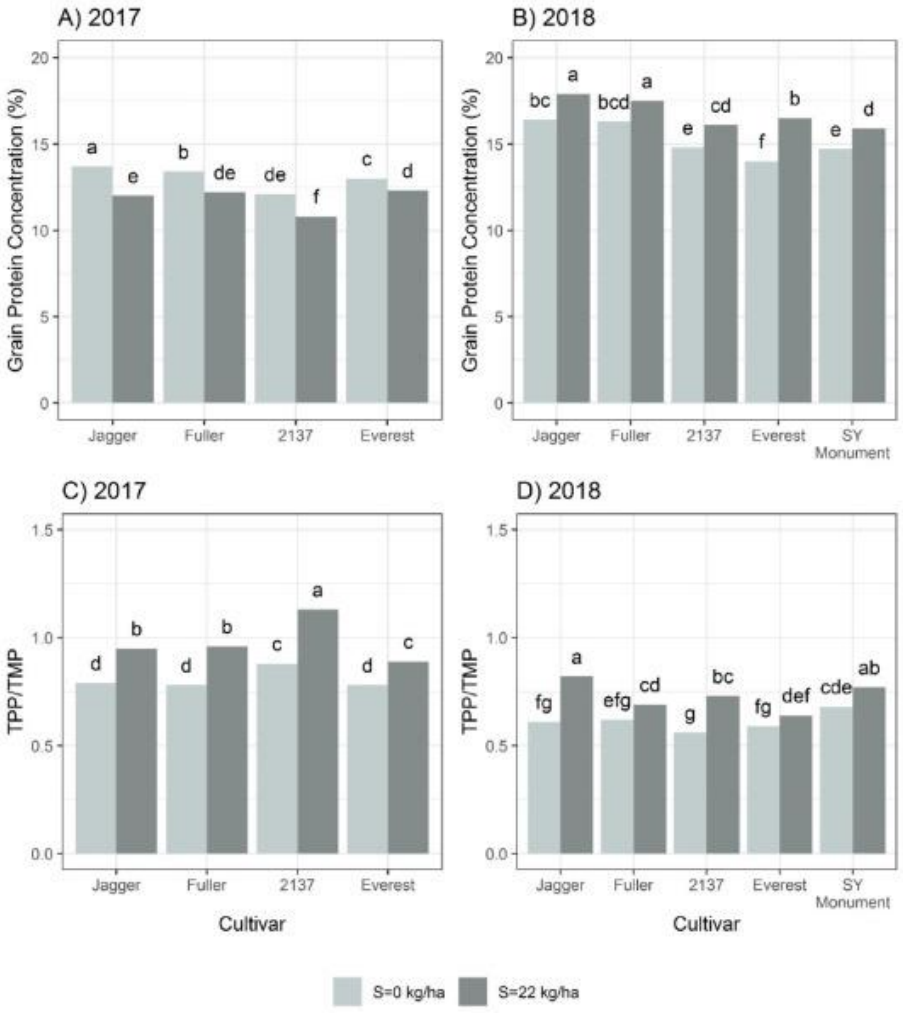

Figure 8. (A&B) Effect of genotype and S application on grain protein concentration in 2017 (A) and 2018 (B). (C&D) Effect of genotype and S application on the ratio of total polymeric protein (TPP) to total monomeric protein (TMP) in 2017 (C) and 2018 (D). Means followed by the same letter are not significantly different according to an LSD test ($\alpha = 0.05$).

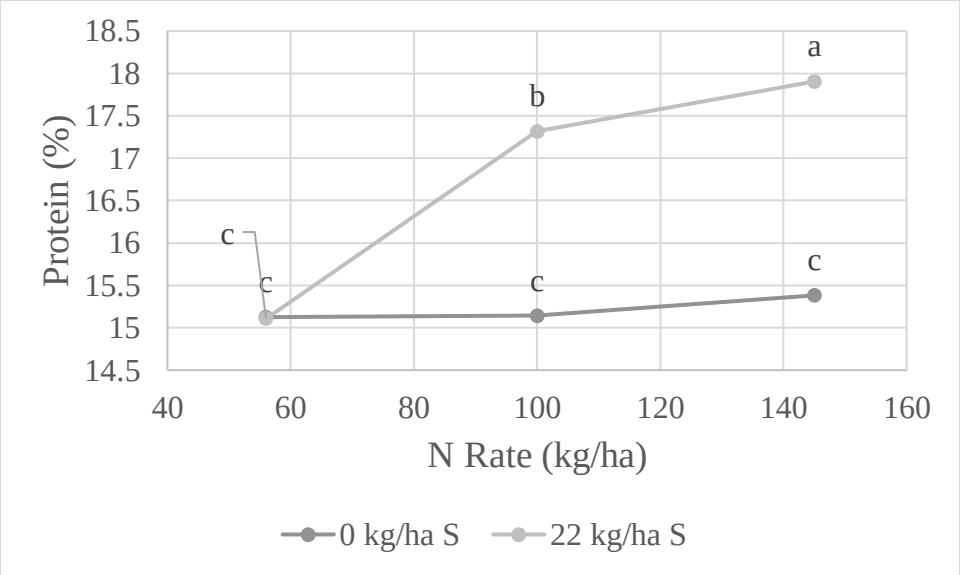

Figure 9. Nitrogen by sulfur interaction effect on grain protein concentration in 2018. (Different letters above bars indicate means are significantly different at p<0.05)

In both 2017 and 2018, genotype and S fertility treatment affected grain protein quality, as measured by the ratio of total polymeric protein (TPP) to total monomeric protein (TMP) and genotypes responded differently to S application (Table 3). The ratio of TPP to TMP was lower in 2018 (average = 0.72) than in 2017 (average = 0.91). The reduced quality of protein in 2018 may be due to the higher temperatures during grain fill. In 2017 and 2018 the ratio of TPP/TMP increased across all genotypes when S was applied (Figure 8 C&D). Nitrogen rate had negligible effect on protein quality: in 2017, protein quality decreased slightly with increasing N rate only in the absence of applied S (Figure 10).

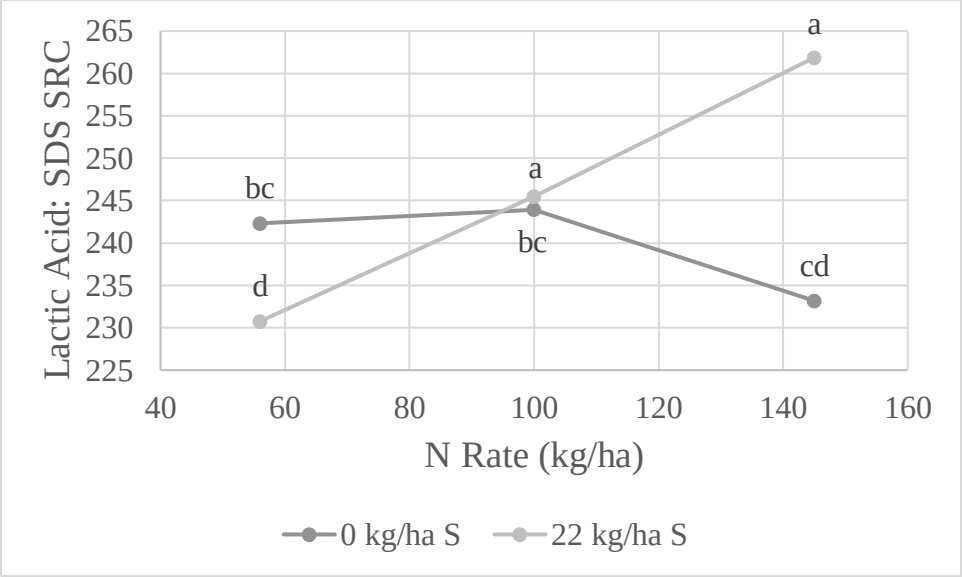

Figure 10 Nitrogen by sulfur interaction effect on grain protein quality in 2017. (Different letters above bars indicate means are significantly different at p<0.05)

Table 7. Effect of S on flour yield, flour ash concentration, farinograph absorption, arrival, MTI, peak, and stability averaged across nitrogen rates in 2018.

					Farinograph									
	Flour extraction		Flour ash (g/kg)		Absorption (%)		Arrival time (min)		MTI		Peak (min)		Stability (min)	
Genotype	0 kg/ ha S	22 kg/ha S	0 kg /ha S	22 kg/ha S	0 kg/ha S	22 kg/ha S	0 kg/ha S	22 kg/ ha S	0 kg/ha S	22 kg/ ha S	0 kg/ha S	22 kg/ha S	0 kg/ha S	22 kg/ ha S
Jagger	650	641	4.82	5.36	59.2	61.9	1.3	2.4	20.6	21.7	4.9	8.6	11.1	15.5
Fuller	624	654	5.04	5.19	58.9	61.1	1.2	1.9	24.1	12.8	4.5	8.6	12.1	17.8
Everest	651	650	5.11	5.25	61.2	61.5	1.5	2.6	35.0	29.1	3.5	6.4	5.6	10.3
2137	662	645	4.89	5.35	59.2	59.5	1.9	2.0	32.5	20.6	5.2	6.6	7.4	13.2
SY Monument	640	646	5.07	5.05	59.4	59.3	1.0	1.4	33.0	16.8	4.2	5.6	9.6	16.0
Mean	645	647 ns	4.99	5.24 ***	59.6	60.7 *	1.4	2.1 ***	29.0	20.2 *	4.5	7.2 ***	9.2	14.6 ***
LSD†$_{(\alpha=0.05)}$	1.6(1.7)		0.02(0.02)		0.9(1.1)		0.3(0.3)		5.6(6.5)		1.4(1.0)		1.5(1.8)	

***, **, * indicate significance of S treatment effect at $p < 0.001$, 0.01, 0.05, respectively.
ns, non-significant
†Values outside parentheses represent least significant differences for comparisons of genotypes within S rates. Values inside parentheses represent LSD for comparisons across S rates.

Free asparagine concentration was greater in 2018 (average = 20.9 μmol/g) than in 2017 (average = 9.8 μmol/g). This is consistent with greater stress in 2018 due to the higher temperature and drought conditions during grain fill. Asparagine concentrations were highly heteroskedastic according to Bartlett's test ($p < 2 \times 10^{-16}$), with greater variance in the absence of S than when S was applied. Data therefore were log-transformed for analysis. Untransformed means are presented in figures and tables, while statistical significance was determined on transformed data. Fertility treatments and genotype affected free asparagine concentration in both years, and genotypes responded differently to S application. Free asparagine concentration decreased significantly in both years across all genotypes when S was applied. Greatest differentiation between genotypes was observed in the absence of S. In the absence of S application, and in both 2017 and 2018, the genotypes Jagger and Fuller, which were rated as low N use efficiency (Dorsey, 2014) had significantly greater free asparagine concentration than the genotypes Everest and 2137, which were rated as high N use efficiency (Dorsey, 2014). When S was applied, asparagine concentration differences between genotypes were minimized (Figure 10 A&B). Increasing N rates also increased asparagine concentration, particularly in the absence of S. Highest asparagine concentrations in both years were observed with 100 or 145 kg ha^{-1} N in the absence of S (Figure 11). Asparagine concentrations ranged from 2.62 μmol/g to 21.0 μmol/g in 2017. Free asparagine concentrations ranged from 5.4 μmol/g to 41.4 μmol/g in 2018. Navrotskyi (2018) previously reported levels of free asparagine ranging from 1.5 μmol/g to 8.32 μmol/g in Nebraska trials, and Curtis (2018) reported concentrations ranging from 0.7

µmol/g to 11.2 µmol/g in 2012-2013 in the United Kingdom. Although there is no statutory threshold for asparagine concentration, lower concentration is preferable.

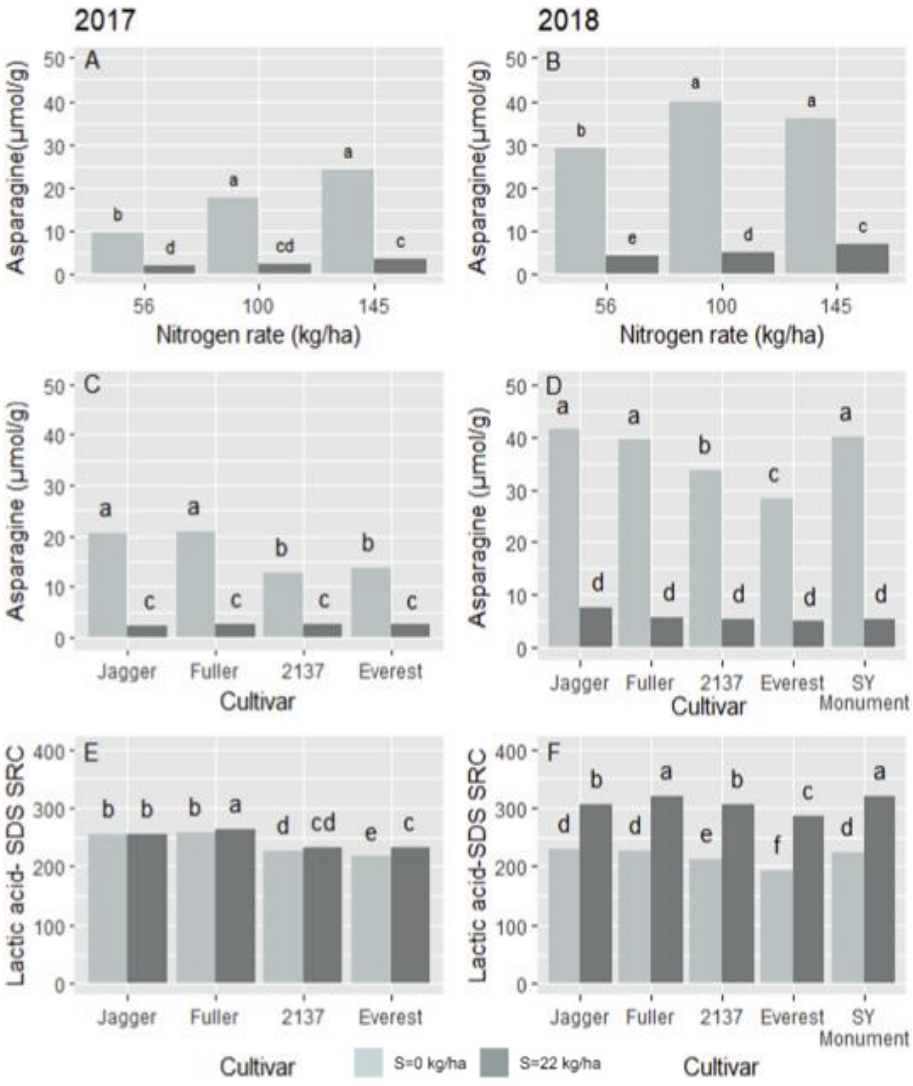

Figure 11 (A&B) Effect of nitrogen rate and S application on free asparagine concentration in 2017(A) and 2018 (B). Means followed by the same letter are not significantly different according to an LSD test (α = 0.05) performed on log-transformed data. (C&D) Effect of genotype and S application on free asparagine concentration in 2017 (C) and 2018 (D). (E&F) Effect of genotype and S application on lactic acid- SDS SRC in 2017 (E) and 2018 (F).

Functional Quality Analysis

The decreased kernel weight observed with S application is concerning from the perspective of flour milling. However, the effect of S application on flour extraction was minimal (Table 5, Table 7). We expected to observe decreased flour extraction when S was applied, yet flour extraction decreased significantly with S application only for Jagger and 2137, while flour extraction increased significantly with S application for Fuller and SY Monument.

Lactic acid SDS SRC was higher in 2018 (average = 262%) than in 2017 (average = 243%). In both years, S application and genotype influenced lactic acid SDS SRC, and genotypes varied in response to S application (Table 7). When S was applied, lactic acid SDS SRC significantly increased (Figure 11 C&D). In both 2017 and 2018, lactic acid SDS SRC increased with increasing N rate when S was applied but decreased when S was not applied (Figure 10, Figure 12). Lactic acid-SDS SRC is a function of both the protein concentration and the protein quality. In 2017, protein concentration decreased substantially with S application, which would be expected to decrease lactic acid-SDS SRC. However, the improvement in protein quality, as demonstrated by the increased TPP/TMP ratio with S application, resulted in a net increase in lactic acid-SDS SRC. Lactic acid-SDS SRC is a rapid, small-scale method to assess bread-making quality by combining the solutions used in the SDS sedimentation method (AACC method 56-70 (AACC, 2000)) with the centrifugation process used in the SRC method (AACC56-11 (AACC, 2000), Seabourn et al., 2012).

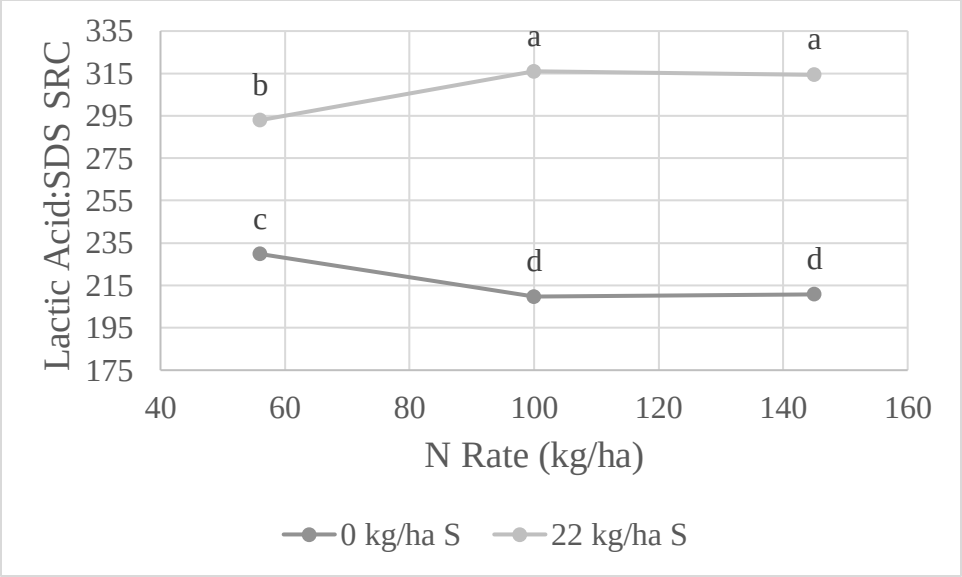

Figure 12. Nitrogen by sulfur interaction effect on grain protein quality in 2018. (Different letters above bars indicate means are significantly different at p<0.05)

Flour ash concentration was greater with S application (average = 5.24 g/kg) than in the absence of S (average = 4.99 g/kg). Sulfur application and genotype affected ash, and genotypes varied in response to S application (Table 7). Flour ash increased significantly for all genotypes except SY Monument when S was applied. We hypothesize that higher flour ash concentration when S was applied was a consequence of decreased kernel weight.

Genotype and S application affected all farinograph parameters (Table 6), and farinograph parameters of the five genotypes responded differently to S application, apart from farinograph stability, for which the S × genotype interaction was non-significant. Mean farinograph parameters for S × genotype combinations are presented in (Table 6). Sulfur application generally increased farinograph absorption and arrival time, and decreased MTI, with relatively minor differences among genotypes in S response. Farinograph peak time increased from 4.5 min to 7.2 min, averaged across N rates and genotypes, when S was applied. Farinograph stability of all genotypes was greatly improved with S application. Averaged across genotypes and N rates, stability improved from 9.2 min to 14.6 min with S application. Farinograph arrival time and water absorption response to N rate followed the pattern observed for grain protein concentration: arrival time and water absorption increased with increasing N rate only when S was applied (Figure 13, Figure 14).

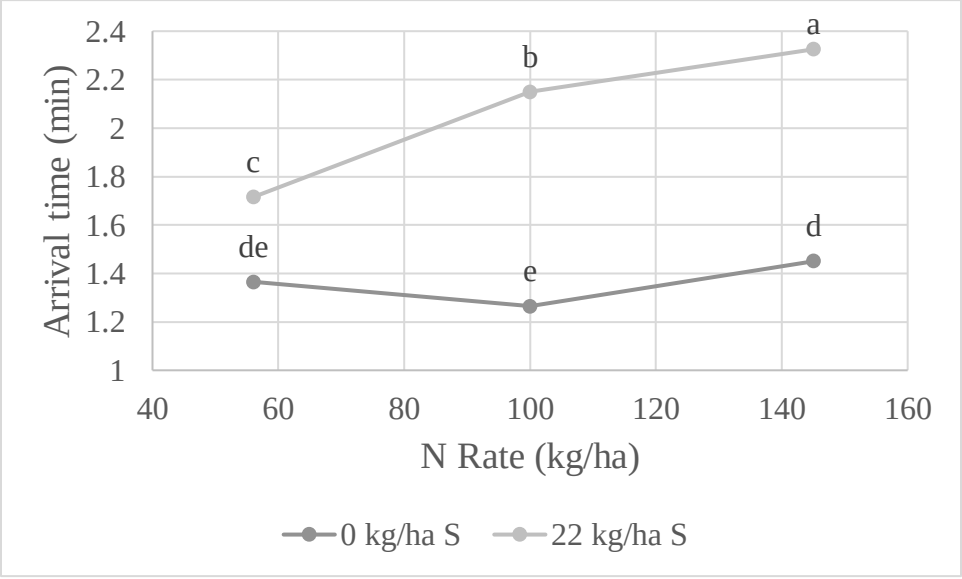

Figure 13. Nitrogen by sulfur interaction effect on farinograph arrival time (min) in 2018. (Different letters above bars indicate means are significantly different at p<0.05)

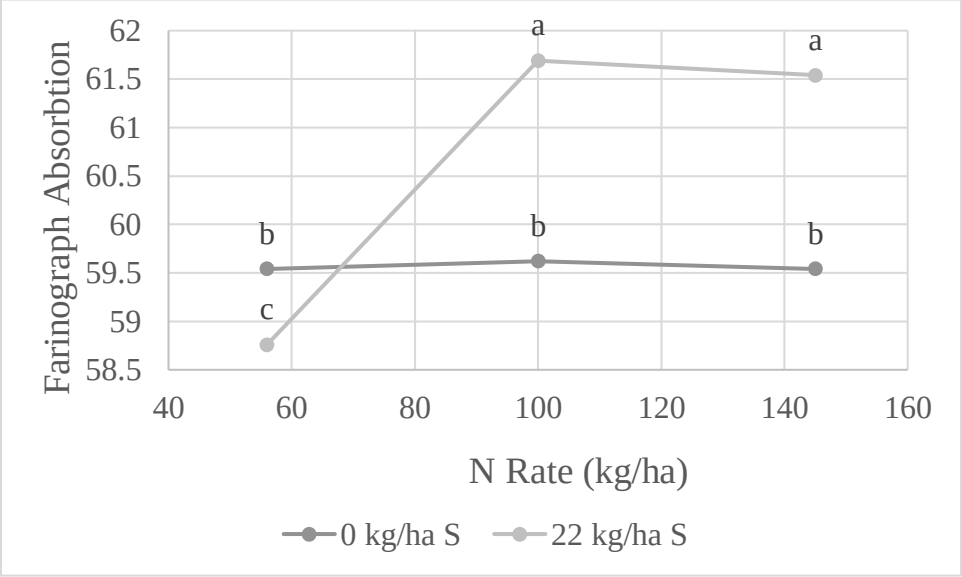

Figure 14. Nitrogen by sulfur interaction effect on farinograph absorption in 2018. (Different letters above bars indicate means are significantly different at p<0.05)

Prediction of farinograph stability is important for millers and end-users of hard winter wheat. In these 120 samples, flour protein concentration and farinograph stability were well correlated (r = 0.46***). The TPP/TMP ratio, an indicator of protein quality, also was well correlated with farinograph stability (r = 0.54***). Lactic acid-SDS SRC was more highly correlated with farinograph stability (r = 0.75***). The lactic acid-SDS SRC appears to integrate both protein quantity and protein quality in its prediction of stability. Averaged across N rates, farinograph stability was effectively predicted by lactic acid-SDS SRC (R^2= 0.78) (Figure 14). This rapid micro-test could have wide application in breeding and agronomic research, and the method could prove useful in flour mills for evaluating incoming grain.

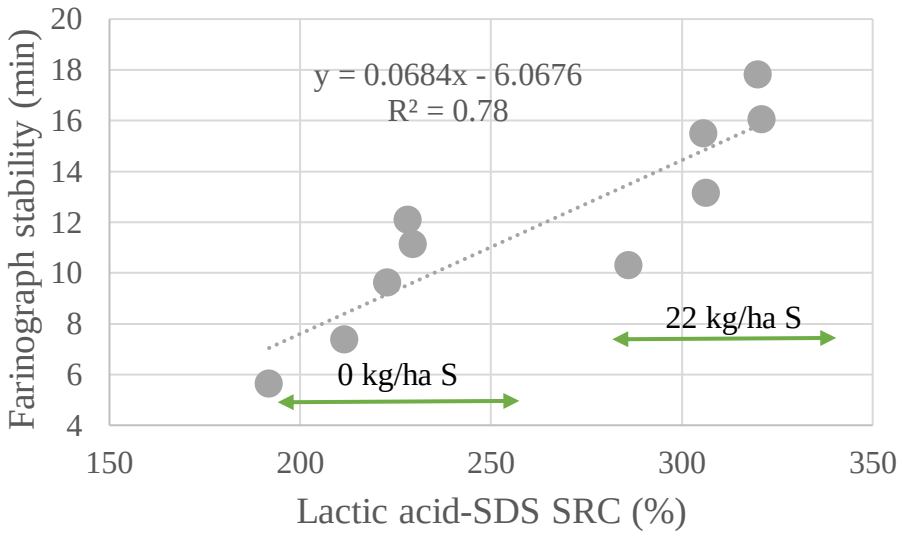

Figure 15. Prediction of farinograph stability by lactic acid–SDS SRC assay in 2018. Data points represent genotype × sulfur treatment combinations. Green arrows indicate sulfur treatments.

Conclusion

Sulfur application significantly increased grain yield in both years of the study, despite decreasing kernel weight. Therefore, when wheat is grown on S-deficient soils, S application appears to improve grain yield by increasing grain number per hectare. Grain protein concentration cannot be assumed to increase with S application due to increasing yields causing a dilution of protein effect. This dilution effect was obviated in 2018 by heat and drought stress, which more significantly reduced kernel weight in 2018. Protein quality improved with S application in both years of the study. Free asparagine concentration in grain grown in low S soils was strikingly high relative to previous studies, and low nitrogen use efficiency genotypes produced grain with greater asparagine concentration than high nitrogen use efficiency genotypes under S-deficient conditions. However, S application decreased asparagine concentration to baseline levels. Farinograph stability was improved with S application in all genotypes and stability was effectively predicted by lactic acid-SDS SRC. The effects of S

application in these studies were observed across a range of N application rates and genotypes. These studies were conducted on sandy soils with low organic matter and low available S, where a wheat crop would be expected to respond most favorably to S application. As soils become increasingly depleted of S, demands for higher yields and improved food safety and end use quality will require producers to conduct yearly soil tests and apply sulfur when needed.

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Chapter 3- Survey of Kansas Soil Sulfur Conditions

Abstract

Changing eco-physiological conditions for winter wheat production on the Great Plains of the United States may have an impact on grain quality. Work by our team and others has demonstrated that wheat grown on Sulfur (S)-deficient soils has decreased yield, protein quality and rheological properties. Sulfur deficiency has a relationship with free asparagine which is a precursor to acrylamide forming potential. This is a potential health concern because acrylamide is a type 2A carcinogen and a known neurotoxin, free asparagine accumulates in wheat grown under S-deficient conditions. Therefore, we undertook to characterize asparagine concentrations in an array of agronomically relevant winter wheat germplasm grown across the state of Kansas in 2018, 2019, and 2020. To parameterize the eco-physiological context in which these crops were grown, we collected soil samples to understand the baseline soil-S availability at each location and analyzed grain from each location for protein content, asparagine concentration and Lactic Acid- Sodium Dodecyl Sulfate (Lactic Acid- SDS SRC) value. Our results to date indicate that the risk of elevated asparagine concentration is, at this time, low across Kansas wheat production environments in the existing germplasm pool. However, there were some locations that resulted in elevated asparagine indicating a need for continued monitoring and development of practices to minimize asparagine concentration in grain. Although there was some indication that higher asparagine concentration had a relationship to lower S concentration in soil, the weak R^2 values further indicate that there are many other factors that also influence asparagine concentrations in grain. It is important that we continue to determine, research and explore factors that increase free asparagine concentration in grain.

Introduction

Wheat (*Triticum aestivum* L.) grain yield, protein concentration and overall quality are dependent on adequate soil fertility. Sulfur deficiency has been recognized as a limiting factor in crop production in many regions of the world (Zaho et al., 1999). Sulfur deficiency also decreases the proportion of polymeric proteins in total proteins but shifts the distribution of polymeric proteins toward lower molecular weight monomeric proteins. These changes in protein composition are closely associated with alterations of dough rheological properties such as farinograph peak time and stability (Wilson et al. 2020). Sulfur (S) fertilization has not been a primary concern in producers minds when they examine their field and see a deficient wheat crop with chlorosis and stunted growth, most producers would automatically think that this deficiency is coming from a nitrogen (N) deficiency (Wilson et al., 2020). One of the main reasons that producers have not had to think of S deficiency is because until 1990, when the Clean Air Act was put into place, S emissions into the environment were very plentiful. The Clean Air Act put National Ambient Air Quality Standards (NAAQS) into place which specified the amounts of sulfur dioxide (SO_2) to be present in outdoor air (USEPA, 2019) including the switch to low-S fuels, and fitting coal burning and oil burning power stations with pre- and post-combustion systems for removing SO_2 (Raffan et al., 2020). Limiting SO_2 in the air protects human health and the environment (USEPA,2019). Crops with greater yield need a greater quantity of nutrients to produce and be vigorous therefore having a much greater demand of soil minerals and nutrients which deplete the reserve in the soil more rapidly (Zaho et al., 1999). The impact these factors had on soil S availability were joined by farmers using more ammonium

nitrate- and ammonium phosphate- based fertilizers in preference to ammonium sulphate or superphosphate (a mixture of $CaSO_4 \cdot H_2O$ and $Ca(H_2PO_4)2 \cdot H_2O$), which contain less nitrogen and phosphorus but also provide S (David et al., 2016).

When S is not available barley grain contains a decreased proportion of storage proteins (hordein) and an increase of non-protein nitrogen (NPN), which is largely present as asparagine. (Shewry et al., 1983). Free asparagine accumulates under states of biological stress such as response to the restriction of protein synthesis. This stress can be caused by drought, pathogen attack, toxicity or nutrient deficiencies (Lea et al., 2007).

Acrylamide is formed from asparagine in the presence of reducing sugars in baked foods via the Maillard reaction (Council for Agricultural Science and Technology, 2006). Free asparagine concentration, therefore, reflects the general acrylamide-forming potential of grain. Acrylamide is classified as a neurotoxin and has been described as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC, 1994). By decreasing asparagine concentration in grain, optimized S availability may decrease the potential health concern of acrylamide in wheat food products (Curtis et al., 2018). A toxicity threshold for acrylamide concentration has not yet been determined, however committees of experts encouraged the food industry to reduce the acrylamide level in their products to obtain a dietary exposure as low as reasonably achievable (Loaëc et al., 2014). Thus, lesser free asparagine concentrations will considerably decrease the acrylamide forming potential therefore grain with lesser free asparagine concentration is preferable.

Soil S-deficiency has been impacting wheat production in many regions of the world, especially Western Europe (Scherer, 2001) but the soil S status of the Great Plains of the USA has not been determined. We have found that in cases of extreme S deficiency, asparagine

concentrations are highly elevated; however, little is known regarding the asparagine concentration in wheat grown on typical soils throughout the state of Kansas. It is important to know the status asparagine concentration in Kansas wheat and to determine if there are regions, soil types, or any other production factors that can be used to predict the threat of elevated asparagine concentration in wheat in the Great Plains. This would be helpful because even if the current risk of elevated asparagine concentration in Kansas wheat is low, it is critical that we can pinpoint locations, varieties, or growing conditions that could potentially increase the risk of elevated asparagine concentrations in wheat grain.

Materials and Methods

Field Experiment

Grain samples from 11 wheat varieties, grown across 15 locations across the state of Kansas in the 2018, 2019, and 2020 harvest years were analyzed in this experiment (Table 8, Table 9, Figure 15). Wheat varieties were selected based on acres currently grown and those that have the potential to be widely grown in the next few years. Some genotypes and locations were lost due to unpredictable weather throughout the three-year study. In each year and location, baseline soil conditions were determined from duplicate composite soil samples collected from the experimental area at 0–15 cm and 15–61 cm during the month of March each year, and analyzed for organic matter by loss on ignition (Combs and Nathan, 1998), SO_4-S by monocalcium phosphate extraction (Combs et al., 1998), and pH in water (Watson and Brown, 1998) conducted by the Kansas State University Soil Testing Laboratory. Available S in the soil profile was computed as shown in Equation 1:

$$AvS = 0.3\times S1\times 6 + 0.3\times S2\times 18 + 2.5\times SOM \quad \text{Equation 1}$$

Where AvS is the available S (lb S acre^{-1}), S1 is the sulfate concentration in soil at 0 to 15 cm deep (mg kg^{-1}), S2 is the sulfate concentration in the soil at 15 to 61 cm deep (mg kg^{-1}), and SOM is the soil organic matter in the soil at 0 to 15 cm deep (%) (Leikam et al., 2003).

Table 8. Listing of year, location, county, region and environment ID for locations from which winter wheat grain samples were collected.

Year	Location	County	Region	Environment ID
2018	Bellville	Republic	Northcentral	Bel18
2018	Colby	Thomas	West	Col18
2018	McPherson	McPherson	Central	Mcp18
2018	Gypsum	Saline	Central	Gyp18
2018	Hutchinson	Reno	Southcentral	Hut18
2018	Lorrain	Ellsworth	Central	Lor18
2018	Ashland	Riley	-	Ash18
2019	Decatur	Decatur	West	Dec19
2019	Hays	Ellis	West	Hay19
2019	Redd	Reno	Southcentral	Red19
2019	Ashland	Riley	-	Ash19
2019	Sumner	Sumner	Southcentral	Sum19
2019	Colby (IR)	Thomas	West	Col19_IR
2019	Colby	Thomas	West	Col19
2020	Ashland	Riley	-	Ash20
2020	Colby	Thomas	West	Col20
2020	Lorrain	Ellsworth	Central	Lor20
2020	Kingman	Kingman	Southcentral	Kin20
2020	Lane	Lane	West	Lan20
2020	McPherson	McPherson	Central	Mcp18
2020	Hutchinson	Reno	Southcentral	Hut20
2020	Solomon	Saline	Central	Sol20
2020	Sumner	Sumner	Southcentral	Sum20
2020	Washington	Washington	Northcentral	Was20

Table 9. Winter wheat genotypes grown at each location during the study (18 indicates genotype was grown at location in 2018, 19 indicates genotype was grown at location in 2019, 20 indicates genotype was grown at location in 2020, and blank boxes indicate genotype was not grown at location).

Location	Genotype										
	Bob Dole	Everest	Gallagher	Joe	LCS Chrome	Larry	Overley	SY Monument	WB 4458	WB 4699	Zenda
Ashland	18, 19, 20	18, 19, 20	18, 19	18, 19	18, 19	18, 19	18, 19, 20	18, 19, 20	18, 19		19
Bellville	18	18	18	18	18	18	18	18	18		18
Colby		18	18	18, 20	18, 19		18	18, 19	18		18, 19
Colby (IR)	18			19				19			19
Decatur				19	19			19			19
Gypsum	18	18	18	18	18	18	18	18	18		18
Hays				19							19
Hutchinson	18, 20	18, 20	18, 20	18, 20	18, 20	18, 20	18, 20	18, 20	18	20	18, 20
Kingman	20	20	20	20	20	20	20	20		20	20
Lane	20	20	20	20	20	20	20	20		20	20
Lorrain	18, 20	18, 20	18, 20	18	18, 20	18, 20	18, 20	18, 20	18	20	18, 20
McPherson	18, 20	18, 20	18, 20	18, 20	20	18, 20	18, 20	18, 20		20	18, 20
Redd	19	19	19	19	19	19	19	19	19		19
Solomon	20	20	20	20	20	20	20	20		20	20
Sumner	19	19	19	19	19	19	19		19		19
Washington	20	20	20	20	20	20	20	20		20	20

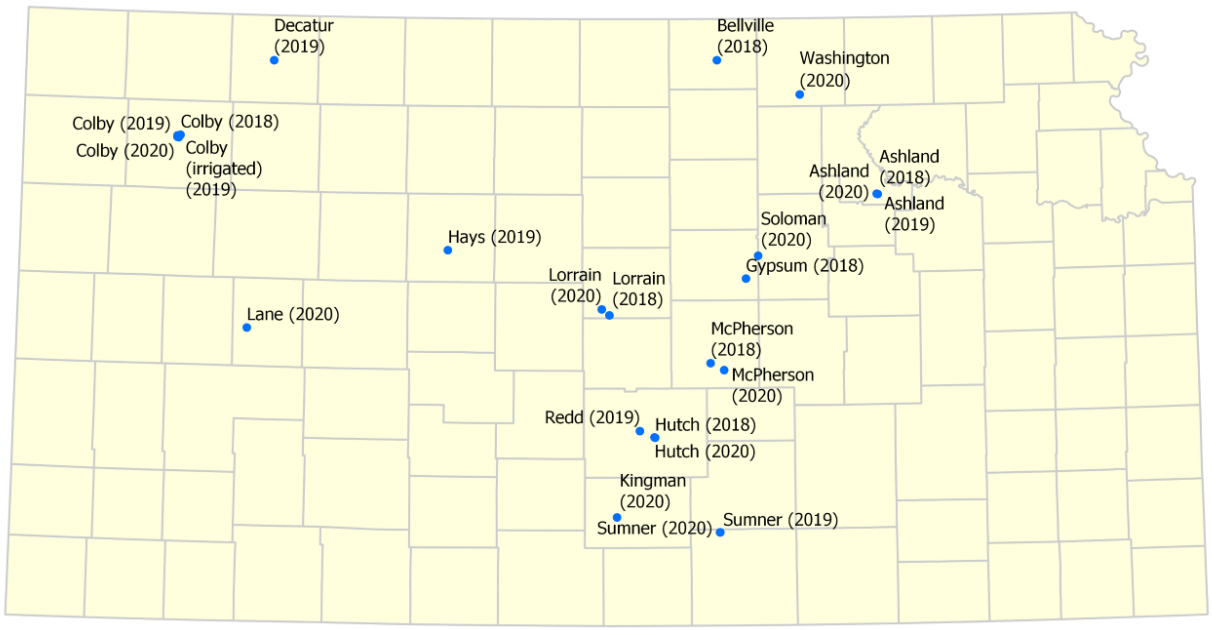

Figure 16. Map of the state of Kansas, with location and year ID for geographic reference.

Grain Compositional Analysis

Whole grain protein concentration and moisture content were analyzed in 2019 and 2020 using near infrared reflectance (DA7250, Perten Instruments North America, Springfield, IL, USA) according to AACC Method 39-25.01. Whole grain samples were ground with a cyclone mill (2010-030 cyclone sample mill, UDY, Ft. Collins, CO, USA) with a 1 mm stainless steel screen. Whole meal protein concentration and moisture content was analyzed in 2018 and 2019 using near infrared reflectance (DA7250, Perten Instruments North America, Springfield, IL, USA).

Free amino acids were extracted from 0.5 g of whole grain flour in 10 mL 0.001 N hydrochloric acid. Extracts were vortexed and then shaken for 10 min. Samples were allowed to rest for 15 minutes, then centrifuged at 6,000 x g for 15 min. An aliquot of the supernatant was transferred to a clean microcentrifuge tube and stored at -20°C until analysis. Samples were thawed, centrifuged at 12,000 x g for 2 min, then derivatized using the EZ:Faast kit (Phenomenx, Torrance, CA, USA) and analyzed by gas chromatography-mass spectrometry (GC-MS) (Agilent 7890B GC and 5977B MS Santa Clara, CA, USA) equipped with a multipurpose auto sampler robot (Gerstel MPS, Mülheim , Germany). The GC-column was a Zebron ZB-AAA (Phenomenex) 10m x 0.25 mm x 0.15 mm. The oven temperature was initially held at 110°C for 1 min, raised at 25°C/min to 320°C, and held for 1 min. The column flow was 22.154 mL/min of helium. The injection volume was 1µL with a run time of 10 min 40 sec. Calibration standards as recommended by the manufacturer and provided with the EZ:Faast kit were supplemented with additional asparagine standards to encompass the range of concentrations observed in the experimental samples.

Lactic-acid sodium dodecyl sulfate (SDS) solvent retention (SRC) (lactic acid- SDS SRC) test is a rapid and economical test used to predict bread-making quality. This test is well correlated to bread loaf volume and Mixograph water absorption and mix time. This test combines the solutions used in the SDS sedimentation method (AACC method 56-70 (AACC, 2000)) with the centrifugation process used in the SRC method (AACC56-11 (AACC, 2000), Seabourn et al., 2012). Higher lactic acid SDS-SRC values are preferable to lower values and indicate higher solvent retention and indicate higher loaf volume potential.

Lactic acid- SDS SRC was measured on whole wheat meal. The method was generally as described by Seabourn et al. (2012). Wheat meal samples (1 g) were accurately weighed into a 50 mL centrifuge tube to which 5 mL of 0.47% lactic acid was then added.

The meal was suspended in the lactic acid solution by mixing on a vortex mixer (Fisher Scientific Fisher brand digital vortex, Waltham, MA) for 6 sec. Then 20 mL of 1.25% (w/v) SDS solution was added to the tube and mixed on vortex mixer for an additional 6 sec. Tubes were then shaken on an orbital shaker for 4 min at 300 rpm (Scientific Industries, Mini- 300 Orbital Genie with adhering mat, Bohemia, NY) then centrifuged (Beckman Coulter Avanti J-E, Indianapolis, IN, USA) for 2 min at 3200 x g. Supernatant was removed by decanting the tubes then placing them upside down on a disposable lab mat for 5 min to drain any remaining liquid. Lactic acid SDS-SRC value was calculated from the weight of the wheat meal and the weight of the final pellet as described in Seabourn et al. (2012). Flour moisture was estimated as 10%.

Statistical Analysis

Data were analyzed by one-way analysis of variance with SAS version 9.4 using the PROC GLIMMIX procedure to determine effect of genotype, location, soil series, region, and environment on free asparagine concentration, lactic SDS-SRC value, and grain protein concentration. Environments represent a unique location and year combination and regions were used to group locations in similar geographic and climatic zones (Table 7). Environment was treated as a random effect when testing the effects of genotype, location, soil series, and region. Genotype was treated as a random effect when testing the effect of environment. All data were log transformed, using the natural log, for statistical analysis and back-transformed means are presented in figures and tables. The location Ashland 2018 was excluded from the data set due to being an outlier with a STD >2 from the means of the other environments. This location also

suffered from serve heat and drought stress which are both precursors to elevated free asparagine forming potential thus, eliminating it from the data set was the best response. The protected LSD method with α=0.05 was used for means comparison. Multiple regression analysis with the SAS PROC STEPWISE procedure was used to identify soil variables that significantly affected asparagine concentration, protein concentration, and lactic SDS-SRC. Soil variables entered in the model were soil organic matter, soil pH, sulfate concentration at 0-15 cm deep, sulfate concentration at 15-61 cm deep, weighted average sulfate concentration from 0-61 cm deep, and available sulfur in the soil profile. Simple regression between asparagine concentration in grain and available S in the soil was computed with SAS version 9.4 PROC REG. Regressions were computed with all data and by year to separate annual variability introduced by growing conditions.

Results and Discussion

Soils

Soil textures and series classification were vastly different across locations and years (Table 9). Soil series did not significantly affect lactic acid SDS-SRC value (SRC), free asparagine concentration, or grain protein concentration (GPC) (p>0.05). The lack of a soil series effect on these measures of grain quality could be because soil series is closely linked to location and environment. Because we did not have many soil series with multiple locations, we did not have a strong test of how soil series would influence grain quality and performance. Each location and site year had vastly different available S, and soil pH (Table 11). Eight growing environments (combinations of location and year) had available S of less than 28 kg ha^{-1} and

were thereby considered to be S deficient (Leikam et al. 2003), which represented 33% of all environments.

Table 10. Web Soil Survey soil series and map unit symbol for site year and location. https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

Year	Location	Soil Series Name	Map Unit Symbol
18	Bellville	Crete silt loam, 1 to 3 percent slopes, loess plains and breaks	3801
18	Colby	Ulysses silt loam, 1 to 3 percent slopes	1857
18	McPherson	Crete silt loam, 0 to 1 percent slopes, loess plains and breaks	3800
18	Gypsum	Longford silt loam, 1 to 3 percent slopes	3401
18	Hutchinson	Ost loam, 0 to 1 percent slopes	5921
18	Lorrain	Crete silt loam, 1 to 3 percent slopes, loess plains and breaks	3801
18	Ashland	Reading silt loam, moderately wet, very rarely flooded	7213
19	Ashland	Reading silt loam, moderately wet, very rarely flooded	7213
19	Redd	Funmar-Taver loams, 0 to 2 percent slopes	5901
19	Sumner	Bethany silt loam, 0 to 1 percent slopes	6320
19	Colby	Keith silt loam, 0 to 1 percent slopes	1619
19	Colby (IR)	Goshen silt loam, rarely flooded	1422
19	Hays	Armo loam, 1 to 3 percent slopes	2518
20	Ashland	Bismarckgrove-Kimo complex, rarely flooded	7107
20	Lorrain	Crete silt loam, 0 to 1 percent slopes, loess plains and breaks	3800
20	Kingman	Blanket silt loam, 0 to 1 percent slopes	6322
20	Lane	Harney silt loam, 0 to 1 percent slopes	2612
20	McPherson	Crete silt loam, 0 to 1 percent slopes, loess plains and breaks	3800
20	Hutchinson	Ost loam, 0 to 1 percent slopes	5921
20	Solomon	Irwin silty clay loam, 1 to 3 percent slopes	4671
20	Sumner	Bethany silt loam, 0 to 1 percent slopes	6320
20	Washington	Crete silty clay loam, 1 to 3 percent slopes	3828
20	Colby	Keith silt loam, 0 to 1 percent slopes	1619

Table 11. Soil analysis from the locations from where winter wheat grain samples were grown and collected.

Year	Location	Organic Matter (%)	pH	S Concentration (ppm)	
				0-15 cm	15-61 cm
2018	Bellville	2.8	6.0	4.7	4.6
2018	Colby	2.5	7.5	6.0	6.5
2018	McPherson	2.8	6.5	6.7	5.0
2018	Gypsum	3.3	6.0	8.9	5.6
2018	Hutchinson	2.5	6.5	8.4	3.8
2018	Lorrain	2.7	6.9	7.6	4.6
2018	Ashland	2.9	6.6	6.1	4.3
2019	Decatur	NA	NA	NA	NA
2019	Hays	2.1	NA	1.4	1.5
2019	Redd	1.5	6.5	3.6	3.7
2019	Ashland	2.2	6.6	1.2	1.0
2019	Sumner	2.3	6.3	3.5	2.6
2019	Colby	1.8	6.9	1.8	1.4
2019	Colby (IR)	3.	6.6	2.7	1.3
2020	Ashland	2.3	7.0	2.1	1.7
2020	Colby	2.3	7.5	3.5	2.4
2020	Lorrain	2.9	6.9	7.0	4.5
2020	Kingman	2.0	7.3	4.0	4.3
2020	Lane	2.4	6.7	4.4	2.0
2020	McPherson	2.8	6.8	8.7	5.8
2020	Hutchinson	2.3	6.5	7.2	4.8
2020	Solomon	3.6	6.3	3.0	1.4
2020	Sumner	3.3	6.2	5.1	3.0
2020	Washington	3.9	6.8	8.8	6.2

One-way ANOVA

Genotype averaged across site year and location did not significantly affect free asparagine concentration (Table 12). Environmental effects were very significant, Ashland and Sumner locations had significantly greater free asparagine concentration in 2020 compared to 2019, McPherson and Hutchinson had significantly greater free asparagine concentration in 2020 than 2018. Colby had significantly higher free asparagine concentrations in 2020 and 2019 Irrigated compared to 2018 (Figure 17). Available soil S may explain some of the differences between location but there are many other factors that influence free asparagine concentration in grain and flour due to free asparagine concentrations link to plant stress.

Table 12. Test for significance of effects based on analysis of variance for free asparagine concentration, lactic acid SDS-SRC value and grain protein composition.

	F- Statistic		
Effect	**Free Asparagine Concentration**	**Lactic Acid SDS-SRC Value**	**Grain Protein Concentration**
Genotype	1.44 ns	65.10 ***	12.33 ***
Region	1.53 ns	2.52 ns	0.39 ns
Environment	9.80 ***	31.98 ***	74.06 ***
Location	0.63 ns	4.44 *	5.08 ns
Soil Series	1.24 ns	1.42 ns	3.17 ns

*, **, *** indicate significance at $p < 0.05$, 0.01, 0.001, respectively. †ns, non-significant

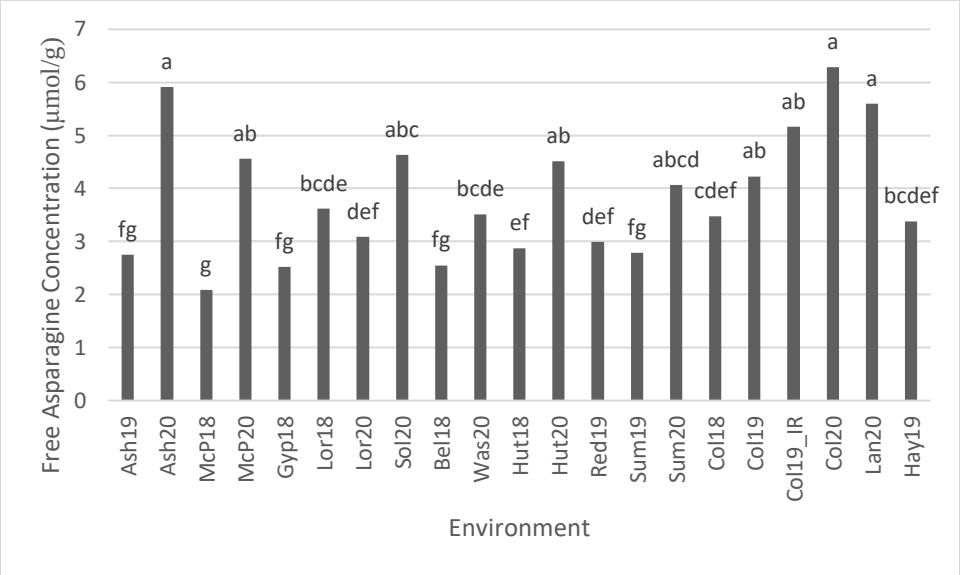

Figure 17. Effect of environment averaged across genotype on free asparagine concentration (µmol/g). (Different letters above bars indicate means are significantly different at p<0.05)

We found that, when averaged across location and year, Bob Dole had the greatest lactic acid SDS-SRC value followed by SY Monument and Overley (Figure 18). Larry and WB 4699 had the lowest lactic acid SDS-SRC values. Although, WB 4699 the genotype with the lowest average lactic acid SDS-SRC value was only evaluated in 2020 and was not planted at multiple locations (Table 9), there is potential for genotype bias decreasing lactic acid SDS-SRC values at the regional and location levels. There was a significant environmental effect, year appears to be a very important component due to changes in weather. Ashland had significantly higher lactic acid- SDS SRC values in 2020 compared to 2019. McPherson and Hutchinson had significantly higher lactic acid- SDS SRC values in 2018 than 2020 (Figure 19).

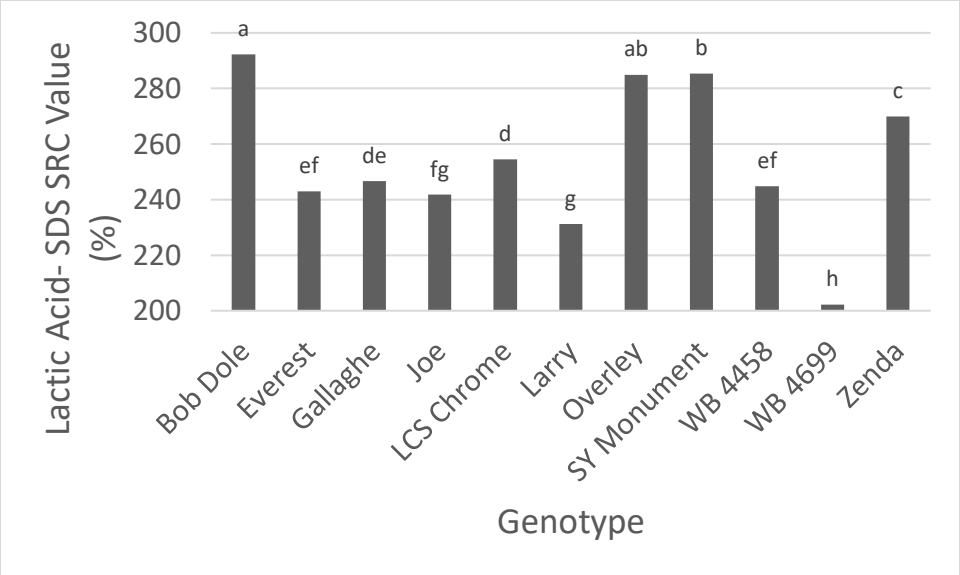

Figure 18. Effect of genotype averaged across location and environment on lactic acid SDS-SRC value (%). (Different letters above bars indicate means are significantly different at p<0.05)

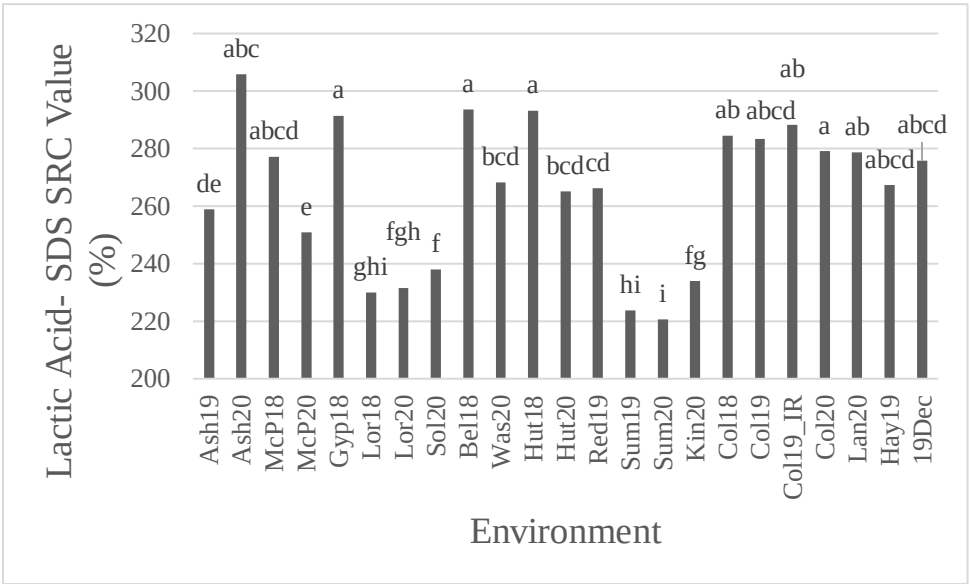

Figure 19. Effect of environment averaged across genotype on lactic acid SDS-SRC value (%). (Different letters above bars indicate means are significantly different at p<0.05)

Grain protein concentration is an important factor when evaluating overall grain quality and producer profitability. Genotype averaged across site year and location significantly affected GPC. Everest, LCS Chrome and Overley exhibited the highest GPC, and were significantly higher than Larry WB 4458, Gallagher, Joe, SY Monument and WB 4699. Bob Dole and Zenda were intermediate (Figure 20). Environmental effects were very significant. Lane and Hutchinson had the highest protein concentration and Lorraine and Sumner exhibited the lowest (Figure 21), differences in environment may be linked to weather changes from year to year.

Differences in grain quality may be linked to many different factors such as, precipitation, temperature during the growing season, biological stresses, soil texture and soil test data.

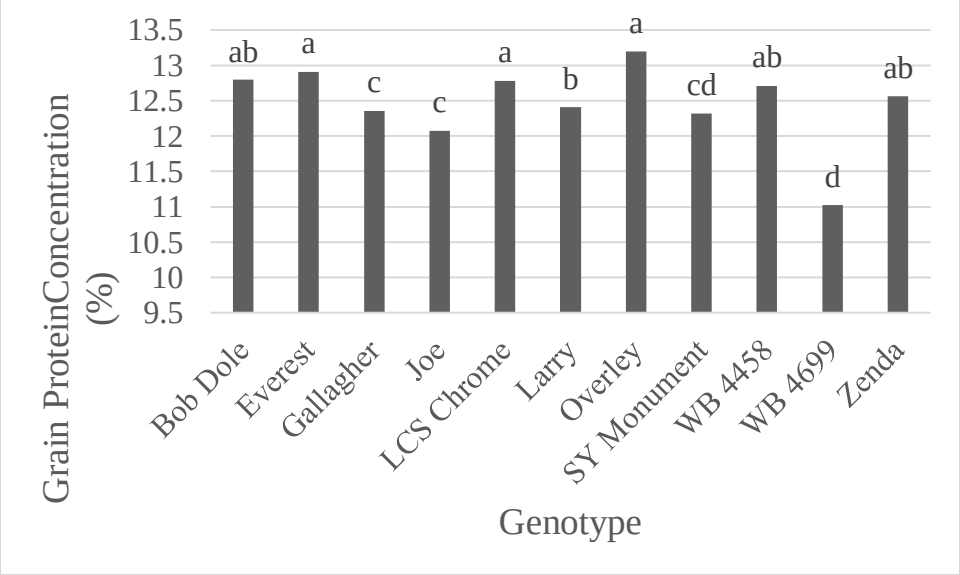

Figure 20. Effect of genotype averaged across location and site year on grain protein concentration (%). (Different letters above bars indicate means are significantly different at p<0.05)

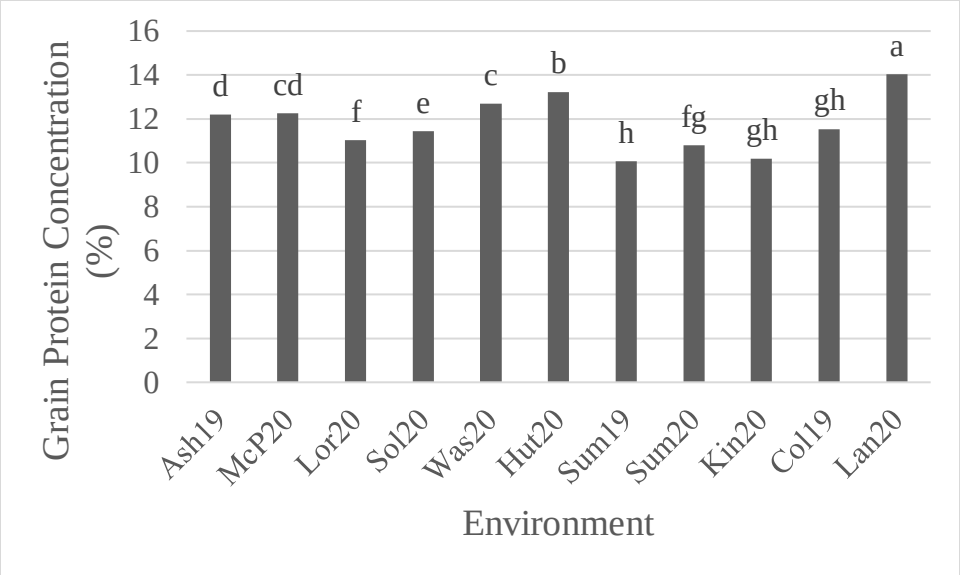

Figure 21. Effect of environment averaged across genotype on grain protein concentration (%). (Different letters above bars indicate means are significantly different at p<0.05)

Regression Analysis

In chapter 2, we found that poor wheat quality in a S deficient soil was increased with S fertilizer application. However, in this study of Kansas trial sites, we did not find a strong correlation between available S in the soil and wheat quality in this study. Although the regression between asparagine and available S in soil for all locations and years had a low *p*-value of 0.06, it was not significant and had a low R^2 (Table 13; Figure 22), indicating that the estimated available S did not explain much of the ove[r]all variability in asparagine concentration in the grain. When regression was done by year, site years 2018 and 2019 did not show a relationship between available S and free asparagine concentration (Table 13). The regression of free asparagine concentration on available S in 2020 had a low, near significant p-value of 0.07 with an adjusted R^2 value of 0.28 (figure 23). This relationship showed that when available S is abundant free asparagine levels tended to decrease. In all three site years there was not a significant relationship between available S and SRC or GPC.

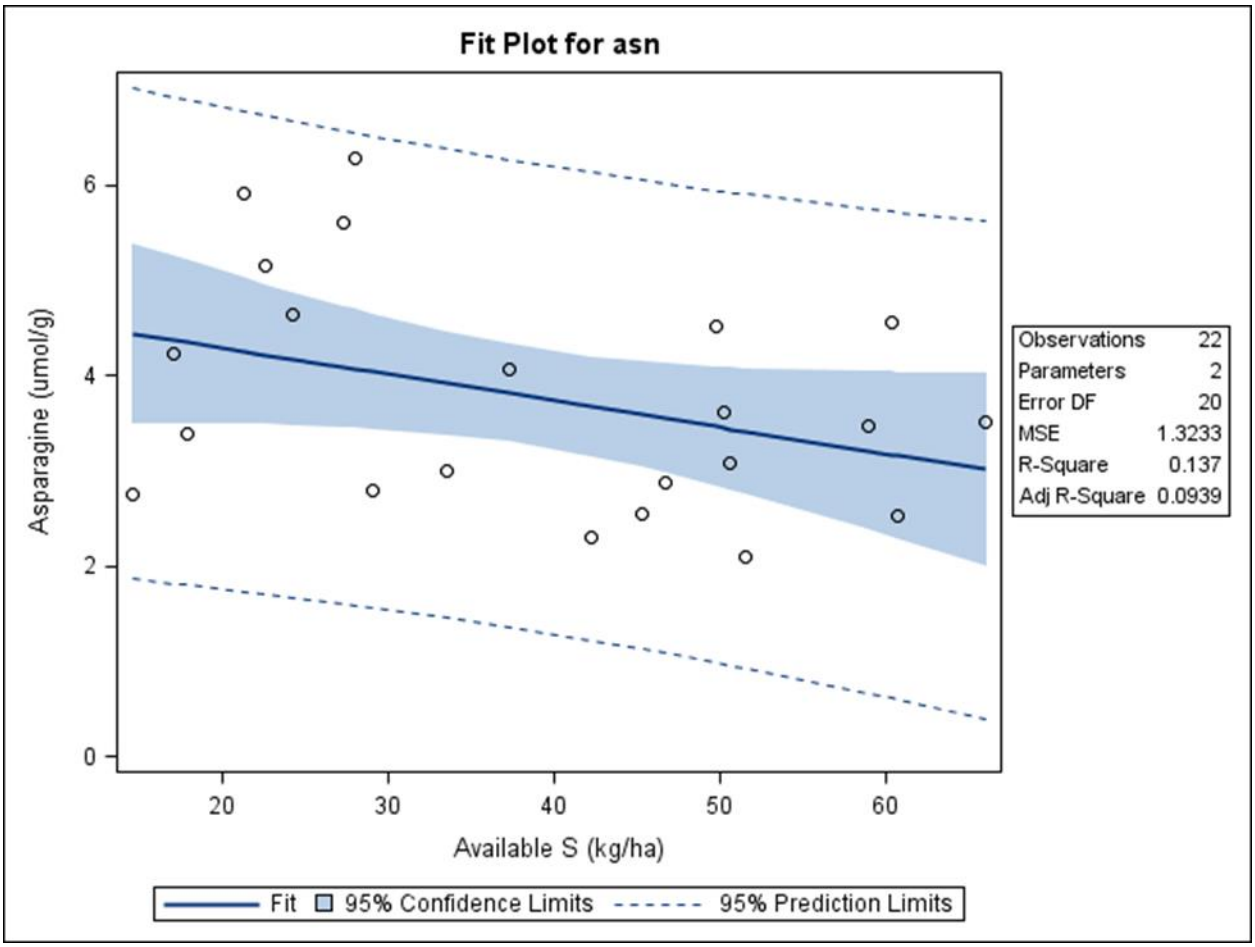

Figure 22. Regression of asparagine concentration in wheat grain on the available S in the soil for winter wheat grown at 15 locations across Kansas between 2018 and 2020 (p-value = 0.0648).

Table 13. Statistical output from PROC REG analysis describing the relationship between available soil S and free asparagine concentration.

Dataset	Intercept†	Slope†	*P*-value (slope)	R^2	Adj. R^2
All locations and years	4.85 (0.66)	-0.028 (0.02)	0.09	0.13	0.09
2018 locations	2.21 (2.43)	0.01 (0.05)	0.80	0.02	-0.23
2019 locations	4.15 (1.48)	-0.03 (0.06)	0.69	0.043	-0.19
2020 locations	6.42 (-0.05)	-0.05 (0.02)	0.0675	0.36	0.28

† (value in parentheses is the standard error)

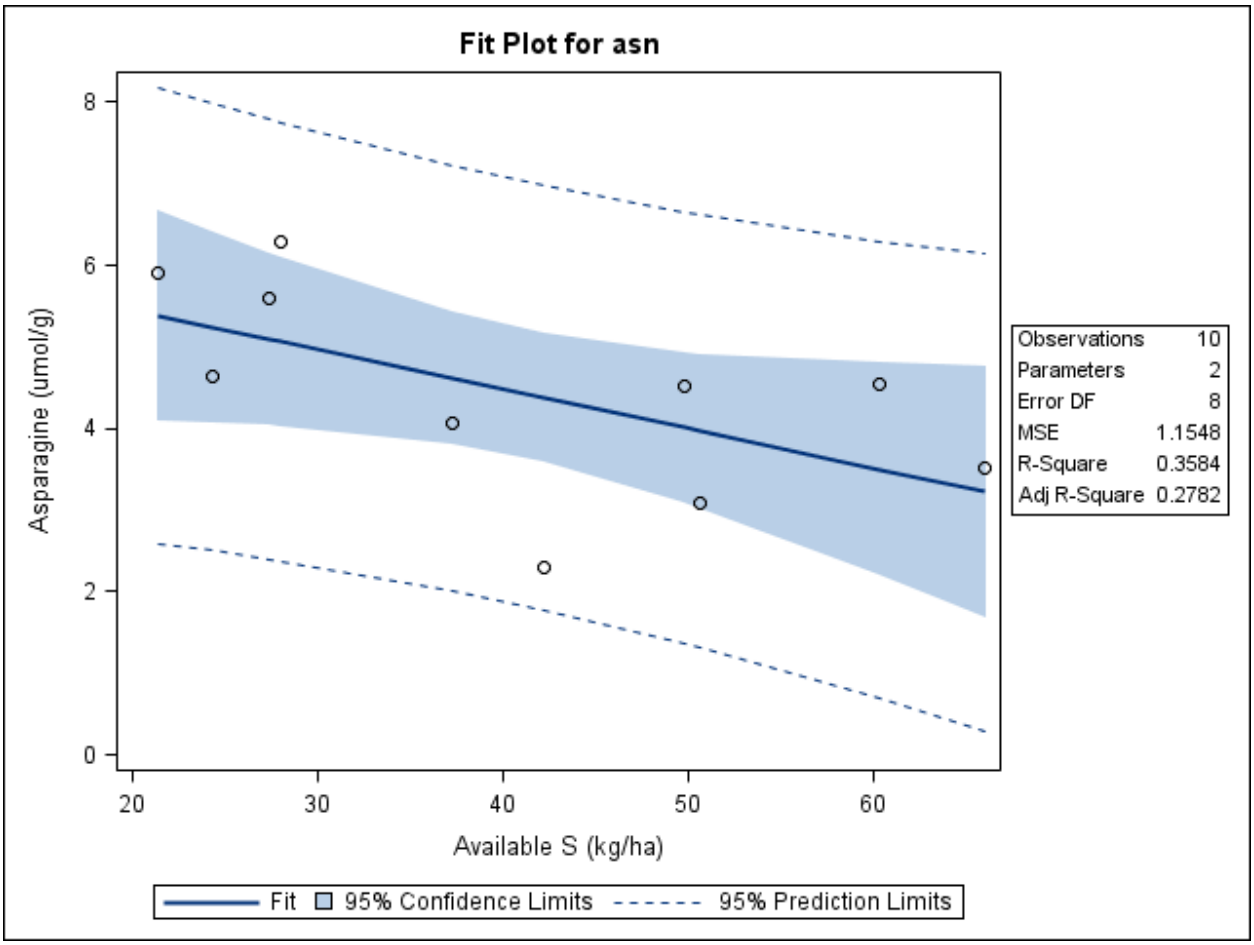

Figure 23. Fit plot from PROC REG analysis describing the relationship between available soil S and free asparagine concentration in 2020.

Use of additional soil parameters in multiple regression analysis identified a significant model (p=0.024) involving sulfate-S concentration in the sub-soil (15 to 60 cm) and the pH of the surface soil (0 to 15 cm) (Table 13). The R^2 for this model is 0.34 with an adjusted R^2 of 0.27. Therefore, the combination of sub-soil sulfate concentration and surface soil pH explained 34% of the variability in asparagine concentration in wheat grain (Figure 24). Other studies have found that asparagine concentrations in grain can be influenced by other stresses, including drought or heat stress (Lea et al., 2007). Future efforts to understand factors influencing wheat asparagine concentrations may benefit by combining the soil properties with climate variables.

Table 14. Parameter estimates for multiple regression of asparagine concentration in wheat grain on the sulfate-sulfur concentration in the soil at 15 to 60 cm deep (mg/kg) and the soil pH at 0 to 15 cm deep.

Variable	Degrees of freedom	Estimate	Standard Error	t-value	p-value
Intercept	1	-2.089	3.727	-0.56	0.5821
SO_4-S (15 to 60 cm)	1	-0.341	0.135	-2.51	0.0217
Soil pH (0 to 15 cm)	1	1.068	0.560	1.91	0.0728

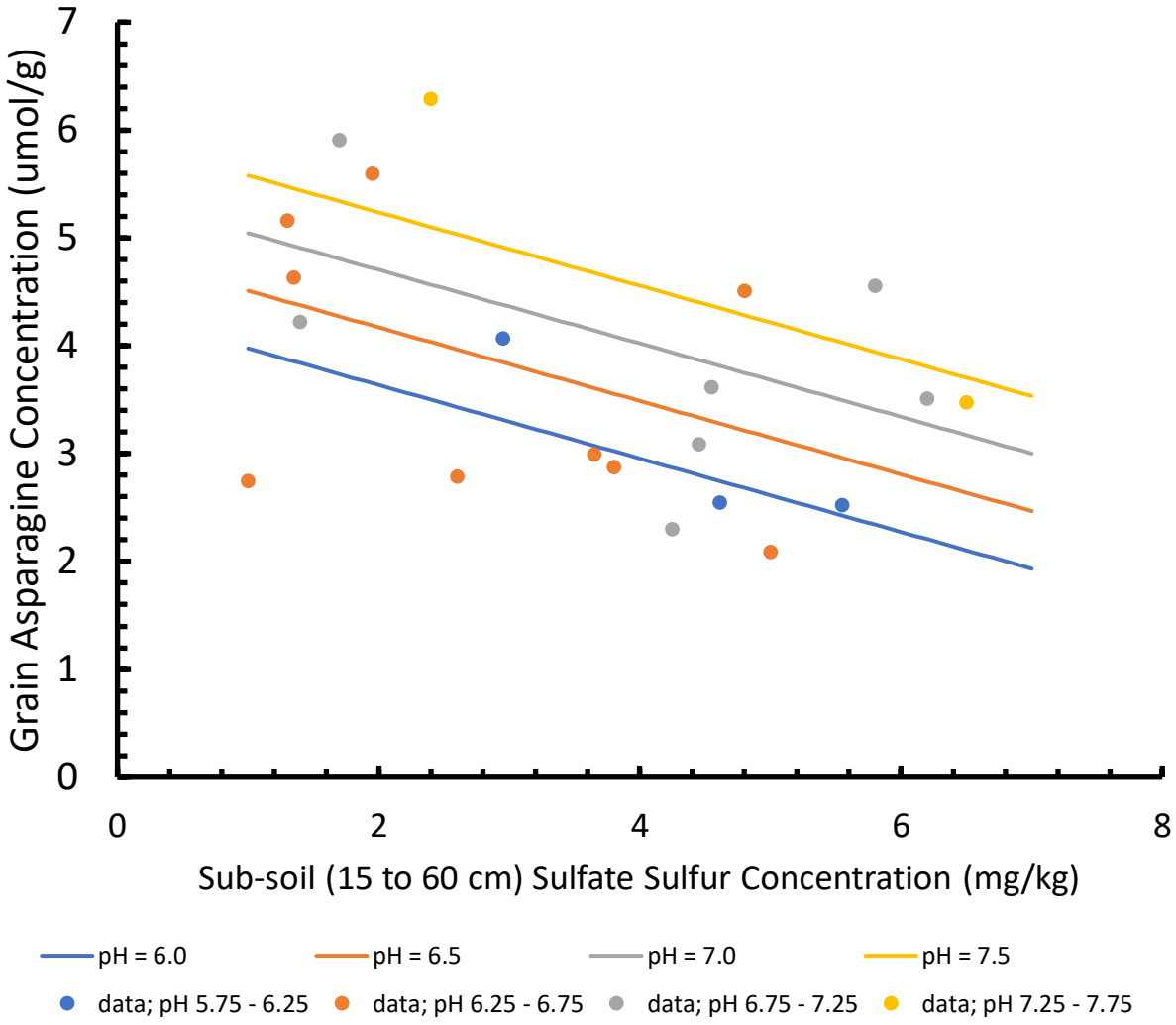

Figure 24. Multiple regression model describing the relationship between asparagine concentration in winter wheat grain and soil properties (sulfate sulfur concentration at 15 to 60 cm deep and soil pH at 0 to 15 cm deep). Solid lines represent regression equations for pH of 6, 6.5, 7, and 7.5 based on regression parameters in Table 13.

Conclusion

Soil S availability throughout the state of Kansas is variable, 1/3 of our locations had S deficiencies that would have required S application to correct the deficiency. In 2020 available soil S had a relationship with free asparagine concentration when soil S was abundant free asparagine concentration tended to decrease. This relationship was able to explain part of the

range of free asparagine concentration across environments, although this relationship was not significant across all years of the experiment. Findings from this study indicate that there are many factors involved in elevated free asparagine concentration and available soil S is only part of the story, precipitation, temperature, and biological stresses also play a part in elevated free asparagine concentration. future research will be necessary to explore all aspects that increase acrylamide forming potential in wheat. Acrylamide forming potential of wheat is important and the discovery of factors that may increase this potential are very important to enable high food safety standards. Information from this study should not be misinterpreted, misunderstood or overexaggerated. It is important for producers, bakers, and end users to acknowledge and educate themselves about the factors that lead to increased acrylamide formation in winter wheat, although further research is needed to identify dangerous thresholds for human health and other factors that increase free asparagine in wheat grain.

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