

PROTEIN, METHIONINE, AND CYSTEINE UPREGULATION IN *PHASEOLUS VULGARIS*
'BLACK TURTLE BEAN' SEEDS THROUGH SULFUR FERTILIZATION AT V2 AND R2 STAGES
OF GROWTH

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ABSTRACT

The purpose of this research was to increase protein, methionine, and cysteine content in *Phaseolus vulgaris* L., common bean *in relation to* the inhibitory compound tannin. Previous research has shown that sulfur fertilization increases total protein, methionine and cysteine content in various crops, but always in tandem with inhibitory compound increases. If successful, the resulting bean seed will have a better nutrient profile for malnourished populations around the world.

Granular gypsum was applied at 0 kg S ha⁻¹ [kilograms of sulfur per hectare], 10 kg S ha⁻¹, 20 kg S ha⁻¹, 40 kg S ha⁻¹, 60 kg S ha⁻¹, and 80 kg S ha⁻¹ in two experiments. The main difference between experiments was timing of application (V2 and R2 stage of growth application respectively). Soluble protein, crude protein, methionine, cysteine, and tannin content were compared to controls. Ratios of soluble protein: tannin, crude protein: tannin, methionine: tannin, and cysteine: tannin were also compared.

Yield depression occurred at 20 kg S ha⁻¹ for both application timings. Fertilization at the V2 stage of growth decreased soluble protein at 10 kg S ha⁻¹ compared to controls. Crude protein increased at 10 and 40 kg S ha⁻¹ compared to 80 kg S ha⁻¹; drought may have influenced this outcome. For V2 application, methionine peaked at 20 kg S ha⁻¹ while cysteine peaked at 80 kg S ha⁻¹. For R2 application, crude protein decreased at 10 kg S ha⁻¹, 40 kg S ha⁻¹, and 80 kg S ha⁻¹, and cysteine was lower at 60 kg S ha⁻¹ compared to controls. Tannin contents were higher at 80 kg S ha⁻¹. These results may suggest nutrient imbalance in the soil.

Sulfur application at the V2 stage of growth produced the highest protein to tannin ratios compared to R2 application, though amino acid to tannin ratios were similar for both fertilization timings. We conclude that sulfur fertilization at V2 stage of growth gives the most improvement in nutritional quality compared to R2 application, with 20 kg S ha⁻¹ giving the best overall quality increases in *Phaseolus vulgaris* bean seeds.

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CHAPTER 1: INTRODUCTION

Food security is a global concern in the 21st century. Unequal distribution allows first-world powers to waste an average 30% of their food annually (Smil, 2002), while over 1 billion people suffer from malnutrition (FAO, 2011). In 2000, the World Health Organization (WHO) labeled iodine, iron, vitamin A and zinc as the world's most serious deficiencies (WHO and FAO, 2006). Post-harvest crop fortification (Brnic et al., 2016), genetic modification (GM) (Hesse et al., 2000), crossbreeding (Nestel et al., 2006) and fertilizer fortification have emerged as methods that directly address these micronutrient issues (Gomez-Galera et al., 2010). However, despite efforts, micronutrient deficiency persists, especially in developing countries where diet diversity is limited.

Diets dominated by plant-based proteins, as is often the case in developing countries, lack essential micro and macronutrients (Mosse, 1990; Reddy and Pierson, 1985) like protein, iron and zinc. Work currently under grant in our lab focuses on remedial health efforts in the Mayan community of La Fortuna Guatemala, where despite fortification efforts, protein energy malnutrition (PEM), vitamin deficiencies, and iron-based anemia are still severe health concerns (Fiedler and Helleranta, 2010). The diet of this Mayan population consists largely of maize (*Zea mays*) and beans (*Phaseolus vulgaris*), infrequently supplemented with low-quality chicken or pork protein as reported by Forbes Walker through multiple visits to the area. The aim of this research is to enter the literary gap in macronutrient fortification toward the goal of addressing the PEM and iron deficiency problem in La Fortuna, Guatemala.

We conducted a literature review to more fully understand relations between macro and micronutrient deficiencies, benefits and drawbacks of current nutrient enhancement methods, how low-quality protein is linked to PEM and iron deficiency, how soils of the reported region contribute to and enhance nutritional gaps in the population's diet, and how upregulation of protein and sulfur-based amino acids in *Phaseolus vulgaris* could assist in filling these gaps. We then outline research objectives, state the hypothesis, give a detailed description of experimental design and procedures, and conclude with results, and recommendations for future studies.

The questions addressed will apply to bean seeds and are as follows: 1) What rate of sulfur significantly increases protein content in relation to the inhibitory compound tannin 2) What rate of sulfur significantly increases sulfur-based amino acids methionine and cysteine in relation to the inhibitory compound tannin? 3) Does timing of sulfur application affect bean seed nutrition?

CHAPTER 2: LITERATURE REVIEW

1. Food Security and Fortification

The first goal of the United Nations' Millennium Development Goals (MDG) in 2005 was to "eradicate extreme poverty and hunger". They define hunger as "having too little to eat to meet daily energy needs" and malnourishment as hunger, disease, and lack of care resulting in growth retardation; both issues are addressed in the stated goal ("The United Nations Millennium Development Goals Report," 2005).

To address this goal, measurable malnourishment indicators –being underweight, stunting, and mortality of children under five– were assessed (WHO, 2015). The WHO and FAO identified micronutrient deficiency as the primary focus to alleviate hunger and malnutrition because they affect health in so many ways (WHO and FAO, 2006; FAO, 2011). However, it is that diversity of interactions that makes it so important to consider all contributing factors (micro and macronutrients) for a truly sustainable solution.

Definition Box 1

Adequate Diet: an appropriate density and amount of nutrients, is sufficiently diverse, is palatable and culturally acceptable, affordable and available year round, and overall supports normal growth and development (WHO and FAO, 2006)

1.A. Malnutrition: Critical Period

It is widely recognized that malnutrition most acutely affects growth and development during early stages of life (WHO, 2015; Heijmans et al., 2008; Lucas, 1991; Rollandcachera et al., 1995). Multiple studies link intrauterine nutrition to *post utero* infant health; the mother's nutritional status during pregnancy greatly influences birth weight, subsequent infection rate, morbidity, and mortality rates in young children (Bhutta, 2006; Branca, 2006; Goulet et al., 2006; Mata et al., 1972) Two years of age is marked as the breaking point from which malnutrition effects are difficult or impossible to reverse (Dewey and Adu-Afarwuah, 2008). A broad-spectrum nutrient remediation effort should therefore be focused on foods that most acutely affect mothers and infants for long-lasting results.

Definition Box 2

Acute PEM: "wasting"; where malnutrition causes altered body composition; discerned from weight for height data (Woodward, 1998)

Chronic PEM: "stunting"; where malnutrition results in linear growth depression; discerned from height to age data (Woodward, 1998)

Stunting: being two standard deviations below the Z score for height and age (Branca, 2006; WHO and FAO, 2006)

In Guatemala, reports indicate fetal growth retardation and stunted growth patterns for children in low-income villages; this stunting is primarily attributed to low quantity and quality food sources coupled with high infection rates (Mata et al., 1971; 1972). Divergence

from normal growth patterns and increases in infection rates was marked at 3-4 months of age when solid food was incorporated into the diet; corn gruel (the complimentary food of choice) has low biologically available protein (Mata et al., 1972) for the infant, as does maize and beans (primary food source of the mother) (WHO and FAO, 2006). Growth pattern standards were compared to the Iowa Standard as compared to well-nourished Guatemalan children (Mata et al., 1972).

Correspondingly, the WHO emphasizes an increase in infant protein needs from 21% to 50% between 6 and 23 months of age (Dewey and Adu-Afarwuah, 2008) making the complementary food quality as well as the quality of the mother's milk important factors to consider (WHO and FAO, 2006). The quality of milk will be directly influenced by the mother's diet and overall health. The quality and quantity of protein in both the child's and mother's diet, and the ability of the body to absorb said protein, therefore directly affects growth patterns and overall health in the Guatemalan children (Bhutta, 2006). Thus, understanding protein recommendations versus actual protein digestion is important.

1.B. Protein Energy Malnutrition

The FAO bases protein recommendations on *metabolic demands* –how much the body needs to function at optimal rate– and *efficiency of utilization* how much food is needed to provide those needs (WHO/FAO/UNU, 2007). No current recommendations for protein have been found though several FAO/WHO/UNU reports dating from 1985-2007 speak in general protein requirement terms. Protein recommendation must be individualized based on level, metabolic demands, and utilization efficiency. These factors will be influenced greatly by age, health, level of activity, and quality of food ingested. In general, young children (<18y/o) require more protein than adults because of growth patterns; people fighting infection or disease have higher protein needs to recover; and active individuals have higher requirements than less-active individuals (FAO/WHO/UNU, 2007). Protein quality, though complex to comprehend fully, is at least partially based in a balanced intake of essential and non-essential amino acids (measured as Nitrogen intake) for bodily function. Enough protein must be ingested and digested to fulfill all essential amino acid needs and have energy left over to create the non-essential amino acids. Lack of fulfilling said requirement (needed levels are dependent on the factors mentioned above) will result in physical inhibition, the severity of which depending on the length of deprivation (WHO, 2007).

Table 1 represents digestibility proportions for various diet and food types. Beans and corn, the main diet (post-weaning) in Guatemala, is only 78% digestible when the individual ingesting it is at optimal health; infection and nutrient deprivation can further reduce nutrient uptake by 40% (Bhutta, 2006; Goulet et al., 2006).

Table 1: Values for the Digestibility of Protein in Humans

Protein Source	True Digestibility	Protein Source	True Digestibility
	%		%
Beans	78	Egg	97
Corn, cereal	70	Meat, fish	94
Corn, whole	87	Milk, cheese	95
Maize	85	Soy flour	86
Maize + beans	78	Wheat flour, white	96
Maize + beans + milk	84	Wheat, whole	86

(WHO and FAO, 2006)

Therefore, individuals eating maize and beans while fighting infection, suffering from nutrient deprivation, or both at the same time are highly likely to absorb less than the needed protein intake for optimal health as supported by the PEM indicators observed there. Consistently failing to meet recommended dietary protein requirements leads to PEM and micronutrient deficiencies, (Goulet et al., 2006; Woodward, 1998) creating a cyclical issue where PEM and infection lead to nutrient depletion and nutrient depletion weakens the body's ability to absorb nutrients and fight off infection. Increases in total protein consumption and amino acid supplementation may be key factors to breaking this cycle as studies indicate both protein and amino acid availability in the body are limiting factors in children recovering from infection (Bhutta, 2006). In other words, increased protein and amino acid absorption in the human body could boost the body's ability to fight infection thereby increasing nutrient absorption potential from the diet and breaking the depletion= infection=depletion cycle.

1.C. Post-Harvest Enrichments

Micronutrient interactions in the body are known to be vitally important and linked to decreased disease and increased overall vigor in the human body (WHO and FAO, 2006). Therefore in efforts to boost nutrient absorption and overall health, direct

Definition Box 3

Post-Harvest Fortification: the addition of micronutrients to a food source after harvest and industrial preparation; typically done immediately before consumption.

Hidden Hunger: inadequate micronutrient intake (Branca, 2006)

Fortification: The addition of micronutrients to a processed food to improve the food's nutritional quality (Fiedler and Helleranta, 2010)

supplementation and food fortification methods have been implemented in low-income areas to combat micronutrient deficiency (FAO, 2011). To be most effective, these efforts have been focused on nursing mothers (review 1A.). In a 2006 study in Guatemala, 49% of lactating mothers and 68% of their infants had low or deficient plasma and B12, but maternal supplementation of B12 positively impacted the critical 1-6 month growing period of nursing children (WHO and FAO, 2006). Direct supplementation for children during complementary feeding periods has also been implemented. However, supplementation is limited by a need for centralized distribution and product availability, and overall fails to recognize the root problem of the deficiency in order to make a long-term solution (FAO, 2011).

Fortification is generally regarded as more cost-effective than supplementation, uses existing infrastructure to reach a large number of people, and requires minimal cultural practice changes. However, its implementation has been slow in low-income areas perhaps due to lack of understanding regarding the severity of micronutrient deficiency, concerns about consumer acceptance, cost, and maintenance of programs (Fiedler and Helleranta, 2010; Shetty, 2011; WHO and FAO, 2006). Furthermore, infection, diarrhea, and the chelating effects of phenolic and polyphenolic compounds (inhibitory compounds) from local diets can reduce or nullify fortification effects (Dewey and Adu-Afarwuah, 2008; FAO, 2011).

Definition Box 4

Supplementation: Short-term remedial efforts for acute nutritional deficiencies through non-food based approaches (FAO, 2011)

Fortification: nutrient enrichment of food (FAO, 2011)

2. Plant Based Solutions

Due to the drawbacks of supplementation and fortification, plant-based intervention is becoming an increasingly utilized nutrient enhancement method in low-income settings (Arimond et al., 2011; Low et al., 2007). The general premise is to somehow make the food more nutrient dense during the growth process so that there is no need for centralized processing, specific dosage requirements, or extra education needed for the program to work long-term (Gibson, 2011; Shetty, 2011). However, the success of any program depends on the sustainability of the method, ease with which it can be culturally implemented, efficacy, effectiveness, and cost effectiveness (Low et al., 2007).

2.A. Biofortification: Crossbreeding and GM Crops

A 2011 study successfully increased β -carotene in microalgae through the upregulation of Phytoene Synthase (PSY) catalases (Couso et al., 2011). This study provided a spring-board for increasing carotenoid levels in crops like “Golden Rice”, a genetically modified rice cultivar that produces carotenoids in the endosperm (Beyer et al., 2002; Burkhardt et al.,

1997). Several studies support the efficacy of bio-fortified crops. A two-year study involving over 800 households in Sub-Saharan Africa, found orange-fleshed sweet potato (higher β -carotene levels) successfully increased serum retinol as compared to control groups (Low et al., 2007). Iron-biofortified rice increased serum ferritin by 17%– though these results were not significant– and significantly increased total body iron for Filipino women (Haas et al., 2005).

However, biofortification efforts can be confounded by co-suppression, sense-suppression, yield loss, loss of plant vigor, and other issues during the research process (Fraser et al., 2009). Furthermore, while some crops are easily modified for specific traits (Gibson, 2011) producing consistent expression of multiple transgenes over time has been a limiting factor for genetic modification (GM) efficacy. Formulation of transgenic libraries (Zhu et al., 2008) and genetic mapping has eased GM application for a wide range of organisms though processes are costly and often produce seeds too expensive for low-income use (Lucht, 2015).

Cultural feasibility and acceptance of genetically modified crops also vary. One review of survey-based studies states a general acceptance of six biofortified foods in low-income settings (Birol et al., 2015) though it and other studies admit that actual integration is typically less than what survey responses generally indicate. Political tides, cost, availability of the resource, public perception of visual changes, taste, and texture, and individual situations all play a part in the actual acceptance of the biofortified crops (de Steur et al., 2013; Govender et al., 2014; Stevens and Winter-Nelson, 2008; Yang et al., 2014). All studies stress the importance of integrative techniques with key educational components for successful integration.

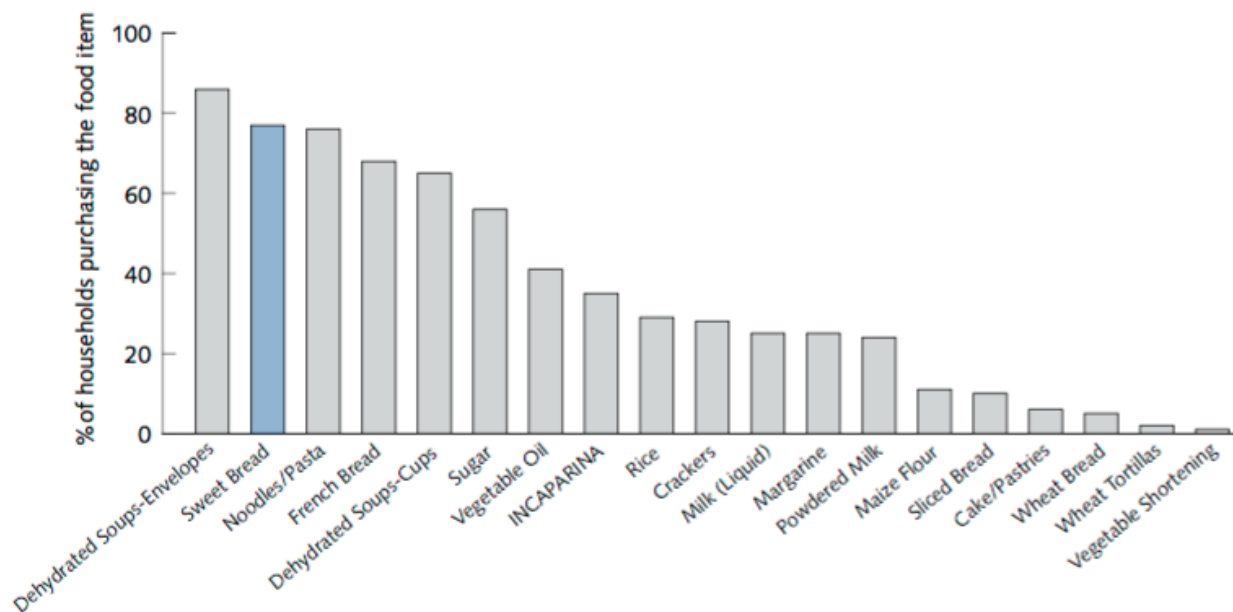
2.B. Fertilizer Fortification

Varied responses to genetically modified crops efforts has led some researchers toward another plant-based fortification idea. It is well accepted that soil nutrient composition directly affects crop nutrient content (White and Broadley, 2009). Adesemoye et al. (2008) support this fact showing that inoculation and fertilization enhanced N content in corn. Vaclav Smil (2002) reports digestible N increases in livestock fodder through fertilization, and multiple other studies have successfully manipulated various nutrients through fertilization efforts (White and Broadley, 2009). However, the success of fertilization efforts is based in three points 1. The applied nutrient of interest must be soil-mobile 2. The nutrient of interest must accumulate in the edible portion of the plant 3. It must accumulate in a bioavailable form (Gomez-Galera et al., 2010). If any one of these points is not fulfilled, then the fortification effort is unsuccessful. Furthermore, nutritional increases can also be confounded by a tandem increase of inhibitory compounds (Raboy et al., 1989). For example, P fertilization in wheat (*Triticum aestivum*) also increased phytic acid ratios, decreasing the bioavailability of zinc (Ryan et al., 2008).

The inhibitory compound of interest in *Phaseolus vulgaris* is tannin and has been shown to increase with fertilizer application (Elsheikh and Elzidany, 1997) but no literature speaks to the ratio of increase in relation to available protein and amino acid contents. Planting location, fertilizer application method, and soil composition will affect the effectiveness of fertilizer fortification.

3. Guatemala: Nutritional Overview

Supplementation, fortification, and biofortification efforts have been implemented to address all FAO micronutrient recommendations in Guatemala. However, there remain significant micronutrient deficiencies in local populations (Fiedler and Helleranta, 2010). Iodine salt effectively reduced iodine deficiency from a severe to mild public health concern, but vitamin A and iron remain moderate and severe public health issues respectively. Perhaps iron deficiency persists because fortification efforts have focused on wheat-based survey data of purchased food items (Figure 1) (Fiedler and Helleranta, 2010) while staple crops of beans and maize, locally grown in traditional *milpa* (intercropping) methods remain unfortified.



* INCAPARINA: a vegetable-based mixture with amino acid bioavailability comparable to that of a meat product; it was implemented by the Institute of Nutrition of Central America and Panama (INCAP) for nutrition remediation efforts

Figure 1: Percentage of household food purchases in the last 15 days, Guatemala 2006 (Fiedler and Helleranta, 2010)

Zinc data has not been quantitatively assessed but is assumed to be a health issue based on stunting and anemia rates (Fiedler and Helleranta, 2010). B12 is also cited as a major health concern (Iannotti et al., 2012) and can only be supplemented or obtained from animal-based food (ASF) (Jones et al., 2007), which is not available at adequate levels in Guatemala (Iannotti et al., 2012) and the La Fortuna Region in particular (Robles et al., 2012).

3.A. Animal Based Food (ASF): Vitamin B12 and Iron

Low consumption of ASF is associated with vitamin B-12, iron, zinc and riboflavin deficiency (Dagnelie and Vanstaveren, 1994; Jones et al., 2007; Murphy and Allen, 2003). These deficiencies are associated with stunting, rickets, anemia and even death (Murphy and Allen, 2003). Weight, length, weight for length and arm and head circumference were significantly depressed by low ASF intake compared to control groups (Dagnelie and Vanstaveren, 1994) in conjunction with PEM symptoms.

Anemia has reportedly dropped in Guatemala though iron deficiency is still moderate to severe and perhaps the greatest micronutrient threat facing the native Guatemalan population (Fiedler and Helleranta, 2010). Continued deficiency in the face of targeted fortification efforts may be caused by low program integration rates, poor plan integration design, and narrow integration methods (Shetty, 2011). Rates of deficiency in Guatemala vary per area but generally remain around 40% for children under five years of age and 20% for both pregnant and non-pregnant women. Similar anemia results were reported in a 2007 study with 9.8% anemia in mothers and 39.4% in infants (Jones et al., 2007). The main causes of anemia and iron deficiency is thought to be inadequate intake of iron or low bioavailability from the diet. Amount of iron ingested, bioavailability, and body iron levels affect iron deficiency and absorption (Towo et al., 2006).

Definition Box 5

Anemia: 1. Hemoglobin concentration below established cutoff levels (WHO and FAO, 2006) 2. Late manifestation of Iron Deficiency (Towo et al., 2006)

Iron Deficiency Anemia (IDA): The most common and severe form of anemia resulting from iron deficiency

Plasma B-12 deficiency was also reported in 49% of infants and 68% of mothers in a peri-urban Guatemalan population. Significantly lower plasma folate and ferritin (iron) levels were reported in vitamin B-12 deficient groups compared to B12 sufficient group. Increased animal source food (ASF) consumption (meat, milk, cheese etc.) would help remediate iron and B12 deficiencies (Jones et al., 2007; WHO and FAO, 2006). However, increasing ASF consumption would require fundamental changes in food availability and price and/or familial financial status (Iannotti et al., 2012) and thus is beyond the scope of this research. Fortunately, another method of increasing iron absorption is available.

3.B. Iron Absorption: Methionine and Cysteine

Plant-based iron sources (Shetty, 2011), tannin content complexation (Reddy and Pierson, 1985) infection and/or diarrhea (Bhutta, 2006) inhibit iron absorption and increase iron excretion from the body. Methionine deficiency reportedly decreased iron absorption in rats (Kaufman et al., 1966). Methionine and cysteine supplementation significantly increased iron absorption from black beans in Venezuelan peasants; cysteine having a greater affect than methionine when supplemented individually as compared to controls (Martinez et al., 1970). Protein levels above 15% was also found to significantly increase total body iron in rats, though the mechanism of protein in iron absorption was unclear (Kalvins et al., 1962). Protein type was also found to influence iron adsorption in rats (Kim et al., 1995). Increasing total protein content in the diet and increasing sulfur-based amino acids methionine and cysteine should therefore increase iron absorption, addressing one of the greatest deficiencies in native Guatemalan communities.

4. Nutritional Facts: Maize and Beans

However, as mentioned previously, increasing protein and iron absorption must be done through sustainable and easily integrated methods. Affected products must be easily accessible, abundant for mothers and young children, and remain within financial means for the target population. Below, we outline the nutritional gaps present in the Guatemalan diet and propose the upregulation of specific amino acids and protein content to sustainably address iron absorption issues in Guatemala.

4.A. Guatemala Food Resources

Maize, domesticated in Guatemala, is a staple of the Guatemalan population. Intercropped with maize in traditional *milpa* are climbing beans (*Phaseolus vulgaris*) and perhaps chilies, and squash plants. While higher-profit crops like broccoli, zucchini, and snow peas were introduced and integrated into the global Guatemalan economy in 1980 by USAID, rural populations hold fast to *milpa* cropping systems relying heavily on traditional *milpa* staples (Morales and Perfecto, 2000; Morales et al., 2001; Ryan et al., 2008). The main sources of protein are therefore beans and maize, which, without supplementation, is potentially a main contributing factor to PEM and low-iron absorption.

4.B. Nutritional Profile: Beans

Beans are not a complete source of dietary protein and eaten in high quantities can lead to macro and micronutrient deficiency (Reddy and Pierson, 1985) including iron. Though relatively high in iron, beans are low in sulfur-based amino acids (methionine and cysteine) (Smil, 2002). Perhaps in part due to the incomplete amino acid profile, beans have low protein digestibility (Huisman et al., 1992) which is exacerbated by high amounts of tannin and phytate complexation (Hesse et al., 2000; Wu et al., 1995). Phenolic compounds were found to reduce iron absorption up to 90% in a Southeast Asian diet, with as little as 5g

reducing iron absorption 75% (Tuntawiroon et al., 1991). In other words, the high phenolic concentration in beans may be a contributing factor to lowering protein digestibility–causing PEM –and iron absorption –causing anemia– in Guatemalan populations.

Tannin, the more abundant inhibitory compound in beans, is primarily found in the hull of the bean seed (Bressani et al., 1983) and is not effectively removed by cooking or soaking (Reddy and Pierson, 1985). There is little reference in the literature to direct tannin effects on human subjects, though it is well documented that tannins decrease overall N retention and protein absorption through non-selective protein and protein enzyme binding (Bressani et al., 1983; Reddy and Pierson, 1985) and reduce iron adsorption in the intestinal tract (Mendoza et al., 1998). Ingesting high levels of tannin can thus exacerbate iron deficiency (Reddy and Pierson, 1985) and contribute to PEM (Woodward, 1998).

Methionine plays a key role in the excretion of tannins from the human body (Contreras, Elias, and Bressani, 1980; Fuller and Potter, 1968). Methionine supplementation significantly counteracted negative effects of tannin in chickens (Potter and Fuller, 1968) and significantly increased weight and net protein retention in rats (Salunkhe et al., 1990). Evidence presented in the previous sections support similar results in humans, though no direct studies have been found in relation to Guatemalan populations.

4.C. Nutritional Profile: Maize

A maize kernel (Figure 2) holds 50-60% of its protein in the “hard starch” vitreous layer in the form of zeins (Chandrashekar and Mazhar, 1999). Zeins are broken into one major class (α -zeins) and three minor classes (β , γ , and δ -zeins). The α fraction is high in cysteine while β and γ are high in methionine. Even so, methionine only makes up approximately 0.9% of the total zein fraction. Limiting amino acids in maize are lysine and tryptophan which are 0.1% and basically nonexistent in the α -zein fraction though there is some of each amino acid in the non-zein fraction (Sofi et al., 2009).

Inhibitory compounds in maize, phytic acid in particular, decrease protein digestibility and micronutrient uptake (Bressani et al., 2004; Raboy, 2002). Phytic acid content varies between variety (Bressani et al., 2004). Wild-type dent corn was reported to have 0.99% phytic acid content (Mendoza et al., 1998) which was slightly higher than the range of 0.5-0.9% phytic acid reported in Wyatt, C.J. and Triana-Tejast A. (1994). Phytic acid was reportedly decreased in maize through cooking and soaking techniques by up to 31% (Bressani et al., 2004).

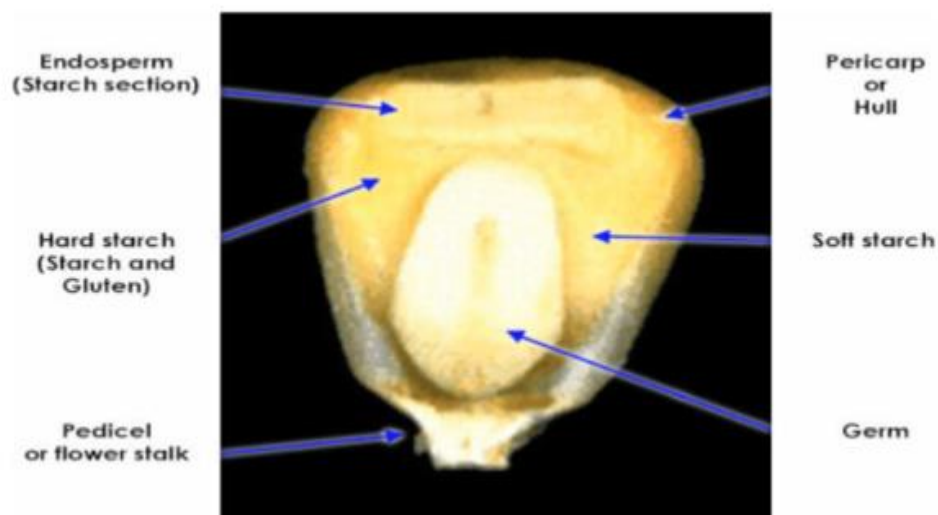


Figure 2: Structure of a Maize Kernel (Sofi et al., 2009)

4.D. Nutritional Profile: Beans and Maize

Consumed together, beans and maize supposedly make a more complete protein than the individual components; maize provides sulfur-based amino acids that beans are low in, and beans provide amino acids maize is deficient in. However, Contreras, Elias, and Bressani (1980) showed the corn and bean diet to be insufficient for optimal growth in swine. They proposed a 70:30 maize:beans ratio for best digestibility, but ultimately found that even the 70:30 ratio provided an incomplete nutrient profile. They also stated that the typical maize and beans diet in Central America more closely adheres to 87:13 ratio providing an even lower nutrient profile than the proposed 70:30 ratio. Supplementation with different treatments of vitamins, minerals and amino acids induced higher growth rates in the swine, which was attributed to a more digestible and complete protein profile (Contreras et al., 1980).

Methionine in maize was found to be limiting (Sofi et al., 2009), but at optimal levels methionine and cysteine enhanced iron absorption (Martinez et al., 1970) bound phenolic compounds (Contreras et al., 1980; Fuller and Potter, 1968) and increased protein absorption (Reddy and Pierson, 1985). Supplementation of methionine in fodder crops promoted optimal growth in non-ruminants leading to multiple studies on how to increase these amino acids without supplementation (Contreras et al., 1980; Hesse et al., 2000).

4.E. Why Not Reduce Inhibiting Compounds Instead?

As noted, phenolic, phytic, and polyphenolic compounds in plant-based diets make proteins and micronutrients less available during digestion (Bressani et al., 1983; Mendoza et al.,

1998; Ramachandra et al., 1977; Reddy and Pierson, 1985; Shetty, 2011; Towo et al., 2006). Some have attempted to reduce these compounds in the seed itself (Raboy et al., 1989; White and Broadley, 2009), but findings show their removal during plant growth severely limits plant survivability (Bartwal et al., 2013; Raboy et al., 1989). Post-harvest tannin removal has also been found relatively ineffective except for hull removal; however, benefits from this technique are negated by the subsequent loss of protein and amino acids also found primarily in or near the hull (Bressani et al., 1983).

5. Soils

We are therefore left with fortifying the seed itself through fertilization. For more comprehensive benefits and drawbacks analysis of fertilizer fortification, see section 2B. The most obvious course of action would be to try and enhance methionine and cysteine in beans– more limited in methionine and cysteine than maize– through sulfur fertilization since they are sulfur-based amino acids. Even so, it is important to understand the native Guatemalan soil profile before finalizing this theory. If the soil is already high in available sulfur for example, more sulfur application would have minimal if any affect. However, given the volatile nature of the sulfur cycle, it is possible to have high sulfur inputs and low sulfur availability.

5.A. Sulfur Cycle

There are several ways sulfur is deposited into the soil and several forms of sulfur that are deposited. Sulfur dioxide (SO_2) is emitted into the atmosphere via anthropogenic sources (industry, power plants etc.) and natural sources (volcanic activity). Once in the atmosphere, it is absorbed into water molecules and then washes into the soil via rainfall. In the soil, sulfurous acid deposits are quickly oxidized into sulfuric acid (H_2SO_4). Sulfides, polysulfides, and elemental sulfur are other forms of sulfur that can be found in the environment, but the biologically important sulfur ion for plants is sulfate (SO_4^{2-}).

The primary source of sulfate (or sulfate containing compounds i.e. gypsum, epsomite etc.) comes from the oxidation of S after the weathering of pyrite (FeS_2) in igneous rock. However, because sulfate is negatively charged, it is highly subject to leaching, making the available quantity within soils dependent on rainfall, temperature, and time. Sulfate is also readily bound in the organic matter layer making it unavailable to plants (Stevenson and Cole, 1999). Therefore, even in areas with high S inputs – industrial developed areas, volcanically active areas etc. – available soil sulfur may still be limiting especially if the environmental conditions are wet and organic matter is high. A basic description of the sulfur cycle can be seen in Figure 3.

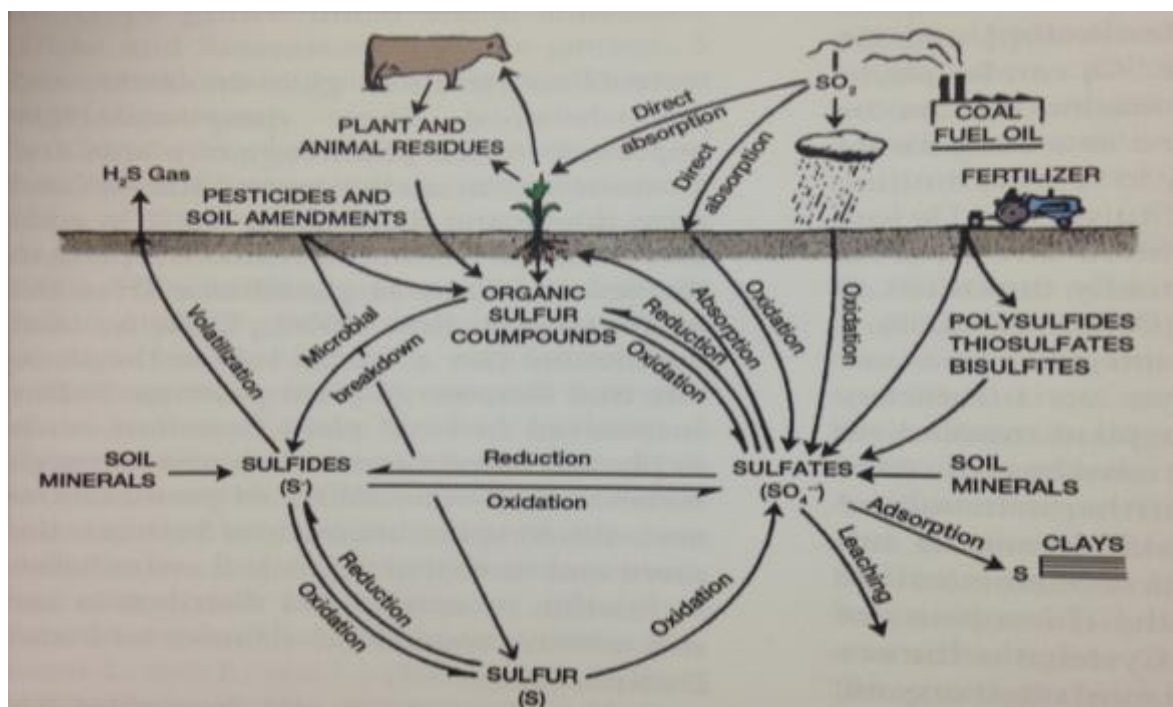


Figure 3: Visual depiction of the sulfur cycle (Kovar and Grant, 2011)

5.B. Soil Sulfur Testing

Given the constant flux between organic and inorganic forms of sulfur within the soil, it is difficult to accurately predict available sulfur content *via* soil testing. For any soil, temperature, moisture, and location in relation to sulfur sources affect available sulfur content at any given time. Even so, there have arisen many soil sulfur tests, leaving much to be desired in homogeneity of results (Kovar and Grant, 2011)..

In Tennessee, sulfur levels are found using the Ammonium Acetate Extraction (AAE) method (Agriculture, 2014). However, like all tests, the AAE method has inherent limitation reducing the level of certainty associated with the results.

5.C. Benefits and Limitations for Ammonium Acetate Extraction

The AAE test is a good representation of actual plant available sulfur compared to other sulfur tests. Unlike Morgan, Mehlich-3, KH_2PO_4 , and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ based extractions, AAE uses a weaker extractant showing less organic-matter-bound sulfur. Thus, the sulfur seen by the test may more closely resemble actual sulfur available to plants. However, this method shows some signs of being soil specific, and has had variable results compared to other sulfur analysis methods (Miyamoto et al., 2011).

It should also be noted, given the volatile nature of sulfur in the soil, that storage and handling conditions may greatly influence final sulfur content results.

5.D. La Fortuna Guatemala

Guatemala has a vast array of soil types (Figure 4) and given that we have no soil nutrient analysis data available, we must draw conclusions based on the type of soil within the area of interest. La Fortuna Guatemala primarily rests on *Andisol* order soils generated from nearby volcanic activity.

5.E. Andisol Soil Order: Basic Breakdown

The Andisol soil order meets the condition of having <25% organic carbon as well as one or both of the following requirements:

1. In the fine-earth fraction, all of the following:
 - a. $\text{Al}_{\text{ox}} + \frac{1}{2} \text{Fe}_{\text{ox}} \geq 2.0$; AND
 - b. A bulk density, measured at 33 kPa water retention $\leq 0.90 \text{ g cm}^{-3}$; AND
 - c. A phosphate retention $\geq 85\%$; OR
2. In the fine-earth fraction, a phosphate retention $\geq 25\%$, $\geq 30\%$ particles 0.02 to 2.0 mm in size, and one of the following:
 - a. $\text{Al}_{\text{ox}} + \frac{1}{2} \text{Fe}_{\text{ox}} \geq 0.4\%$ and, in the 0.02 – 2.0 mm fraction, $\geq 30\%$ volcanic glass; OR
 - b. $\text{Al}_{\text{ox}} + \frac{1}{2} \text{Fe}_{\text{ox}} \geq 2.0\%$ and, $\geq 5\%$ volcanic glass; OR
 - c. $\text{Al}_{\text{ox}} + \frac{1}{2} \text{Fe}_{\text{ox}}$ totaling between 0.4 – 2.0%; and there is at least a proportional content of volcanic glass in the 0.02 to 2.0 mm fraction between 30 and 50% (Dahlgren et al., 2004)

In general terms, Andisol soils are young, have high phosphate retention, and are thus generally limited in productivity by Phosphorus availability, have high OM content, excellent tilth, stable aggregates, and excellent physical properties for crop production (Dahlgren et al., 2004; Doerner et al., 2010; Rahman et al., 2008; Rahman et al., 2003; T and S, 2002). Further classification is based on a variety of chemical, physical, and mineralogical differences, making specific classification without *in situ* testing difficult. A detailed description of Andisol classification can be found in Distribution and Classification of Volcanic Ash Soils and Advances in Agronomy vol 82 p 113-182.

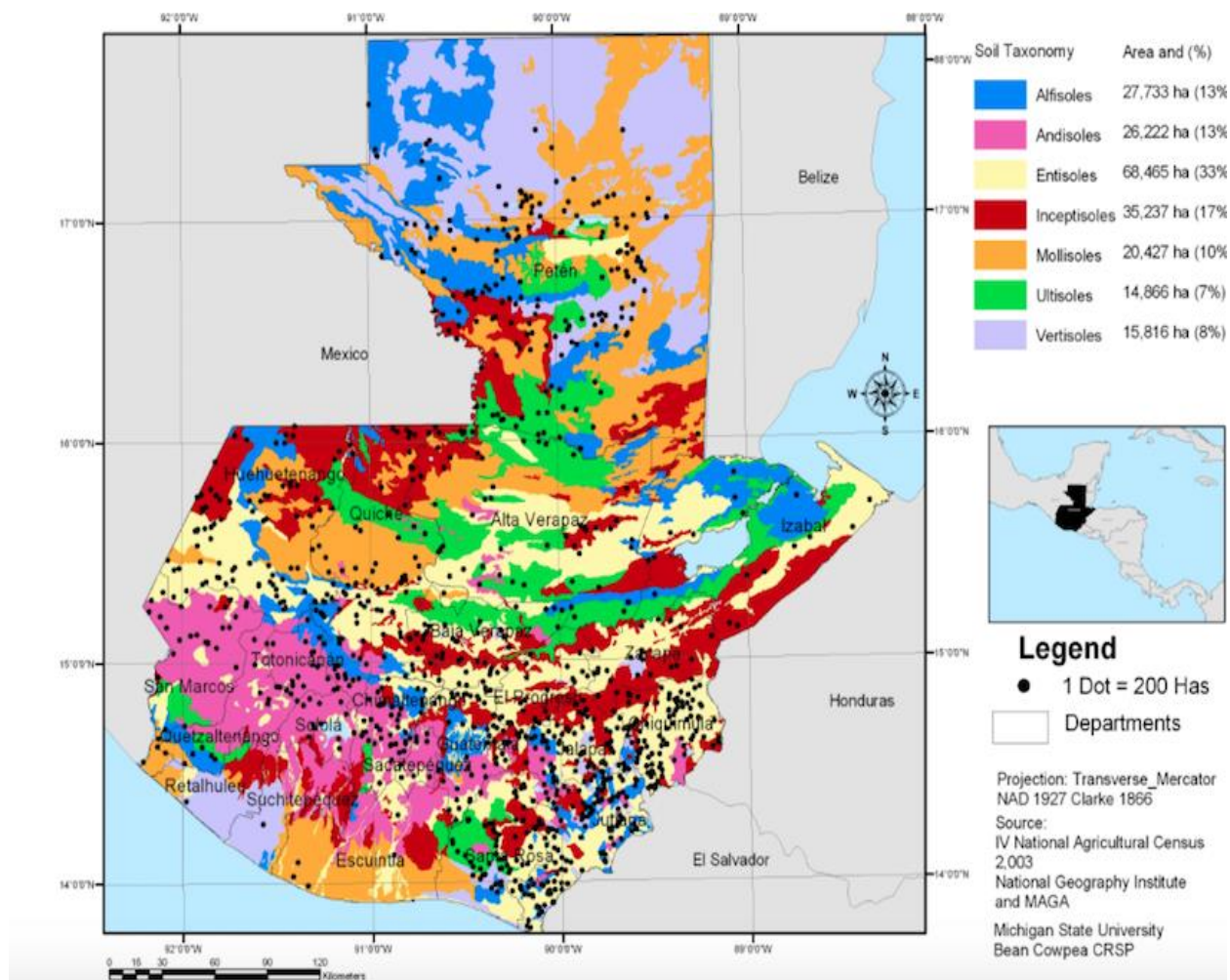


Figure 4: Soils of Guatemala (University, 2003)

5.F. Andisols: Sulfur

The need for sulfur (S) application in agricultural efforts on Andisols is somewhat unclear. While volcanic ash is high in S and causes an immediate influx of elemental sulfur (S) upon deposit, this influx stabilizes quickly (<50 d) (Dahlgren et al., 2004) perhaps due to occlusion or Al complexation. The amount and availability of the S fractions vary by location and soil management (Tanikawa et al., 2009) and may be linked to OM content levels. Dissolved OM acts as a pool for mineralizable N, P, and S through microbial activity (Haynes, 2005) though the relative effects of OM composition and soil properties are not cited.

For Andisols specifically, some reports suggest addition of sulfur through fertilizer application increases crop productivity and nutritional content – specifically methionine and cysteine (Habtegebrial and Singh, 2009; Mora et al., 1999) but these studies note that the reported sites had been extensively farmed beforehand, perhaps indicating lower initial sulfur rates. Gypsum was shown to be an especially useful sulfur application in Andisols as it raised soil pH slightly, limited Al toxicity by balancing Al:S ratios and increased available SO_4^{2-} for optimal plant growth in an acidic Chilean Andisol soil. However, pH changes due to gypsum application varies between sites depending on initial zero-point charge (ZPC) (Mora et al., 1999).

The easily adsorbing, desorbing, precipitating, and oxidative-reductive nature of sulfate anions make it hard to accurately measure true sulfur availability.

6. Sulfur Fertilization: Increases in Yield, Protein, and Amino Acids

6.A. Sulfur

Sulfur is known to be an integral part of Nitrogen fixation in leguminous crops. Yield, protein quality, and S-containing amino acids were negatively impacted by S-deficient soils (Gayler and Sykes, 1985). Sulfur application at 30 and 60 kg ha⁻¹ resulted in significantly increased grain yield, and protein yield in faba beans (*Vicia faba*), though protein changes were attributed to total grain yields and not to grain chemical composition differences (Cazzato et al., 2012). Blackgram (*Phaseolus radiatus* L.) showed significant seed protein content increase with gypsum application at 30 kg ha⁻¹ (Singh and Aggarwal, 1998). Sulfur application increased S-containing amino acid in wheat (*Triticum aestivum*) (Zhao et al., 1999), and positively impacted total protein and amino acid contents in *Phaseolus vulgaris* leaves (Ruiz et al., 2005) independent of yield increases. Improved seed protein in faba bean (*Vicia faba*) from S fertilization is supported by Elsheikh and Elzidany, 1997. No literature has been found outlining increased protein quality and S-containing amino acids in *Phaseolus vulgaris* seeds from sulfur application.

7. Caveats

7.A. Integrated Approach

As with all complex problems, alleviating nutrient deficiencies in low-income areas is multi-faceted. Multiple strategies have been implemented as outlined non-exhaustively above, and no single strategy is fully able to address the problem. In the case of iron and PEM remediation through methionine and cysteine fertilizer fortification, efforts are complicated by the methyl-folate trap.

7.B. Methyl-Folate Trap

Vitamin B12 is integral in the synthesis of methionine from homocysteine in the human body. During either methionine or B12 deficiency (which is recognized as a methionine deficiency) the body attempts to conserve methionine by entering a cyclical process known as the Methyl-folate trap. In true methionine deficiency, this trap conserves and recycles methionine for essential body functions. However, because B12 is essential for methionine production, the body incorrectly recognizes vitamin B12 deficiency as a methionine deficiency, entering the methyl-folate trap unnecessarily to the detriment of the individual.

In this misdiagnosed situation, extra methionine is wasted or converted into taurine, of which there are no detrimental reports and perhaps even beneficial effects (Abd-Allah et al., 2005; Hanna et al., 2004; Kerai et al., 1998; Scott and Weir, 1981; Sun et al., 2011; Sun and Xu, 2008). However, the wasting of methionine could potentially drive the individual into a true methionine deficiency, reducing the body's ability to excrete complexing agents like tannin and thus decreasing macro and micronutrient absorption. While methionine supplementation should increase absorption to some extent, if B12 levels remain inadequate, the methyl-folate cycle cannot be broken and methionine supplementation effects will be limited at best. Therefore, the following proposal, should it have positive results, should be implemented with vitamin B12 supplementation efforts for best results. As is reported numerous times in the literature and is re-emphasized here, a complete solution is only possible through a multidisciplinary (Arimond et al., 2011) and multi-faceted focus including but not limited to the following method.

7.C. Guatemala Soil

Though we must consider the implications of Guatemalan soil to understand potential deficiencies and what fertilization methods should be applied, we feel that during this proof of concept phase, it is enough to proceed on the native soils of Knoxville TN with full awareness that results may or may not be representative once applied in the Guatemalan field setting.

The soils that will be used for the proposed project are Heiskell Silt Loam with 2 to 5% slope, severely eroded and rocky with a rating of clayey, mixed, active, thermic, shallow, Inceptic Hapludalfs.

CHAPTER 3: PROTEIN, METHIONINE, AND CYSTEINE UPREGULATION IN *PHASEOLUS VULGARIS* ‘BLACK TURTLE BEAN’ SEEDS THROUGH SULFUR FERTILIZER AT V2 STAGE OF GROWTH

8. Objectives

OBJECTIVE 1: Assess the effects of sulfur application at the V2 stage of growth on yield in *Phaseolus vulgaris* L ‘black turtle bean’ seeds

OBJECTIVE 2: Assess the effects of sulfur application at V2 stage on total protein and the sulfur-containing amino acids Methionine and cysteine content in *Phaseolus vulgaris* L ‘black turtle bean’ seeds in relation to inhibitory compound (tannin) increases

9. Hypothesis

HYPOTHESIS 1: Sulfur fertilization at the V2 stage of growth of *Phaseolus vulgaris* L ‘black turtle bean’ will significantly increase yield compared to controls

HYPOTHESIS 2: Sulfur fertilization at the V2 stage of growth of *Phaseolus vulgaris* L ‘black turtle bean’ will significantly increase protein and the sulfur-based amino acids methionine and cysteine

HYPOTHESIS 3: Sulfur fertilization at the V2 stage of growth of *Phaseolus vulgaris* L ‘black turtle bean’ will significantly increase protein content and amino acid content in relation to tannin content increases in the seeds

10. Methods

10.A. Site of Experiment

A plot experiment was run at the University of Tennessee Knoxville’s East Tennessee Research and Education Center (ETREC), 3215 Alcoa Highway Knoxville, TN 37920. The season before, a white potato crop *Solanum tuberosum* was planted and harvested—exact fertilization records are unknown. The soil classification is Heiskell Silt Loam with 2 to 5% slope, severely eroded and rocky with a rating of clayey, mixed, active, thermic, shallow, Inceptic Hapludalfs.

Soil cores were taken on June 29, 2016 and analyzed at the Soil Plant and Pest Center at 5201 Marchant Drive Nashville TN for pH using Adams Evans buffer solution. Levels of nitrogen, phosphorus, potassium, calcium, magnesium, zinc, iron, born, sodium and sulfur were also measured with the Mehlich 1 test. Phosphorus levels were “low” (defined as 0-22.4 kg P ha⁻¹) at 15.7 kg P ha⁻¹ and pH levels were also below the recommended 6.5 to 7 pH reading an average 5.8 pH (Figure 5).

Figure 5: Soil test report for plots with V2 application (samples R14B3 and R14 BF) and with R2 application (samples R482 and R481)

SOIL TEST REPORT

FORBES WALKER

, TN

Deborah K. Joines
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Manager
Soil, Plant and Pest Center
5201 Marchant Drive
Nashville, TN 37211-5112
(615) 832-5850
soilplantpestcenter@utk.edu

Date Tested: 8/2/2016

County: Knox

Lab Number: 529382

Mehlich 1 SOIL TEST RESULTS and RATINGS

Sample ID	R14B3	(Pounds Per Acre)										
Water pH	Buffer Value	P Phosphorus	K Potassium	Ca Calcium	Mg Magnesium	Zn Zinc	Fe Iron	Mn Manganese	B Boron	Na Sodium	S-NH4OAC Sulfur	Nitrates-ISE (ppm)
5.9	7.7	14 L	69 L	1154 S	93 S	0.9 S	8 S	14 S	0.4	12	76.2	
		Organic Matter %	Soluble Salts PPM**									

RECOMMENDATIONS

R14B3 Fertilizer/Lime Application Rate and Timing

Beans, Snap or Lima

N / P₂O₅ / K₂O

Nitrogen/Phosphate/Potash: 45 / 90 / 60 pounds per acre

Limestone: Lime is not recommended at this time

Use only 15 pounds of nitrogen per acre when a Blue Lake variety of snap beans is used.

Apply 2 pounds of zinc per acre when zinc tests deficient. If zinc is not tested, apply two pounds of zinc per acre when soil pH is above 6.0 or anytime lime is applied.

County: Knox

Lab Number: 529383

Mehlich 1 SOIL TEST RESULTS and RATINGS

Sample ID	R14BF	(Pounds Per Acre)										
Water pH	Buffer Value	P Phosphorus	K Potassium	Ca Calcium	Mg Magnesium	Zn Zinc	Fe Iron	Mn Manganese	B Boron	Na Sodium	S-NH4OAC Sulfur	Nitrates-ISE (ppm)
5.7	7.7	13 L	61 L	1117 S	105 S	0.7 S	10 S	12 S	0.3	11	75.2	
		Organic Matter %	Soluble Salts PPM**									

WALKER - Page 1

See back of this report for interpretation and detailed explanation of results and recommendations.

If you have questions about this report, please contact us or your County Extension Office.

Visit us on the web at ag.tennessee.edu/spp or Facebook at SoilPlantandPestCenter.

We appreciate your business!

Figure 5 continued

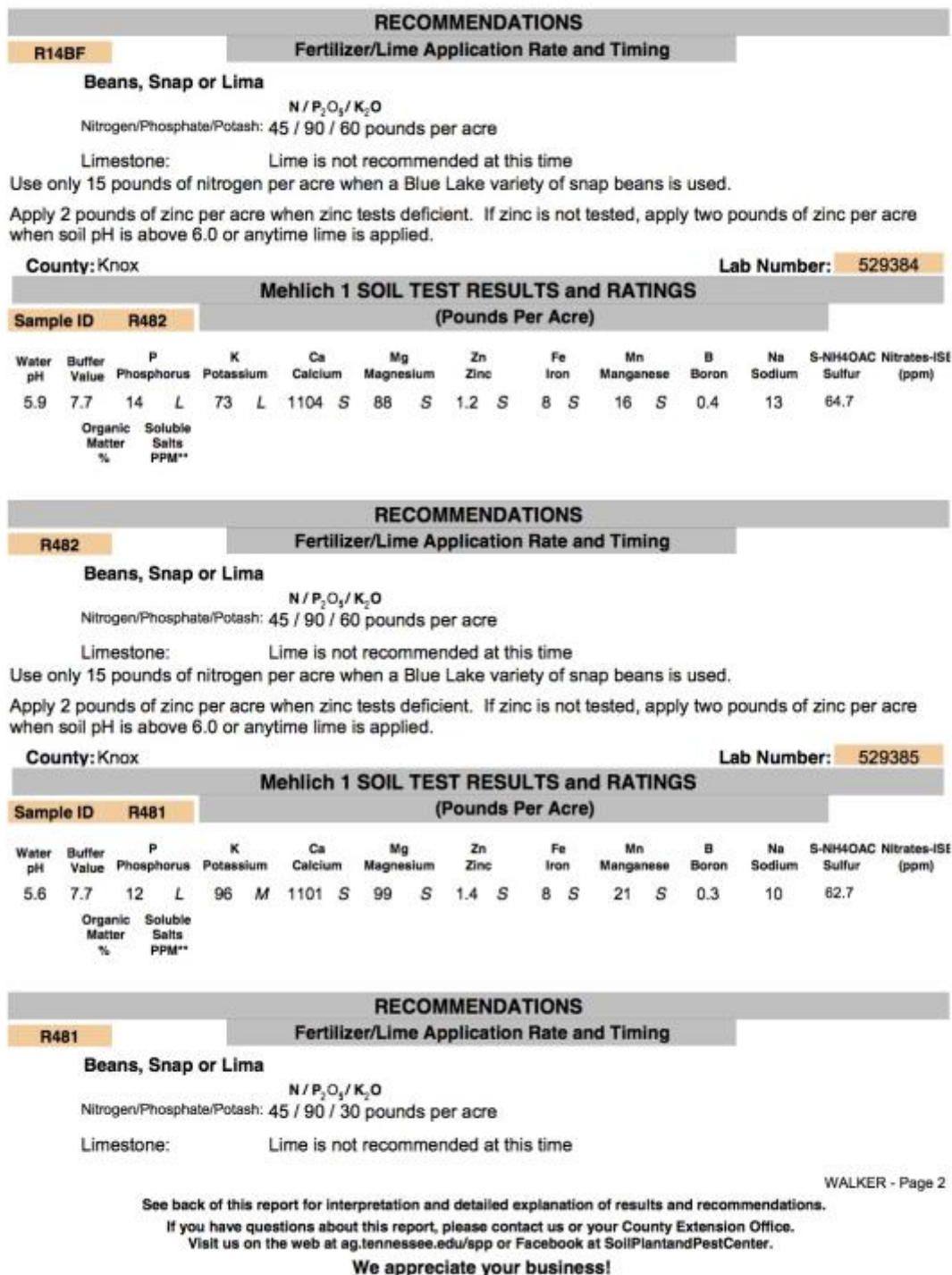


Figure 5 continued

Site preparation included full tillage of 0.1 hectare and four rows of seeds were planted at 2.54 cm depth via a John Deer Max-Emerge 7200 planter on 76 cm row spacing. The planting plates were calibrated to soybean (*Glycine max*) seed size, thus planting rate was variable given the larger and irregular shape of common bean seeds. Seeds were sown on June 10, 2016 and thinned to 2.5 to 8 cm spacing on June 26, 2016 for an estimated rate of 128 thousand plants per hectare.

10.B. Experimental Design and Treatments

We used a randomized complete block design with six treatments and four replicates. Each treatment plot encompassed a 2.6 meter width by 1.8 meter length area for a total area of 4.5 m² or 0.0005 hectares.

Sulfur application was modified from Togay et al., 2008 and Habtegabriel and Singh, 2009. Application was done by hand in the form of granular gypsum (16% S) on July 11, 2016 at the following rates of elemental sulfur: control (0 kg ha⁻¹), low (10 kg ha⁻¹) intermediate (20 kg ha⁻¹), medium (40 kg ha⁻¹), intermediate 2 (60 kg ha⁻¹) and high (80 kg ha⁻¹) (Togay et al., 2008; Habtegabriel and Singh, 2009). The application was during the vegetative stage 2 or “V2 growth” when the “second trifoliate leaf has unfolded at node 4” (Schwartz and Langham, 2010).

10.C. Harvest

After reaching the reproductive harvest stage (RH) when 80% or more of the pods have reached harvest maturity (Schwartz and Langham, 2010), we harvested all pods from each plant within the middle two rows of each plot. The number of plants harvested was recorded for later data analysis. Pods, seeds, and diseased seeds were separated, counted, and diseased seeds were discarded. Diseased seeds were ones with noticeable mold, insect damage, were still immature, or were >1 mm in diameter. Non-diseased seeds were kept for analysis of protein, tannins, and amino acid content.

10.D. Moisture Analysis

Approximately 15 grams of seed from each harvested plot were ground with a Cuisinart Supreme Grind Automatic Burr Mill Coffee Grinder on the “fine” setting. Roughly 10 grams of bean flour was transferred into pre-weighed metal tins, mass was recorded (+/- 0.01 g) and samples were dried in a small oven at 60°C for 24 hours or until constant mass. Moisture content was found by the following equation:

$$\text{Moisture Content} = \frac{\text{Wet Weight (g)} - \text{Dry Weight (g)}}{\text{Wet Weight (g)}}$$

We then converted to a standard 13% moisture assumption (Berrios, 1999; Dorn, 2009; Russo, 2003) via the following equation:

$$\text{Moisture Content 13\% adjustment} = \frac{\text{Dry Weight (g)}}{(1-0.13)}$$

Adjusted moisture content was used to calculate kg ha⁻¹ dry matter and then divided by the area harvested to get kg plot⁻¹ dry matter at an adjusted moisture of 13%.

Approximately 5 g of the remaining, non-oven-dried bean flour from each sample was sieved through a 590-µm E.H. Sargent sieve and stored in plastic containers with lids at room temperature for further nutritive testing.

10.E. Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data. Significance was tested at both $P < 0.05$ and $P < 0.1$ (Bonser et al., 1996; Kimura et al., 2004). Correlation (Proc Corr) analysis was also run (Cartwright et al., 2012).

CHAPTER 4: PROTEIN, METHIONINE, AND CYSTEINE UPREGULATION IN *PHASEOLUS VULGARIS* 'BLACK TURTLE BEAN' SEEDS THROUGH SULFUR FERTILIZER AT R2 STAGE OF GROWTH

11. Objectives

OBJECTIVE 1: Assess the effects of sulfur fertilization at the R2 growth stage of *Phaseolus vulgaris* L 'black turtle bean' on grain yield

OBJECTIVE 2: Assess the effects of sulfur fertilization at the R2 growth stage of *Phaseolus vulgaris* L 'black turtle bean' on total protein and sulfur containing amino acids Methionine and cysteine content in relation to tannin content

12. Hypothesis

HYPOTHESIS 1: Sulfur application at R2 stage of growth will have no effect on grain yield of *Phaseolus vulgaris* L 'black turtle bean'

HYPOTHESIS 2: Sulfur application at the R2 stage of growth will not affect total protein and sulfur-based amino acid methionine and cysteine content in relation to tannin content for *Phaseolus vulgaris* L 'black turtle bean'

13. Methods

Though experiments for V2 and R2 application are separate experiments, we will compare the two indirectly.

13.A. Site of Experiment

Site history, preparation, and planting were the same as the V2 application experiment. Soil test results for plots in the R2 application experiment are shown in figure 5. The R2 application experiment was also on a Heiskell Silt Loam with 2 to 5% slope with a rating of fine-loamy, mixed, semiactive, thermic, aquic Hapludalfs.

13.B. Experimental Design and Treatments

A randomized complete block design with three replicates was used. Plot size of each treatment was 2.6 meter width by 1.5 meter length for a total of 3.9 m² or 0.0004 hectares a piece. Sulfur application was modified from Togay et al. (2008) and Habtegabriel and Singh (2009). Togay et al. (2008) called for 10, 50, and 100 kg S ha⁻¹, and Habtegabriel and Singh (2009) called for 0, 10, 20, 40, and 60 kg S ha⁻¹

Granular gypsum (16% S) was applied by hand at the following rates of elemental sulfur: control (0 kg ha⁻¹), low (10 kg ha⁻¹), intermediate (20 kg ha⁻¹), medium (40 kg ha⁻¹),

intermediate 2 (60 kg ha⁻¹), and high (80 kg ha⁻¹) on July 29, 2016 (Togay et al., 2008; Habtegabriel and Singh, 2009). Plants were fertilized during the R2 stage of growth when 50% or more of flowers had opened (Schwartz and Langham, 2010).

13.C. Harvest

After reaching the RH stage, 80% of pods at harvest maturity (Schwartz and Langham, 2010), we harvested all pods from each plant within the middle two rows of each plot. Number of plants harvested was recorded for later data analysis. Once harvested, pods, seeds, and diseased seeds were separated, counted, and diseased seeds were discarded. Diseased seeds were ones with noticeable mold, insect damage, were still immature, or were >1 mm in diameter. Non-diseased seeds were kept for analysis of protein, tannins, and amino acid content.

13.D. Moisture Analysis

Approximately 15 grams of seed from each harvested plot were ground with a Cuisinart Supreme Grind Automatic Burr Mill Coffee Grinder on the “fine” setting. Roughly 10 grams of bean flour was transferred into pre-weighed metal tins. Mass was recorded (+/- 0.01 g) and samples were dried in a small oven at 60°C for 24 hours. After 24 hours, mass was retaken and samples were dried for another 24 hours or until constant mass was reached. Moisture content was found by the following equation:

$$\text{Moisture Content} = \frac{\text{Wet Weight (g)} - \text{Dry Weight (g)}}{\text{Wet Weight (g)}}$$

We then converted to a standard 13% moisture assumption (Berrios, 1999; Dorn, 2009; Russo, 2003) via the following equation:

$$\text{Moisture Content 13\% adjustment} = \frac{\text{Dry Weight (g)}}{(1-0.13)}$$

Adjusted moisture content was used to calculate kg ha⁻¹ dry matter and then divided by the area harvested to get kg plot⁻¹ dry matter at an adjusted moisture of 13%.

Approximately 5 g of the remaining, non-oven-dried bean flour from each sample was sieved through a 590-micron E.H. Sargent sieve and stored in plastic containers with lids at room temperature for further nutritive testing.

13.E. Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data at both P<0.05 and P<0.1 (Bonser et al., 1996; Kimura et al., 2004). Correlation analysis (Proc Corr) was also run (Cartwright et al., 2012).

CHAPTER 5: PROTEIN, AMINO ACIDS, AND TANNIN ANALYSIS

14. Physical Composition Analysis

Protein, amino acid, and tannin analysis for the V2 and R2 application experiments were identical.

14.A. Total Soluble Protein

Total soluble protein was analyzed using a modified Bradford assay (Bradford, 1976). Ten milligrams of sieved, air-dried, bean flour were weighed into 1 mL centrifuge tubes (+/- 0.01 mg), 1.0 mL of Tris Buffered Saline (TBS) solution was added to each vial and shaken for 90 minutes at 3,000 rpm. Each vial then was centrifuged at 21°F and 8,000 rpm for 30 minutes. Supernatant was decanted into clean 1.5 mL centrifuge tubes, diluted X 50 with DI water and vortexed before use. To create the standard curve, 5µL Bovine Serum Albumin (BSA) was added to 495µL deionized water and diluted to create a range of concentrations: 20 µg µL⁻¹, 10 µg µL⁻¹, 5 µg µL⁻¹, 2.5 µg µL⁻¹, 1.25 µg µL⁻¹. From each sample and standard 100 µL of supernatant was transferred to a 96-well plate in duplicate via a micropipette. All samples and standards then received 100 µL of Bradford Reagent (color indicator) via an automatic pipette and came to completion for five minutes at room temperature. Samples were read calorimetrically on a Cabrex ELx808 spectrophotometer from bioMONTR labs at 630nm and results were calculated as µg protein mL⁻¹.

14.B. Crude Protein Analysis (Nitrogen)

Crude protein was measured via total Nitrogen combustion analysis with a Leco-NS2000 purchased from Leco Instruments, Inc., St. Joseph, MI Nitrogen content was converted to a crude protein estimate by using a factor of 6.25 since protein is 16 percent nitrogen (100/16=6.25). These procedures can be found in the Association of Official Analytical Chemists (AOAC) manuals (AOAC 968.06, 1990).

14.C. Tannin Content Analysis

Total tannin content was measured using a modified Vanillin Assay (Stanly, 1992; Villavicencio, 2000; Mojica, 2015). Ten milligrams of bean flour were weighed into 1 mL centrifuge tubes (+/- 0.01 mg), 1.0 mL of Tris Buffered Saline (TBS) solution was added to each vial and shaken for 90 minutes at 3,000 rpm. Each vial then was centrifuged at 21°F and 8,000 rpm for 30 minutes. Supernatant was decanted into clean 1.5 mL centrifuge tubes and frozen at -40°C until use. To make the standard, 1mg catechin was mixed with 1mL methanol and diluted to the following concentrations: 1,000 µg µL⁻¹, 500 µg µL⁻¹, 250 µg µL⁻¹, 125 µg µL⁻¹, 62.5 µg µL⁻¹, and 0 µg µL⁻¹. To analyze, 20µL of supernatant from each sample and standard concentration was added to a 96-well plate in triplicate. Then, 30µL

methanol and 150 μ L working reagent (1:1 Vanillin: 8% HCL 12N) was added to each sample and standard and the reaction came to completion at room temperature for 10 minutes. Samples were then read calorimetrically on a Cabrex EL_x808 spectrophotometer from bioMONTR labs at 490nm and results were calculated as μ g catechin equivalents mL⁻¹ from the standard curve.

14.D. Methionine and Cysteine Content

Methionine and cysteine content were measured using adapted methods from Kwanyuen (2010). Two milligrams of un-homogenized bean meal were hydrolyzed in evacuated hydrolysis tubes for 24 hours with 1.6 mL of 6N HCL and 1% phenol, premixed and stored as stock solution in a clear glass container with a lid. Samples were then dried under vacuum at 60°C for 12 hours or until no liquid was visible in the bottom of the hydrolysis tubing. After drying, 160 μ L 2:2:1 Ethanol(ETOH): Deionized water (H₂O) : TriEthylAmine (TEA) was used to stop the reaction, samples were re-dried under vacuum, and 160 μ L 7:1:1:1 ETOH:H₂O:TEA:Pheno Isothycyanate (PITC) was used to derivatize amino acids into the more stable PTC amino acid form. The reaction came to completion for 20 minutes at room temperature and samples were completely dried under vacuum and then stored in the freezer until use.

Due to light and temperature sensitivities, 1mL Na₂(HPO)₄ buffer, pH 7.4 containing 5% acetonitrile was added to each sample one at a time in the dark and filtered through a 0.45- μ m membrane. Ten microliters of sample were injected and analyzed with an Agilent Technologies 1200 Series High Performance Liquid Chromatography (HPLC) system using a reverse-phase C18 column. Temperature was maintained at 38°C and the PTC amino acids were separated and eluted by a gradient resulting from mixing eluents A and B. Eluent A was 150 mM CH₃COONa•3H₂O with 0.05% TEA and 6% acetonitrile, pH 6.4, and Eluent B was 6:4 acetonitrile to water. Flow rate was 1 mL min⁻¹ throughout the analysis and the gradient consisted of the following profiles: 100% A at start, 80% A and 20% B at 5.5 min, 54% A and 46% B at 10 min, 100% B at 10.5-12.5 min, 100% A at 13 min. The amino acids eluted from the column were detected at 254 nm via a Diode Array and Multiple Wavelength Detector and recorded. Analysis was run 20 minutes a piece and 15-17 samples were run at one time.

Standards for both methionine and cysteine were made according to Kwanyuen (2010) with modifications. A stock solution of 1,000 ppm cysteine was diluted to 10, 25, 50, 100 ppm and 10, 25, 50, 100, 200 ppm concentrations were prepared for methionine with 6N HCL and 1% phenol, dried under vacuum and then continued in preparation as with the samples; no hydrolysis step was necessary for the standards. Concentrations for standards were based on the lowest and highest methionine and cysteine concentrations found in the bean samples. Standards and samples were run together in the HPLC machine with no modifications between the two.

CHAPTER 6: RESULTS AND DISCUSSION V2 APPLICATION

15. Results and Discussion

15.A. Grain Yield

Sulfur fertilization during the V2 stage of growth affected grain yield of *Phaseolus vulgaris* L. 'black turtle bean' (Table 2, Figure 6).

Table 2: Effect of sulfur fertilization on *Phaseolus vulgaris* 'black turtle bean' during V2 stage of growth on grain yield, soluble protein, crude protein (N), methionine and cysteine, and tannin

Sulfur	Grain Yield			Soluble Protein			Crude Protein			Methionine			Cysteine		Tannin			
kg ha ⁻¹	kg ha ⁻¹			µg g ⁻¹			µg g ⁻¹			µg g ⁻¹			µg g ⁻¹		µg g ⁻¹			
	*	**		*	**		*	**		*	**		*	**		*	**	
0	449.8	ab	ab	8.62	ns	a	25.98	ab	ab	2.37	b	c	0.89	ns	b	6.27	b	b
10	335.6	ab	ab	6.97	ns	b	26.57	ab	a	2.86	ab	abc	1.02	ns	ab	7.24	ab	b
20	261.0	b	b	7.97	ns	ab	25.87	ab	ab	3.91	a	a	1.12	ns	ab	6.17	b	b
40	289.4	ab	b	7.72	ns	ab	27.06	a	a	2.88	ab	abc	1.34	ns	ab	7.82	ab	ab
60	415.3	ab	ab	7.4	ns	ab	26.37	ab	ab	3.64	ab	ab	1.09	ns	ab	7.83	ab	ab
80	515.1	a	a	7.24	ns	ab	24.57	b	b	2.58	ab	bc	1.71	ns	a	10.83	a	a

* Significant at the 0.05 probability level

** Significant at the 0.1 probability level

† ns=not significant

Grain yields were significantly higher at the 80 kg S ha⁻¹ level compared to the 20 kg S ha⁻¹ level (at P < 0.05) and at the 40 kg S ha⁻¹ level (at P < 0.1) (Table 2; Figure 6). Overall yields were low on these plots.

Our results disagree with Togay et al. (2008), who found no yield difference in dry bean (*Phaseolus vulgaris* L.) from phosphorus and sulfur treatments and Cazzato et al. (2012) who found increased grain yield in faba bean (*Vicia faba* L.) with sulfur application between 30 and 60 kg ha⁻¹. Tiecher et al. (2012), found no dry matter yield response to sulfur fertilization over a range of soil and plant types and including common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max*), and castor bean (*Ricinus communis*). However, each of these studies had irrigation, controlled nitrogen levels, and worked on different soil types.



Figure 6: Sulfur application at V2 stage effect on yield of *Phaseolus vulgaris* 'black turtle bean'

Grain yields were significantly higher at the 80 kg S ha⁻¹ level compared to the 20 kg S ha⁻¹ level (at $P < 0.05$) and at the 40 kg S ha⁻¹ level (at $P < 0.1$) (Table 2; Figure 6). Overall yields were low on these plots.

Our results disagree with Togay et al. (2008), who found no yield difference in dry bean (*Phaseolus vulgaris* L.) from phosphorus and sulfur treatments and Cazzato et al. (2012) who found increased grain yield in faba bean (*Vicia faba* L.) with sulfur application between 30 and 60 kg ha⁻¹. Tiecher et al. (2012), found no dry matter yield response to sulfur fertilization over a range of soil and plant types and including common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max*), and castor bean (*Ricinus communis*). However, each of these studies had irrigation, controlled nitrogen levels, and worked on different soil types.

Our results could in part be due to poor stand establishment, disease and insect pressure, or poor rainfall distribution during the growing period. Southern blight was identified on some of the plants and all infected plants were dug up and removed from the field. In some treatments plant removal was up to 97% of the plot and disease most acutely affected the 10 kg S ha⁻¹, 20 kg S ha⁻¹, and 40 kg S ha⁻¹ plots. While yield was adjusted based on seeds given per plant, lack of variability in some plots may have skewed the data.

Poor rainfall distribution may have also negatively affected yields. In June, July, and August of 2016 there were 11.25 cm, 12.19 cm, and 5.92 cm of rainfall respectively. According to the National Oceanic and Atmospheric Administration records taken less than 10 miles from the site location at 2055 Alcoa Hwy, Alcoa, TN 37701, June, July, and August of 2015 experienced 13.4 cm, 16.48 cm, and 9.30 cm of rainfall respectively. It is well established

that drought conditions negatively impact grain yields in the field (Mejía et al., 2003). These drought conditions would have been exacerbated in the 10 kg S ha⁻¹, 20 kg S ha⁻¹, and 40 kg S ha⁻¹ plots as more exposed ground would have encouraged soil evaporation.

15.B. Soluble and Crude Protein

Soluble protein was significantly lower at 10 kg S ha⁻¹ compared to control treatments but no other differences were observed between treatment levels (at P< 0.1) (Table 2). These results were unexpected as Elsheikh et al. (1999), and Fiel et al. (2002) showed increased protein content in faba bean (*Vicia faba* L.) particularly in the soluble fraction with less than 50 kg S ha⁻¹ + mycorrhiza treatments. This could in part be due to poor rainfall distribution as water regime was shown to affect protein fractions in Fiel et al. (2002).

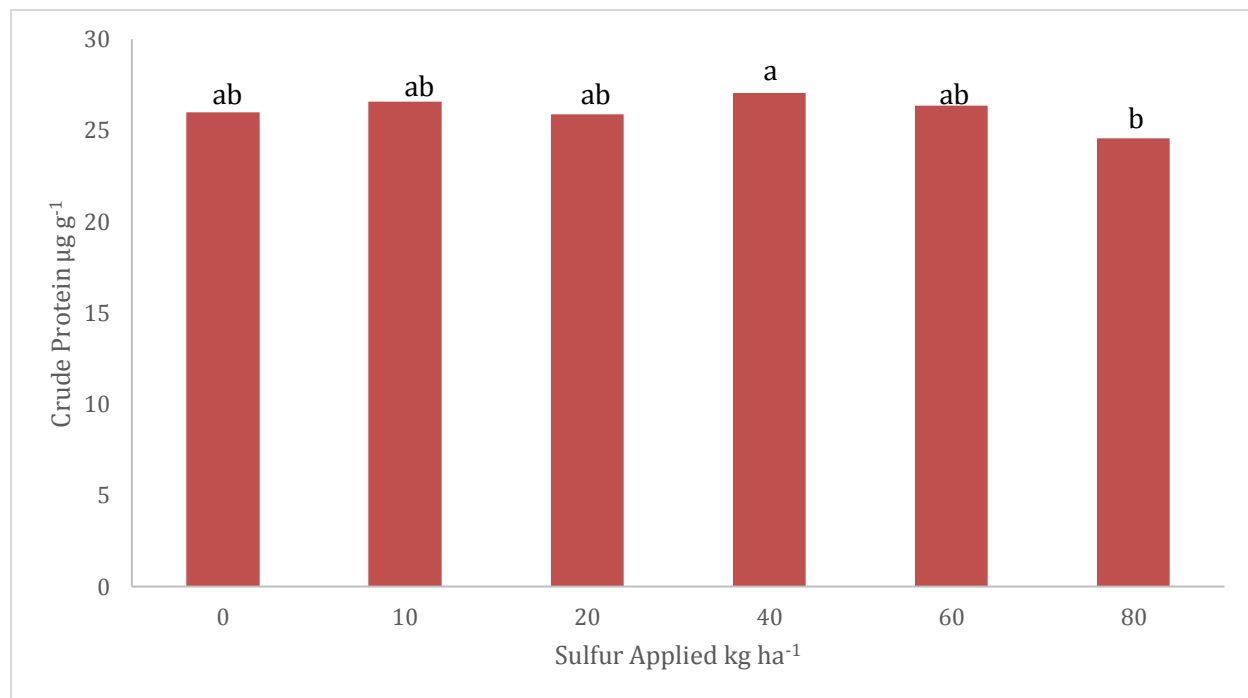


Figure 7: Sulfur application at V2 stage effect on crude protein and tannin of *Phaseolus vulgaris* 'black turtle bean' seed

Crude protein was significantly higher at 40 kg S ha⁻¹ (at P<0.05) and 10 kg S ha⁻¹ and 40 kg S ha⁻¹ (at P<0.1) compared to 80 kg S ha⁻¹ (Table 2, Figure 7). The increase in protein at 40 kg S ha⁻¹ agrees with some previous work including Bahadur and Tiwari (2014), who showed increased protein in mung bean (*Vigna radiate*) at 30 kg ha⁻¹ sulfur application compared to controls. We expected protein to continue to rise as sulfur application increased; however, there is some literature to suggest that nutrient ratios could negatively affect protein synthesis at higher sulfur levels. Pucek and Pys (1996) showed decreased

protein levels under extreme sulfur conditions with nitrogen application of 25 kg N ha⁻¹ and higher. Their work agrees with Schmidt, de Bona, and Monterio (2013) who state that nitrogen to sulfur ratio outside of the expected 20:1 range can cause decreased protein synthesis and an accumulation of non-protein nitrogen (Schmidt, de Bona, and Monterio, 2013). A lack of observable nodulation would have equalized nitrogen availability in the soil of our study, but varying rates of sulfur would change nitrogen to sulfur demands over treatment levels, effectively changing N:S ratios in the plant tissue, affecting protein synthesis and nitrogen accumulation. We did not test for N:S ratios in plant tissues directly. All previous work, including Bahadur and Tiwari, (2014) supplemented nitrogen with their sulfur fertilization, which would have kept their N:S ratios in optimal ranges for protein synthesis.

15.C. Methionine and Cysteine

Methionine content was higher than controls at 20 kg S ha⁻¹ (at P<0.05 and P<0.1). Methionine content at 20 kg S ha⁻¹ was also higher than 80 kg S ha⁻¹ treatments (at P<0.1) (Table 2, Figure 8). Cysteine content was higher at 80 kg S ha⁻¹ compared to controls (at P<0.1) (Table 2, Figure 9).

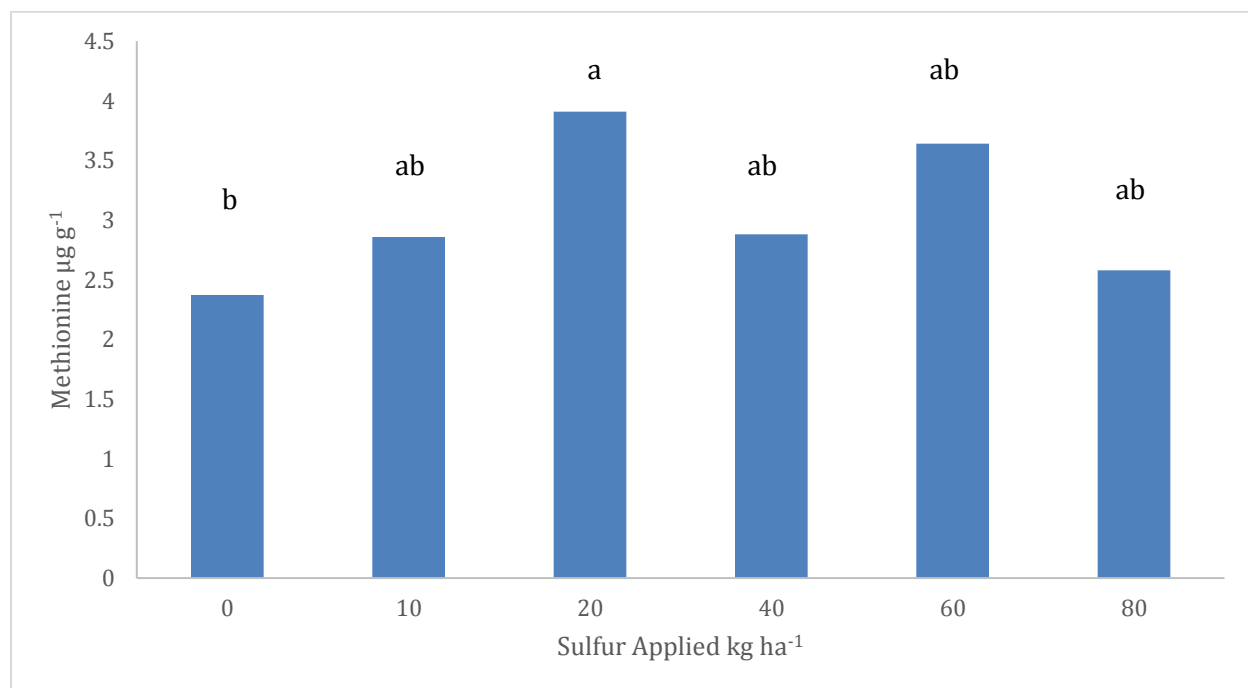


Figure 8: Sulfur application at V2 stage effect on methionine of *Phaseolus vulgaris* 'black turtle bean' seed

Similarly, Klikocka et al. (2016) found significantly higher methionine and cysteine content in wheat (*Triticum aestivum*) at 50 kg ha⁻¹ sulfur fertilization compared to unfertilized controls. It was hypothesized that methionine and cysteine would increase as sulfur application increased, but this was not observed. Klikocka et al. (2016), noted that most significant methionine and cysteine increases occurred when sulfur was supplemented with nitrogen. In our study, no nitrogen was supplemented and no nodulation was observed in any treatment which could cause N:S ratio in plant tissues and affect methionine and cysteine production (Jamal, et al., 2010; Schmidt, de Bona, and Monterio, 2013).

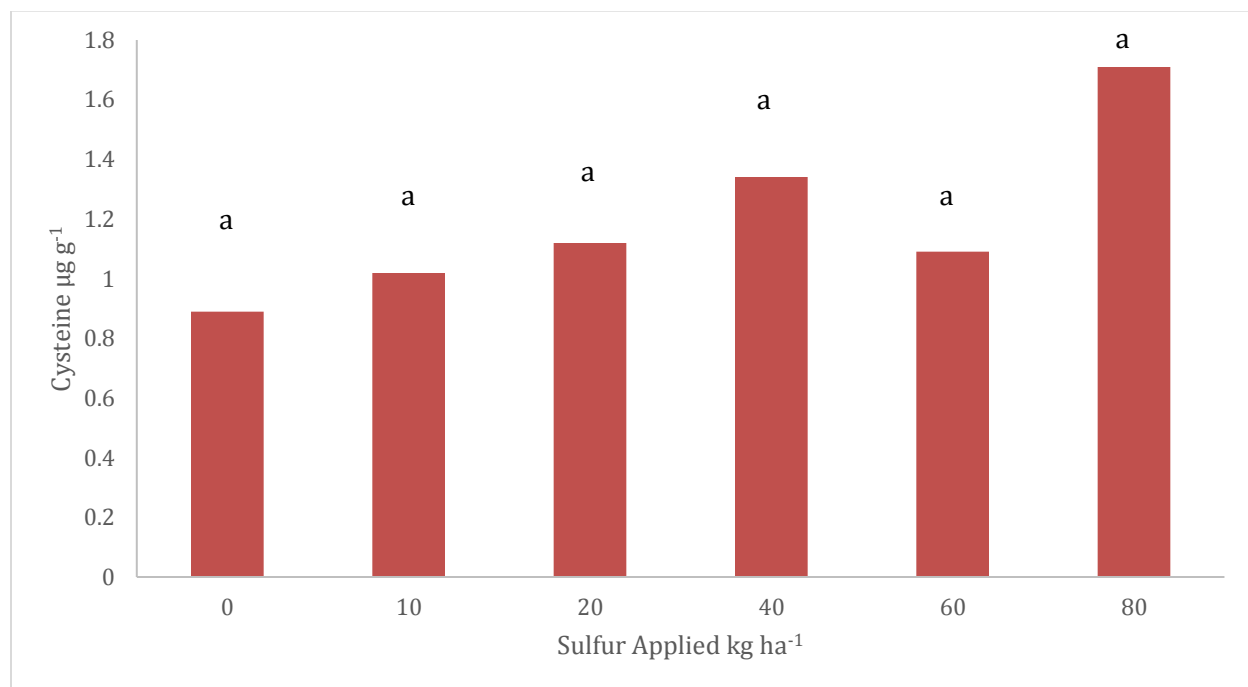


Figure 9: Sulfur application at V2 stage effect on cysteine of *Phaseolus vulgaris* 'black turtle bean'

15.D. Tannin

Tannin content within controls and at 20 kg S ha⁻¹ was lower than 80 kg S ha⁻¹ treatments (at P<0.05). Tannin was higher at 80 kg S ha⁻¹ than all other treatment levels (at P<0.1) (Table 2, Figure 10).

The significant increase in tannin content at 80 kg S ha⁻¹ was expected and agrees with Elsheikh et al. (1999), who showed increased tannin content with sulfur fertilization of faba bean (*Vicia faba*).

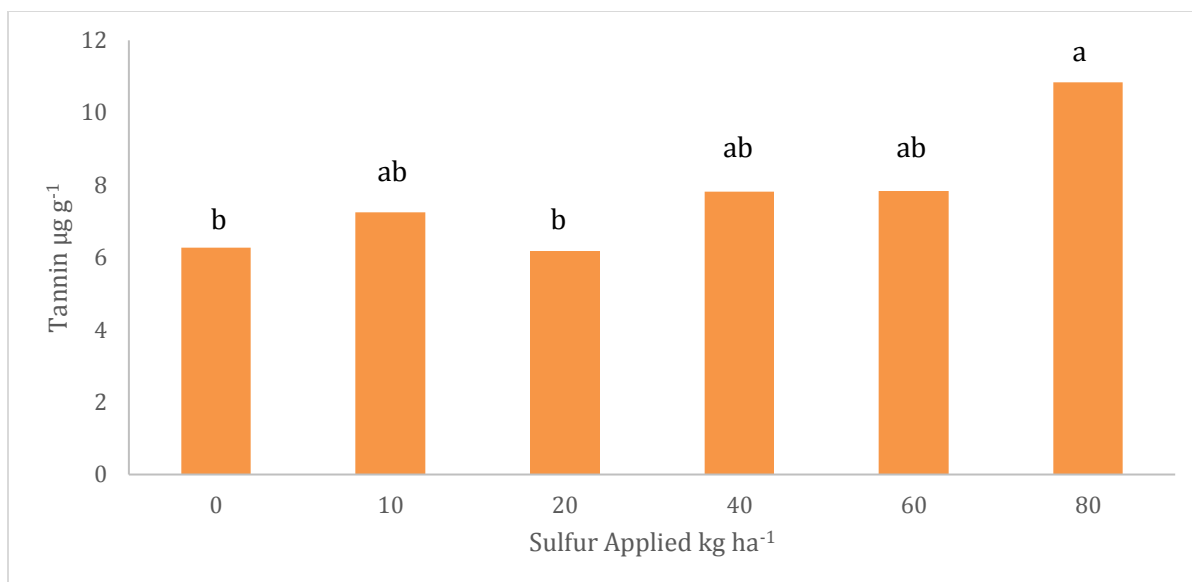


Figure 10: Sulfur application at V2 stage of growth effect on tannin content of *Phaseolus vulgaris* 'black turtle bean' seed

15.E. Ratio of Protein to Tannin

Soluble and crude protein to tannin ratios were lower with 80 kg S ha⁻¹ sulfur treatments compared to control and 20 kg S ha⁻¹ treatments (at $P < 0.05$ and $P < 0.1$) (Table 3, Figure 11, and Figure 12).

Protein to tannin ratio results somewhat disagree with the work of Elsheikh et al. (1999), who found improved seed quality with sulfur treatment, though Elsheikh et al. (1999), did not compare ratios of protein to tannin directly

Table 3: Effect of V2 sulfur fertilization on *Phaseolus vulgaris* 'black turtle bean' seed on the ratio of soluble protein, crude protein, methionine and cysteine, to tannin content

Sulfur	Soluble Protein:Tannin			Crude Protein:Tannin			Methionine:Tannin			Cysteine:Tannin		
kg ha ⁻¹	μg μg ⁻¹			μg μg ⁻¹			μg μg ⁻¹			μg μg ⁻¹		
	*	**		*	**		*	**		*	**	
0	1.38	a	a	4.19	a	a	0.38	bc	bc	0.15	ns	ns
10	1.0	ab	ab	3.72	ab	ab	0.39	bc	bc	0.14	ns	ns
20	1.32	a	a	4.25	a	a	0.63	a	a	0.18	ns	ns
40	1.02	ab	ab	3.59	ab	ab	0.38	bc	bc	0.17	ns	ns
60	1.02	ab	ab	3.62	ab	ab	0.52	ab	ab	0.16	ns	ns
80	0.81	b	b	2.72	b	b	0.25	c	c	0.16	ns	ns

* Significant at the 0.05 probability level

** Significant at the 0.1 probability level

† ns= not significant

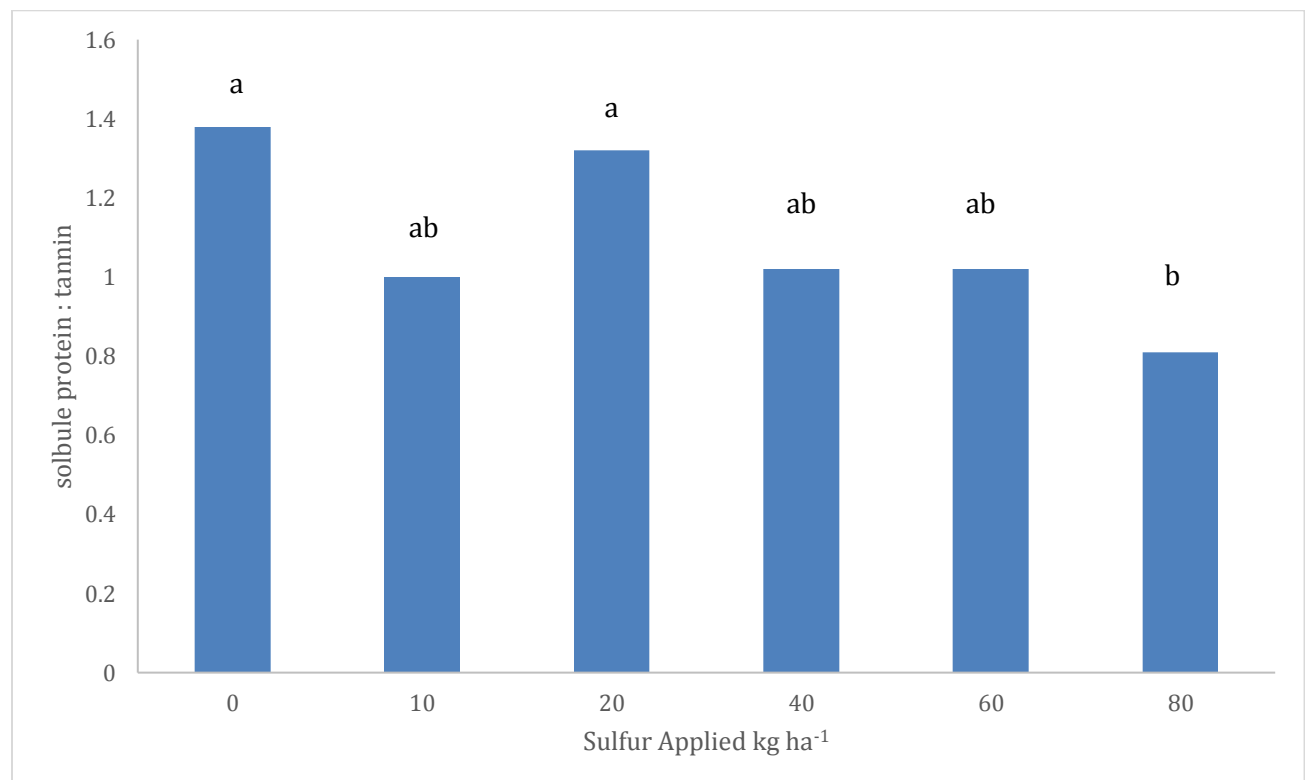


Figure 11: Sulfur application during V2 stage of growth effect on soluble protein to tannin ratios in *Phaseolus vulgaris* 'black turtle bean' seed

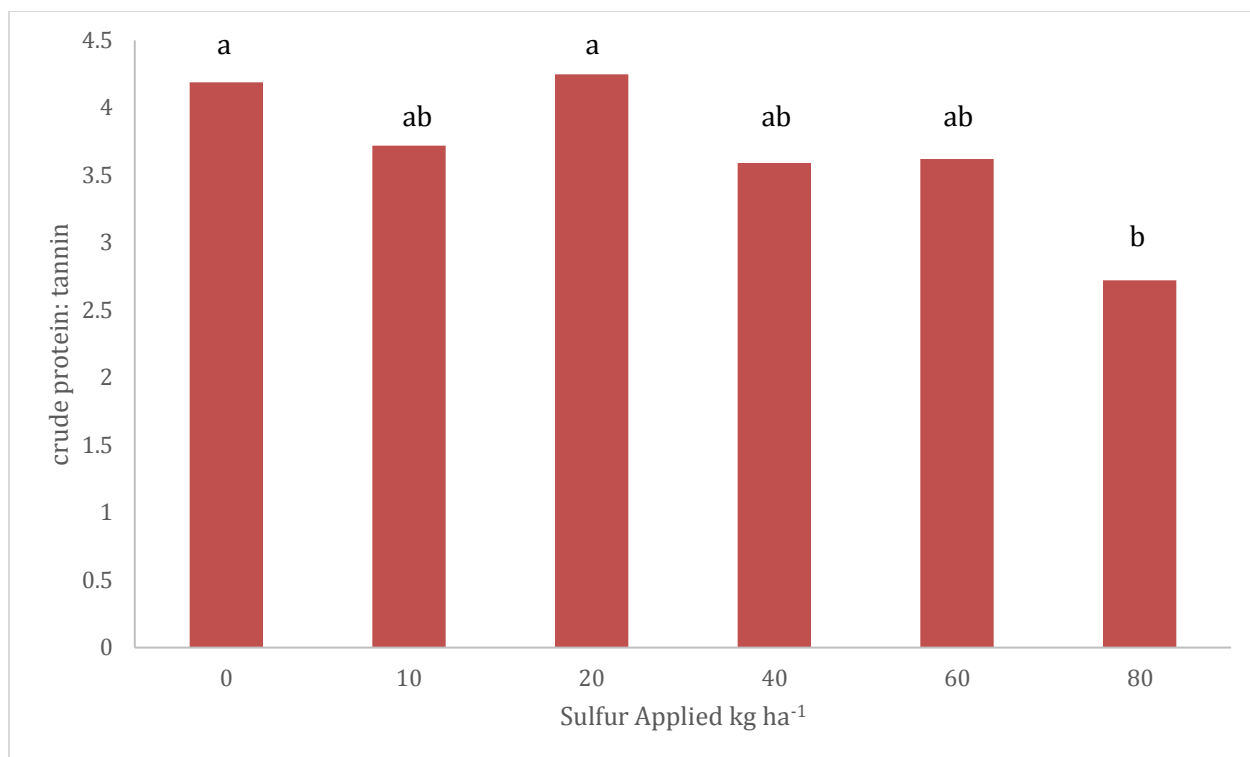


Figure 12: Sulfur application during V2 stage of growth effect on crude protein to tannin ratios in *Phaseolus vulgaris* 'black turtle bean'

15.F. Ratio of Methionine and Cysteine to Tannin

Methionine to tannin ratios were significantly higher at 20 kg S ha⁻¹ compared to control, 10 kg S ha⁻¹, 40 kg S ha⁻¹, and 80 kg S ha⁻¹ treatment levels. At 80 kg S ha⁻¹ methionine to tannin ratios were lower than 60 kg S ha⁻¹ treatments but were not significantly different from control, 10 kg S ha⁻¹ and 40 kg S ha⁻¹ levels (at $P < 0.05$ and $P < 0.1$) (Table 3, Figures 13 and 14).

All ratios of cysteine to tannin were the same between treatment levels (at $P < 0.05$ and $P < 0.1$) (Table 3, Figure 14). Amino acid to tannin ratio results somewhat agree with and build upon the work of Elsheikh et al. (1999), who found improved seed quality with sulfur treatment, though Elsheikh et al. (1999), did not compare ratios amino acids and tannin directly.

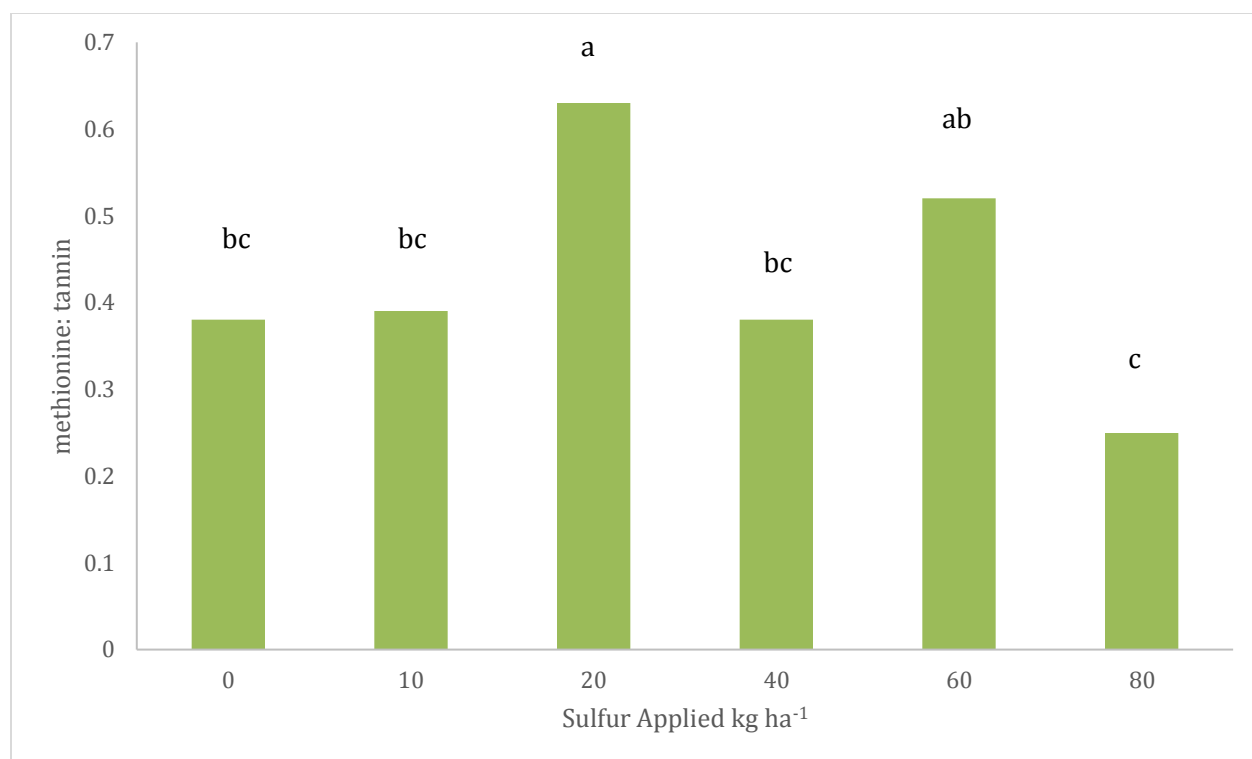


Figure 13: Sulfur application during V2 stage of growth effect on methionine to tannin ratios in *Phaseolus vulgaris* 'black turtle bean'

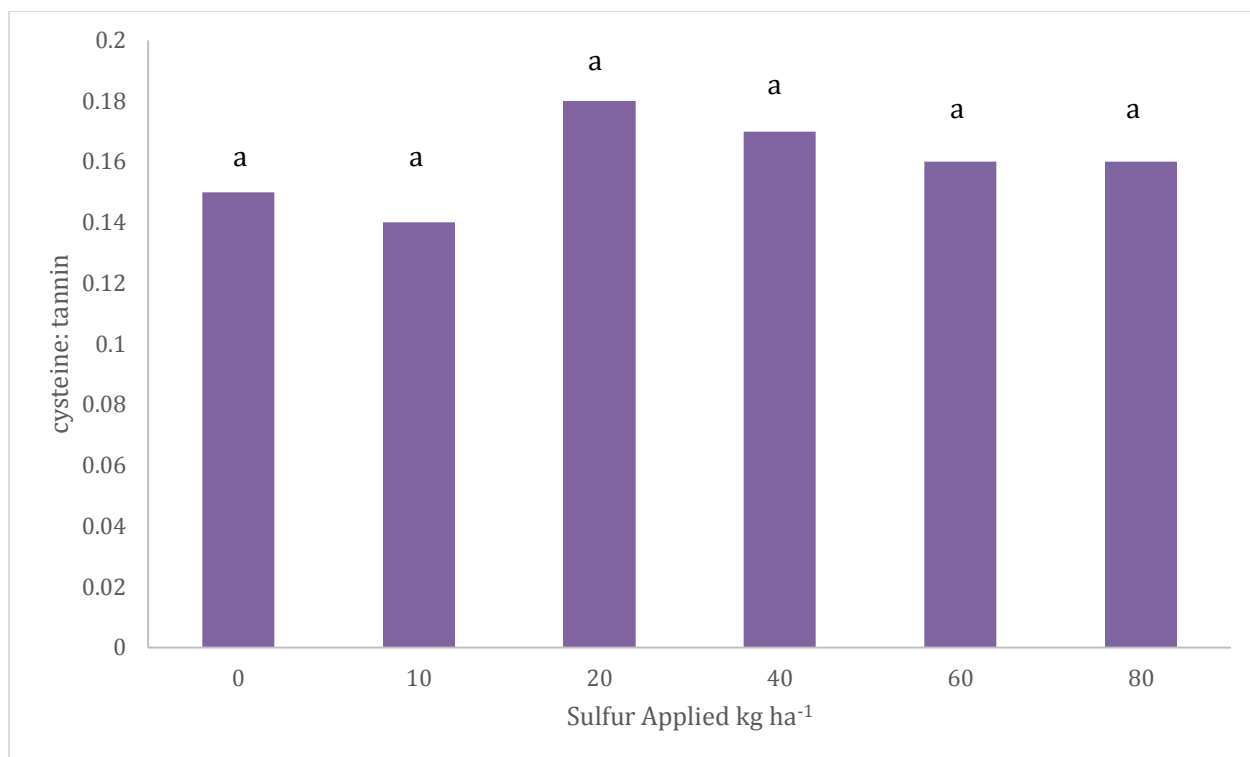


Figure 14: Sulfur application during V2 stage of growth effect on cysteine to tannin ratios in *Phaseolus vulgaris* 'black turtle bean'

15.G. Correlation (Proc Corr)

There was significant and strong positive correlation between tannin content and cysteine content ($P < 0.001$) (Table 4) showing that sulfur application during V2 stage of growth increased both tannin and cysteine content. All other correlations were nonsignificant, except a moderate correlation between soluble protein and cysteine content ($p < 0.1$). No literature has been found to confirm this correlation.

Table 4: Correlation between yield, protein, crude protein, tannin, cysteine, and methionine content for V2 stage of growth application of sulfur

		Yield	Soluble Protein	Crude protein	Tannin	Cysteine	Methionine
		kg ha ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹
Yield	kg ha ⁻¹		0.21	-0.17	-0.19	-0.26	-0.24
	*		ns	ns	ns	ns	ns
Soluble Protein	µg g ⁻¹			-0.11	-0.33	-0.39	-0.08
	*			ns	ns	*	ns
Crude protein	µg g ⁻¹				-0.18	-0.18	0.29
	*				ns	ns	ns
Tannin	µg g ⁻¹					0.67	0.25
	*					***	ns
Cysteine	µg g ⁻¹						0.34
	*						ns
Methionine	µg g ⁻¹						
	*						

* Significant at the 0.1 probability level

** Significant at the 0.05 probability level

*** Significant at the 0.001 probability level

† ns = not significant

CHAPTER 7: RESULTS AND DISCUSSION R2 APPLICATION

16. Yield Results

16.A. Grain Yield

Sulfur fertilization during the R2 stage of growth affected grain yield of *Phaseolus vulgaris* L. 'black turtle bean'. Yield was significantly lower at 20 kg S ha⁻¹ compared to all other treatments except 40 kg S ha⁻¹ where the yield was the same. Yield did not differ at 40 kg S ha⁻¹ from all other treatments (at P<0.05 and P<0.1) (Table 4, Figure 15).

Table 5: Effect of sulfur fertilization on *Phaseolus vulgaris* 'black turtle bean' during R2 stage of growth on grain yield, soluble protein, crude protein (N), methionine and cysteine, and tannin

Sulfur	Grain yield			Soluble protein			Crude protein			Methionine			Cysteine			Tannin		
kg ha ⁻¹	kg ha ⁻¹			µg g ⁻¹			µg g ⁻¹			µg g ⁻¹			µg g ⁻¹			µg g ⁻¹		
	*	**		*	**		*	**		*	**		*	**		*	**	
0	1181.1	a	a	5.13	ns	ns	24.15	ns	ns	4.12	ns	ns	1.41	ns	a	5.93	ns	ns
10	1109.5	a	a	5.74	ns	ns	22.54	ns	ns	4.26	ns	ns	1.10	ns	ab	5.80	ns	ns
20	815.4	b	b	6.35	ns	ns	23.80	ns	ns	3.61	ns	ns	0.94	ns	ab	5.60	ns	ns
40	978.6	ab	ab	5.89	ns	ns	23.58	ns	ns	3.03	ns	ns	1.36	ns	ab	5.30	ns	ns
60	1147.2	a	a	5.61	ns	ns	23.82	ns	ns	3.12	ns	ns	0.64	ns	b	6.06	ns	ns
80	1123.3	a	a	5.75	ns	ns	23.64	ns	ns	3.00	ns	ns	1.18	ns	ab	5.09	ns	ns

* Significant at the 0.05 probability level

** Significant at the 0.1 probability level

† ns= not significant

Togay et al. (2008) found no yield difference from phosphorus and sulfur treatments in *Phaseolus vulgaris* and Cazzato et al. (2012) found increased grain yield in faba bean (*Vicia faba* L.) with sulfur application between 30 and 60 kg S ha⁻¹. Tiecher et al. (2012) found no dry matter yield response to sulfur fertilization in common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max*), and castor bean (*Ricinus communis*).

Rainfall distribution and weed pressure may have contributed to our results. In June, July and August of 2016 there were 11.25 cm, 12.19 cm, and 5.92 cm of rainfall respectively. According to the National Oceanic and Atmospheric Administration records taken less than 10 miles away at 2055 Alcoa Hwy, Alcoa, TN 37701, showed higher rainfall in June, July, and August of 2015 (13.4 cm, 16.48 cm, and 9.30 cm respectively).

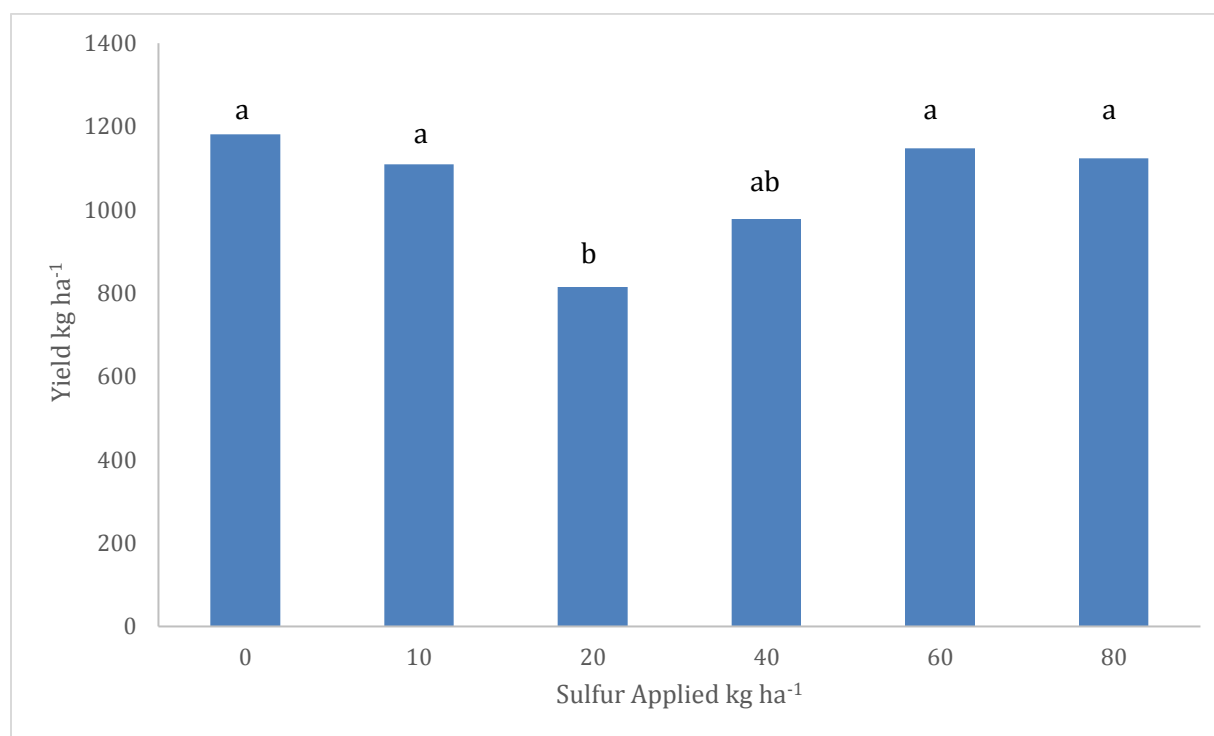


Figure 15: Sulfur application at R2 stage effect on yield of *Phaseolus vulgaris* 'black turtle' bean

Drought conditions have been shown to negatively affect yields (Mejía et al., 2003). Weeding and thinning did not occur in these plots until July 29, 2016 at time of fertilization when flower set had already occurred—weed pressure was not categorized on a per-plot basis but it is well documented that competition for resources can reduce yield (Ostergard et al., 2008; Saberali and Mohammadi 2015).

16.B. Soluble and Crude Protein

There were no differences in soluble protein (at $P < 0.05$ and $P < 0.1$). There were no differences in crude protein levels (at $P < 0.05$ or $P < 0.1$) (Table 4).

Protein results agree with Eriksen and Mortenson (2002) who found no difference in protein concentrations of barely grain (*Hordeum vulgare* L.) with sulfur application at grain set in a pot study. Hrivna, Kotkova, and Buresova (2015) and Steinfurth et al. (2012) also found no difference in protein concentrations in wheat grain (*Triticum aestivum*) with sulfur fertilization at grain set in a field and pot study respectively. No studies on sulfur fertilization timing in common bean were found.

16.C. Methionine and Cysteine

There were no differences in methionine content between treatment levels (at $P < 0.05$ and $P < 0.1$) (Table 4). Cysteine content was significantly lower at 60 kg S ha⁻¹ compared to controls (at $P < 0.1$) (Table 4, Figure 16).

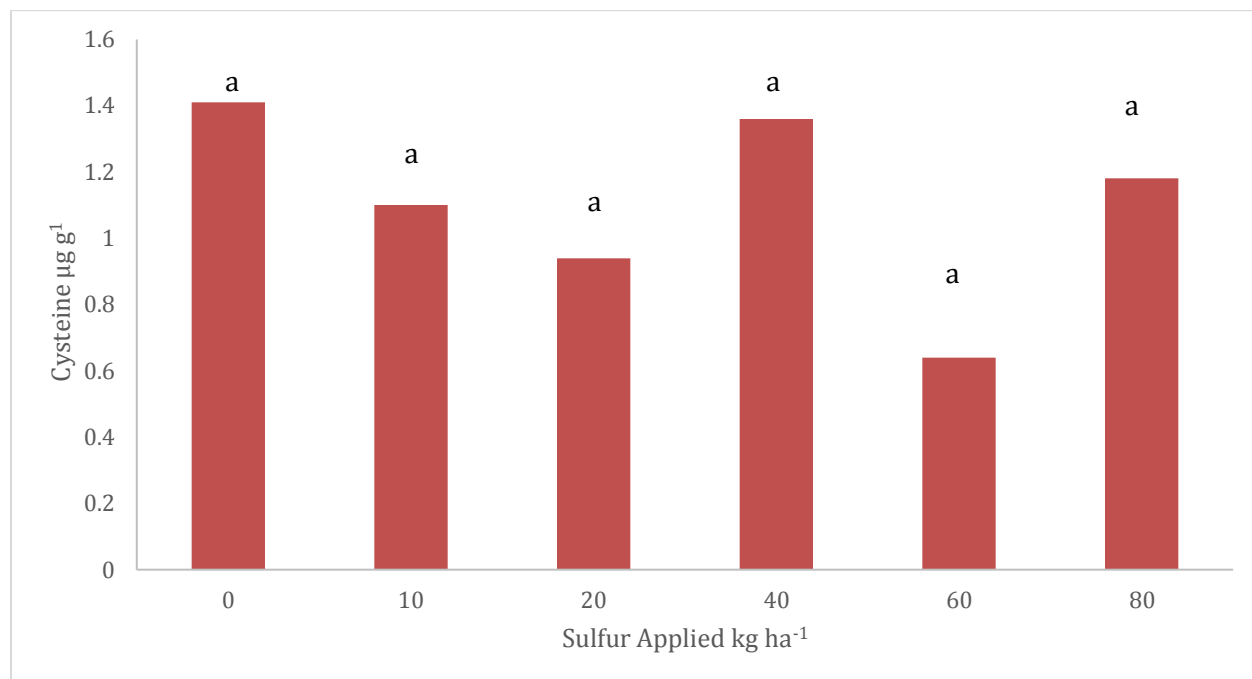


Figure 16: Sulfur application at R2 stage effect on cysteine of *Phaseolus vulgaris* 'black turtle' bean seed

The decrease in cysteine at higher sulfur rates disagrees with Zhao et al. (1999) and Eriksen and Mortensen (2002) who found sulfur application significantly increased S-containing amino acids in wheat (*Triticum aestivum* L.) compared to controls in a pot study. Ruiz et al. (2005) also found increased methionine and cysteine in (*Phaseolus vulgaris*) bean leaves in a pot study, and Klikocka et al. (2016) found more methionine and cysteine production in spring wheat (*Triticum aestivum* L.) especially when nitrogen was also applied in a field experiment. Differences in our results may have been influenced by initial weed pressure, and N:S dynamics in the soil. Weeding and thinning happened July 29, 2016 at time of fertilization when flower set had already occurred—weed pressure was not categorized on a per-plot basis but was observably heavy throughout the plots; it is well documented that heavy weed pressure influences nutrient availability (Ostergaard et al., 2008, Saberali and Mohammadi, 2015) and uptake which would affect amino acid production.

Nitrogen to sulfur dynamics in the soil increase in relation to each other; as nitrogen becomes more abundant, sulfur demand raises and as sulfur becomes more abundant, nitrogen demands rise (Schmidt, de Bona, and Monterio, 2013). So, as sulfur levels increased nitrogen demand would have gone up as well. Given that no nodulation was observed, nitrogen would have been used up more quickly at higher sulfur treatment rates, decreasing the efficiency with which sulfur could be used, and ultimately curbing cysteine accumulation in the 60 kg S ha⁻¹ treatments. We did not test for N:S ratios in plant tissues to confirm this theory. Zhao et al. (1999), and Erikson and Mortenson (2005) supplemented nitrogen with their sulfur fertilization, which would have kept their N:S ratios in optimal ranges.

16.D. Tannin

There were no differences in tannin content between treatments (at $P < 0.05$ and $P < 0.1$) (Table 4).

These results disagree with Elsheikh et al. (1999) who showed increased tannin content with sulfur fertilization of faba bean (*Vicia faba*). However, timing of sulfur application is a glaring difference between the two studies as Elsheikh et al. (1999) applied sulfur at planting and we applied sulfur at the R2 stage of growth.

16.E. Ratio of Protein to Tannin

There were no differences in soluble or crude protein to tannin ratios (at $P < 0.05$ and $P < 0.1$) (Table 5) showing that protein and tannin production was not affected, at the R2 stage of growth.

Overall, protein to tannin ratios were much lower for plots fertilized at the R2 stage compared to plots fertilized at the V2 stage of growth indicating that sulfur fertilization had less impact on protein levels when applied at the later growth stage. This difference could be influenced by the slight change in elevation between the two plots, affecting overall moisture in the soil. While moisture content was not directly measured in our study, plots fertilized during the R2 stage of growth were noticeably moist and muddy compared to dry soil up to 2.5-5 cm depth in the V2 plots. Mejía et al. (2003) showed that drought conditions result in production of drought proteins in (*Phaseolus vulgaris* L.) bean seeds, resulting in an overall increase in total protein content compared to seeds formed under non-drought conditions (Mejía et al., 2003).

No literature has been found correlating tannin and protein ratios directly. Further research is needed to show repeatability of these results.

Table 6: Effect of Sulfur Fertilization on *Phaseolus vulgaris* 'black turtle bean' During R2 Stage of Growth on the Ratio of Soluble Protein, Crude Protein (N), Methionine and Cysteine, to Tannin Content

Sulfur	Soluble Protein:Tannin			Crude Protein:Tannin			Methionine:Tannin			Cysteine:Tannin		
kg ha ⁻¹	µg µg ⁻¹			µg µg ⁻¹			µg µg ⁻¹			µg µg ⁻¹		
	*	**		*	**		*	**		*	**	
0	0.86	ns	ns	4.10	ns	ns	0.35	ns	abc	0.24	ns	ns
10	0.99	ns	ns	3.95	ns	ns	0.25	ns	bc	0.20	ns	ns
20	1.15	ns	ns	4.46	ns	ns	0.26	ns	abc	0.19	ns	ns
40	1.15	ns	ns	4.58	ns	ns	0.42	ns	a	0.27	ns	ns
60	0.93	ns	ns	3.97	ns	ns	0.21	ns	c	0.10	ns	ns
80	1.17	ns	ns	4.82	ns	ns	0.39	ns	ab	0.24	ns	ns

* Significant at the 0.05 probability level

** Significant at the 0.1 probability level

† ns = not significant

16.F. Ratio of Methionine and Cysteine to Tannin

There was a significantly higher methionine to tannin ratio at 40 kg S ha⁻¹ compared to 10 kg S ha⁻¹ and 60 kg S ha⁻¹ treatments (Table 5, Figure 17). Methionine to tannin ratios were also higher at 80 kg S ha⁻¹ compared to 60 kg S ha⁻¹ treatments (at P<0.1) (Table 5, Figure 18). There were no differences found between cysteine to tannin ratios (at P<0.05 and P<0.1) (Table 5).

These results show that sulfur fertilization at the R2 stage of growth improved methionine production more rapidly than tannin production, especially at higher sulfur concentrations. No literature has been found correlating methionine and cysteine to tannin ratios.

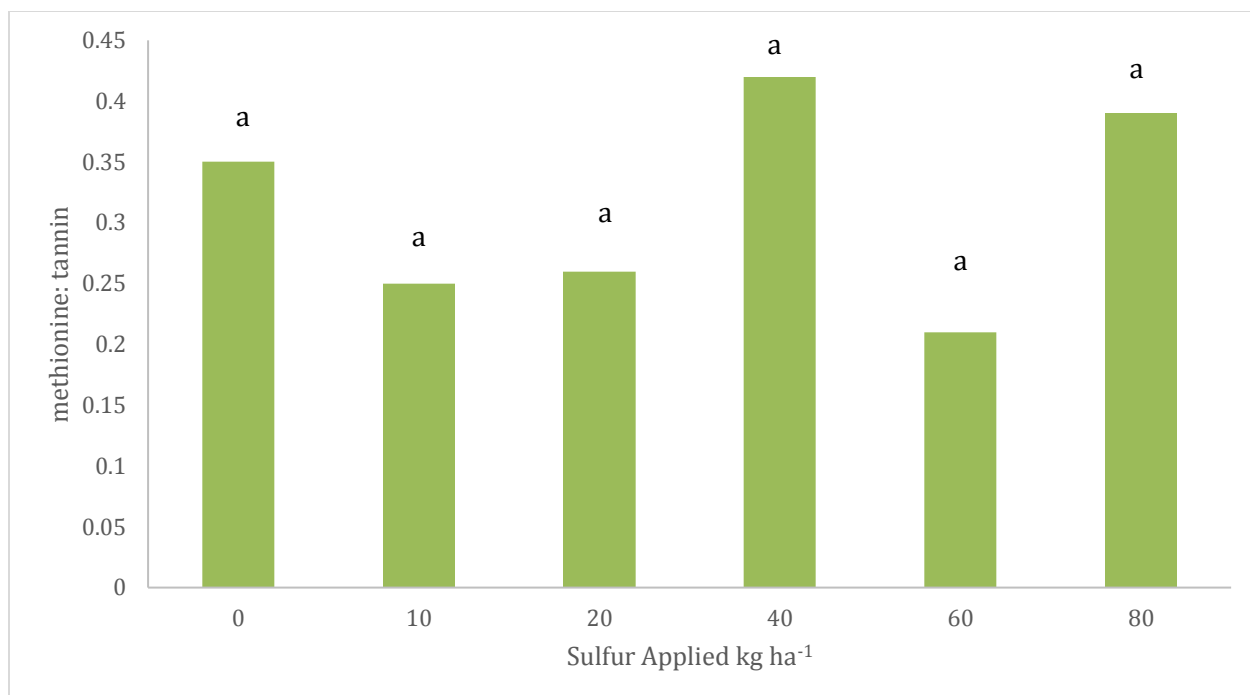


Figure 17: Sulfur application during R2 stage effect on methionine to tannin ratios in *Phaselous vulgaris* 'black turtle bean' seeds

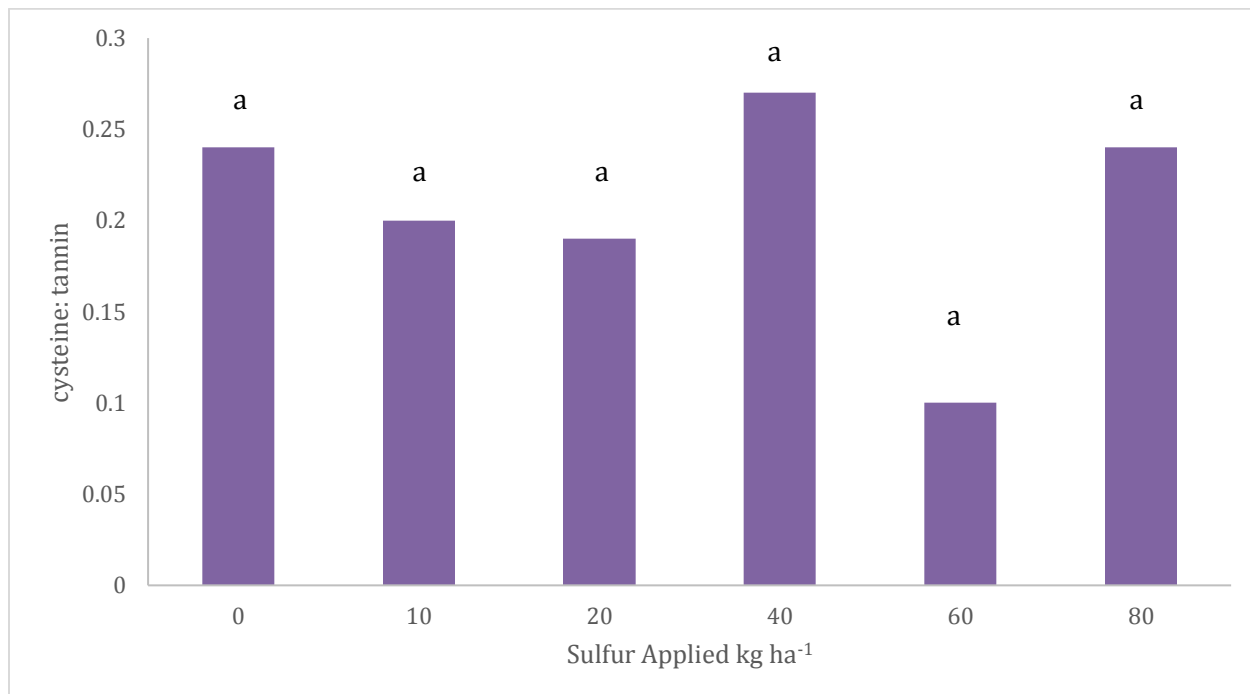


Figure 18: Sulfur application during R2 stage effect on cysteine to tannin ratios in *Phaselous vulgaris* 'black turtle bean' seeds

16.G. Correlation (Proc Corr)

Significant, strong, positive correlations were found between tannin and yield, cysteine and crude protein, and cysteine and methionine content ($P < 0.05$). Moderate positive correlation was found between soluble protein and crude protein ($P < 0.1$). And strong negative correlation was found between tannin and cysteine content ($P < 0.05$) (Table 5).

Table 7: Correlation between yield, protein, crude protein, tannin, cysteine, and methionine content in *Phaseolus vulgaris* 'black turtle bean' seeds at R2 stage of growth sulfur application

		Yield	Soluble Protein	Crude Protein	Tannin	Cysteine	Methionine
		kg ha ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹
Yield	kg ha ⁻¹						
	*		-0.16 ns	-0.32 ns	0.56 *	-0.30 ns	-0.25 ns
Soluble Protein	µg g ⁻¹			0.43	0.26	0.06	-0.002
	*			*	ns	ns	ns
Crude protein	µg g ⁻¹				-0.26	0.65	0.33
	*				ns	**	ns
Tannin	µg g ⁻¹					-0.53	-0.12
	*					**	ns
Cysteine	µg g ⁻¹						0.51
	*						**
Methionine	µg g ⁻¹						
	*						

* Significant at the 0.1 probability level

** Significant at the 0.05 probability level

† ns = not significant

It is unknown what caused these trends. While it is possible that reduction in condensed tannins could result in increased pest damage (Bartwal et al., 2013) there is no evidence in our data to support such a claim. The reduction in yield at 20 kg S ha⁻¹ does not correspond with a significant tannin decrease, and there was no difference in the number of diseased seeds at 20 kg S ha⁻¹ compared to controls ($P < 0.05$ and $P < 0.1$) (Table 6).

Table 8: Analysis of variance (ANOVA) for number of diseased seeds between treatment levels of sulfur at R2 stage of growth

Number Diseased Seeds		P<0.05	P<0.1
0 kg ha ⁻¹	193.0	a	a
10 kg ha ⁻¹	126.0	ab	ab
20 kg ha ⁻¹	121.5	ab	ab
40 kg ha ⁻¹	103.3	b	b
60 kg ha ⁻¹	164.5	ab	ab
80 kg ha ⁻¹	98.0	b	b

Soluble protein to crude protein correlation is expected and cysteine to crude protein positive correlation was expected and agrees with the literature since cysteine and protein production are linked systems (Hesse et al.,2001; Brosnan and Brosnan, 2006). Positive cysteine and methionine correlation was also expected and agrees with the literature as cysteine is a precursor to methionine production (Hesse et al.,2001; Brosnan and Brosnan, 2006). It is not well understood why there was a significant, strong, negative correlation between tannin and cysteine, especially since there was a significant, strong, positive correlation when sulfur was applied at the V2 stage of growth.

CHAPTER 8: CONCLUSIONS

There were positive and negative effects from fertilization at the V2 and R2 stage of growth. It was found that sulfur at 20 kg S ha⁻¹ reduced grain yield regardless of when the sulfur is applied. Methionine to tannin ratios increased with 20 kg S ha⁻¹ compared to controls when applied at V2 stage of growth and 40 kg S ha⁻¹ compared to 10 kg S ha⁻¹ when applied at R2 stage of growth. We therefore concluded that 20 to 40 kg S ha⁻¹ had positive effects on methionine to tannin ratios regardless of when the sulfur was applied. However, overall there were pronounced differences in how the timing of application affected nutritional components in the seed.

Fertilization at the V2 stage of growth decreased soluble protein production at 10 kg S ha⁻¹ compared to controls and crude protein increased at 10 and 40 kg S ha⁻¹ compared to 80 kg S ha⁻¹. Methionine production peaked at 20 kg S ha⁻¹ while cysteine increased as more sulfur was applied, peaking at 80 kg S ha⁻¹. At R2 application, crude protein was lower at 10, 40, and 80 kg S ha⁻¹, and cysteine was lower at 60 kg S ha⁻¹ compared to controls. These results all point toward nutrient limitation at the stated fertilizer rates, perhaps due to nutrient imbalance in the soil. Interestingly, crude protein, methionine, and cysteine to tannin ratios peaked at 20 kg S ha⁻¹ for V2 fertilization. This result indicates that 20 kg S ha⁻¹ gave the greatest nutritional increase for the bean seed, given the conditions of for the V2 application experiment. Also, fertilization during V2 stage of growth had overall higher protein to tannin ratios compared to R2 application, though amino acid to tannin ratios were similar between application timing. We conclude that sulfur fertilization at the V2 stage of growth gives the most improvement in nutritional content compared to sulfur application at the R2 stage of growth with 20 kg S ha⁻¹ having the most beneficial effect at V2 application in (*Phaseolus vulgaris* L.) bean seeds.

CHAPTER 9: RECOMMENDATIONS FOR FUTURE STUDIES

In evaluating nutritional content in common beans (*Phaseolus vulgaris* L.), future studies should further compare sulfur application timing effects on protein and tannin content; specific attention should be given to controlling soil moisture as drought believed to affect protein synthesis.

Given the potential effect of nutrient ratios in the soil for our study, future research should focus on controlling N:S soil ratios more closely to see how they affect yield and total protein content of *Phaseolus vulgaris* L. seeds. Previous research suggests that N:S ratios at a 20:1 ratio can result in methionine and cysteine upregulation compared to controls. No research has been found showing how N:S ratios effect methionine and cysteine production *in relation to* tannin or other inhibitory compound production. Future research should focus on comparing beneficial and inhibitory compound production given optimal and suboptimal N:S ratios in the soil.

Future research should also expand the fertilization regime to include levels of phosphorus treatment, which has been shown to affect protein synthesis, and include analysis of protease inhibitors.

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