ABSTRACT

Title of Thesis:	SULFUR MANAGEMENT TO ENHANCE YIELD AND PROTEIN QUALITY OF GRAIN LEGUMES
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Sulfur (S) is an essential macronutrient and a key component in essential amino acids, methionine and cysteine (MET+CYS) that are the building blocks of protein. For a number of reasons, including difficulties in analysis for S, soil testing and fertility management has largely ignored this essential plant macronutrient. Trials were carried out over three years to evaluate the role of S fertility on the yield, seed S content, S yield and seed MET+CYS content of three types of grain legumes: double crop soybeans (*Glycine Max)*, full season soybeans, and common dry beans (*Phaseolus vulgaris*). Sulfur fertility management significantly increased yield, seed S content, S yield, and seed MET+CYS content on low S soils. Additionally, four soil extractions were evaluated as potential methods to improve S fertility recommendations. Calcium phosphate extractions more accurately identified sites that had a yield or seed s content response to applied S compared to Mehlich 3 and Calcium Chloride.

SULFUR MANAGEMENT TO ENHANCE YIELD AND PROTEIN QUALITY OF GRAIN LEGUMES

by

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Chapter 1: Introduction

Definition of Problem

It is not new information that plants need S for healthy growth, however the focus on S fertility is a relatively recent phenomenon. Traditionally, farmers satisfied plant S needs through application of organic amendments such as compost and manure, impurities in common chemical fertilizers. In recent times farmers have moved away from organic amendments on a large scale in favor of targeted, more pure chemical fertilizers. In the past, fertilizers such as superphosphate and ammonium sulfate, used to supply N and P, contained sufficient amounts of S as "impurities" to satisfy crop demand, however increasing purity in fertilizers has contributed to S deficiency in fields (Scherer, 2001). Until recently, despite lack of application, farmers in industrialized areas received sufficient S through atmospheric deposition as a result of SO_2 emissions from coal-fired power plants. An amendment to the Clean Air Act in 1990 that regulated SO_2 emissions, a toxic air pollutant, drastically improved air quality but reduced S application to crops (Ketterings et al., 2011; Klimont et al., 2013).

Improved air quality is overall a positive for humans and the environment, but it is requiring farmers to pay more attention to *all* the macronutrients that they need, rather than just nitrogen (N), phosphorous (P), potassium (K), magnesium (Mg), and calcium (Ca). In Maryland, S deficiency is mainly observed on sandy soils that have low anion exchange capacity, however if farmers continue to deplete soil S reserves without reapplication, finer textured soils will become deficient as well. Sulfur deficiency is also a significant problem in non-industrialized countries that have been continuously

depleting their reserves without ever having received S from atmospheric deposition (Weil and Mughogho, 2000).

Sulfur deficiency can significantly impact crop yield and may also have impacts on the nutritional quality of the crop. All crops have S requirements, but requirements are higher for certain crops than others. This project will specifically look at grain legumes which have higher S demands because of the role that S plays in the N fixation process. Sulfur is a key component of S-containing amino acids methionine and cysteine (MET+CYS) which are essential amino acids that often limit the nutritional quality of vegetable proteins. When MET+CYS are limiting, human and non-ruminant animal health are affected because they cannot synthesize MET+CYS on their own and must receive them from a dietary source (Jez and Fukagawa, 2008). Research has shown that S fertilization on deficient soils not only improves yield but also protein quality through increased MET+CYS content in the seed (Weil and Notto, 2018). This discovery could open a market for soybeans with improved protein quality, but current methods for testing amino acid content are slow and costly.

Although there is a general understanding of the causes of S deficiency and the important role it plays in plant growth and nutritional quality, methods for determining deficiency in the soil and seed are lacking. Plant tissue analysis to determine nutrient status generally focuses on mid-season leaves, partially because the seed composition of many crops is quite constant regardless of soil or plant nutrient status. However, there is limited evidence suggesting that soybean seed S content could be used as a sensitive indicator of plant S status (Hitsuda et al., 2004).

Developing a method to rapidly test for crop quality has significant implications for driving a quality-based market rather than a quantity-based market that exists currently for soybeans grown in the mid-Atlantic. In the mid-Atlantic region, commodity legume farmers are paid based on the quantity they deliver to the consumer, therefore the only way to increase income is to produce higher yields. A rapid method, like portable X-Ray fluorescence (XRF), to test seed elemental composition could be used at a grain elevator or other purchasing hub to test incoming product. If this were implemented farmers would have an incentive to produce higher quality grain legume crops because a rapid test would mean they could be compensated based on the quality rather than just the quantity. Without such quality incentives, farmers will likely not change their soil fertility practices unless yields are boosted.

The main objectives of this project are to (1) evaluate the yield, seed S content, and seed MET+CYS content response of soybeans (*Glycine Max*) to applied S, (2) evaluate the yield, seed S content, and seed MET+CYS content response of common dry beans (*Phaseolus Vulgaris*) to applied S and (3) evaluate the effectiveness of four soil extractions for predicting legume crop response to S.

Justification of Research

Legumes are some of the most widely cultivated crops worldwide and serve as an integral source of protein for both humans and animals(Foyer et al., 2016). This research aims to improve the quality of grain legume protein through enhancing the amino acid profile by managing S fertility, finding an effective soil test well-correlated with crop response to S application, and developing a protocol to rapidly test for seed S content as a proxy for protein quality.

Preliminary data indicate soil S applications increase legume yields, seed S content, and seed MET+CYS content (Weil and Notto, 2018). Our research has significant implications for human health, especially in developing countries that rely on plant protein as a main protein source. To date, the majority of research on MET+CYS content of the seeds themselves focus on breeding, not S fertilization. Enhancing MET+CYS content through S fertilization provides a relatively low-cost way to improve nutritional content of a crop that is fundamental to human and animal health.

In order to implement appropriate S management, an accurate and reliable soil test is necessary in order to provide farmers with S fertilizer recommendations. Using several years of samples across a wide range of soil types in Maryland, this project will seek to develop a test that can accurately predict sites that will respond to S application, that is vital to appropriate soil S management.

The current methods for amino acid analysis are expensive and time consuming (~$200 per sample). Due to this, measuring nutritional composition of grain legume seeds is not feasible for routine farmer marketing of legume seeds. An alternative, emerging method for rapid plant tissue analysis is use of portable X-Ray fluorescence devices (XRF). This type of rapid nondestructive analysis could provide a cost-effective way to perform nutritional analysis. The XRF could provide a way for farmers to have their product tested at purchasing hubs, like grain elevators, and be compensated for higher quality crops in addition to higher yield. Such a quality-based market would be new for the commodity legume market and incentivize improved soil health practices that in turn benefit human and animal nutritional health.

Research Approach

To meet project goals one and two, we established experiments on 12 fields (8 soybeans and 4 common dry beans) throughout the 2017-2019 growing seasons at the Central Maryland Research and Education Center (CMREC) in Beltsville and Upper Marlboro, MD. Field trials were carried out as randomized complete block trials with split plots. The main plots were with (B1) or without gypsum (B0) broadcast at the time of planting and the subplots were with (F1) or without (F0) Epsom salt (MgSO4) applied as a foliar spray at the beginning of the reproductive growth stage (R1). Thus, the four-way factorial treatments were: control (B0F0); foliar S (B0F1); broadcast S (B1F0); and combined broadcast + foliar S (B1F1), with at least four replications at each site. Soil samples were collected at the time of planting, and yield was collected either by hand harvest or from the combine yield measurements in order to determine yield response for each treatment. Seed samples were analyzed with the XRF to determine effect of treatments on seed S content. A select sample of seeds were analyzed for MET+CYS content and total S by ICP. Sulfur values measured by ICP were used to confirm correlation between XRF seed S photons and seed S content by ICP, a method that is accepted as reliable.

To meet project goal three, soil samples collected from a total of 23 fields throughout the 2017-2019 growing season were used to evaluate four different soil extraction methods. The four different soil extraction methods used were 0.01 M $CaCl_2$ solution, 0.002M $Ca(H_2PO_4)_2$ in water, 0.002M $Ca(H_2PO_4)_2$ in 2M HOAc, and Mehlich3. Field sites were categorized into four categories based on their response to S application. The categories were non-responsive, significant yield response, significant S response, and significant yield and S response. Results were analyzed using a Cate-Nelson analysis

to determine the critical value above which a response would not be expected for each soil test using the topsoil alone, subsoil alone, and the weighted mean of the whole sample vs. four response variables (relative yield, relative S yield, yield response, and S response). The most effective soil test was determined based on the percentage of sites correctly identified as responsive or unresponsive using the critical x value determined by the Cate-Nelson analysis.

Research Goals and Hypotheses

Project Goal 1

Objectives

1. Evaluate the potential for applied S to improve yield of soybeans
2. Determine the relative effectiveness of soil-applied gypsum and foliar-applied Epsom salt as methods of applying S to soybeans
3. Evaluate the effect of applied S on MET+CYS content of soybeans
4. Confirm the relationship between total S by ICP and total S by XRF

Hypotheses

1. S treatment will increase yield of soybeans on low-S soils
2. S treatment will increase S content of soybeans seeds on low-S soils
3. Foliar applied Epsom salt will have a greater effect on yield and seed S content than soil applied gypsum
4. S treatment will increase the concentration of MET+CYS in the seed
5. Total S content of seed will correlate with the MET+CYS concentration.

Project Goal 2

Objectives

1. Evaluate the potential for applied S to improve yield of common dry beans

2. Determine the relative effectiveness of soil-applied gypsum and foliar-applied Epsom salt as methods of applying S to common dry beans
3. Evaluate the effect of applied S on MET+CYS content of common dry beans

Hypotheses

1. S treatment will increase yield of common dry beans on low-S soils
2. S treatment will increase S content of common dry beans seeds on low-S soils
3. S treatment will increase the concentration of MET+CYS in the seed
4. the total S content of seed will correlate with the MET+CYS concentration.
5. Foliar applied Epsom salt will have a greater effect on yield and seed S content than soil applied gypsum

Project Goal 3

Objectives

1. Evaluate the effectiveness of four different soil extracting solutions at predicting crop response to applied S
2. Determine the critical level for soil S above which there will be no further crop response to applied S

Hypotheses

1. Calcium phosphate or CaCl2 extractable-S will be more correlated with crop response than Mehlich3 extractable-S, the current standard in the mid-Atlantic
2. Extractable S concentration in the surface and subsoil will be better at predicting crop response than extractable S from the surface layer alone.

Chapter 2: Literature Review

Sulfur (S) deficiency is becoming more severe throughout the eastern United States and several other areas across the world. Historically, farmers relied on S-containing organic amendments such as compost or manure to meet crop S needs. Prior to the 1850s farmers regularly applied gypsum to their fields and post-1850s, farmers regularly applied superphosphate fertilizers that contained sufficient S impurities to meet crop demand (Gilbert, 1951; Russel and Williams, 1977). Beginning in the early to mid 1900s up until the passage of the Clean Air Act, farmers, especially in the eastern United States, received sufficient S through atmospheric deposition on their fields to meet crop demand. This allowed farmers to neglect using any additional S fertilizer (Gilbert, 1951). The main source of this S was sulfur dioxide emissions from coal-fired power plants, which then would be deposited on farmer fields during precipitation events (Gilbert, 1951).

After the implementation of amendments to the Clean Air Act in 1990, S emissions from coal-fired power plants were drastically reduced, leading to increased incidence of S deficiency in the United States (Ketterings et al., 2011; Klimont et al., 2013). Prior to the passage of the Clean Air Act in 1990 total S deposition rates (including both dry and wet deposition) reached as high as 21.5 kg/ha in some parts of the eastern United States (Baumgardner et al., 2002). However, after the Clean Air Act was successfully passed, S deposition drastically decreased max deposition rate to around 12-13 kg/ha by 2010 in the highest deposition areas of the eastern US ((Baumgardner et al., 2002; National Atmospheric Deoposition Program, 2011). Additionally, farmers began to regularly use higher analysis fertilizers such as diammonium phosphate and urea

that contain little or no sulfur (Scherer, 2001). This drastic reduction in S deposition coupled with increased crop requirements and harvest removals has led to inadequate S supplies for optimal crop growth in many soils (Scherer, 2001). These environmental and cultural changes mean that, increasingly, farmers need to include S-containing fertilizers as part of their routine nutrient management practices.

Sulfur in Soil

Sulfur is an essential nutrient that is critical to agricultural crops with respect to both their yield and protein quality. Sulfur is one of the six essential macronutrients along with nitrogen (N), potassium (K), phosphorous (P), calcium (Ca), and magnesium (Mg) that plants need from the soil for growth. Sources of S in the soil are primarily from the soil parent material, S gases in the atmosphere, and soil organic matter (Prasad and Singh, 2016). Sulfur in the soil is present in four chemical forms (1) sulfide, (2) sulfate, (3) organic S, and (4) elemental S. However, only a small amount of the S that is present in most soil is readily available to plants in the form of sulfate (SO_4^{2-}) (Prasad and Singh, 2016).

Plants take up S in the inorganic form of SO_4^{2-}, which can be highly soluble and mobile in the soil. Sulfate concentration is effected by the balance of atmospheric deposition, decomposing organic material, fertilizer inputs, S leaching, plant uptake and microbial activity, and can fluctuate throughout the growing season (Eriksen, 2008). Once taken up by plants, the S in SO_4^{2-} is first reduced and then assimilated into organic compounds, which can be utilized by different plant or animal physiological processes. These organic compounds are eventually returned to the soil through death or defecation which allows soil microorganisms to cycle some of the S back to SO_4^{2-} ions that become

available again for plant uptake. Sulfur is generally mineralized from organic material in the top layer (A horizon) of soil, which includes both humus (stabilized organic matter) and recent crop residues left on or in the surface soil (Schoenau, 2008). The rate at which this mineralization occurs is moderated by temperature, pH, moisture, and aeration. The plant-available SO_4^{2-} typically accounts for less than 5% of the total S in humid region soils. Plant available SO_4^{2-} includes both SO_4^{2-} in the soil solution as well as adsorbed SO_4^{2-} (Scherer, 2009).

Sulfate adsorption is regulated mainly by the presence of other anions in the solution, the anion exchange capacity of the soil, and the pH of the soil. Sulfate is adsorbed onto the surfaces of clay particles as well as those of iron and aluminum oxides that often coat soil particles. Therefore we expect that sandy soils, especially those without significant iron and aluminum oxide coatings, will exhibit low anion exchange capacity and will be more susceptible to SO_4^{2-} leaching (Eriksen, 2008). Sulfur adsorption also increases with decreasing pH, therefore when farmers amend their soil with calcium carbonate to increase the pH they decrease SO_4^{2-} adsorption which, in turn, increases the plant availability but also the leaching potential of SO_4^{2-} (Scherer, 2009).

Typically, soils that have low organic matter content, are well drained, and coarser textured are more susceptible to S deficiency due to their susceptibility to leaching (Dick et al., 2008). Many soils on the Eastern Shore of Maryland and other parts of the eastern US exhibit these types of characteristics and are thought to be susceptible to S deficiency. Sulfate that leaches out of the sandy surface soil layers can be adsorbed onto the surface of the more plentiful amounts of clays and iron oxides in the subsoil. This adsorbed SO_4^{2-} in the subsoil can serve as a significant source of plant available S

(Ketterings, Miyamoto, Mathur, Dietzel, and Gami, 2011; Westermann, 1974). In many countries S deficiency is also exacerbated by the common practice of burning crop residue. When crop residues are burned most of the S is lost to the atmosphere, and soil S reserves therefore become depleted.

Sulfur in Plants

Sulfur is an essential macronutrient that is responsible for plant physiological functions, growth, and overall development and is taken up in quantities similar to those for P. Unlike P, S has become more limiting to crop production in recent decades (Mia, 2015). Typically sulfur concentration in plant tissue ranges from about 0.1 – 0.4 % with legumes having particularly high S contents (Gilbert, 1951; Mia, 2015). Sulfur is relatively immobile in plants and S deficiency effects chlorophyll production causing S deficient plants to be stunted, spindly, and chlorotic on the new leaves (Friedrich and Schrader, 1978; Scherer, 2008).

Sulfur plays important roles in several plant growth processes, including photosynthesis, protein synthesis, nitrogen fixation, and oil synthesis (Epstein and Bloom, 2005). Sulfur is a component of the essential amino acids, methionine and cysteine (MET+CYS). The S in these amino acids is responsible for bonds that stabilize the three-dimensional molecular folding that is key to protein function. The key role that S plays in biological N fixation explains why legumes have a relatively high S requirement compared to grasses. The combined assimilation of both N and S is integral to the ability of the plant to synthesize MET+CYS; the supply of these amino acids in turn often limits plants' ability to synthesize required proteins (Ruiz et al., 2005). In a cropping system that is limited by N it would be unlikely to see a yield or amino acid

response to applied S (Rendig, 1986). However, when S is limited plants accumulate non protein N (Friedrich and Schrader, 1978). This accumulation of non-protein N and inhibited MET+CYS production can reduce crop yield (Scherer, 2008). In a survey of 24 studies done on seed protein and cereals, legumes consistently showed a higher increase in S containing amino acid response to applied S than cereals (Rendig, 1986). While plants can utilize S in the inorganic SO_4^{2-} form, humans and livestock have to receive S through MET+CYS in their diet. Therefore it is essential that protein sources in human and animal diets contain the correct amino acid balance, otherwise human and animal health will be compromised (Gilbert, 1951).

Soil Testing for Sulfur

Recommendations for S application and management have lagged behind other macronutrients due to difficulties in testing soil S levels and lack of calibration for crop response to applied S. The first documentation of soil testing can be traced back to 50 B.C in Rome in which soil was tasted to assess acidity and salinity (Allen et al., 1994). Modern day soil testing can be divided into three distinct time periods (Anderson, 1960). The first period, which spans from 1845 to 1860, focuses on the work of Daubeny, Liebig, Hilgard, and Dyer. In 1845, Daubney developed quick soil test methods that were based on the concepts of "active" and "dormant" nutrients referring to the solubility of nutrients and relied on carbonated water as the extracting solution (Anderson, 1960; Allen et al., 1994). Liebig later built on the procedure and used dilute acids to extract nutrients from soils. Hilgard further built on their work, in 1906, by providing the first approximate values for making fertilizer recommendations to farmers. These

recommendations were made around the same time as Dyer showed that the amount of phosphorous extracted by citric acid is related to crop response (Anderson, 1960).

The second fundamental period spans from 1906 to 1925 and marks the time period in which background information on a range of soils was developed in order to inform nutrient management recommendations. This data was taken on a wide range of soils, mostly looking at P, where it was coming from in the soil, its movement within soils and how that might influence crop P demands. This was important in developing baselines for recommendations that were not previously known (Anderson, 1960).

The final period examined the common soil test methods that were practiced at various laboratories around 1951. This was done to determine further background values for various soil types and crop implications in order to better inform management decisions. This process highlighted the importance of taking prior land management into consideration when making a fertilizer recommendation rather than using the soil test value as the sole piece of information.

Beginning around 1951 through the 1980s there was a focus on both development of a universal extract for multi elemental analysis as well as development of methods for single elemental analysis (Allen et al., 1994). During this period both the Morgan and Mehlich extracts were developed which are common multi element extracts still used today. Finally, beginning in the 1970s and continuing today attention has mainly been focused on standardization of the various methods developed in preceding decades (Allen et al., 1994).

A well standardized soil S test should be able to give an S reading that is correlated with plant uptake of S and predictive of crop response to S application. The S

level from the soil test reading should be predictive of S available as SO_4^{2-} and soluble organic S compounds in the soil solution, as well as SO_4^{2-} adsorbed onto the surface of clays and iron oxides that could become available throughout the growing season (Ketterings et al., 2011).

Standardization of soil sampling methods is another important factor for soil test procedures. For routine soil analysis farmer's typically only sample the top 15 cm of their soil (Moebius-Clune et al., 2016). However, much of the potentially available adsorbed SO_4^{2-} may not be included when only the top 15 cm of the soil is sampled (Scherer, 2008; Ketterings et al., 2011). In a study done on two different climatic regions in Iran, on both irrigated and non-irrigated fields that received applied S in the form of gypsum, soil samples were collected at two depths 0-30 cm and 30-60 cm and extracted with three different solutions: water, 0.01 M $CaCl_2$ and 0.01 M $Ca(H_2PO_4)_2$. The study found that the SO_4^{2-}S levels at both depths increased with increasing application of S and was higher in the subsoil than in the topsoil (Shahsavani et al., 2006). Another study done by Calvo, Echeverría, & Rozas (2009) sampled twenty- two fields in Argentina at 0-20 cm, 20-40 cm, and 40 – 60 cm and extracted the soil using $Ca(H_2PO_4)_2$. The study found that SO_4^{2-}S levels in the subsoil horizons (0-40 cm) were better predictors for plant available S than just the surface (0-20 cm). A 1972 study done on five different Iowa soils found that total S decreased significantly with increasing depth and was highly correlated with Organic C (Tabatabai and Bremner, 1972). These studies illustrate that sampling for S just at the top 15 cm, as is commonly done, may not be telling the whole story when it comes to plant available S. Sulfur is more likely to be adsorbed in the subsoil for reasons that include

higher clay content in most Alfisol and Ultisol subsoils (Bt horizon) as well as iron and aluminum oxide accumulations and lower pH conditions in subsoils.

In addition to standardizing extraction and sampling methods, S soil test methods need to be well calibrated to crop response. A well calibrated soil test will be able to accurately estimate the amount of plant available nutrient that the plant will be able to access throughout the growing season and provide a recommendation based on additional nutrients needed to meet crop demand. Therefore, a soil test needs to be able to estimate soil S that the plant will be able to access throughout the growing season that might not be currently available as sulfate in the soil solution. Commonly used S extracting solutions can be categorized into three groups based on the type of S they extract. Week salts such as $CaCl_2$ and water typically only extract sulfate that is readily soluble (part of the soil solution). Phosphate containing extractants are able to extract S that is readily soluble in addition to adsorbed S. Finally, phosphates in week acids, extract readily soluble S, adsorbed S, as well as some organic S Reisenauer (1975).

In the mid-Atlantic region of the United States routine soil testing is commonly performed using the Mehlich3 extraction (Sims et al., 2002). The Mehlich3 extract procedure was introduced in 1984 and is now widely used as the standard soil test procedure by soil testing laboratories in the mid – Atlantic region (Rao and Sharma, 1997; Sims et al., 2002; Wolf and Beegle, 2011; Ketterings et al., 2014; Seth et al., 2018). The Mehlich3 extracting solution contains multiple components that have been shown to be effective at extracting both macro and micro nutrient ions across a wide range of soils and pH conditions (Mehlich, 1984; Shahandeh et al., 2017). The Mehlich3 extraction has been shown to effectively predict soil P and K supply however, there has been little work

done to evaluate its effectiveness at determining soils on which crops will be responsive to S fertilization (Sims et al., 2002).

Most labs using Mehlich3 soil test results report S levels as "plant available S" and give interpretations such as "low," "medium," or "high" which would appear to be based on the critical levels determined by the soil test calibration curves. The limited research done by Ketterings et al (2011), and Sahrawat et al. (2009) suggest that Mehlich3 extractable S is not consistently related to plant S uptake across soils types. A study done in British Columbia looked at the ability of 5 different extracting solutions, that are widely used to extract a range of nutrients, at extracting different fractions of S. The intention of this study was to identify one test that could provide accurate recommendations to farmers for a wide range of nutrients including S (Kowalenko et al., 2014). The main findings of the study suggest that timing of the sampling did not have a noticeable effect on extractable S, that pH was a useful variable in identifying pedogenic processes that might cause a high extractable S level, and that although Mehlich3 would be a useful way to measure a wide range of elements and would simplify the soil test process more work needs to be done to evaluate the relationship between extracted S and plant response. The study concluded that previously researched soil tests for S, specifically the $CaCl_2$ and $Ca(H_2PO_4)_2$ tests, show more promise for correlation with crop response. However, more research needs to be done before wide reaching adoption of any of the tested methods (Kowalenko et al., 2014).

Ketterings et al. (2011) found that a 0.01 *M* $CaCl_2$ extract was the least impacted by soil variability, meaning that the test responded consistently to S additions, whereas the 1.0 mol L^{-1} NH_4OAc, 0.016 mol L^{-1} KH_2PO_4, 0.01 mol L^{-1} $Ca(H_2PO_4)_2$, Morgan

NaOAc, and the Mehlich3 solution did not respond consistently across the soil types to the same S additions. The $CaCl_2$ extract also showed the most sensitivity to the different treatments of applied S which was determined to be beneficial because it indicates more sensitivity when identifying deficient soils (Ketterings et al., 2011).

A study done on tropical soils in India, reported that $CaCl_2$ and $Ca(H_2PO_4)_2$ solutions extracted similar amounts in neutral to alkaline soils, however the $Ca(H_2PO_4)_2$ solution extracted significantly higher S in low pH soils (Sahrawat et al., 2009). This difference was attributed to PO_4^{2-} being able to replace SO_4^{2-} more readily than Cl^- because PO_4^{2-} ions have a higher replacing power than Cl^- and are strong enough to replace SO_4^{2-} ions adsorbed to soil surfaces (Sahrawat et al., 2009). This is important for soil S tests because the adsorbed SO_4^{2-} is thought to be a significant source of plant available SO_4^{2-}.

A field study done on 49 soils across Wisconsin evaluated the effectiveness of six different soil extractions on alfalfa (*Medicago saliva*). The six tested extracts were 500 ppm $Ca(H_2PO_4)_2$ in both water and 2*N* HOAc, 0.03*M* $NaH_2PO_4 \bullet H_2O$, 0.025*N* $CaCl_2$, 0.25*N HCl* + 0.03*N* NH_4F, and 0.1*N* H_2PO_4. The results of the experiments suggested that only the $Ca(H_2PO_4)_2$ extractions were correlated with percent S in the first cutting of alfalfa and the $Ca(H_2PO_4)_2$ in HOAc was most well correlated with yield response (Hoeft et al., 1973a). In addition to finding that the $Ca(H_2PO_4)_2$ in HOAc was most well correlated with S percent and yield response, their results showed that including pH in the regression equation gave the most predictive results for yield response. The results of this experiment showed that sites testing above 10 ppm were not likely to respond to S

treatment, sites testing below 6 ppm were likely to respond and 39% of sites testing between 6-10 ppm responded (Hoeft et al., 1973a).

The fluctuating availability of S as well as SO_4^{2-} susceptibility to leaching contributes to difficulty in testing for S. Soil testing is typically only done at one point during the year but uses the data to predict fertility needs in the coming growing season. Therefore, an effective S soil test needs to be able to extract an amount of S from the soil that is correlated with the amount that plants can access throughout the season. Some of the methods that have been used to evaluate S status in the soil include measuring extractable S (typically in the form of SO_4^{2-}), S released during soil incubation, or measurements of plant growth and microbial growth. However, effectiveness of these measurements have been inconsistently effective at predicting crop response to applied S (Jones, 1986). In order to combat S deficiency moving forward, it is critical to calibrate a S soil test that will accurately predict where crops will positively respond to applied S.

Effectiveness of Sulfur Treatments

There has been little work done to evaluate the rate, timing, and sources of S used for S fertilization. The limited literature on the amount of S needed to satisfy crop demand indicates that rates around 10 kg/ha are sufficient to meet crop demand (Weil and Mughogho, 2000; Camberato and Casteel, 2017). Sulfur is relatively mobile in near neutral soil but relatively immobile in the plant. Therefore, the timing and source of S may affect the ability of the plant to assimilate S.

Two common sources of applied S are gypsum ($CaSO_4 \bullet 2H_2O$) and Epsom salt ($MgSO_4$). Soil applied $CaSO_4 \bullet 2H_2O$ is a relatively low-cost option for farmers to apply S. Gypsum is widely available as a mined mineral and is also created as a byproduct of

many industrial processes, importantly as a result of the scrubbers used to remove sulfur dioxide (SO_2) from coal-fired power plant emissions (Miller and Sumner, 1997). Flue Gas Desulfurization gypsum (FDG) is a relatively pure source of gypsum that is a byproduct of the scrubbers used to remove SO_2 from coal-fired power plant emissions and comes in the form of a powder that can be applied to the soil surface. Typically, FDG contains three components in varying proportions based on the scrubbing process used by the individual plant. The three main components in FDG are SO_2 in the form of $CaSO_4$, unreacted sorbent which is typically highly alkaline, and coal combustion ash (Chen et al., 2005). Flue Gas Desulfurization gypsum is more soluble than other byproduct gypsums and thus makes it suitable for agricultural uses.

The main soil fertility use of gypsum is to supply calcium for peanut crops and to supply S to canola crops (Miller and Sumner, 1997). The other main use of gypsum in agriculture is for the soil flocculation properties. In dispersive soils gypsum is often used to increase water infiltration. In these dispersive soils, the yield increase seen from gypsum has been attributed to increased water infiltration and holding capacity, especially in dry seasons. However, in low S soils more work needs to be done to determine the impact of applied gypsum on yield as well as quality of grain legumes.

Gypsum is a moderately soluble powder (~2.0–2.5 g/l at 25 °C) that can be applied at the time of planting to allow for the plants to access it over the growing season. In the experiments done by Chen et al. in Ohio (2005) researchers looked at the effectiveness of FGD as a fertilizer for alfalfa and soybeans and potential environmental impacts from use of FGD as a fertilizer. The study found that applied gypsum increased yields of soybeans over the control by 3.3 – 11.6 % which was highly significant across

several treatments. The main conclusions from the study were that FGD has good potential as a S fertilizer for both alfalfa and soybeans and may also have additional benefits gained from other elemental impurities contained in the gypsum (Chen et al., 2005).

A study done in Brazil looked at the effect of lime and gypsum on corn and soybean yield under no till systems. The study found that there was a significant effect of applied gypsum on corn yield but not on soybean yields (Caires et al., 2011). Another study done in India on Typic Haplustert soils in 1992 looked at the effect of applied S at four levels (0, 20, 40, and 60 kg S/ha) as gypsum on soybean yield, nodule production and leaf chlorophyll content. The study found that up to 20 kg S/ha nodule production increased but above that nodule production reached a plateau, similarly leaf chlorophyll content and seed yield increased significantly with the 20 and 40 kg S/ha applications but above that the rates reached a plateau (Ganeshamurthy and Sammi Reddy, 2000).

Applied S as gypsum (either mined of as FGD) is only one source of S that could be used for S fertilization. Foliar applied $MgSO_4$ is another possible source of S. Due to the slow solubility of gypsum, application early in the season is necessary to allow enough time for it to move into the soil and be assimilated by the plant roots. However, the potential for S loss is higher with this method due to high susceptibility of S leaching, especially in sandy soils. Epsom salt is highly soluble (250 g/L 20°C) and can be applied as a foliar spray at the beginning of the reproductive stage (R1) of growth. This is the time of seed production and filling which corresponds with highest S demand (Bender et al., 2015). Research has shown that soybean S demand drastically increased at the beginning of the reproductive stage and that S deficient conditions at this time can be

detrimental to yield and protein quality ((Wang et al., 2008a; Bender et al., 2015). Previous work demonstrated that a rate of 86 kg Epsom ha $^{-1}$ (which corresponds to 12 kg S ha $^{-1}$) was used successfully on soybeans without any damage to soybean foliage in Maryland (Weil and Notto, 2018). However, more research is needed to determine appropriate timing and rate of both methods of applications.

Importance of Grain Legumes and their Protein Quality

Legumes are second only to cereal grains as the most widely grown crop type worldwide and serve as a main protein source for many of the world's people, especially in developing countries (Duranti, 2006). Legumes are rich in proteins, carbohydrates, and dietary fibers as well as an assortment of other essential nutrients (Bouchenak and Lamri-Senhadji, 2013) However, due to the limiting amounts of MET+CYS, legumes are often considered an inferior source of protein despite the multitude of other health benefits that come from human consumption of legumes (Nwokolo and Smartt, 1996). Grain legumes are particularly important because of the complementary role they play to cereal grains (Eggum and Beams, 1983). In a typical animal feed ration that is made up predominantly of corn and soybean the ratio of the two is formulated to balance out the essential amino acids. Corn is typically high in methionine and low in lysine whereas soybeans are often low in methionine but high in lysine. This allows for the mix of the two to provide a complete amino acid balance. However, in S deficient systems the quantities of methionine are often too low to meet the needs of non-ruminant animals such as poultry and pigs (Krishnan and Jez, 2018).

Soybeans (*Glycine max)*, are unique in their high protein and oil content compared to other legumes. Modern soybean varieties typically grown throughout the

United States can contain approximately 38% protein and 18% oil (Krishnan, 2008). Soybeans contain almost double the concentrations of protein and many amino acids compared to other common legumes, however, their nutritional value is also limited by MET+CYS. Soybeans also have relatively high lysine content, compared to other legumes, which is also commonly limiting in grain legume protein. If protein is limited by any one amino acid, the protein will not perform correctly in human and non-ruminant diets (Uversky and Uversky, 2015). The usability (quality) of legume protein is usually limited by the low amounts of S-containing amino acids in the seed (Paek et al., 2000). Humans and non-ruminant animals (chickens and pigs) cannot synthesize MET+CYS, and therefore must get it from dietary sources.

Soybean records date back to writings by Chinese Emperor Sheng Nung as early as 2838 B.C and were considered one of the five sacred grains essential to Chinese civilization (Morse, 1949). Soybean production in the United states can be traced back to the 1890s when the United States Department of Agriculture introduced a large number of soybean varieties and since then the amount of acreage planted into soybeans has continued to increase (Morse, 1949). As of 2018 the United States accounts for approximately 34% of worldwide soybean production and has over 83.7 million acres of soybean cultivation that is worth approximately $38 billion (Krishnan and Jez, 2018).

Approximately 85% of the world's soybean supply goes directly to animal consumption or is processed into oil (Krishnan, 2008). However, due to high growth rate methods for growing animals such as poultry and swine, soybeans are not able to supply adequate amounts of MET+CYS. This means that feed companies have to synthetically add MET+CYS to meet dietary requirements for feed rations (Krishnan, 2008). There has

been some work done to genetically engineer a soybean with increased MET+CYS content (Kastoori Ramamurthy et al., 2014), however the idea that soil or foliar applied S can improve both yield and MET+CYS content is novel. Due to the role that legumes play in both human and animal nutrition there is great interest in improving the MET+CYS content of the seed (Hayat et al. 2013) instead of synthetically adding MET+CYS to food and feed products.

Unlike their soybean relative, common dry beans (*Phaseolus vulagaris*) are thought to be native to the New World, primarily Mexico, Central America, and northern South America. Common dry beans are the most widely grown and consumed leguminous human food around the world (The State of Food and Agriculture, 1991). Common dry beans are a good source of many nutrients and contain between 20 – 30% protein. Most of the protein in common dry beans consist of storage proteins which are often low in MET+CYS (Nwokolo and Smartt, 1996). Therefore, although common dry beans are a good source of most amino acids, it is necessary for humans and non-ruminant animals to obtain supplementary MET+CYS from another source. Limited work has been done to evaluate the role of sulfur fertility in common dry beans, this project will seek to fill that gap in knowledge to answer the question of whether or not common dry beans respond similarly, both in yield and seed S content, to applied S as soybeans.

Limited research has been done in Denmark to assess the nutritional value of field beans (*Vicia faba)* based on the amino acid content of the seed. Field beans grown in Great Britain and Denmark are primarily used in feed rations for non-ruminant animals but their feed value is limited by MET+CYS content (Eppendorfer, 1971). In the study

conducted by Eppendorfer (1971) field beans were grown in pots treated with S, N, and P applied as $Na_2SO_4 \bullet 10H_2O$, $Ca(NO_3)_2$ and $Ca(H_2PO_4) \bullet H_2O$. Total amino acid, MET+CYS content, and nutritional value through a feeding experiment were evaluated based on four levels of S, N, and P treatments that were fed to rats. The results showed that yield was significantly affected by S and P when the soil was deficient and seed N concentration increased with applied S. They found that total N could be used to get an approximate estimate of amino acid content and that the ratio of amino acids was largely unaffected by fertilizer applications despite change in total individual amino acid concentrations (Eppendorfer, 1971). Another rat feeding experiment done by Porter, Maner, Axtell, & Keim (1974) examined five commercial varieties of dry beans that were incorporated into a formulated feed ration for 21 day old rats that were fed the diet twice a day. Their protein efficiency ratio (PER) was calculated over a four-week period to evaluate the amount of weight gained based on total protein consumed. The five dry bean varieties had total MET+CYS values ranging from 2.15% to 3.16%. The results showed that the MET+CYS content was significantly correlated with PER for all varieties of dry beans.

It has been estimated that in order to meet the food demands of the growing population, expected to reach at least 9 billion by 2050, with the predominant population increase in developing countries, global food production will need to increase by at least 60 – 70% by 2050 (Cazcarro et al., 2019). The high concentration of people living in developing countries means that a large majority of the world's population relies on a plant based diet with legumes comprising the main protein source (Cazcarro et al., 2019). Due to current trends in agriculture many plants are lacking in essential amino acids and

thus provide a diminished quality of protein. According to the Food and Agriculture Organization (FAO) approximately 10% of the world's population suffers from protein malnutrition, making it one of the most common forms of malnutrition worldwide (Cazcarro et al., 2019).

Variable Sulfur Content of Soybeans

A preliminary survey done of soybeans across MD, found S content and MET+CYS content of soybeans to vary considerably (Weil and Notto, 2018). This indicates a wide range in nutritional quality of the beans. However, current methods for testing amino acids cost approximately $200/sample and take weeks to get results. Even total S analysis by ICP requires acid digestions and several days in the lab. A 1974 study that evaluated the nutritional value of grain legumes based on their total sulfur content looked at the ability to use total sulfur as an indicator of nutritional quality of five different varieties of common dry beans, one variety of mung beans (*Vigna radiata*) and one variety of cowpeas **(***Vigna unguiculate)* (Porter et al., 1974). The study compared the total S determined by nitric acid-perchloric acid digestion with the percent protein that was present in the form of MET+CYS and found that the correlation between the two was highly significant. This is important because measuring total S is a much easier process and may be a valuable tool for evaluating the nutritional value of legumes.

One potential alternate method for measuring total S is XRF which as able to do total elemental analysis. This method of analysis utilizes photons of energy created by x-rays that are passed through a sample (Towett et al., 2016; Byers et al., 2019). The photons from the x-ray transfer energy to an atom within the sample which displaces an electron from its preferred, stable shell and leaves a vacancy that an electron from an

outer shell then occupies (Byers et al., 2019). As the outer shell electron drops down to the lower shell it releases energy. The XRF measures the released energy and is able to determine the elemental composition of the sample by the total amount of energy released by a sample at a given wavelength. The XRF is a rapid, nondestructive way to measure total elemental composition once a calibration curve is established (Byers et al., 2019).

Use of the XRF to measure elemental composition of lithologic material is a well-established and understood method (Byers et al., 2019). The use of XRF for plant tissue analysis is in its infancy (Towett et al., 2016). Although, XRF devices were developed for heavy metal analysis, with the use of a vacuum the XRF is able to produce reliable readings for lighter elements such as S and P that are relevant for plant tissue analysis (Towett et al., 2016). This project will add to the limited body of knowledge by confirming the correlation between total S from XRF with total S from ICP as well as the relationship between total S and MET+CYS content.

Researchers believe that the amino acid balance of soybeans is more important for human and animal feed than total protein content, therefore improving MET+CYS content remains a focus for breeding, but is also thought to be influenced by other environmental and farmer cultural practices (Krishnan and Jez, 2018). The main focus of improved MET+CYS content has been limited to the soybean seed itself, but there are additional soybean byproducts and other protein rich substances that could also benefit from enhanced S content. Continued research into S deficiency could have a significant impact on the world's food supply (Krishnan and Jez, 2018). The results of this study

could result in shifts in soil S management that would be an alternative to traditional breeding methods that would still lead to improved feed value of legumes.

Preliminary research in Maryland (Weil and Notto, 2018) found that on the most responsive sites S fertilization could lead to 10-15% yield increases and close to doubling of MET+CYS content. Further, the soybean studies found a significant and close correlation between total S ppm from the XRF and MET+CYS content by extraction and high-pressure liquid chromatography. This correlation allows total S readings by XRF to be used as a proxy for protein content.

Conclusion

Despite a general understanding that S is an essential crop macronutrient that is needed in amounts similar to P, there is a lack of consensus around effective soil test methods that will accurately predict a crop response which is integral to the ability to make recommendations to farmers. Current trends in agriculture indicate that S deficiency will continue to be an increasing problem due to higher analysis fertilizers, increasingly high yield production methods, and reduced atmospheric S deposition.

Chapter 3: Sulfur Management to Enhance Yield and Protein Quality of *Glycine Max*

Abstract

Sulfur (S) is an essential plant macronutrient and a key component of the S-containing amino acids methionine and cysteine (MET+CYS) that are essential for non-ruminant animals and often limit the nutritional value of grain legumes. For a number of reasons, including difficulties in analysis for S, soil fertility management has largely ignored this essential plant macronutrient. High crop yields, in combination with decreased levels of atmospheric S deposition, are depleting soil S reserves and leading to widespread soil S deficiencies. Field trials were conducted in Maryland, USA using four S treatments: (B0F0) no-amendment control; (B1F0) 560 kg/ha gypsum ($CaSO_4$; 17% S) broadcast at planting; (B0F1) 86 kg/ha Epsom salt ($MgSO_4$; 11% S) as a foliar spray at first flower; (B1F1) the combination of broadcast and foliar S application. In each of two years this experiment was conducted on full season and double crop soybeans on two soil types (relatively coarse and fine), for a total of eight site-years Soybean yield and seed S concentration were measured in all eight site-years and MET+CYS in the seed was measured for two coarse soil sites in one year. Soybean seed yield, S content, or S yield were significantly ($P<0.10$) increased with the S treatment on the four coarse soils ($p<0.10$), but not on the four fine soils. S application increased seed MET+CYS content by 32 to 78% ($p<0.05$) on the two coarse soil sites. We show that applying S can improve the MET+CYS content of the soybean protein, as well as the yield.

Introduction

Sulfur is an essential macronutrient assimilated by soybeans in similar quantities to P (Bender et al., 2015). Yet, compared to nitrogen (N), phosphorous (P) and potassium (K), little attention has been paid to managing S in most cropping systems. One reason for this lack of attention is that for most of the twentieth century S was inadvertently included in chemical fertilizers popular at the time (mainly ammonium sulfate and superphosphate) and sulfur dioxide emissions from coal-fired power plants resulted in large amounts of S reaching farm soils as atmospheric deposition (especially in the Eastern USA, northern Europe and Central China) (Eriksen, 2008). After the implementation of air pollution control policies in many industrial countries, S emissions from coal-fired power plants and other industries were drastically reduced (Klimont et al., 2013). The combination of (1) the reduction of S deposition due to the successful regulation of S dioxide emissions, (2) use of chemical fertilizers with lower amounts of S impurities (e.g. diammonium phosphate and urea), and (3) reduced reliance on organic soil amendments has led to increasing occurrence of S deficiency in most industrial countries. In other parts of the world, particularly sub-Saharan Africa and South America, S deposition has historically been low and significant S has been lost from soils by annual biomass burning (Zhong et al., 2020).

Sulfur deficiency may significantly impact crop yield. Soybean yield responses to S fertilization, especially on low-organic matter soils, are increasingly common in major soybean growing regions of the world (Salvagiotti et al., 2012; Kaiser and Kim, 2013). Research in the USA (Ohio), India, and Argentina showed that S applied as gypsum or ammonium sulfate on low S soils increased soybean yields between 4-14% with no significant differences between sources of S (Ganeshamurthy and Sammi Reddy, 2000;

Chen et al., 2005; Gutierrez Boem et al., 2007; Caires et al., 2011). Soils that are low in organic matter and coarse in texture are most likely to be responsive to S application because of the low potential for mineralization and high potential for leaching loss of sulfate ions (Dick et al., 2008). Such sandy soils that are likely to have limited S supplying capacity, are common in soybean producing areas of the mid-Atlantic coastal plain region of the USA.

Sulfur plays an important role in many plant physiological functions and growth processes including photosynthesis, protein synthesis, nitrogen fixation, and oil synthesis (Epstein and Bloom, 2005). Legumes, such as soybeans, have an especially high S demand because of the S required for N fixation. Sulfur is a key component of methionine and cysteine, which are essential amino acids that often limit the nutritional quality of legume proteins (Eriksen, 2008). When the sum of these two amino acids (MET+CYS) is limiting, the health of humans and non-ruminant animals is affected because they cannot synthesize MET+CYS on their own and must receive them from a dietary source (Ruiz et al., 2005; Jez and Fukagawa, 2008). Although the protein and amino acid contents of the seed are commonly considered to be a characteristic of the soybean crop (Baker et al., 2014), there have been efforts for decades to increase the MET+CYS content of soybean seeds through genetic manipulation (Krishnan and Jez, 2018). For cereal crops like corn, foliar analysis rather than seed analysis is used to determine the S status of the plant. In contrast, the S status of soybeans may be indicated by the S content and S/N ratio in the seed, as well as in the foliage (Hitsuda et al., 2004; Salvagiotti et al., 2012).

Due to the fact that most of the S in soybean seeds is present in MET+CYS, we propose that S deficiency might decrease the concentration of MET+CYS. Conversely, fertilization with S might improve the amino acid profile of soybean seeds by increasing the concentration of MET+CYS. A preliminary survey of commercial soybean fields in Maryland found the content of S and MET+CYS within the seed to be highly variable (Weil and Notto, 2018). Limited research on S uptake and mobility within the soybean plant suggests that S demand increases greatly at pod filling (R1-3 growth stages) and that S deficiency at this time could reduce yield and protein quality (Fehr et al., 1971; Wang et al., 2008b; Bender et al., 2015). We, therefore, propose that applying S as a foliar spray when pods begin to form may be more effective in enhancing the amino acid profile of soybean seeds than the more traditional broadcast application of S to soil at planting time.

We report on field experiments designed to test the following hypotheses: (1) S application will increase the yield of soybeans on low- S soils, (2) S application will increase S content of soybean seeds, (3) Foliar application of S will have a greater impact on yield and S in the seed than broadcast S applied to soil, (4) S application will increase the concentration of MET+CYS in soybean protein, and (5) The MET+CYS concentration will correlate with total S content of the seed.

Materials and Methods

Field sites

Replicated field experiments were conducted in 2017 and 2018, at the Central Maryland Research and Education Center (CMREC) in Beltsville, facility (within a 2 km radius of coordinates 39.012162, -76.833329). This region has a humid temperate climate

with mean annual minimum and maximum temperatures of 5°C and 25°C, respectively. On average, this location receives approximately 1075 mm of precipitation per year evenly spread among 12 months (NOAA, 2020; Figure 1).

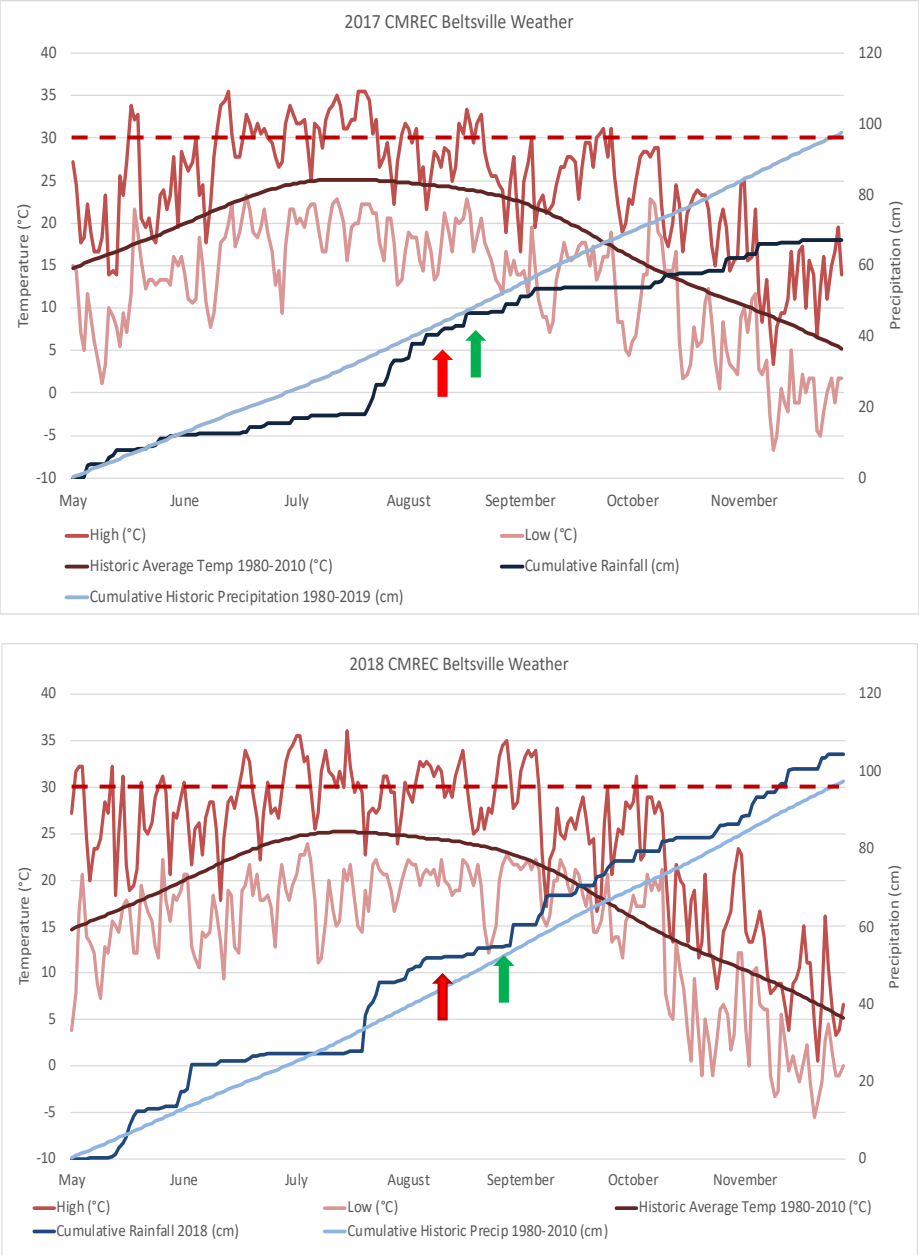


Figure 1. Daily high temperature (°C), daily low temperature (°C), and daily precipitation (cm) at Beltsville for 2017 (left) and 2018 (right)) and the 1980-2010 average temperature(°C) and average precipitation (cm) at Baltimore (BWI NOAA weather station). The red arrow indicates when Epsom salt was sprayed for full season soybeans and the green arrow indicates when Epsom salt was sprayed for double crop soybeans. The dashed red line indicates higher temperature stress above 30°C.

Field experiments were conducted for a total of eight site-years, four using double crop (DC) soybeans (planted after winter wheat harvest), and four using full season (FS) soybeans (Table 1). For each of these soybean crop types, two site-years were on soils formed from coarse sandy sediments (Downer-Hammonton complexes referred to hereafter as "coarse" soils) and two site-years on soils formed from silty to clayey sediments (Russet-Christiana complexes referred to hereafter as "fine" soils). All eight sites were located within the Northern Coastal Plain region of the Eastern US in which soil parent materials consist of deep fluviomarine deposits (Soil Survey Staff, 2014; Table 2). Specific soil series present from among those indicated in the Web Soil Survey (USDA/NRCS, 2020) mapping units were determined by examining the texture, Munsell color, and structural features of two profiles at each site using bucket augers to a depth of one meter.

Table 1 Agronomic practices and timing of operations at the eight study sites. No Insecticides or fungicides, other than seed treatment, were applied, all fields under no-till management for at least the past five years. All fields received four treatments: B1F0: Gypsum applied at a rate of 560 kg/ha broadcast at time of planting, B0F1: Epsom Salt applied at a rate of 86 kg/ha as a foliar spray between R1-R3, B1F1: combined gypsum and Epsom Salt, and B0F0: No treatment control. DC=Double Crop Soybean planted after cereal grain (usually wheat), FS=Full Season Soybean.

Year	Field ID	Crop type	Variety	Prior S Application (year - kg-S/ha)[1]	Crop Rotation	Herbicide Application	Plant Date	Gypsum Applied	Epsom Applied	Harvest Date
2017	5-43A	DC	TA3959R2S	2017-32 2016-20 2015-0	2017 Wheat/ DC Soybean 2016 FS Soybean 2015 Sorghum/FS Soybean 2014 Wheat DC Soybean 2013 FS Soybean 2012 Corn	glufosinate @ 0.85 L Ammonium Sulfate @1.3 kg glyphosate	7/11/17	4/11/17	8/31/17	10/31/17
2017	5-39B	DC	TA3959R2S	2017-20 2016-0 2015-20	2017 Wheat/ DC Soybean 2016 FS Soybean 2015 Wheat/ DC Soybean 2014 FS Soybean 2013 Corn 2012 Barley/ DC Soybean	glufosinate @ 0.85 L 1.3 kg Ammonium Sulfate, glyphosate	7/11/17	4/11/17	8/31/17	10/31/17
2017	5-43B	FS	Pioneer 40T84X	2017-1120 kg/ha gypsum (190kg-S) 2016-32 2015-20	2017 FS Soybean 2016 Corn 2015 Wheat /DC Soybean 2014 FS Soybean 2013 Corn	0.95L glyphosate,0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide, Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/10/17	4/12/17	8/10/17	10/18/17
2017	5-18O	FS	Pioneer 40T84X	2017-0/32 2016-0/32 2015-0/32	2017 FS Soybean 2016 Corn 2015 FS Soybean 2014 Corn 2013 Wheat/ DC Soybean 2012 FS Soybean	0.95L glyphosate,0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide, Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/10/17	4/12/17	8/26/17	10/26/17

2018	5-17C	DC	Asgrow 4135	2018- 20 2017-32+1120 kg/hs gypsum (190kg-S) 2016-20 2015-32	2018 Wheat/ DC Soybean 2017 Corn 2016 Wheat/ DC Soybean 2015 Corn 2014 Wheat/ DC Soybean 2013 Corn	0.85L glufosinate , 0.17 L Clethodim, 1.3 kg Ammonium Sulfate,1.4L Glyphosate,	7/10/18	7/1/18	9/4/18	12/6/18
2018	5-25A	DC	Asgrow 4135	2018 – 0/32 2017-0/32 +1120 kg/hs gypsum (190kg-S) 2015-0/32	2018 Wheat/ DC Soybean 2017 FS Soybean 2016 Corn 2015 Wheat/ DC Soybean 2014 FS Soybean 2013 Corn	0.85L glufosinate , 0.17 L Clethodim, 1.3 kg Ammonium Sulfate,1.4L Glyphosate,	7/10/18	7/1/18	9/4/2018	11/29/18
2018	5-39C	FS	Pioneer 31A22	2018 – 0 2017-32 2016-32 2015-32	2018 FS Soybean 2017 Corn 2016 Corn 2015 Corn 2014 Wheat/ DC Soybean 2013 Corn	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/18/18	5/25/18	8/5/18	10/9/18
2018	5-18	FS	Pioneer 31A22	2018 - 0/32 2017-0/32 2016-0/32 2015-0/32	2018 FS Soybean 2017 Corn 2016 FS Soybean 2015 Corn 2014 Wheat/ DC Soybean 2013 FS Soybean	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/18/18	5/25/18	8/17/18	12/10/18

[1]All prior S treatments applies as 22-0-0-5S analysis fertilizer

Table 2. Soil properties and classification for fields at CMREC Beltsville research facility used in 2017 and 2018. Soil organic matter (SOM) determined by loss on ignition (LOI), pH measured in water, Mehlich3 extractable ,P,K, and S. A1= 0-10 cm, A2= 10- bottom of A horizon, B = bottom of A– 30 cm, mean = weighted average of three depths based on the bulk density data for a representative "fine" or "coarse" textured field at CMREC Beltsville.

Field Code	Textural Class	Soil Series	Taxonomy	Depth	pH	SOM	P	K	S	Est. CEC
						(%)	----- (mg/kg)--------			(meq/100g)
5-43A	Coarse	Downer-Hammonton Complex	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	A1	5.5	1.25	58	124	11.2	5.2
				A2	5.5	0.55	55	59	8.6	3.9
				B	5.7	0.45	12	57	5.7	3.6
				Mean	5.6	0.72	43	77	8.6	4.2
5-39B	Fine	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults Fine, kaolinitic, mesic Aquic Hapludults	A1	5.6	0.6	72	74	11.9	5
				A2	5.7	0.75	74	59	11.1	3.2
				B	6	0.2	24	43	7.3	2.9
				Mean	5.8	0.58	62	60	11	3.7
5-43B	Coarse	Downer-Hammonton Complex	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	A1	5.9	1.4	60	100	10	4.6
				A2	5.7	0.65	62	58	9.4	3.7
				B	5.6	0.3	38	43	6.4	3.1
				Mean	5.7	0.79	56	67	9.3	3.8
5-18O	Fine	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults Fine, kaolinitic, mesic Aquic Hapludults	A1	6.5	2.5	199	90	22.1	4.6
				A2	6.6	1.7	178	58	25.5	3.7
				B	5.5	0.85	3	43	36.4	3.1
				Mean	6.2	1.65	128	62	25.5	3.8
5-17C	Coarse	Downer-Hammonton Complex	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	A1	5.7	1.7	41	44	7.6	5.2
				A2	5.7	1.7	45	31	7	3.9
				B	5.7	0.4	25	30	5.9	2.7
				Mean	5.7	1.19	36	34	6.9	3.8
5-25A	Fine	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults Fine, kaolinitic, mesic Aquic Hapludults	A1	6.2	2.35	181	83	11.4	7
				A2	6.1	1.15	113	36	11	5.9
				B	5.9	0.85	10	37	22	5.4
				Mean	6.1	1.36	90	49	14	6.1
5-39C	Coarse	Downer-Hammonton	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	A1	5.6	0.6	71	74	12	5
				A2	5.7	0.75	74	59	11.1	3.2
				B	6	0.2	24	43	7.2	2.9
				Mean	5.8	0.53	58	58	11	3.6
5-18	Fine	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults Fine, kaolinitic, mesic Aquic Hapludults	A1	6.4	3.35	226	80	53.9	14.5
				A2	6.2	2.15	173	37	21.9	12.4
				B	6.5	1.45	68	31	19.2	8.8
				Mean	6.4	2.2	145	47	30.4	11.5

Experimental Design and Treatments

The experiment used a randomized complete block design with split-plots. Depending on the size of the field, each of the eight site-years included between three and five replications. The whole plot treatment factor was broadcast S applied at a rate of zero or 560 kg gypsum/ha (100 kg S/ha) at the time of planting (B0 or B1). The subplot treatment factor was foliar S applied at a rate of zero or 86 kg Epsom salt/ha (11 kg S/ha) dissolved in 150 liters of water applied as a foliar spray at the beginning of the soybean R1 growth stage (F0 or F1). Thus, the four factorial treatments were control (B0F0), broadcast S (B1F0), foliar S (B0F1), and broadcast S + foliar S (B1F1). Soybeans were no-till planted in 37.5 cm rows in plots that were on average 14 m wide and 20 m long (plot length varied somewhat with the space available).

Soil sampling

Soil samples were collected near the time of soybean planting, but before any S treatments were applied. Four 30 cm deep cores were collected from each control (no S added) plot using a 1.8 cm cutting diameter push probe and divided into three segments referred to hereafter as A1, A2, and B (0-10 cm, 10cm- bottom of the A horizon, and bottom of A horizon to 30 cm). The length of each core segment was recorded and the four segments from each depth increment were composited within each replication. Thus, A1 was always 10 cm deep, but the depth and thickness of the A2 and B samples varied with depth of the genetic A horizon boundary, which was easily visible in these soils. After collection, soil was transported on ice back to the lab, fan-dried at room temperature for 24 – 48 hours, ground, and passed through a 2mm sieve before being stored for analysis. For site characterization of each field (Table 2), soil samples from

two replications from each field were sent to University of Delaware Soil Test Lab for routine soil analysis including pH_{water}, soil organic matter (SOM) by loss on ignition, Mehlich3 extractable P, K and S, and estimated cation exchange capacity (CEC) by the sum of the cations.

Plant Sampling

Seed samples for each plot were collected by hand immediately before combine harvest by cutting all the plants 2 cm above the soil surface from three, 3-m long sections of row. After harvesting, the plants were dried at 40° C for at least 48 hours. The seeds were then threshed from the plant and a subsample of seeds for each plot stored for analysis. Seed yield and moisture content from all plots were measured by a calibrated combine yield monitor and all yields were normalized to 13% moisture content. The S yield (kg/ha) was calculated for each plot based on the seed S content (%) as determined by XRF (following the procedures outlined below):

$$S\ yield\ (kg/ha)\ =\ yield\ (kg/ha)\ *\ \frac{seed\ S\ content\ (\%)\ by\ XRF}{100}$$

Seed S and Amino Acid Content

The total S content of the seed was determined by two methods. A subset of seed samples from 32 plots chosen to represent a range of seed S contents were ground to pass a 1 mm sieve and sent to a commercial lab (Waypoint Analytics, Richmond, VA) for total S analysis using digestion followed by ICP measurement.

All seed samples were analyzed for total S content using x-ray fluorescence (XRF) analysis (Bruker Tracer 3-SD, Bruker AXS Handheld, Kennewick, WA) as follows. Seeds were ground in a household coffee grinder (Proctor Silex, E160BYR) for

90 seconds prior to XRF analysis. A random subset of 10 ground samples were also ground to pass through a 1 mm mesh sieve in order to evaluate the effect of sieving on S content by XRF analysis. There was no significant difference between sieved and unsieved ground samples therefore subsequent XRF S measurements were made using unsieved samples. Samples were placed in a 28 mm inside diameter sample cup that was open on top and sealed with a 4μm thick prolene film on the bottom before analysis. Enough sample was used to create a layer at least 3 mm thick. This thickness was determined to be sufficient to provide an "infinite" absorption of x-rays based on our preliminary analyses and others' research (Towett et al., 2016; Sapkota et al., 2019). A 60g solid cylinder 17mm in diameter was placed on the sample to uniformly compact it. Spectra were created using a 120-second irradiation period with a voltage of 15 kev, an anode current of 25μA and a pulse length of 200. The readings were taken with the instrument head under a vacuum of <5 torrs to reduce air attenuation (Towett et al., 2016; Sapkota et al., 2019).

Spectra files were generated using SP1XRF software (Bruker, 2008) and were downloaded as .csv files. Spectra files for 88 plant tissue samples, including 24 soybean seed samples from this study, were then loaded, along with corresponding S values from ICP analysis into the CloudCal software (Drake, 2018) website to generate calibration curves. The Lucas Tooth model built into CloudCal (Lucas-Tooth and Pyne, 1963; Drake, 2018) was used to normalize the XRF data taking into account non-linear inter-element effects to predict S content values. Linear regression was then used to define the relationship between the predicted S from XRF and the S measured independently by ICP

(Figure 2). The calibration model was then applied to the remaining samples that did not have ICP values to determine S content of all samples.

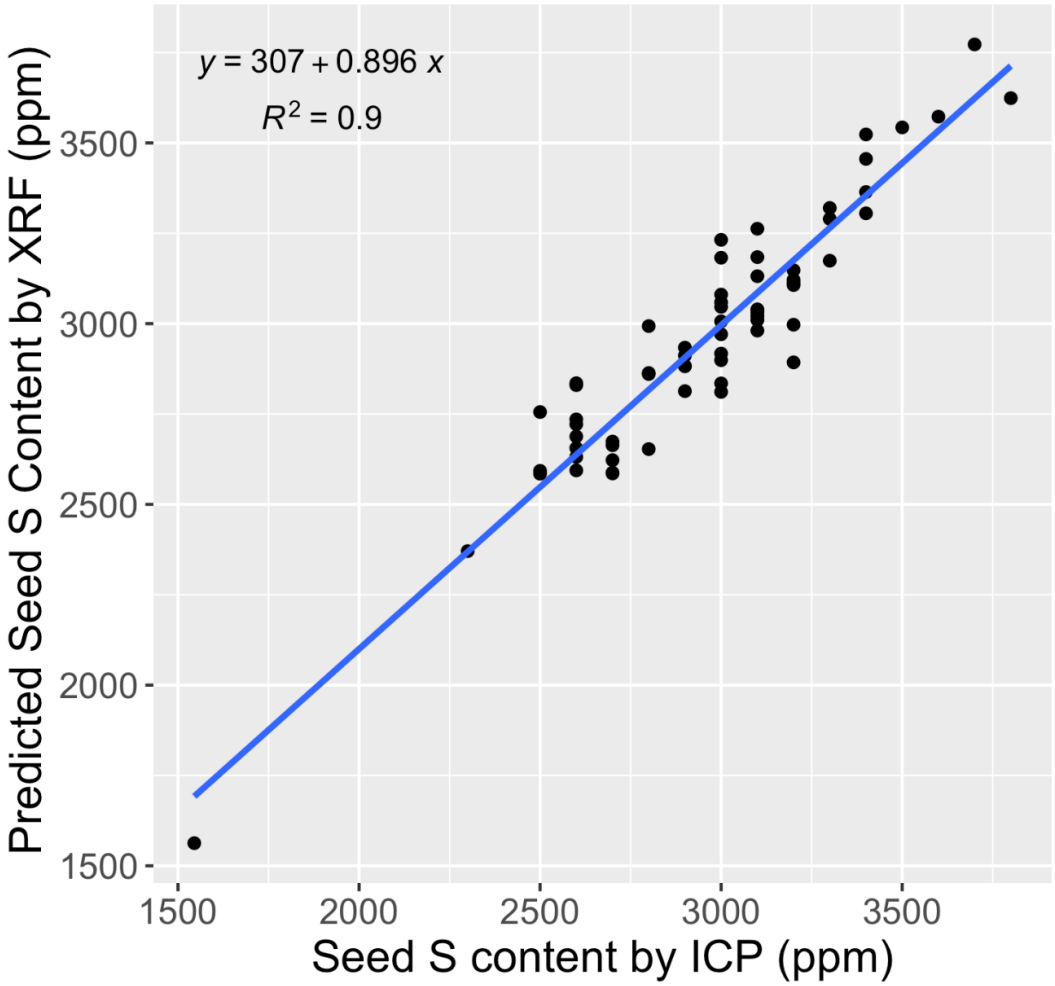


Figure 2 Calibration curve developed using spectral normalized net photon values from XRF spectra and S concentration values obtained by independent ICP analysis for a set of 88 plant tissue samples. Calibration was smoothed using the Lucas Tooth mode (Lucas-Tooth and Pyne, 1963)) and CloudCal software (Drake, 2018)

Twenty-four samples representing three replications of the four S treatments at both 2017 coarse sites (one with DC and one with FS soybeans) were sent for amino acid analysis (AAA) to the Molecular Structure Facility of the University of California, Davis,

CA. After de-lipidization, the amino acids were determined with two separate analyses using a Hitachi L8900 Amino Acid Analyzer (Hitachi, USA, Santa Clara, CA) with post-column, ninhydrin derivatization. The first analytical run quantified all the common amino acids except for MET, CYS, and Tryptophan (Trp). (Hitsuda et al., 2004, 2005). The second analysis was performed on a separate oxidized aliquot of each sample to detect cysteic acid (which is the combination of cysteine and cystine) and met-sulfone (oxidized/hydrolyzed stable form of methionine). Results for each amino acid were expressed as a percent of the extracted protein (g amino acid/100 g protein). The total protein content of the samples was calculated from total N content as determined on separate subsamples by high-temperature combustion/gas chromatography (LECO, St. Joseph, MI) as recommended by Tabatabai and Bremner, 1991 and an N-to-protein conversion factor for soy protein of 5.71 (FAO, 2003) as:

$$g\ total\ protein\ /\ g\ seed\ = \frac{g\ N}{g\ dry\ matter} * \frac{5.71\ g\ soy\ protein}{g\ N}$$

and the content of the amino acid in the seed was calculated as:

$$g\ amino\ acid/100 g\ seed\ = \frac{reported\ \%\ amino\ acid}{100}\ *\ Crude\ Protein * 100 g$$

The same 24 samples were also analyzed for total seed S content (%) by acid digestion and ICP (Waypoint Analytical Labs, Richmond, VA). The N/S ratio was also calculated for each of the 24 samples as a potential indicator S deficiency (Hitsuda et al., 2004).

Statistical analysis

Effects of S treatments on response variables (yield, seed S concentration, S yield, and seed MET+CYS concentration) were determined by a split-plot ANOVA in R, using

the ‘agricolae’ package (de Mendiburu, 2020). Broadcast gypsum application was the main plot factor and foliar Epsom salt application was the subplot factor, with both factors considered to be fixed. Replications were considered to be random. Unless otherwise indicated, a significance level of $\alpha = 0.05$ was used to determine significant differences between treatments. An F-protected post hoc Tukey HSD test was conducted to determine the significance levels between groups.

Linear regression was used to determine how well seed S content predicted crude protein. Additionally, linear regression was used to predict how well seed S concentrations, seed N concentrations, and N:S ratios predicted seed MET+CYS concentrations (g/100g protein).

Linear and nonlinear regression was also used to evaluate the relationship between Mehlich 3 extractable S content of the soil, SOM, pH, and yield response calculated as the difference between the average of the highest yielding treatment and the yield of the control plot for each site-year. All statistics were performed in the R for Mac (R Core Team, 2019).

Results

Site Characterization Results

The coarse sites used for the replicated experiments had average pH values of 5.7 (A1), 5.7 (A2), and 5.8 (B), average SOM values of 1.23 (A1), 0.91 (A2), and 0.33 (B), and average Mehlich3 extractable S content of 10.2 (A1), 9.0 (A2) and 6.3 (B). The fine sites used for the replicated experiments had average pH values of 6.2 (A1), 6.2 (A2), and 6.0 (B), average SOM values of 2.20 (A1), 1.44 (A2), and 0.84 (B), and average

Mehlich3 extractable S content of 24.8 (A1), 17.4 (A2) and 21.22 (B). The average yield response to S application on the coarse textured sites was 496 kg/ha and the average yield response on the fine textured sites was 138 kg/ha.

There was no significant relationship between Mehlich3 extractable S and pH or SOM in the coarse sites. There was no significant relationship between Mehlich 3 and pH for the fine sites. There was a significant positive relationship between Mehlich 3 extractable S and SOM for the fine sites ($p<0.01$, $R^2=0.41$). For all the fields combined there was a significant negative exponential relationship between yield response and Mehlich3 extractable S content ($p<0.05$, $R^2=0.64$) and a significant positive relationship between SOM and Mehlich3 extractable S content ($p<0.05$, $R^2=0.4$) (Figure 3).

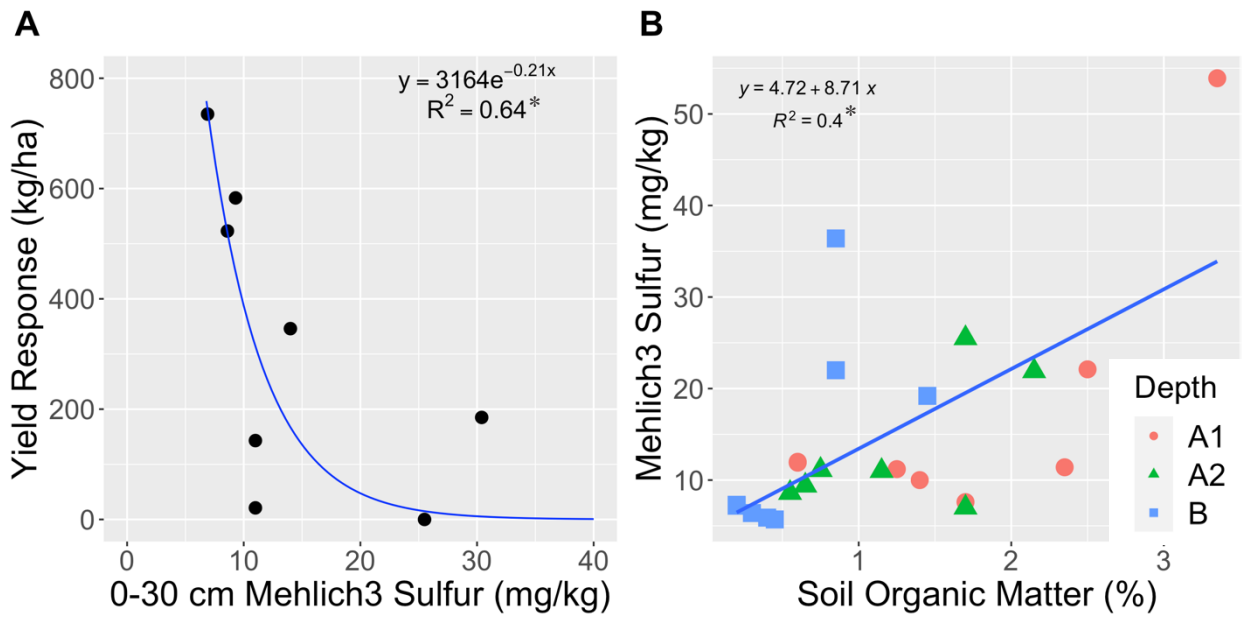


Figure 3 Relationship between (A) Mehlich3 Extractable S content (mg/kg) for the weighted average of the 0-30 cm soil layer and yield response to applied S and (B) soil organic matter content and Mehlich3 extractable S content for three soil depths (0-10 cm, 10-bottom of A, bottom of A to 30 cm). Samples represent averages of samples taken from 3-4 reps in each of eight site years used for replicated experiments. Soil organic matter content was determined by loss on ignition and yield response was calculated as the difference between the averages of the highest yielding treatment and the yield of the control. Relationships were both significant at p<0.05.

Yield Results

Yields for DC soybeans grown in this study ranged from 1354 to 3315 kg/ha and yields for FS soybeans ranged from 2195 to 4726 kg/ha. There was no significant effect of applied S on yields on any of the fine textured soils. Yield increases from S application were significant on all four coarse soils and ranged from 2 to 35%. Broadcast-applied S increased yield by 3% compared to the no S application control at one site (FS on coarse soil in 2018), while foliar S application increased yield at all four coarse soil sites. There was a significant main effect of foliar applied S on three out of the four coarse textured sites ($p<0.10$). There was a significant broadcast x foliar interaction effect on three out of the four coarse textured fields ($p<0.10$) (Table 3).

Table 3 Average yield and split plot ANOVA results for soybean yields from eight site-years grown in 2017-2018. Whole plot treatment was with or without broadcast S applied as gypsum at the time of planting at a rate of 560 kg/ha(B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at the beginning of the R1 growth stage at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences at $\alpha = 0.1$. FS= full season soybeans, DC=double crop soybeans planted after winter wheat harvest.

		DC	**FS**	**DC**	**FS**	**DC**	**FS**	**DC**	**FS**
		Coarse Soil		**Fine Soil**		**Coarse Soil**		**Fine Soil**	
		2017				**2018**			
		Soybean Yield, kg/ha							
Broadcast S (Whole plot effect)	B0	2348	3127	1903	3899	2383	4360	1917	2753
	B1	2525	3375	1684	3808	2744	4400	2005	2656
Foliar S (Subplot effect)	F0	2263b	3130b	1788	3912	2377b	4385	1955	2645
	F1	2609a	3371a	1799	3795	2752a	4375	1967	2764
Broadcast S x Foliar S	B0F0	2136	2840b	1892	3933	2087b	4314b	1794	2661
	B0F1	2560	3414a	1914	3866	2680a	4407a	2040	2846
	B1F0	2391	3422a	1684	3892	2667a	4457a	2140	2630
	B1F1	2659	3328ab	1684	3725	2822a	4345a	1870	2683
Source of Variation	**Df**	**P > F**							
Rep	3	0.996	0.514	0.154	**0.007**	0.630	0.446	0.942	0.661

Broadcast S	1	0.141	0.213	0.134	0.199	0.295	0.681	0.739	0.483
Error a	3								
Foliar S	1	**0.017**	**0.064**	0.923	0.259	**0.014**	0.857	0.936	0.317
Broadcast S x Foliar S	1	0.429	**0.018**	0.923	0.616	**0.090**	**0.085**	0.130	0.568

Seed S Content Results

The Seed S content of DC soybeans ranged from 0.264-0.389% S and the seed S content of FS soybeans ranged from 0.228-0.381% S. Of the eight site-years, there was a positive main effect of broadcast S on two, a negative main effect of foliar S on two, and a broadcast x foliar interaction effect on one (Table 4). Seed S content was significantly increased by broadcast S application by 4-18%. In the 2017, DC, coarse soil experiment, foliar and broadcast S applied separately each increased seed S concentration, but only in the absence of the other.

Table 4 Average seed S content and split plot ANOVA results for soybean seed S content (%) from eight site-years grown in 2017-2018.E Whole plot treatment was with or without broadcast S applied as gypsum at the time of planting at a rate of 560 kg/ha(B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at the beginning of the R1 growth stage at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences at $\alpha = 0.1$. *FS= full season soybeans, DC=double crop soybeans planted after winter wheat harvest.*

		DC	**FS**	**DC**	**FS**	**DC**	**FS**	**DC**	**FS**
		Coarse Soil		**Fine Soil**		**Coarse Soil**		**Fine Soil**	
		2017				**2018**			
		Seed S content (%)							
Broadcast S (Whole plot effect)	B0	0.344	0.275b	0.332	0.358	0.352b	0.351	0.36	0.347
	B1	0.367	0.326a	0.331	0.357	0.365a	0.337	0.371	0.351
Foliar S (subplot effect)	F0	0.346	0.314a	0.371a	0.361	0.354	0.338	0.365	0.351
	F1	0.365	0.287b	0.286b	0.354	0.367	0.351	0.366	0.347
Broadcast S x Foliar S	B0F0	0.314b	0.297	0.372	0.363	0.345	0.345	0.361	0.35
	B0F1	0.374a	0.247	0.293	0.353	0.360	0.358	0.359	0.345
	B1F0	0.377a	0.335	0.37	0.358	0.364	0.331	0.369	0.351
	B1F1	0.357ab	0.319	0.275	0.356	0.366	0.345	0.373	0.35
Source of Variation	**Df**	**P>F**							
Rep	3	0.532	0.883	0.375	**0.021**	0.769	0.782	0.597	0.342
Broadcast S	1	0.356	**0.060**	0.865	0.706	**0.038**	0.359	0.296	0.513
Error a	3								
Foliar S	1	0.298	**0.031**	**0.000**	0.288	0.109	0.396	0.930	0.683
Broadcast S x Foliar S	1	**0.070**	0.939	0.151	0.458	0.174	0.957	0.694	0.766
Error b	6								

S Yields Results

The DC soybean S yields ranged from 4 – 12 kg-S/ha and the FS soybean S yields ranged from 7 – 16 kg-S/ha. There was a significant effect on S yield from broadcast application on one out of the eight site-years ($p<0.05$). There was a

significant effect from foliar S on three out of the eight site-years ($p<0.05$). There was a significant broadcast x foliar interaction effect for the DC on coarse soil in both years, indicating that broadcast and foliar S applications both increased S yield, but only in the absence of the other (Table 5).

Table 5 Average S yield and split plot ANOVA results for soybean S yield (kg-S/ha) from eight site-years grown in 2017-2018.E Whole plot treatment was with or without broadcast S applied as gypsum at the time of planting at a rate of 560 kg/ha (B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at the beginning of the R1 growth stage at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences at $\alpha = 0.1$. FS= full season soybeans, DC=double crop soybeans planted after winter wheat harvest.

		DC	FS	DC	FS	DC	FS	DC	FS
		Coarse Soil		Fine Soil		Coarse Soil		Fine Soil	
		2017				2018			
		Seed S Yield (Kg-S/ha)							
Broadcast S (Whole plot effect)	B0	8.15	8.39b	6.33	13.99	10.02	15.09	7.43	9.75
	B1	9.25	11.07a	5.59	13.61	8.41	14.89	6.89	9.32
Foliar S (subplot effect)	F0	9.52a	9.91	6.64a	14.13	8.46b	14.76	7.17	9.42
	F1	7.88b	9.55	5.22b	13.48	9.99a	15.22	7.15	9.6
Broadcast S x Foliar S	B0F0	6.74b	8.43	7.03	14.3	7.18b	14.81	6.44	9.66
	B0F1	9.57a	8.34	5.62	13.68	9.64a	15.37	7.34	9.81
	B1F0	9.02a	11.75	6.23	13.95	9.72a	14.71	7.89	9.25
	B1F1	9.48a	10.52	4.61	13.28	10.32a	15.07	6.97	9.39
Source of Variation	**Df**	**P>F**							
Rep	3	0.645	0.481	0.219	**0.001**	0.681	0.537	0.958	0.381
Broadcast S	1	0.178	**0.015**	0.190	0.105	0.247	0.784	0.579	0.294
Error a	3								
Foliar S	1	**0.033**	0.307	**0.031**	0.201	**0.007**	0.536	0.986	0.746
Broadcast S x Foliar S	1	**0.084**	0.441	0.365	0.956	**0.047**	0.893	0.125	0.498
Error b	6								

Sulfur-Containing Amino Acid Content

The percent MET+CYS in the soybean protein ranged from 0.615% for the no-S control to 1.049% for the foliar S application in DC soybeans and from 0.533% in the no S applied control to 0.953% in the foliar S treatment for FS soybeans. Thus, S foliar

application stimulated a relative increase in seed MET+CYS content by 71% in DC soybeans and 79% in FS soybeans (Table 6). Broadcast S application did not significantly affect seed MET, CYS, or MET+CYS in DC soybeans, but did increase CYS and MET+CYS in FS soybeans when foliar S was not also applied. There was a significant foliar S application main effect on MET, CYS, and MET+CYS on both DC and FS soybeans. There was a significant broadcast x foliar S interaction effect on CYS and MET+CYS content in FS soybeans such that foliar S application increase these amino acid percentages only when S was not also broadcast applied.

The linear regression revealed that MET+CYS was weakly related to seed N content (R^2=0.32, $p<0.05$). In contrast, MET+CYS content (g/100g protein) showed a strong positive relationship with crude protein (R^2=0.66, $p<0.05$), seed S content (R^2=0.62, $p>01$), and a strong negative relationship with N/S ratio in the seed (R^2=0.63, $p<0.01$). (Figure 4).

Table 6. Split plot ANOVA results for Methionine (MET), Cysteine (CYS), and Methionine + Cysteine (MET+CYS) content (% of extracted protein) of double crop (DC) and full season (FS) soybean seeds grown in three replications on coarse soils in 2017 (N=24.site years. The whole plot treatment was with or without broadcast application of S as gypsum at a rate of 560 kg/ha (B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at a rate of 86 kg/ha (F1 or F0). Means followed by the same lowercase letter are not significantly different at α = 0.10.

		MET	CYS	MET+CYS	MET	CYS	MET+CYS
			DC			FS	
				---------- % ----------			
Broadcast S	B0	0.298	0.535	0.832	0.265	0.478	0.743
(Whole plot effect)	B1	0.296	0.538	0.836	0.275	0.495	0.770
Foliar S	F0	0.261b	0.450b	0.711b	0.223b	0.377b	0.601b
(subplot effect)	F1	0.333a	0.623a	0.956a	0.317a	0.596a	0.912a
Broadcast S x Foliar S	B0F0	0.233	0.383b	0.615b	0.206	0.327c	0.533c
	B0F1	0.363	0.686a	1.049a	0.323	0.630a	0.953a
	B1F0	0.290	0.517ab	0.807ab	0.240	0.427b	0.670b
	B1F1	0.233	0.560ab	0.865ab	0.310	0.563a	0.870a
Source of Variation	**Df**			**P>F**			
Rep	2	**0.075**	**0.026**	**0.045**	0.464	0.285	0.299
Broadcast S	1	0.899	0.807	0.885	0.734	0.751	0.734
Error a	2						
Foliar S	1	**0.071**	**0.020**	**0.031**	**0.003**	**0.000**	**0.000**
Broadcast S x Foliar S	1	0.117	**0.049**	**0.065**	0.190	**0.004**	**0.007**
Error b	4						

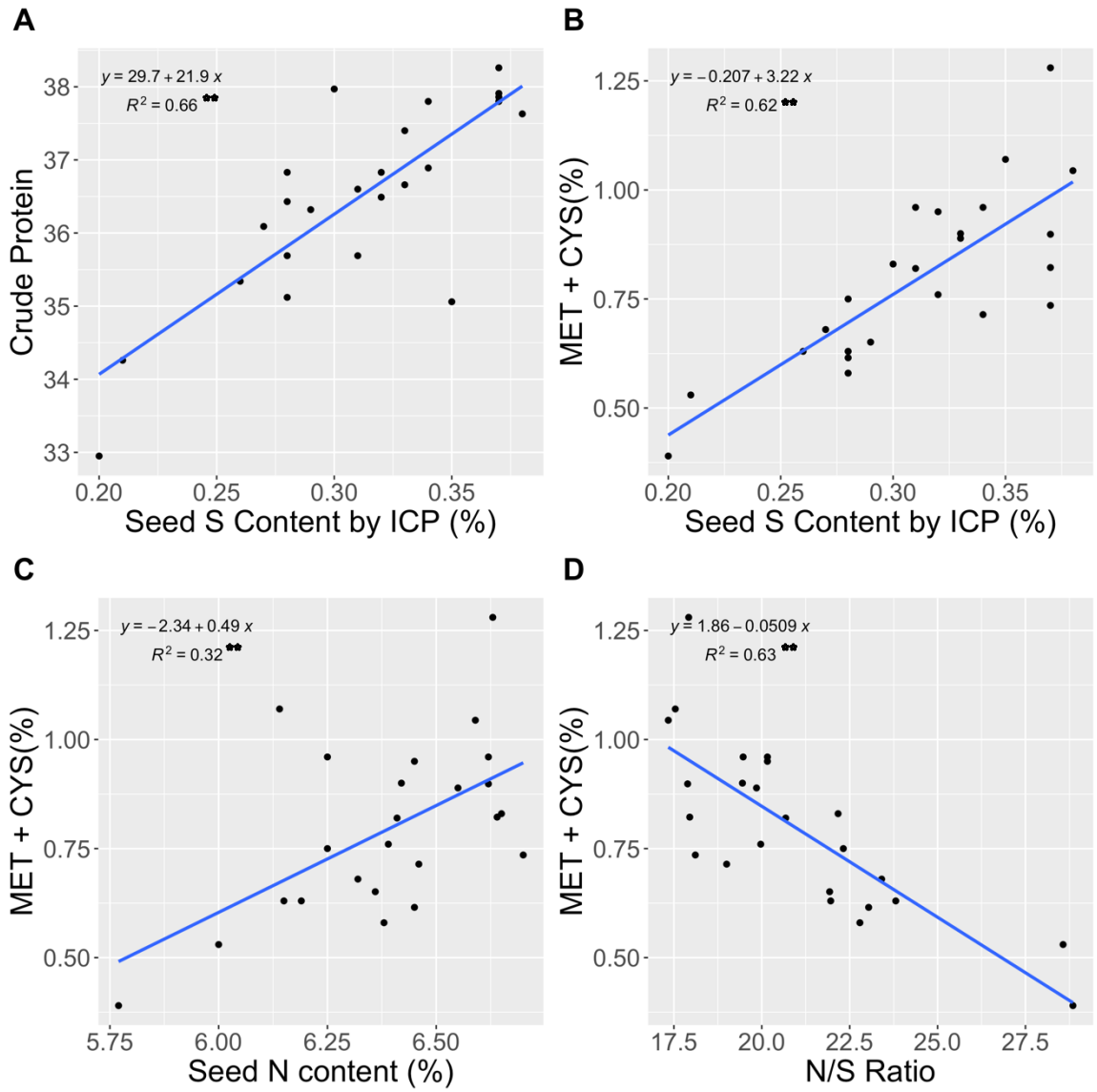


Figure 4 Significant linear relationships between crude protein content and seed S content (A) and between MET+CYS content (g/100g extracted protein) and seed S (B), seed N content (C), and N/S ratio (D). Data are for a total of 24 samples from replicated DC and a FS soybean experiments on two coarse soil site years in 2017.

Discussion

The average yields of the soybeans were comparable to average soybean yields reported for Maryland, which are approximately 3,000 kg/ha for FS soybeans and 2,000 kg/ha for DC soybeans (USDA, 2020a). None of the soybeans grown in this study exhibited the upper leaf chlorosis associated with severe S deficiency and the seed S

concentrations were almost all above the critical level of 0.23% S identified by Hitsuda et al., (2004). Therefore, it is unlikely that severe S deficiency occurred in the control treatments in any of the eight site-years.

Contrasting weather patterns and soil differences likely affected soybean growth and response to S in different site-years (Figure 1). The 2017 growing season with 670 mm cumulative rainfall was near the long-term average of 660 mm, while 2018 with 1004 mm cumulative rainfall was much wetter than an average growing season. No leaf injury was observed from the foliar S applications in either year, even though in 2018 the weather was hot (>30^0C) and dry (no significant rain) for the week prior to and the week after spraying. However, the foliar application main effect was not significant in 2018 but was significant on the coarse soils in 2017 when it was cooler and wetter before and after spraying. These contrasting conditions also occurred during flowering when plants were starting to put more energy into reproductive production. Sufficient amounts of nutrients and water during the flowering stage are critical for the plant to reach maximum yield potential. In addition, wet soil conditions in fall 2018 caused harvest to occur about a month later than in 2017.

Our results show differing responses to the two modes of S applications on soybean yield, seed S content, and S yield. While yield was significantly increased by foliar S on four out of eight site-years, broadcast S increased yield in only one site-year, and only when foliar S was not applied. In contrast, seed S content was positively increased by broadcast S application and negatively affected by foliar S application in two site-years. The decrease in seed S content on two soils in 2017 may be indicative of some unobserved salt injury. In the future, we suggest a somewhat lower S rate of about 8

kg S/ha should be compared to determine if the same response may be stimulated with less risk of osmotic damage to the plant.

We chose to apply Epsom salt as a foliar spray at the beginning of the plant reproductive stage (between R1-R3) because Epsom salt is highly soluble (similar amounts of S as potassium sulfate clogged sprayers in preliminary trials), most farmers are well equipped to apply foliar sprays (many told us they did not have equipment to spread small amounts of powdered gypsum), and because plants are rapidly accumulating S and new leaves are still expanding at that stage of growth. Additionally, some plants, including soybeans, may not be able to effectively translocate S from older to younger, more photosynthetically active leaves or to the seeds where storage proteins are synthesized (Sunarpi and Anderson, 1997; Paek et al., 2000; Naeve and Shibles, 2005). The younger leaves in the upper soybean canopy may be more effective than older leaves at assimilating SO_4^{2-} taken up by the plant into amino acids and other essential compounds (Naeve and Shibles, 2005). Therefore, a spray that supplies S directly to the younger leaves that are assimilating SO_4^{2-}, could be an effective method for ameliorating S deficiency in the plant and increase S in the seeds.

In addition to timing and source of applied S, soil type and S present in the soil may also have had an effect on S uptake and mobilization within the plant. Sulfate ions are highly susceptible to leaching unless adsorbed onto the positively charged surfaces of certain clays and Fe or Al oxides (Schoenau and Malhi, 2008). The coarse textured soils, which are lower in clay and Fe or Al oxides than finer textured soils, have lower anion exchange capacity, and thus are more susceptible to losing SO_4^{2-} by leaching. The coarse soils used in this study were Downer-Hammonton complexes which are characterized by

loamy sands over a relatively deep Bt horizon of sandy loam texture, over a loamy sand to sand C horizon. Although the Bt horizon has an accumulation of clay and Fe and Al oxides that can contain adsorbed SO_4^{2-}, it may occur too deep in the soil for the roots to effectively access the sorbed S. The deep Bt horizon, coupled with very sandy, low organic matter A horizons helps to account for the apparent low S supplying capacity of these coarse textured soils.

The finer textured soils that characterized four of the sites in this study likely released sufficient S to meet crop demand from soil organic matter mineralization and desorption from subsoil iron-coated clay, so crop growth at these sites may not have been limited by S. The fine textured soils used in this study were Russet-Christiana complexes characterized by sandy loam or silt loam A horizons over distinctly more clayey Bt horizons. These soils had higher organic matter content in the surface horizon than the coarse soils and would be expected to have higher adsorbed S that is within the plant root zone (Table 2). The addition of S (either broadcast or foliar) may have failed to significantly increase yields on these soils because S was probably not limiting.

The soil test results (Table 2) for the eight sites showed that Mehlich3 extractable S content increased with increasing SOM content and was somewhat able to predict where a response to S would be likely to occur (Figure 4). Prior research suggests that the Mehlich3 soil test does not accurately predict where crop responses to S will occur (Sims, 1989; Sahrawat et al., 2009; Ketterings et al., 2011; Kowalenko et al., 2014). However, the mean Mehlich3 S for the coarse textured sites was 8.5 mg/kg which is below the critical level of 18-22 mg/kg Mehlich3 extractable S that has been identified by prior

studies (Soil Fertility Management, 2010; Seth et al., 2018). The mean Mehlich3 S for the fine textured sites was 21.1 mg/kg, near the reported critical value.

While total S is a relatively easy measurement to make on plant material, measuring MET+CYS content is expensive, time-consuming and requires specialized equipment. Porter et al. (1974) reported the total S contents of grain legume seeds (dry beans; *Phaseolus vulgaris*, mung beans; *Vigna radiata*, and cowpeas; *Vigna unguiculate*) were positively correlated with the percent MET+CYS in the protein (Porter et al., 1974). Our data show that this holds true for soybeans, as well. Therefore, total S, could potentially be used as a proxy measurement for seed MET+CYS content and thus an indicator of protein nutritional quality.

The strong correlation between plant tissue S determined by XRF and by ICP found in our study (Figure 2) suggests that XRF is a promising method to analyze S in plant tissue samples, including seeds. Although we did not test this possibility, the work of Sapkota et al., (2019) suggests that because of the small sample size (<0.1g) used for ICP analysis, the correlation might have been improved if the material had been more finely ground (to pass a 0.5 mm rather than 1.0 mm screen) before subsampling for ICP analysis. However, for XRF analysis, we did compare coarsely ground material to samples passed through a <1mm screen and found that the fineness of grind did not affect the XRF photon counts for S, probably because of the relatively large sample size (> 1.0 g) and the averaging of many repeated readings taken during a 120 second period. We conclude that portable XRF is a convenient, inexpensive, rapid, non-destructive method. As such, we suggest that it could potentially be used by wholesale grain purchasers to evaluate the S concentration of soybeans. Further, the significant correlations between S

and MET+CYS content suggest that total S could potentially be used as a proxy measurement for protein quality at the point of sale. Additional work needs to be done to confirm these relationships and create a calibration equation between total S and MET+CYS content that includes a wider range of plant genotypes and growing environments.

Both foliar and broadcast S treatments increased the proportion of MET+CYS in soybean protein in our study, with the greatest increase from the foliar S treatment (Table 6). This outcome supports our hypothesis that the soybean seed protein quality can be enhanced by S fertility management. Prior efforts to improve MET+CYS content of the soybean protein largely focused on breeding for improved MET+CYS content (Krishnan, 2008; Krishnan and Jez, 2018). Our study indicates that soybean protein composition is more variable than often assumed and can be influenced by the S fertility status of the plant which suggests that S fertility management can maintain or improve the nutritional quality of soybeans. Increases in seed MET+CYS content of the magnitudes observed in this study could have very large impacts on the economic value of soybeans as feed (McVey et al., 1995). Our results warrant further research into the potential for S fertility management to enhance the nutritional quality of soybean, and possibly other grain legumes, for humans and non-ruminant livestock.

Conclusion

This study confirmed that, in the absence of a definitive soil test, application of S to soybeans is likely to be justified on sandy coastal plain soils. The rate and timing of Epsom salt foliar application were effective, but further research needs to be done to be able to determine if lower rates, earlier timing, or other sources (such as potassium sulfate

or ammonium sulfate) might be equally or more effective. The results are inconclusive as to whether broadcast S at the time of planting or foliar S at R1 is a more effective mode of S application. While foliar S more often increased the soybean yields, when seed S content and S yield are included in the objectives, the advantage of foliar application is less clear because broadcast S increased seed S content in more site-years than foliar S alone.

Although the two years during which these experiments took place were characterized by contrasting growing season weather conditions, further studies should be carried out on a wider range of environmental conditions including soil sulfur levels, soil types, and weather regimes. It is also possible that S supplying capacity of the soils in our study may have been inadequate, but the crop did not respond significantly to S application because drought, extreme rainfall, or other nutrient deficiencies were more growth-limiting than the S deficiency. For example, a drought-stressed crop might not respond to S fertilization, despite having an inadequately low supply of S, until its water needs were met.

The highly significant linear relationship (Figure 2) between tissue S content measured by standard digestion / ICP analysis and S content determined by XRF scan suggests that XRF has potential for rapid, inexpensive seed S analysis. Our amino acid data show that S nutrition of the plant affects protein quality in the seed. With further research to refine the technique for portable use, XRF has the potential to be applied to market differentiation that could pay farmers for improved nutritional quality of the crop. This research also has particular significance for the organic farming sector because

organic regulations only allow for restricted amounts of synthetic methionine in organic poultry production ("USDA," 2020b).

The results of this study support continued research on S fertility management for soybeans, especially in regions with low soil organic matter, sandy textured soils. The combination of continually increased yields and reduced ancillary S inputs can be expected to deplete the S supplying capacity of soils in the mid-Atlantic and other soybean-producing regions. The documentation of a nearly 2-fold increase of MET+CYS concentrations in soybean seed suggests that S fertilization should be investigated as a potential new tool in achieving enhanced food security with grain legumes in many regions of the world.

Chapter 4: Sulfur Management to Enhance Yield and Protein Quality of *Phaseolus Vulgaris*

Abstract

Common dry beans *(Phaseolus vulgaris)* serve as a principle protein source for many of the world's people especially in developing countries. However, the protein quality of common dry beans is often limited by the content of essential sulfur (S) containing amino acids methionine and cysteine (MET+CYS). Prior research on soybeans has shown positive relationships between S application and seed yield and S content. This project sought to expand those results to common dry beans used for human consumption. Field trials were carried out in Maryland, USA using four S treatments: (B0F0) no-amendment control; (B1F0) 560 kg/ha gypsum ($CaSO_4$; 17% S) broadcast at planting; (B0F1) 86 kg/ha Epsom salt ($MgSO_4$; 11% S) as a foliar spray at soybean R1 growth stage; (B1F1) the combination of broadcast and foliar S application. In each of two years, this experiment was conducted on common dry beans on two soils of contrasting texture (coarse and fine) for a total of four site years. Common dry bean yield, seed S concentration, and seed S yield were measured for all site years. Seed MET+CYS content was measured for a subset of 32 samples from all four site years. Broadcast S significantly increased yield by 46% on one site year and significantly increased seed S content by 8-12% on two site years ($p<0.10$). We show that applying S can increase both the yield and seed S content of common dry beans.

Introduction

Legumes are the second most widely grown crop type worldwide and serve as a main protein source for many of the world's people, especially in developing countries (Duranti, 2006). Legumes are rich in protein, carbohydrates, dietary fiber and an assortment of other essential nutrients (Bouchenak and Lamri-Senhadji, 2013). Although soybeans (*Glycine Max)* are the most commonly grown legume worldwide, common dry beans (*Phaseolus vulgaris)* are considered one of the most important food sources for low income people worldwide and provide significant health benefits including: lowering cholesterol, protecting against kidney disease and cancer, diabetes, and obesity (Kudre et al., 2013).

The usability of legume protein is often limited by the S-containing amino acids methionine and cysteine (MET+CYS) which cannot be synthesized by humans and must come from a dietary source (Nwokolo and Smartt, 1996; Sathe, 2002). In the typical omnivorous Western diet, MET+CYS are consumed in adequate amounts from the combined consumption of plant and animal proteins. However, in vegan and plant-based diets which are common in developing countries, MET+CYS deficiency can negatively affect human and animal health (Jez and Fukagawa, 2008).

Common dry bean protein has lower biological value and digestibility for humans than animal protein which is attributed to deficiency of MET+CYS (Sathe, 2002). Common dry beans contain around 20-30% protein. Approximately 50% of the protein in common dry beans consists of storage proteins, which are often low in MET+CYS (Nwokolo and Smartt, 1996; Sathe, 2002). This project will explore the potential for S fertilization to improve the MET+CYS content of common dry beans, which would in turn improve the biological feed value of common dry beans.

It has been known for over a century that S is an essential macronutrient that is assimilated by common dry beans in quantities similar to Phosphorous (P) (Sachs, 1865; Bender et al., 2015) however in recent times it has largely been ignored from routine nutrient management planning. For most of the twentieth century S was inadvertently added to fields in North America through use of organic amendments such as compost and manure, "impurities" in common chemical fertilizers, or through atmospheric deposition resulting from sulfur dioxide emissions from coal fired power plants (especially in the Eastern USA, northern Europe, and Central China) (Eriksen, 2008).

After the implementation of amendments to the Clean Air Act in 1990 that regulated S dioxide emissions from coal-fired power plants, atmospheric deposition of S was greatly reduced leading to increased incidence of S deficiency (Ketterings et al., 2011). According to the National Trend Network the S deposition rates in the US decreased from an average of 11 kg S ha^{-1} in 1979 to 1981 to 5 kg S ha^{-1} in 2008 (National Atmospheric Deoposition Program, 2011). The combination of reduced atmospheric deposition of S, chemical fertilizers with lower amounts of S impurities (e.g. diammonium phosphate and urea), and movement away from organic soil amendments on a large scale is depleting S reserves and leading many fields to supply inadequate S for optimal crop growth. In other parts of the world, such as sub-Saharan Africa and South America that rely heavily on legumes as a major protein source, S deposition rates have been historically lower than in more industrialized countries and significant S has been lost from soils due to annual biomass burning (Zhong et al., 2020). Rates of S deficiency will continue to increase if farmers continue to ignore S fertilization as part of their

routine nutrient management practices. This research project will add to the current body of research on S management in common dry bean systems.

Efforts to improve the MET+CYS content of grain legumes have mostly been focused on plant breeding (Sathe, 2002; Krishnan and Jez, 2018). While most of the breeding work has been focused on soybeans, plant-based proteins are becoming increasingly popular and further research on improving their amino acid balance is warranted. Production of common dry beans has been on the rise since the early 1960s and has steadily increased worldwide from 11 million metric tons in 1960 to 30 million metric tons in 2018 (FAOSTAT, 2020). With growing human populations, increasing concern about environmental impacts of food production, and declining consumption of animal based proteins in many Western diets, more research on improvement of common dry bean protein quality is warranted (Sathe, 2002).

Sulfur plays several important roles in plant growth processes, including being a component of essential MET+CYS. The S atoms in these amino acids are responsible for bonds that stabilize the three-dimensional molecular folding that is key to protein function. Additionally, S is one of 18 essential elements for plants and is required for plant functions such as nitrogen (N) fixation and photosynthesis (Epstein and Bloom, 2005). The combined assimilation of both N and S is integral to the ability of the plant to synthesize MET+CYS; the supply of these amino acids in turn often limits plants' ability to synthesize required proteins (Ruiz et al., 2005).

There has been limited research on the response of legumes to applied S, with the majority of studies focused on soybeans. Recent research in Maryland (Chapter 3, Rushovich and Weil, 2020) indicates that on S deficient soils S applied to soybeans can

increase yield, seed S content and seed MET+CYS content. Plants growing under S deficient conditions, may restrict their MET+CYS synthesis thus leading to variation in MET+CYS content of seeds grown under different environmental factors (Schumacher et al., 2011). The majority of the S in legume seeds is present in MET +CYS, this suggests that fertilization with S could improve the amino acid profile of the seed by increasing the concentration of MET+CYS within the seed.

We report on four field experiments designed to test the following hypotheses (1) S treatment will increase yield of common dry beans on low-S soils, (2) S treatment will increase S content of common dry bean seeds on low-S soils, (3) S treatment will increase the concentration of MET+CYS in the seed, and (4) the total S content of the seed will correlate with the MET+CYS concentration.

Materials and Methods

Field Sites

Replicated field trials were conducted in 2018-2019 at the Central Maryland Research and Education Center (CMREC) in Beltsville, MD (39.012162, -76.833329), and Upper Marlboro, MD (38.859454, -76.777549). This region has a humid temperate climactic zone with mean annual minimum and maximum temperatures of 5°C and 25°C, respectively. The sites receive on average 107.5 cm of precipitation per year evenly spread among 12 months (NOAA, 2020; Figure 6).

Field experiments were conducted for a total of four site years. In each of two years common dry beans were grown on two contrasting soil types, relatively coarse and fine. The coarse soils at CMREC Beltsville were a complex of Downer and Hammonton series with loamy sand surface horizons over sandy loam subsoil. At Beltsville the fine

soils consisted of a complex of Russet and Christiana series with fine sandy loam to silt loam surface horizons over sandy clay loam to clay loam subsoil. The field at Upper Marlboro was dominated by “fine” soils of the Donlonton and Annapolis series with fine sandy loam surface horizons over clay loam subsoils (Table 7). Site management histories are provided in Table 8. Specific soil series present from among those indicated in the Web Soil Survey (USDA/NRCS, 2020) mapping units were determined by examining the texture, Munsell color and structural features of two profiles at each site using bucket augers to a depth of one meter. All four site years were located in the Northern Coastal plain region of the Eastern US in which soil parent material consists of deep fluviomarine deposits (Soil Survey Division Staff, 2017).

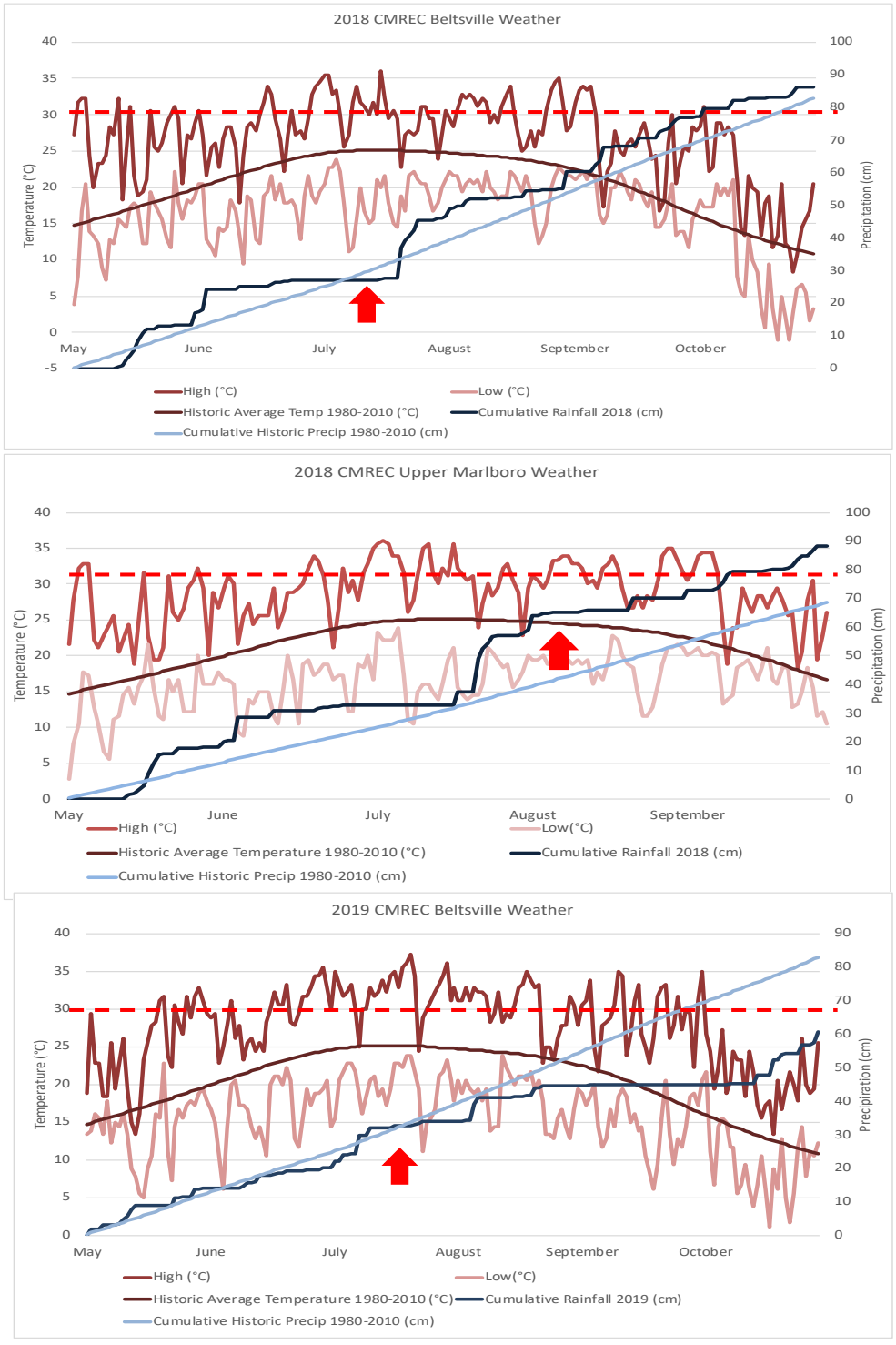


Figure 5 Daily high temperature (°C), daily low temperature (°C), and daily precipitation (cm) at Beltsville for 2017 (left) and 2018 (right)) and the 1980-2010 average temperature(°C) and average precipitation (cm) at Baltimore (BWI NOAA weather station). The red arrow indicates when Epsom salt was sprayed. The dashed red horizontal line indicates high temperature stress above 30°C

Table 7. Soil characterization data for 2017 and 2018 fields at CMREC Beltsville and Upper Marlboro research facilities. Soil organic matter (SOM) determined by loss on ignition (LOI), pH measured in water, and Mehlich3 extractable P, K, and S and estimated Cation Exchange Capacity (CEC). A1 = 0-10 cm, A2 = 10 –the bottom of the A horizon, B = bottom of A horizon– 30 cm, Mean = weighted average for all three horizons based on the bulk density of a representative "fine" and "coarse" textured field at CMREC Beltsville

Field	Soil Series	Taxonomy	Horizon	pH	SOM (%)	P	K	S	Est. CEC (meq/100g)
						-------(mg/kg)----			
UMBB	Annapolis-Donlonton	Fine-loamy, glauconitic, mesic Typic Hapludults	A1	6.3	3.05	41	151	11.9	11.7
		Fine-loamy, glauconitic, mesic Aquic Hapludults	A2	6.3	1.9	24	75	11.1	9.8
			B	5.1	1.3	14	80	7.3	11.4
			Mean	5.9	2.03	26	98	10.1	11
5-39B	Downer-Hammonton	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	A1	5.6	0.6	71	74	10.1	5
		Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	A2	5.7	0.75	74	59	9.2	3.2
			B	6	0.2	24	43	8.8	2.9
			Mean	5.8	0.53	57	58	9.3	3.6
5-39A	Hammonton	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	A1	6	1.3	57	94	9.7	5.1
			A2	5.8	0.7	79	67	9.2	4
			B	6	0.4	28	68	5.8	3.4
			Mean	5.9	0.77	57	75	8.3	4.1
5-7A	Christiana	Fine, kaolinitic, mesic Aquic HapludultsCoarse	A1	5.8	1.6	34	109	8.4	5.7
			A2	5.9	1.1	18	74	8.3	4.6
			B	6.1	0.85	6	91	6.7	4.7
			Mean	5.9	1.15	18	90	7.7	4.9

Table 8. Management history for all sites, including sulfur variety, tillage, rotation, and S application history for the past five years, and any irrigation used on crop grown for study. None of the fields received any manure application in the past five years. FS= full season soybean, DC= double crop soybean grown after a cereal grain (typically wheat).

Year	Field Code	Variety	Tillage History (past 5 years)	Crop Rotation (past 5 years)	Sulfur Application[1] (Past 5 years)	Irrigation
2018	UMBB	Midnight Black Turtle	2018-surface till, prior five years no till	2018 Black Bean 2017 No Crop 2016 Soybeans 2015 Soybeans 2014 corn 2013 Soybeans	NA	1.5 cm weekly from 6/25/2018-7/18/2018
2018	5-39B	Midnight Black Turtle	2018 Turbo Till Rest No-Till	2018 Black Bean 2017 Barley DC Soybeans 2016 Wheat DC Soybeans 2015 Corn 2014 Wheat DC Soybeans 2013 Corn	2019-20 2018-0 2017-20 2016-0 2015-20	Yes
2019	5-39A	Eclipse	2019 Turbo Till Rest No-Till	2019 Black Bean 2018 Wheat DC Soybeans 2017 FS Bean 2016 Corn 2015 Wheat DC Soybeans 2014 FS Soybeans	2019-20 2018-32 2017-0 2016-32 2015-20	No
2019	5-7A	Eclipse	2019 Turbo Till Rest Chisel Plow	2019 Black Bean 2018 FS Soybeans 2017 Vegetables 2016 Vegetables 2015 Sweet Corn 2014 Vegetables	2019-0 2018-32 2017-0 2016-32 2015-0	Yes

[1]All prior S treatments applied as 22-0-0-5 S analysis fertilizer

Table 9 Agronomic practices and timing of operations at the four study sites. All fields were inoculated with Bradyrhizobium japonicum, Bradyrhizobium sp. (Vigna), Rhizobium leguminorsarum biovar viceae and Rhizobium leguminosarum biovar phaseoli at the time of planting. All four fields received the same S treatments;B0F0= Control, B0F1 = foliar S applied as Epsom salt at a rate of 86 kg/ha (11 kg-S/ha) at the beginning of reproductive stage of growth, B1F0= broadcast S applied as gypsum at the time of planting at a rate of 500 kg/ha (100 kg-S/ha), and B1F1= combined broadcast and foliar S.

Field Code	Weed Control	Pesticide Application	Other Fertilizers	Planting Date and Row width	Gypsum Applied	Epsom Applied	Harvest Date
UMBB	Hand Weeded on 8/8, 8/13, 8/15	NA	NA	6/19/18, 50 cm	6/19/18	8/8/18	9/11/18
5-39B	**5-30-2018-**950 ml Paraquat Dichloride, 30 ml Halosulfuron-methyl, methyl 3-chloro-5-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl) -1-methylpyrazole-4-carboxylate), 473 ml Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide **6-24-2018**-414 ml Clethodim	NA	50 kg-N/ha as Ammonium Nitrate	5/30/18, 37.5 cm	6/1/19	7/13/18	9/11/18
5-39A	**6-7-2019**-15 ml, Halosulfuron-methyl, methyl 3-chloro-5-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl) -1-methylpyrazole-4-carboxylate), 414 ml- Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide	8-6-2019 189 ml. Bifenthrin	50 kg-N/ha as Ammonium Nitrate	6/6/19, 37.5 cm	6/6/19	7/20/19	10/4/19
5-7A	**6-7-2019-**15 ml, Halosulfuron-methyl, methyl 3-chloro-5-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl) -1-methylpyrazole-4-carboxylate), 414 ml- Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl) acetamide. Hoop-hoe weeded Rep 2 on 6/21/19	8-6-2019 189 ml. Bifenthrin	50 kg-N/ha as Ammonium Nitrate	6/6/19, 37.5 cm	6/6/19	7/20/19	10/19/19

Experimental design and Treatments

The experiment used a randomized complete block design with split-plots. Depending on the size of the field, each of the four site years included between four and six replications. The whole plot treatment factor was broadcast S applied at a rate of zero or 560 kg gypsum/ha (100 kg S/ha) at the time of planting (B0 or B1). The subplot treatment factor was foliar S applied at a rate of zero or 86 kg Epsom salt/ha (11 kg S/ha) dissolved in 150 liters of water applied as a foliar spray at the beginning of the soybean R1 growth stage (F0 or F1). Thus, the four factorial treatments were control (B0F0), broadcast S (B1F0), foliar S (B0F1) and broadcast S + foliar S (B1F1) (Table 8). Experimental plots varied in size depending on the size of the field available with an average plot size of 55 m^2. Row width was 50 cm at Upper Marlboro and 37.5 cm at Beltsville. Gypsum was hand applied at Beltsville and applied with a 1.5 m wide drop spreader at Upper Marlboro. Epsom was applied with a tractor drawn boom sprayer at Beltsville and with a backpack sprayer at Upper Marlboro. At Upper Marlboro one hand weeding was performed in mid-August due to herbicide restrictions on certified organic land.

In 2018, seeds at both locations were inoculated with *Bradyrhizobium* (Guard – N brand, www.johnnyseeds.com). However, after growth of several true leaves no nodules were visible on most plants observed. Therefore, to ensure N sufficiency, the beans at Beltsville were fertilized with 50 kg/ha N as ammonium nitrate. Due to organic certification rules at the Upper Marlboro site, N could not be applied in a form that would not also apply additional S. In 2019, to prevent N deficiency, all plots at Beltsville were

inoculated with *Bradyrhizobium* (Guard-N and Exceed brand) and fertilized with 50 kg/ha N as ammonium nitrate at the time of planting

Soil Sampling

Soil samples were collected near the time of planting, but before any S was applied. In 2018 four soil cores were collected from each control plot and in 2019 twelve soil cores were collected from each rep. Soil cores were collected with a 1.8 cm cutting diameter push probe and divided into three segments referred to hereafter as A1, A2, and B (0-10 cm, 10cm- bottom of the A horizon, and bottom of A horizon to 30 cm). The length of each core segment was recorded and the four segments from each depth increment were composited within each replication. Thus, A1 was always 10 cm deep, but the depth and thickness of the A2 and B samples varied with depth of the genetic A horizon boundary, which was easily visible in these soils. After collection, soil was transported on ice back to the lab, fan-dried at room temperature for 24 – 48 hours, ground, and passed through a 2mm sieve before being stored for analysis. For site characterization of each field (Table 2), soil samples from two replications from each field were sent to University of Delaware Soil Test Lab for routine soil analysis including pH_{water}, soil organic matter (SOM) by loss on ignition, Mehlich3 extractable P, K and S, and estimated cation exchange capacity (CEC) by the sum of the cations.

Plant Sampling

Seed samples and yield measurements were collected by hand for all plots in 2018. Whole plants from a 6.75 m^2 area from each rep were cut 2 cm above the surface. A hanging digital field scale was used to get the total wet weight of all the plants from the harvest area. Then a subsample of 25 plants was weighed and saved for future analysis.

The 25-plant subsamples were dried at 40°C for 5-7 days until mass was constant. The beans were then threshed from the pods and any chaff was winnowed away. The dry mass of the bean seed from each 25-plant sample was then recorded. Using the ratio of dry bean weight to wet plant weight the total yield was calculated and reported on a kg/ha basis.

$$Yield\ kg/ha\ = \left(\frac{g\ dry\ seed\ 25\ plants}{g\ wet\ 25\ plant\ sample}\right) * \left(\frac{g\ whole\ wet\ sample}{area\ harvested(m^2)}\right) * \frac{10{,}000\ m^2}{ha} * \frac{1\ kg}{1000\ g}$$

In 2019, yields and seed samples were harvested by mechanical plot combine (Wintersteiger Classic, Ried im Innkreis, Austria). Bean weights and moisture content were recorded by the calibrated combine yield monitor. Yields were standardized to 13% moisture and recorded on a kg/ha basis. The S yield (kg/ha) was calculated for each plot based on the seed S content (%) as determined by XRF (following the procedures outlined below):

$$S\ yield\ (kg/ha)\ =\ yield\ (kg/ha)\ *\ \frac{seed\ S\ content\ (\%)\ by\ XRF}{100}$$

Seed S and Amino Acid Content

The total S content of the seed was determined by two methods. Subsamples of seeds from 32 plots chosen to represent a range of expected seed S contents were ground to pass a 1 mm sieve and sent to a commercial lab (Waypoint Analytics, Richmond, VA) for total S analysis using digestion followed by ICP measurement.

Seed samples from all 72 plots were analyzed for total seed S content x-ray fluorescence (XRF) analysis (Bruker Tracer 3-SD, Bruker AXS Handheld, Kennewick, WA) as follows. Prior to XRF analysis all seeds were ground in a household coffee grinder (proctor silex, F160BYR) for 90 seconds. A random subset of 10 ground seed

samples were also sieved through a 1 mm mesh sieve in order to evaluate the effect of sieving on S content by XRF analysis. There was no significant difference between sieved and unsieved samples therefore subsequent XRF S measurements were taken on unsieved samples. Prepared samples were placed in a 28mm inside diameter sample cup that was open on top and sealed with a 4μm prolene film on the bottom prior to analysis. Enough sample was used to create a layer at least 3 mm thick. This thickness was determined sufficient to provide an "infinite" absorption of x-rays based on our preliminary analyses and others' research (Towett et al., 2016; Sapkota et al., 2019). A 60g solid material cylinder 17mm in diameter was placed on the sample to uniformly compact it. Spectra were created using a voltage of 15 kev, an anode current of 25μA and a pulse length of 200. The readings were taken under a vacuum of <5 torr to reduce air attenuation (Towett et al., 2016; Sapkota et al., 2019).

Spectra files were generated using SP1XRF software (Bruker, 2008) and were downloaded as .csv files which were then loaded, into the CloudCal software (Drake, 2018). The Lucas Tooth model built into CloudCal (Lucas-Tooth and Pyne, 1963; Drake, 2018) was used to normalize the XRF data taking into account non-linear inter-element effects to predict S content values. The S photon counts as generated by the XRF where then converted to predicted S (%) values using an already developed calibration model. The model for the relationship between predicted S (%) by XRF and observed S (%) by ICP was: $y=307+0.896x$, $R^2=0.89$, N= 88 (Chapter 3, Rushovich and Weil, 2020, Figure 2).

A subset of 32 samples representing all four fields where common dry beans were grown in 2018 and 2019 were sent for amino acid analysis (AAA) to the Molecular

Structure Facility of the University of California, Davis, CA. Out of the 32 samples, 24 samples were sent from the 2018 site years (16 from the 2018 coarse experiment and 8 from the 2018 fine experiment) and 8 samples from the 2019 site years (4 from the 2019 coarse experiment and 4 from the 2019 fine experiment). These samples were chosen to represent the full range of seed S content as measured by ICP and XRF. After de-lipidization, the amino acids were determined with two separate analyses using a Hitachi L8900 Amino Acid Analyzer (Hitachi, USA, Santa Clara, CA) with post-column, ninhydrin derivatization. The first analysis run quantified all the common amino acids except for MET+CYS and Tryptophan (TRP). The second analysis run was performed on a separate oxidized aliquot of each sample and detected cysteic acid (which is the combination of cysteine and cystine) and met-sulfone (oxidized/hydrolyzed stable form of methionine). Results for each amino acid were expressed as a percent of the extracted protein (g amino acid/100 g protein). The crude protein content of the samples was calculated from total N content as determined on separate subsamples by high-temperature combustion/gas chromatography (LECO, St. Joseph, MI) as recommended by Tabatabai and Bremner, (1991) and an N-to-protein conversion factor for common dry beans of 6.25 (FAO, 2003) as:

$$g\ total\ protein\ /\ g\ seed\ = \frac{g\ N}{g\ dry\ matter} * \frac{6.25\ g\ common\ bean\ protein}{g\ N}$$

and the content of the amino acid in the seed was calculated as:

$$g\ amino\ acid\ /100g\ seed\ = \frac{reported\ \%\ amino\ acid}{100} * Crude\ Protein * 100g$$

The same subset of 32 samples were also analyzed for total seed S content (%) by acid digestion and ICP (Waypoint Analytical Labs, Richmond, VA). The N/S ratio was

also calculated for each of the 24 samples as a potential indicator S deficiency (Hitsuda et al., 2004)

Data Analysis

Effects of S treatments on response variables (yield, seed S concentration, S yield, and MET+CYS concentration) were determined by a split plot ANOVA in R, using the 'agricolae' package (de Mendiburu, 2020). Broadcast gypsum application was the main plot factor and foliar Epsom salt application was the subplot factor, with both factors considered to be fixed effects. Replications were considered to be random effects. Unless otherwise indicated, a significance level of $\alpha = 0.05$ was used to determine significant differences between treatments. An F-protected post hoc Tukey HSD test was conducted to determine significance levels between groups. All statistical analyses were performed using R for mac (R Core Team, 2019).

A linear regression was used to determine how well seed S content by ICP and XRF predicted Crude Protein. Additionally, a linear regression was used to predict how well seed S content by ICP and XRF, seed N content, and N:S ratio predicted seed MET+CYS content (g/100g seed).

Results

The coarse sites used for this experiment had average pH values of 5.8 (A1), 5.75 (A2), and 6 (B), average SOM values of 0.95 (A1), 0.72 (A2), and 0.3 (B), and average Mehlich3 extractable S content of 9.9 (A1), 9.2 (A2) and 7.3 (B). The fine sites had average pH values of 6.05 (A1), 6.1 (A2), and 5.6 (B), average SOM values of 2.32 (A1), 1.5(A2), and 1.075 (B), and average Mehlich3 extractable S content of 10.15 (A1), 9.7 (A2) and 7(B). The average yield response to S application on the coarse textured sites

was 567 kg/ha and the average yield response on the fine textured sites was 136 kg/ha. There was no significant relationship between Mehlich3 extractable S and pH or SOM response in the coarse sites. There was no significant relationship between Mehlich 3 and pH for the fine sites. There was a significant relationship between Mehlich 3 extractable S and SOM for the fine sites ($p<0.05$, $R^2=0.82$). For all the fields combined there was no relationship between Mehlich3 extractable S and yield response. There was a significant positive relationship between SOM and Mehlich3 extractable S content ($p<0.05$, $R^2=0.37$) (Figure 6).

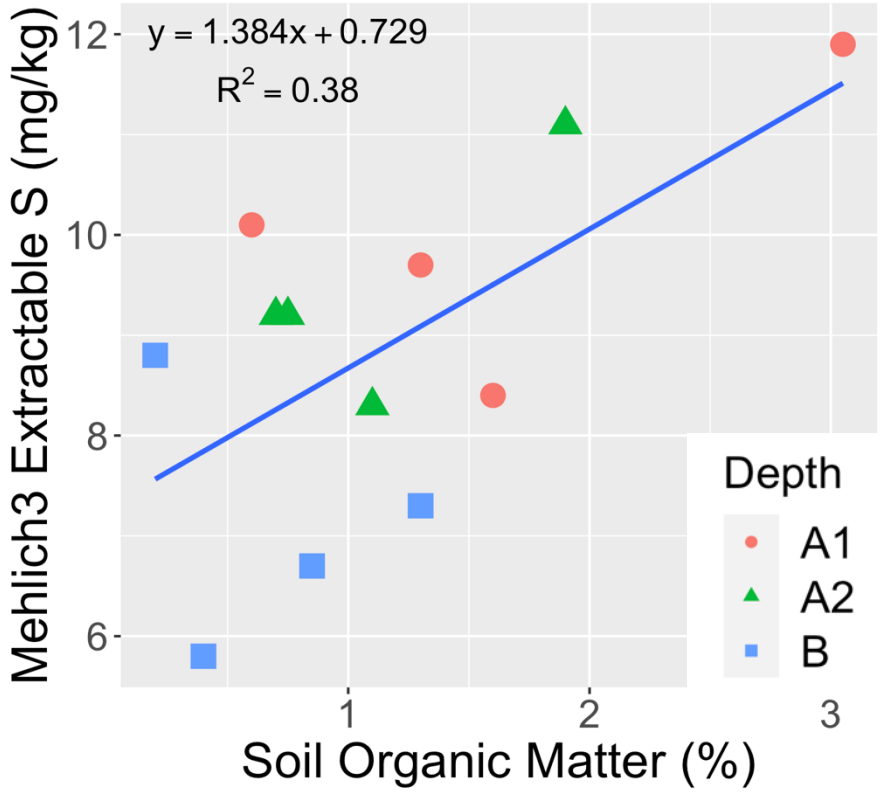


Figure 6 soil organic matter content and Mehlich3 extractable S content for three soil depths (0-10 cm, 10-bottom of A, bottom of A to 30 cm). Samples represent averages of samples taken from 3-4 reps in each of four site years used for replicated experiments. Soil organic matter content was determined by loss on ignition.

Yield Results

Yields for common dried beans grown in this study ranged from 408-2960 kg/ha. There was no significant foliar S effect or broadcast x foliar interaction effect on any of the site years. There was a significant increase from broadcast S application on the 2018 coarse field ($p<0.05$). On the 2018 coarse field there was a 46% increase in yield from the broadcast S application (Table 10).

Table 10 Split plot ANOVA results for common dry bean yields(kg/ha) from four site years grown in 2018-2019 at the Central Maryland Research and Education Center in Beltsville and Upper Marlboro, MD. Whole plot treatment was with or without broadcast s applied as gypsum at the time of planting at a rate of 560 kg/ha (B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at first flower at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences.

		Coarse Soil	Fine Soil	Coarse Soil	Fine Soil
		2018		2019	
		Common Dry Bean Yield, kg/ha			
Broadcast S (Whole plot effect)	B0	1630b	825	1613	1508
	B1	2389a	883	1674	1626
Foliar S (subplot effect)	F0	2113	963	1617	1509
	F1	1906	744	1670	1624
Broadcast S x Foliar S	B0F0	1570	1054	1623	1494
	B0F1	1691	596	1603	1523
	B1F0	2243	873	1610	1525
	B1F1	2536	894	1738	1726
Source of Variation	**Df**			**P>F**	
Rep	5	0087	**0.024**	0.165	0.255
Broadcast S	1	**0.014**	0.304	0.486	0.297
Error a	5				
Foliar S	1	0.309	0.215	0.502	0.319
Broadcast S x Foliar S	1	0.662	0.180	0.362	0.452
Error b	10				

Seed S Content Results

Seed S content for common dry beans grown over the four site years ranged from 0.231-0.323% S. Out of the four site years there was a positive main effect of broadcast S on two site years, a negative main effect of foliar S on one site year, a

positive main effect of foliar S on one site year, and a broadcast x foliar interaction effect on one site year. Broadcast S significantly increased seed S content on both 2019 site years ($p<0.05$) by around 8%. In the 2019 coarse soil site year foliar and broadcast S applied separately each increased seed S concentration, but only in the absence of the other. The seed S for the combined broadcast x foliar S treatment was 12% higher than the control for the 2019 coarse site year (Table 11).

Table 11 Split plot ANOVA results for common dry bean seed S content(%) from four site years grown in 2018-2019 at the Central Maryland Research and Education Center in Beltsville and Upper Marlboro, MD. Whole plot treatment was with or without broadcast s applied as gypsum at the time of planting at a rate of 560 kg/ha (B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at first flower at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences.

		Coarse Soil 2018	Fine Soil 2018	Coarse Soil 2019	Fine Soil 2019
		Seed S content (%)			
Broadcast S (Whole plot effect)	B0	0.263	0.292	0.273b	0.279b
	B1	0.268	0.300	0.295a	0.303a
Foliar S (subplot effect)	F0	0.270a	0.296	0.277b	0.287
	F1	0.263b	0.296	0.291a	0.295
Broadcast S x Foliar S	B0F0	0.269	0.291	0.262b	0.270
	B0F1	0.258	0.293	0.285a	0.288
	B1F0	0.271	0.301	0.292a	0.303
	B1F1	0.267	0.299	0.297a	0.303
Source of Variation	**Df**			**P>F**	
Rep	5	0.793	0.271	0.607	0.773
Broadcast S	1	0.597	0.180	**0.001**	**0.034**
Error a	5				
Foliar S	1	**0.058**	0.969	**0.007**	0.274
Broadcast S x Foliar S	1	0.619	0.593	**0.044**	0.275
Error b	10				

Sulfur Yield

Sulfur yields for the four site years ranged from 1.26-7.69 kg-S/ha. There was no significant main effect from foliar S or broadcast x foliar S interaction effect on

any of the four site years. There was a significant main effect from broadcast S on three out of the four site years. Broadcast S increased S yield by 12-48% (Table 12).

Table 12 Split plot ANOVA results for common dry bean S yield (Kg-S/ha) from four site years grown in 2018-2019 at the Central Maryland Research and Education Center in Beltsville and Upper Marlboro, MD. Whole plot treatment was with or without broadcast s applied as gypsum at the time of planting at a rate of 560 kg/ha (B1 or B0) and the subplot treatment was with or without foliar S applied as Epsom salt at first flower at a rate of 86 kg/ha (F1 or F0). Lowercase letters indicate significant differences.

		Coarse Soil	Fine Soil	Coarse Soil	Fine Soil
		2018		2019	
		S yield (Kg-S/ha)			
Broadcast S (Whole plot effect)	B0	4.30b	2.39	4.40b	4.18b
	B1	6.40a	2.64	4.93a	4.93a
Foliar S (subplot effect)	F0	4.81	2.83	4.86	4.31
	F1	5.77	2.2	4.47	4.79
Broadcast S x Foliar S	B0F0	4.22	3.03	4.24	4.01
	B0F1	4.38	1.75	4.56	4.35
	B1F0	5.60	2.62	4.71	4.63
	B1F1	6.88	2.65	5.16	5.23
Source of Variation	**Df**		**P>F**		
Rep	5	**0.040**	**0.017**	0.111	0.234
Broadcast S	1	**0.007**	0.111	**0.037**	**0.040**
Error a	5				
Foliar S	1	0.303	0.223	0.133	0.130
Broadcast S x Foliar S	1	0.645	0.203	0.786	0.654
Error b	10				

Amino Acid Composition

Seed MET+CYS content ranged from 0.070-0.180 g/100 g seed. There was no significant effect of applied S on any of the four site years.

The linear regression analysis between seed S content and crude protein was significant ($p<0.05$) for seed S content measured by XRF and seed S content by ICP with R^2 values of 0.35 and 0.44 respectively. Additionally, seed S content by ICP and XRF were both significantly correlated ($p<0.05$) with MET+CYS content (g/100 g seed) with R^2 values of 0.22 and 0.27, respectively. Seed N content was significantly correlated

($p<0.05$) with MET+CYS content (g/100 g seed) with an R^2 value of 0.59 and the N/S Ratio was significantly correlated ($p<0.05$) with MET +CYS content (g/100 g seed) with an R^2 value of 0.13 (Figure 7).

Table 13 ANOVA results for MET + CYS content (g/100gseed) and MET+CYS yield (kg MET+CYS/ha) for a set of 32 black bean samples from four site years grown at CMREC Beltsville and Upper Marlboro during 2018-2019. S1= plots that received broadcast S as gypsum applied at a rate of 100 kg-S/ha at the time of planting or combination of broadcast S and foliar S and S0= no S applied. Lower case letters denote statistical significance between treatments for year-soil combination at p<0.1 level from post hoc Tukey HSD test.

	Coarse Soil	**Fine Soil**	**Coarse Soil**	**Fine Soil**
	2018		**2019**	
	MET+CYS Content			
	---------- g/100 g seed ----------			
Treatment	n=4	n=4	n=2	n=2
S0	0.104	0.144	0.136	0.107
S1	0.098	0.141	0.139	0.096
P>F	0.523	0.856	0.201	0.855

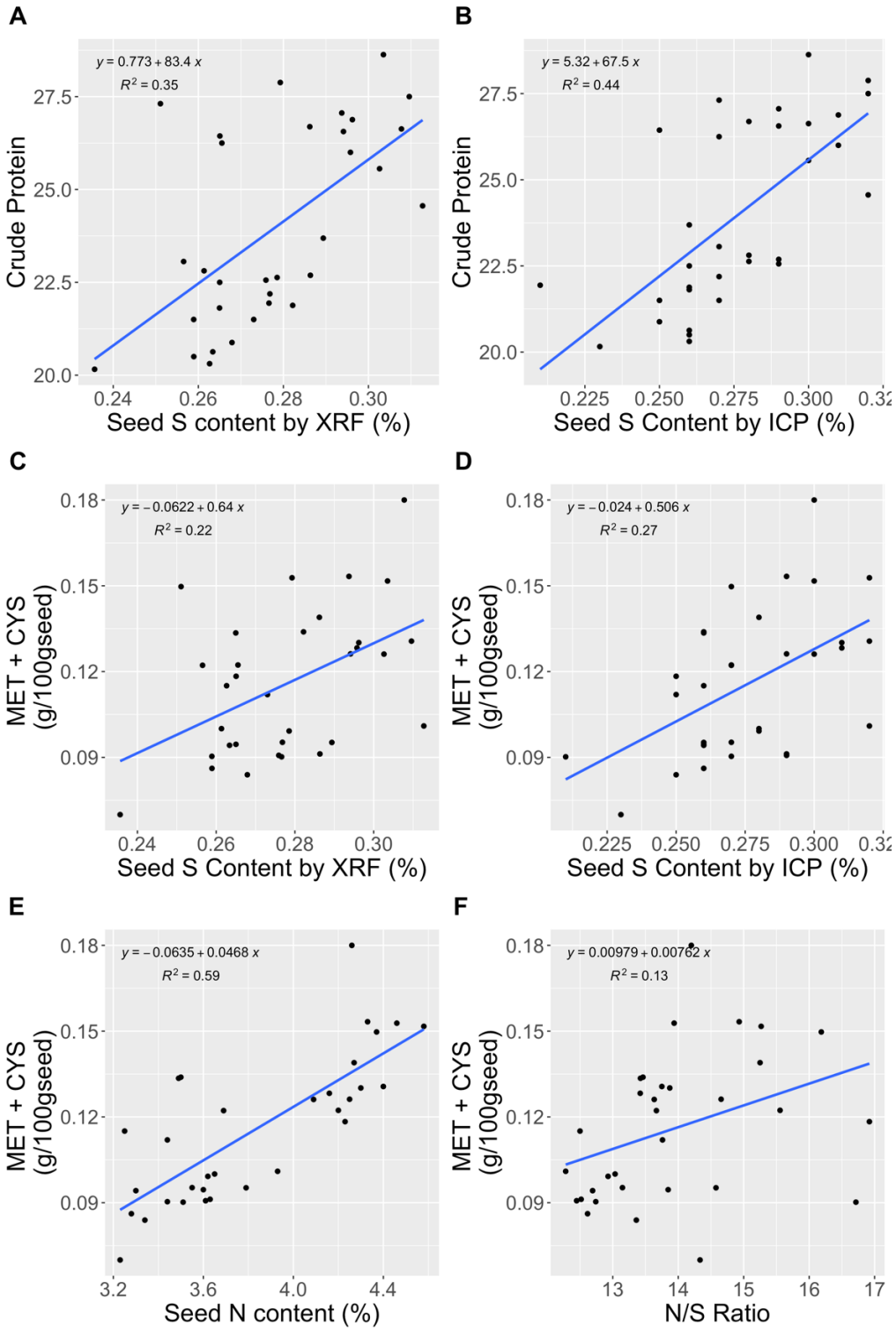


*Figure 7. Relationship between Crude Protein content and seed S content by XRF (A) and ICP (B). Crude Protein calculated as total N % (by high temperature combustion) * 6.25 (top left). Linear Relationship between MET+CYS content (g/100gseed) and seed S by XRF (C), seed S by ICP (D), seed N content (E), and N/S ratio (F.) Data are for a total of 32 common dry bean samples grown over four site years at CMREC Beltsville and Upper Marlboro in 2018-2018. All samples were analyzed for MET+CYS content (expressed as g/100g seed) by HPLC and for S in the seed by ICP and by XRF. All relationships are significant at p<0.05.*

Discussion

Except for the Upper Marlboro site, the yields of the common dry beans grown in this study were comparable to average yields of common dry beans grown throughout the US which are approximately 2,000 kg/ha. All four site years used in the experiment had Mehlich3 soil test values below the critical values identified by prior research of 18-22 mg/kg (Table 7), however there was only a significant yield response in the 2018 coarse field. (Soil Fertility Management, 2010; Seth et al., 2018; Chapter 5, This thesis). These results are in agreement with prior research that suggests that the Mehlich3 soil test does not accurately predict where crop responses will occur (Sims, 1989; Sahrawat et al., 2009; Ketterings et al., 2011; Kowalenko et al., 2014).

Soil type and S present in the soil may have also had an effect on S uptake and translocation within the plant. The significant positive correlation with SOM may be a useful indicator when predicting where to apply S, as S deficiency is more likely to occur on coarse soils with lower SOM that are susceptible to S leaching. Prior research has also used an N/S ratio of greater than 16-18 to identify S deficiency on common dry beans and soybeans (Ligero and Lluch, 1982; Hitsuda et al., 2004; Orman and Kaplan, 2017). One study found that common dry bean plants grown in a low S solution produced seeds with N/S = 19 while seeds grown in a high S solution the N/S ratio =16. Common dry beans grown for this study had N/S ratios between 12-17 suggesting that the plants were not severely S deficient.

The lack of yield response at three out of the four site years to applied S was likely due to several abiotic factors other than S deficiency that occurred throughout the growing season. The 2018 growing season was unseasonably wet with cumulative

rainfall significantly higher than the historic average for most of the growing season (100.4 cm vs 66.0 cm average). The wet conditions interfered with mechanical weed control at the Upper Marlboro site which was under organic management. Due to the nearly constant wet conditions in the spring, cultivation was delayed and failed to kill many of the weeds. To mitigate the excessive weed pressure, plots were hand weeded in early August, but by then bean yield was most likely already reduced by weed competition. Common dry beans are not efficient competitors against weeds and production typically relies heavily on either chemical herbicides or mechanical interventions to maintain weed-free conditions during the critical 3-6 weeks after planting (Burnside et al., 1998).

The common dry beans at Upper Marlboro may have also experienced N deficiency and osmotic stress. Due to certified organic management protocols, the Upper Marlboro plots did not receive any applied N fertilizer when the lack of good root nodulation was discovered. As a result, growth of the beans at the Upper Marlboro site were likely limited by weed competition and N deficiency, thus restricting the possibility of positive responses to S application. Additionally, the timing of the foliar application at Upper Marlboro (which occurred later than at Beltsville in 2018) coincided with a period of very hot (>30°C) and dry conditions. The reduction in yield and seed S content from the foliar S treatment may have been a result of osmotic stresses incurred from the Epsom salt foliar spray even though foliar symptoms of salt burn were not observed. Common dry beans are particularly sensitive to salt stress which can lead to reduced yields (Geetanjali and Garg, 2008; Çiftçi et al., 2011). In the future, we suggest trials of a

somewhat lower rate of 8 kg/ha-S to determine the effect of foliar S on yield and seed S content with a lower risk of osmotic stress to the plant.

In contrast, the 2019 growing season experienced average rainfall from May through mid-August and below average rainfall after August with an extended drought period from mid-August through late October. The beginning of the 2019 drought period also coincided with several days of high temperatures (above 30°C) and extremely high temperatures (above 35°C). It is unlikely that the 2019 sites experienced osmotic stress as the Epsom spraying in 2019 occurred before the drought period that began in mid-August but the prolonged drought likely caused water to be a limiting factor during the plants reproductive growth stages, thus limiting the possibility of a response to S application. Prior research on fava beans suggests that the time and length of water stress has an effect on overall protein quality (Barłóg et al., 2019).

Although there was only a yield response in one site year, there was a significant seed S content and S yield response in three out of the four site years. These results are in agreement with prior research done by Pandurangan et al. (2015) that found no significant effect on yield of common dry beans grown under low and high S treatments both in the field and under controlled conditions but did find a significant effect on seed S concentrations.

Prior research has shown that while concentration of a nutrient in plant tissue usually responds positively to increased supply of that nutrient, the nutrient content of the mature crop may be influenced by the availability of all nutrients in the soil (Wortmann et al., 2018). In agreement with prior research (Naeve and Shibles, 2005) it appears that applying S early in the season is important to allow the plant adequate time during the

growing season to incorporate the nutrient into the seed. In agreement with the results seen in soybeans, where soybean yields were positively increased with foliar S application while seed S content was not, (Chapter 3, Rushovich and Weil, 2020), it appears that the foliar S application may not have been well-timed to improve the S concentration of the seed (Sunarpi and Anderson, 1997; Paek et al., 2000; Naeve and Shibles, 2005). Research on the early-season S requirements of eight different crops (including common dry beans and soybeans) found that S applied early to groundnuts (*Arachis hypogaea)* significantly increased yield and dry weight, but S applied at the beginning of the reproductive stage did not (Hago and Salama, 1987; Hitsuda et al., 2005). This suggests that common dry beans may not be able to efficiently incorporate S applied as a foliar spray mid-season into the seed. A good S supply throughout the entire growing season, as provided by a broadcast soil amendment, may be necessary to encourage enhanced seed S content of common dry beans.

Common dry bean MET+CYS content was not significantly affected by S application. These results are in contrast to the results seen in soybeans that showed a significant increase in MET+CYS content and an almost doubling in MET+CYS content from foliar S application (Chapter 3, This Thesis). The dramatic results seen in soybeans warrant further research on other varieties of legumes. As the soybean results suggest that legume seed MET+CYS content is variable and susceptible to improvement through S fertilization, which could impact human and animal nutritional health.

One significant factor limiting research and implementation of improved seed MET+CYS content is the difficulty of analyzing total S and MET+CYS content of the seed. Amino acid analysis is an expensive, time-consuming process that cannot easily be

implemented on a large scale. Even analysis of total S is fairly expensive and time-consuming; it can take a week or longer to get results and cost approximately $20 each to send samples to a commercial lab to digest and run ICP analysis. This project has further confirmed the results presented in the previous chapter that the XRF is an appropriate replacement tool for ICP to measure total S content in a rapid, nondestructive manner. Further the significant correlations between seed S content by XRF and ICP and crude protein and MET+CYS content warrant further research to refine a calibration equation that could use total S as a proxy measurement for crude protein and MET+CYS concentration. This would allow for market differentiation that could compensate farmers for higher quality products. This rapid testing method could thus incentivize farmers to improve their fertility practices to ultimately improve the nutritional quality of the crops they are growing.

Conclusion

The literature on S fertilization has been focused mainly on soybeans and cereal grains (which generally have higher MET+CYS concentration than legumes). However, common dry beans are an important staple crop worldwide that should not be ignored. The significant yield response in one out of four site years despite similar soil test levels for all four fields suggests that there were several non-treatment factors that had an impact on the common dry bean yields in both years. However, the significant seed S content response on three out of four site years suggests that seed S content of common dry beans is variable and can be enhanced through S fertility management. These results warrant. Future research to evaluate additional S sources, methods, and rates of application that may maximize the yield, seed S content, and MET+CYS content of

common dry beans and other food legumes on low S soils. Additionally, better methods for predicting where S application is needed could have positive impacts on bean yield and protein quality.

Chapter 5: Soil Tests to Predict Crop Response to Sulfur in Mid-Atlantic Soils

Abstract

Sulfur (S) deficiency rates are increasing mainly due to higher yielding production practices and reduced atmospheric deposition of S necessitating an improved soil S test. We compared the efficacy of four candidate soil test extracting solutions using data on relative yield, relative S yield, seed S content response to S application, and yield response to S application from 122 plots across 23 fields growing soybeans (*Glycine Max)* and common dry beans (*Phaseolus vulgaris*) in the mid-Atlantic coastal plain region of the Eastern US. The four extracting solutions compared were 0.01*M* calcium chloride ($CaCl_2$), 0.002*M* calcium phosphate ($Ca(H_2PO_4)_2$) in water (CaP), 0.002*M* $Ca(H_2PO_4)_2$ in 2*M* acetic acid (HOAc) (acid CaP) and Mehlich 3. Soil samples down to a depth of 30 cm from each plot were divided into three segments (0-10 cm, 10- bottom of A horizon, bottom of A horizon – 30 cm). Each segment was analyzed separately as well as combined to form a weighted average S content for the entire sampling depth. A Cate-Nelson analysis was performed to identify the critical level above which a response to applied S would be unlikely. Using the entire 0-30 cm sample depth, the critical values from the Cate-Nelson analysis based on 90% of relative yields were 26, 13.2, 14.7 and 16.2 mg S/kg soil for $CaCl_2$, CaP, acid CaP, and Mehlich3, respectively. The CaP extraction correctly identified 90% of sites based on the critical X value from the Cate Nelson analysis. These results suggest that CaP extracts are better able to predict where S is needed than Mehlich3, the current standard in MD.

Introduction

Effective management of sulfur (S) fertility with S-containing amendments would be greatly aided by a reliable and accurate soil test for S. A reliable S soil test would provide values that correlate with plant uptake of S and identify fields where crops are likely to respond positively to S application. Sulfur becomes plant available mainly by the release of sulfate (SO_4^{2-}) ions and soluble organic S compounds from decomposition of soil organic matter and by the desorption or dissolution of sulfate ions from iron and aluminum oxide coated clay surfaces. A reliable soil S test should dissolve an amount of S that is related to what could become available throughout the growing season from both of these sources in the soil (Ketterings et al., 2011).

Plant- available S concentration in the soil is affected by atmospheric deposition, decomposing organic material, fertilizer inputs, S leaching, plant uptake, and microbial activity. Plants take up S as sulfate (SO_4^{2-}), which is relatively mobile in the soil and easily leached down the soil profile. Sulfur in the surface soil horizons (A horizon) is generally mineralized from organic material, which includes both humus (stabilized soil organic matter) and recent crop residues left on or in the surface soil (Schoenau, 2008). The rate at which this mineralization occurs is moderated by temperature, pH, moisture, aeration and the C:S ratio of the material (Weil and Brady, 2017). The plant-available SO_4^{2-}, including both SO_4^{2-} in the soil solution and SO_4^{2-} adsorbed onto mineral surfaces, accounts for less than 5% of the total S in humid region soils (Scherer, 2009).

Soil testing for S has lagged behind other essential macronutrients for several reasons. One, until relatively recently, farmers received agronomically sufficient S from impurities in common fertilizers (mainly ammonium sulfate and superphosphate) and atmospheric deposition from sulfur dioxide emissions from coal fired power plants, so S

deficiency was a relatively uncommon phenomenon. After the implementation of air pollution control policies in many industrialized countries, S atmospheric deposition was drastically reduced, especially in the northeastern United States, northern Europe, and central China (Baumgardner et al., 2002; Eriksen, 2008). Decreased atmospheric deposition coupled with higher yielding crops and chemical fertilizers with lower amounts of S impurities (such as diammonium phosphate and urea) have led to increased S removal from soil without replenishment leading to increased reports of S deficiency (Klimont et al., 2013).

Retention of plant available SO_4^{2-} relies on the anion exchange capacity of the soil which is greater in soils with higher clay and iron/aluminum oxide content (Ensminger, 1954; Reisenauer and Dickson, 1961; Metson, 1979). The sandy surface soils that are characteristic of the Coastal Plain region in the mid-Atlantic United States generally have low organic matter and low anion exchange capacity and are thought to be susceptible to S deficiency (Soil Survey Division Staff, 2017). However, SO_4^{2-} that leaches from the surface soil can be adsorbed onto subsoil clays and iron/aluminum oxides and serve as a significant source of plant available S later in the growing season (Metson, 1979). Using soil samples to a depth of 30 cm, this work will evaluate the efficacy of four soil extractions at correctly identifying where soil S application is needed.

The current standard method for S soil testing in the mid-Atlantic US is the Mehlich3 extraction which was introduced as a multi-element extracting solution in 1984 (Rao and Sharma, 1997; Sims et al., 2002; Wolf and Beegle, 2011; Ketterings et al., 2014; Seth et al., 2018). The Mehlich3 extracting solution contains multiple components that are proven to be effective at extracting both macro- and micronutrient ions across a

wide range of soils and pH conditions (Mehlich, 1984; Shahandeh et al., 2017). Most labs using the Mehlich3 soil test results report S levels as "plant-available S" and give interpretations such as "low," "medium," or "high," which would imply that critical levels had been determined by the soil test calibration studies. Mehlich3 extractability has been shown to effectively predict soil P and K supply but there is limited research evaluating its effectiveness at predicting where crops will respond to S fertilization (Sims et al., 2002).

The limited research done by Ketterings et al (2011), and Sahrawat et al. (2009) suggest that Mehlich3 extractable S is not consistently related to plant S uptake across soil types. However, more work needs to be done to evaluate the relationship between Mehlich3 extracted S and plant response to applied S. Using Mehlich3 to extract available S would be convenient and would simplify the soil test process, since it is already widely used for P, K, Ca, Mg and other nutrients. Kowalenko et al., (2014) reported that soil extracting solutions such as $CaCl_2$ and $Ca(H_2PO_4)_2$ showed more promise than Mehlich3, however they concluded that more research needs to be done before widespread adoption of any of the tested methods. The goal of this project is to identify an alternate soil test to Mehlich3 that is more consistently related to plant S uptake.

A study done on New York soils that are characterized by higher pH, SOM content, and 2:1 clay minerals than soils in the mid-Atlantic coastal plant suggested that the 0.01 *M* $CaCl_2$ extraction responded more consistently to S additions across the studied soils, as compared to extraction with 1.0 mol L^{-1} NH_4OAc, 0.016 mol L^{-1} KH_2PO_4, 0.01 mol L^{-1} $Ca(H_2PO_4)_2$, Morgan, NaOAc, and Mehlich3 extracting solutions (Ketterings et

al., 2011). The $CaCl_2$ extract also showed the most sensitivity to different types of applied S indicating a greater ability to identify S deficient soils (Ketterings et al., 2011).

Research done by Sahrawat et al (2009) on tropical soils in India, reported that $CaCl_2$ and $Ca(H_2PO_4)_2$ solutions extracted similar amounts in neutral to alkaline soils, however $Ca(H_2PO_4)_2$ extracted significantly higher S in low pH soils. They attributed this difference to PO_4^{3-} being able to replace SO_4^{2-} more readily than Cl^-, a more weakly adsorbed anion. Calcium phosphate is thought to be a suitable extracting reagent for SO_4^{2-} because PO_4^{2-} ions are more strongly adsorbed than SO_4^{2-} ions on soil mineral surfaces. An additional advantage is that Ca^{2+} ions flocculate soil colloids, making filtration easier (Sahrawat et al., 2009). Research by Reisenauer (1975) categorized extractants based on the type of S they extract. Dilute neutral salts (such as $CaCl_2$) and water typically extract only SO_4^{2-} that is readily soluble (or already part of the soil solution), but phosphate-containing extractants are able to extract adsorbed S in addition to S that is readily soluble (e.g. plant-available). This is important for soil S tests because the adsorbed SO_4^{2-} is thought to be a significant source of plant available SO_4^{2-}.

A field study done in Wisconsin evaluated the effectiveness of six different soil extractions at predicting response to S by alfalfa (*Medicago saliva)*. The tested extracts were 0.002 *M* $Ca(H_2PO_4)_2$ in water and 2M HOAc, 0.015*M* $NaH_2PO_4 \bullet H_2O$, 0.025*N* CaCl2, 0.25*M HCl* + 0.0075*M* NH_4F, and 0.05*M* H_2PO_4. The results of the experiments showed that only the $Ca(H_2PO_4)_2$ extractions were correlated with tissue S concentration in the first cutting of alfalfa and the $Ca(H_2PO_4)_2$ in HOAc was most closely correlated with yield response (Hoeft et al., 1973a). In their study, sites testing above 10 mg/kg were not likely to respond to S treatment, sites testing below 6 mg/kg were likely to

respond and sites testing between 6-10 ppm responded 27% of the time to S treatment (Hoeft et al., 1973a).

In addition to a well calibrated extracting solution, standardization of sample collection procedures is also important. Since different forms of S are prevalent in different soil horizons, depth is an important consideration for sample collection (Ensminger, 1954; Metson, 1979). For routine soil analysis, typically only the top 15 to 18 cm of soil is sampled, which is the typical tillage depth (Moebius-Clune et al., 2016). However, much of the potentially available adsorbed SO_4^{2-} may be located in deeper layers enriched in iron and aluminum oxide coated clays (Scherer, 2008; Ketterings et al., 2011). A study done on 22 fields in Argentina found that $Ca(H_2PO_4)_2$ extractable SO_4^{2-}-S levels in the subsoil horizons (20-40 cm) were better predictors for plant available S than just the surface (0-20 cm) (Calvo et al., 2009). Additionally, a study done in Iowa, USA on five different soils found that total S decreased significantly with increasing depth and was highly correlated with Organic C (Tabatabai and Bremner, 1972). These studies suggest that sampling for S just at the top 15 cm, as is commonly done, may not be telling the whole story when it comes to plant available S.

The next important step in soil test development is soil test calibration to divide the extracted S into low, medium, or high interpretative levels for making recommendations for S application (Dahnke and Olson, 1990). One method for interpreting soil test data into recommended fertilizer levels is to use the Cate-Nelson method for dividing bivariate data into four quadrants of a graph based on a critical level that separates responsive and unresponsive sites (Cate and Nelson, 1971; Dahnke and Olson, 1990). This method relies on having a wide range of test sites that include

different soil types in order to establish an accurate and widely applicable critical level. This critical level can then be used to make a fertilizer recommendation. In addition to the use of the critical level to implement a fertilizer recommendation other soil factors, crops grown, climate, yield goals, and economics should be considered (Dahnke and Olson, 1990).

The goal of the present research was to identify an effective soil test procedure and critical level for soil S in order to make appropriate fertilizer recommendations for soybeans grown in Mid-Atlantic coastal plain soils. This project aimed to develop a S soil test calibration curve using soil samples and crop response data collected over three years from 23 Maryland sites with a wide range of soil properties , The two main objectives of the project were to (1) Compare the effectiveness of four different soil extracting solutions at predicting crop response to applied S and (2) to determine the critical level for extracted soil S above which a crop response to applied S is unlikely.

The two main hypotheses were (1) $Ca(H_2PO_4)_2$ or $CaCl_2$ extractable-S will be more correlated with crop response than Mehlich3 extractable-S, the current standard in the Mid-Atlantic and (2) extractable S concentrations in both the surface and subsoil will be better at predicting crop response than extractable S from the surface layer alone.

Materials and Methods

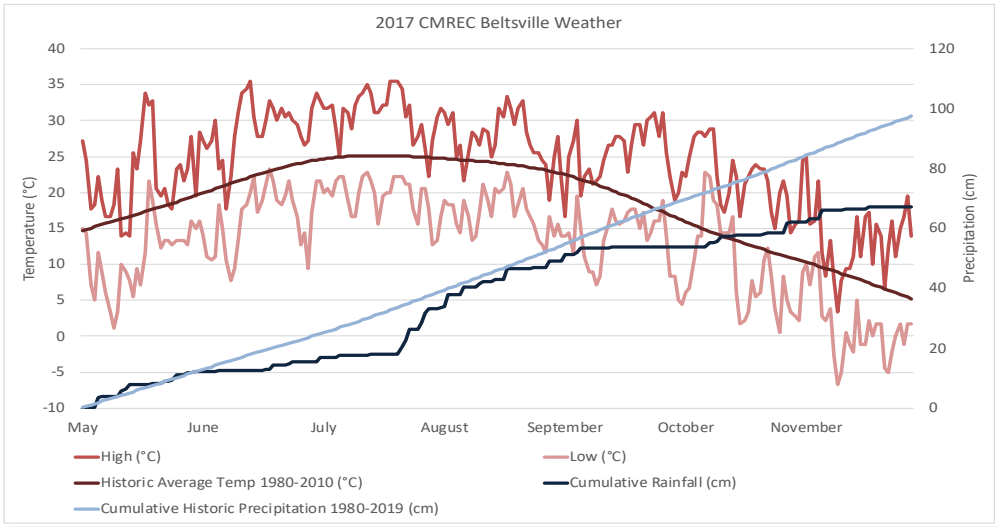
Field Sites

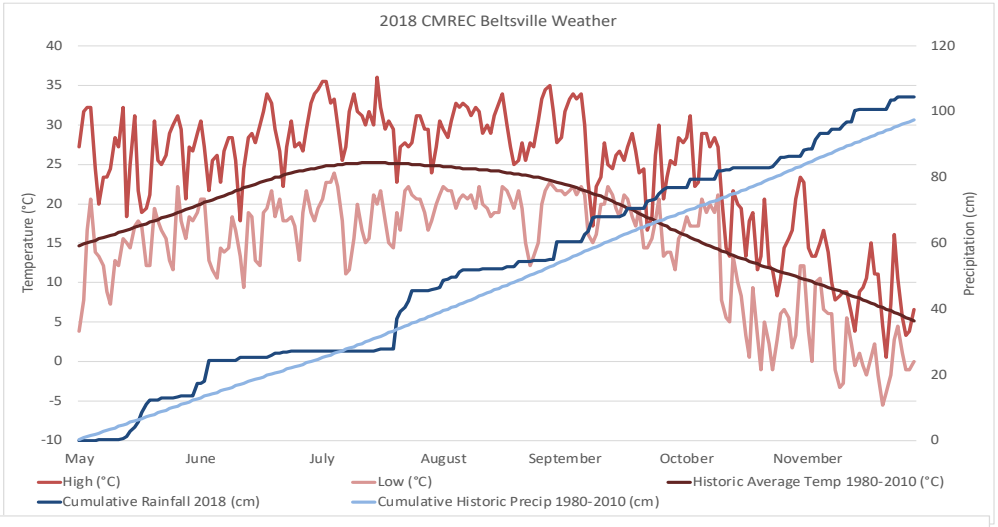
Sulfur response trials were conducted on a total of 23 fields encompassing 122 plots during the 2017, 2018, and 2019 growing season spread throughout the Central Maryland Research and Education Center (CMREC) Beltsville and Upper Marlboro locations and the Eastern Shore of Maryland at three farm collaborator fields (Appendix

1). This region has a humid temperate climate with mean annual minimum and maximum temperatures of 5°C and 25°C, respectively. On average, this location receives approximately 1075 mm of precipitation per year evenly spread among 12 months (NOAA, 2020; Figure 8). Field soil characterization information was determined by University of Delaware Soil test Lab, including pH measured in water, soil organic matter (SOM) by loss on ignition, Mehlich3 extractable phosphorous (P) and potassium (K), and estimated CEC (Table 14). All fields were located within the Northern Coastal plain region of MD in which soil parent material consists of deep fluviomarine deposits (Soil Survey Staff, 2014).

The experiments used a randomized complete block design with split plots with three to six replications per field and were planted with one of three different grain legumes: Double crop (DC) soybeans (*Glycine Max)* planted after a cereal grain (typically winter wheat; *Triticum aestivum*), full season (FS) soybeans planted after winter cover crop burn down, or common dry beans (*Phaseolus vulgaris)* (CB). Twelve of the 23 fields contained plots with and without broadcast S applied as gypsum ($CaSO_4$) around the time of planting at a rate of 560 kg/ha (100 kg S/ha). All fields contained plots with and without foliar S applied as Epsom salt ($MgSO_4$) at the beginning of the reproductive stage (R1) at a rate of 86 kg/ha (11 kg S/ha) dissolved in 150 liters of water. On fields that received both treatments broadcast S was the whole plot treatment and foliar S was the subplot treatment. Farm scale equipment was used for all planting, spraying, and harvest operations. On farm collaborator field sizes varied significantly based on the size of the field and the equipment available. Common dry bean plots

averaged 7m x 10 m. Applications to CB fields were done both by hand and with farm scale equipment.





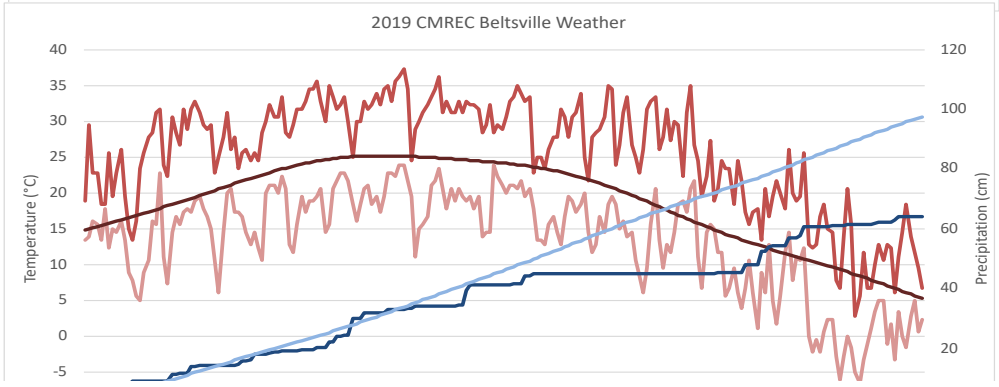


Figure 8 Weather data for the 2017, 2018, and 2019 growing seasons (May – November) for CMREC Beltsville. Includes historic average daily temperature and cumulative precipitation from the BWI weather station. Daily High and low temperature (C), and cumulative daily precipitation (cm) are shown.

Table 14. Soil characterization data for all fields used during 2017, 2018, and 2019 seasons. Soil organic matter (SOM) determined by loss on ignition (LOI), pH measured in water, Mehlich3 extractable, P and K and estimated CEC. A1=0-10 cm, A2=10- bottom of A horizon, B = bottom of A horizon– 30 cm, Mean = weighted average for the 0-30 cm sample based on the depth of the sample and the bulk density of a representative "Coarse" and "Fine" field at CMREC Beltsville

Field Code	Horizon	Soil Series	Taxonomy	pH	SOM (%)	P --(mg/kg)--	K	Est. CEC (meq/100g)
5-18O	A1	Downer	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	6.5	2.50	199	90	4.6
	A2			6.6	1.70	178	58	3.7
	B			5.5	0.85	3	43	3.1
	Mean			6.2	1.65	128	62	3.8
5-39B	A1	Downer-Hammonton Complex	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	5.6	0.60	71	74	5.0
	A2			5.7	0.75	74	60	3.2
	B		Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	6.0	0.20	24	43	2.9
	Mean			5.8	0.58	62	60	3.7
5-43A	A1	Christiana	Fine, kaolinitic, mesic Aquic Hapludults	5.5	1.25	58	124	5.2
	A2			5.5	0.55	55	59	3.9
	B			5.7	0.45	12	57	3.6
	Mean			5.6	0.72	44	77	4.2
5-43B	A1	Downer	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	5.9	1.40	60	100	4.6
	A2			5.7	0.65	62	58	3.7
	B			5.6	0.30	38	43	3.1
	Mean			5.7	0.79	56	67	3.8
5-17C	A1	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults	5.7	1.70	41	44	5.2
	A2			5.7	1.70	45	31	3.9
	B		Fine, kaolinitic, mesic Aquic Hapludults	5.7	0.40	25	30	2.7
	Mean			5.7	1.19	36	34	3.8
5-18	A1	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults	6.4	3.35	226	80	14.5
	A2			6.2	2.15	173	37	12.4
	B		Fine, kaolinitic, mesic Aquic Hapludults	6.5	1.45	68	31	8.8
	Mean			6.4	2.20	145	47	11.5
5-39B	A1	Downer-Hammonton	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	5.6	0.60	71	74	5.0
	A2			5.7	0.75	74	60	3.2
	B		Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	6.0	0.20	24	43	2.9
	Mean			5.8	0.53	57	58	3.6
UMBB	A1	Annapolis-Donlonton	Fine-loamy, glauconitic, mesic Typic Hapludults	6.3	3.05	41	151	11.7
	A2			6.3	1.90	24	75	9.8
	B		Fine-loamy, glauconitic, mesic Aquic Hapludults	5.1	1.30	14	90	11.4
	Mean			5.9	2.03	26	98	11.0
5-25C	A1	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults	6.2	2.35	181	83	7.0
	A2			6.1	1.15	113	36	5.9
	B			5.9	0.85	10	37	5.4

	Mean		Fine, kaolinitic, mesic Aquic Hapludults	6.1	1.36	90	49	6.1
5-39A	A1	Downer	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	6.7	1.15	90	91	3.9
	A2			6.6	0.45	109	51	2.4
	B			6.8	0.20	45	53	1.7
	Mean			6.7	0.56	83	63	2.5
5-40	A1	Downer-Hammonton	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	6.2	1.50	74	28	4.3
	A2			5.8	0.70	103	22	3.3
	B			5.9	0.18	76	24	2.7
	Mean		Coarse-loamy, siliceous, semiactive, mesic Aquic Hapludults	5.9	0.77	87	24	3.4
5-43A	A1	Christiana	Fine, kaolinitic, mesic Aquic Hapludults	5.8	1.55	68	42	5.2
	A2			5.8	0.75	68	34	3.7
	B			6.1	0.30	29	33	3.0
	Mean			5.9	0.83	55	36	3.9
5-39A	A1	Downer	Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults	6.0	1.30	57	94	5.1
	A2			5.8	0.70	79	67	4.0
	B			6.0	0.40	28	68	3.4
	Mean			5.9	0.77	57	75	4.1
5-7A	A1	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults	5.8	1.60	34	109	5.7
	A2			5.9	1.10	18	74	4.6
	B			6.1	0.85	6	91	4.7
	Mean		Fine, kaolinitic, mesic Aquic Hapludults	5.9	1.15	18	90	4.9
5-7F	A1	Russett-Christiana	Fine-loamy, mixed, semiactive, mesic Aquic Hapludults	6.2	2.55	20	67	6.2
	A2			6.4	1.30	9	36	4.7
	B			6.3	1.05	2	31	5.4
	Mean		Fine, kaolinitic, mesic Aquic Hapludults	6.3	1.55	9	43	5.4
DS1	A1	Hambrook-Woodstown	Fine-loamy, mixed, active, mesic Aquic Hapludults	6.2	0.85	99	106	3.9
	A2			6.1	0.70	100	63	3.5
	B			6.2	0.40	53	64	2.6
	Mean			6.2	0.64	83	75	3.2
JL1	A1	Hambrook	Fine-loamy, siliceous, semiactive, mesic Typic Hapludults	6.7	1.75	94	173	6.5
	A2			6.6	0.85	40	111	4.3
	B			6.6	0.60	12	87	3.6
	Mean			6.6	1.01	45	119	4.7
SK1	A1	Queponco	Fine-loamy, mixed, semiactive, mesic Typic Hapludults	5.6	2.00	156	181	7.0
	A2			6.3	1.10	201	130	4.9
	B			5.9	1.00	43	143	5.3
	Mean			5.9	1.34	155	147	5.6

Soil and Seed Sampling

Soil samples were collected near the time of soybean planting, but before any S treatments were applied. Four 30 cm deep cores were collected from each control (no S added) plot in 2017 and 2018 and twelve 30 cm deep cores were collected from each rep in 2019 using a 1.8 cm cutting diameter push probe and divided into three segments referred to hereafter as A1, A2, and B (0-10 cm, 10cm- bottom of the A horizon, and bottom of A horizon to 30 cm). The length of each core segment was recorded and the segments from each depth increment were composited within each replication. Thus, A1 was always 10 cm deep, but the depth and thickness of the A2 and B samples varied with depth of the genetic A horizon boundary, which was easily visible in these soils. After collection, soil was transported on ice back to the lab, fan-dried at room temperature for 24 – 48 hours, ground, and passed through a 2mm sieve before being stored for analysis. The depth to B horizon (increment thickness of the A2 and B samples), along with a representative bulk density (BD) measurement determined for one coarse textured and one finer textured field at CMREC Beltsville were used to calculate a weighted average of soil per depth increment. An example calculation of the weighted average is shown below:

1. $\frac{kg\ soil}{core\ segment} = BD\left(\frac{g}{cm^3}\right) * volume\ soil\ (cm^3) * \frac{1\ kg}{10000g}$

2. $\frac{mg\ S}{core\ segment} = \frac{mg\ S}{kg\ soil} * BD\left(\frac{g}{cm^3}\right) * volume\ soil(cm^3) * \frac{1\ kg}{1000}$

3. $Weighted\ average\ soil\ S\ (0-30cm) = \frac{mg\ S\ A1+mg\ S\ A2+mgS\ B}{kg\ soil\ A1+kg\ soil\ A2+kg\ soil\ B}$

Measurements of yield and seed moisture content were made for each plot by calibrated combine yield monitor. Yields were all standardized to 13% moisture. In 2017 and 2018, seed samples for S analysis were collected manually by cutting all plants in three, 3-m sections of row per plot and then sub sampling 25 plants per plot to be threshed and winnowed for the seed sample. In 2019, seed samples for analysis were collected at CMREC Beltsville from the combine. Seed samples from collaborating farmers' fields were obtained by mechanically threshing and subsampling seed from 100 plants collected randomly throughout the plot.

Analysis of Seed S Content

All seed samples were analyzed for total S content using x-ray fluorescence (XRF) analysis (Bruker Tracer 3-SD, Bruker AXS Handheld, Kennewick, WA) as follows. Seeds were ground in a household coffee grinder (Proctor Silex, E160BYR) for 90 seconds prior to XRF analysis. A random subset of 10 ground samples were also ground to pass through a 1 mm mesh sieve in order to evaluate the effect of sieving on S content by XRF analysis. There was no significant difference between sieved and unsieved ground samples therefore subsequent XRF S measurements were made using unsieved samples. Samples were placed in a 28 mm inside diameter sample cup that was open on top and sealed with a 4μm thick prolene film on the bottom before analysis. Enough sample was used to create a layer at least 3 mm thick. This thickness was determined to be sufficient to provide an "infinite" absorption of x-rays based on our preliminary analyses and others' research (Towett et al., 2016; Sapkota et al., 2019). A 60g solid cylinder 17mm in diameter was placed on the sample to uniformly compact it. Spectra were created using a 120-second irradiation period with a voltage of 15 kev, an

anode current of 25μA and a pulse length of 200. The readings were taken with the instrument head under a vacuum of <5 torrs to reduce air attenuation (Towett et al., 2016; Sapkota et al., 2019).

Spectra files were generated using SP1XRF software (Bruker, 2008) and were downloaded as .csv files. Spectra files for 88 plant tissue samples, including 24 soybean seed samples from this study, were then loaded, along with corresponding S values from ICP analysis into the CloudCal software (Drake, 2018) website to generate calibration curves. The Lucas Tooth model built into CloudCal (Lucas-Tooth and Pyne, 1963; Drake, 2018) was used to normalize the XRF data taking into account non-linear inter-element effects to predict S content values. The linear calibration model previously established (Figure 2) was used to convert XRF photon counts to S percent values for each sample ($y=307+0.896x$, $R^2=0.89$) (Chapter 3, This Thesis).

Soil Extraction procedures

Soil S levels were analyzed using four different extraction methods (Table 15). The four extraction methods used were (1) 0.01 *M* $CaCl_2$ (CaCl2) (2) 0.002 *M* $Ca(H_2PO_4)_2$ in water (CaP) (3) 0.002 *M* $Ca(H_2PO_4)_2$ in 2 *M* HOAc (acid CaP) and (4) Mehlich3. For the CaCl2 extraction, 25 ml of solution was added to 5 grams of soil, the solution was then agitated for 30 min at 180 rpm on a shaker table, then left to settle for 15 min before being filtered through Whatman no. 42 filter paper. For the CaP and acid CaP extractions, 25 ml of solution was added to 10 g of soil, the solution was then agitated at 180 rpm for 30 min, left to settle for 15 min before filtering through Whatman no. 42 filter paper. For the Mehlich3 extraction 20 ml of solution was added to 2 g of soil,

the solution was then agitated for 5 minutes at 180 rpm and then immediately filtered through Whatman no. 41 filter paper.

After extraction 10-12 ml of each extracted solution were transferred to a 15 ml centrifuge tube and then frozen. All the frozen samples along with 500 ml of the extracting solution used were then sent with freezer-packs in an insulated box to the Pennsylvania State University Agricultural Analytical Services Laboratory in University Park, Pennsylvania to be analyzed for total S content by ICP.

Table 15. Characteristics of the three methods used for sulfur soil testing.

Extractant	Soil : Solution Ratio (g:ml)	Extraction Shake Time	Shake Speed	Abbreviation	Reference	S Analysis Method
0.01 *M* $CaCl_2$	1:5	30 min	180	CaCl2	(Williams and Steinbergs, 1959)	ICP
0.002 *M* $Ca(H_2PO_4)_2$ in water	1:2.5	30 min	180	CaP	(Singh et al., 1995)	ICP
0.002 *M* ppm $Ca(H_2PO_4)_2$ in 2*M* HOAC	1:2.5	30 min	180	Acid CaP	(Hoeft et al., 1973b)	ICP
Mehlich3	1:10	5 min	180	Mehlich3	(Mehlich, 1984)	ICP

Calculations

For each plot yield, seed S content by XRF (%), and S yield were measured. Four response variables (relative yield, relative S yield, yield response, and S response) were then calculated based on yield and S yield. Example calculations shown below:

$$\textbf{S yield } (\frac{kgS}{ha}) = seed\ S\ content \frac{mg\ S}{kg} * seed\ yield\ \frac{kg}{ha} * \frac{1\ kg}{1\ x\ 10^6\ mg}$$

$$\textbf{Relative Yield} = \frac{Yield\ of\ Control\ Plot\ (\frac{kg}{ha})}{Highest\ yield\ for\ that\ Crop\ x\ Year}$$

$$\textbf{Relative S Yield} = \frac{S\ yield\ of\ Control\ Plot\ (\frac{kgS}{ha})}{Highest\ S\ yield\ for\ that\ Crop\ x\ Year}$$

$$\textbf{\textit{Yield Response}}\ (\%) = \frac{Highest\ yielding\ S\ fertilized\ plot\ \frac{kg}{ha} - control\ plot\ yield\ \frac{kg}{ha}}{Control\ plot\ yield\ \frac{kg}{ha}} * 100$$

$$\textbf{\textit{S Response}}\ (\%) = \frac{Highest\ S\ yield\ plot\ \frac{g\ S}{kg} - control\ plot\ S\ yield\ \frac{g\ S}{kg}}{Control\ plot\ \frac{g\ S}{kg}} * 100$$

Data Analysis

Four categories of sites were identified based on crop responses to S as determined with a split plot ANOVA performed in R, using the 'agricolae' package with broadcast S as the main plot factor and foliar S as the subplot factor (de Mendiburu, 2020). The four site categories were:1) non-responsive (NR), 2) significant yield response (YS), 3) significant seed S content response (SS), and 4) both a yield response and seed S content response (YSS). Unless otherwise indicated, a significance level of $\alpha = 0.05$ was used to determine significant differences between treatments. An F-protected post hoc Tukey HSD test was conducted to determine significance levels between groups. All statistics were performed using R for MAC (R Core Team, 2019).

The Cate-Nelson method of dividing bivariate data into responsive and unresponsive sites was used to determine the critical value of S that would predict the highest crop response for four response variables: relative yield, relative S yield, yield response, and S response in the whole 0-30 cm soil profile as well as just the A1 (0-10 cm) and B (bottom of A or 20cm – 30 cm) horizons (Cate and Nelson, 1971). This was done using the "rcompanion" package in R (Mangiafico, 2020). For relative yield and relative S yield the critical x value was determined using a y value of 90%. For yield response and S response the critical x value was determined using a y value of 5%.

Using the critical level identified by the Cate-Nelson analysis for all 122 plots, the number of fields correctly identified by each soil test were determined for the A1 horizon, B horizon and weighted mean of the whole 0-30 cm sample based on four criteria (1) fields with no response and S level above critical level, (2) fields with no response and S level below critical level, (3) fields with significant response (YS, SS, or YSS) and S level below critical level, and (4) fields with significant response and S level above critical level. The % correctly identified by soil test was then defined as:

$$\frac{\#fields\ with\ no\ response\ above\ critical\ level + \#fields\ with\ significant\ response\ below\ critical\ level}{total\ number\ of\ fields}$$

Results

The ANOVA identified 8 out of 23 fields in which crops showed a significant response (YS, SS, or YSS) to applied S. Average yields for FS soybeans ranged from 1510-6705 kg/ha, relative yield ranged from 20-92%, and yield response ranged from 1-18%. Average yields for DC soybeans ranged from 1420-3624 kg/ha, relative yield ranged from 36-92%, and yield response ranged from 2.5-45%. Average CB yields ranged from 1054-2026 kg/ha, relative yields ranged from 35-78%, and yield response ranged from 17-76% (Table 16).

Sulfur yield for FS soybeans ranged from 4.20-20.8 kg-S/ha, relative S yield ranged from 30-87%, and S response ranged from 0-20%. Sulfur yield for DC soybeans ranged from 4.30-12.6 kg-S/ha, relative S yield ranged from 30-87% and S response ranged from 0-23%. Sulfur yield for CB ranged from 3.0-5.3 kg-S/ha, relative S yield ranged from 32-64% and S response ranged from 3.50-16.5%.

Soil S levels averaged by field for each horizon (A1, A2, B, and weighted average) as determined by the four extraction methods are summarized in Table 18.

Calcium Chloride extractable S ranged from 0.6-100.5 in the A1 horizon, 0.32-10.15 in the A2 horizon, and 0.15-18.5 in the B horizons with average CaCl2 extractable S values equaling 10.06 in the A1, 3.15 in the A2, and 3.97 in the B. Calcium phosphate in water extractable S ranged from 2.56-120 in the A1 horizon, 2.23-18.5 in the A2 horizon, and 2.26-34.27 in the B horizon with average CaP extractable S values equaling 20.8 in the A1, 6.87 in the A2, and 12.82 in the B. Acid CaP extractable S levels ranged from 1.93-55.25 in the A1 horizon, 1.37 – 79.02 in the A2 horizon, 1.28 -79.9 in the B horizon with average acid CaP values equaling 10.17 in the A1, 11.7 in the A2, and 15.9 in the B. Mehlich3 extractable S levels ranged from 9.02-88.1 in the A1 horizon, 10.26 – 35.8 in the A2 horizon, and 7.5 – 38.3 in the B horizon with average Mehlich3 extractable S levels equaling 21.3 in the A1, 15.4 in the A2, and 16.3 in the B.

The Critical x values as determined by the Cate-Nelson analysis are shown in Figures 9-12. Only extractable S results for the A1, B, and weighted mean of all three soil layers (0-30cm) are shown. The A2 segment was considered a transition horizon that may have had some mixing between the surface and subsoils. It is not shown separately but is included in the weighted average. The critical x values for CaCl2 extractable S in the A1 were not significant (NS) for any of the response variables. The critical x value for the B was only significant for relative S yield which was 13.8. The critical x values were significant for the weighted mean for relative yield and yield response which were 26 and 8.3 respectively ($p<0.05$). The critical x values for CaP extractable S were 10.4 (A1), 26.9 (B), and 13.2 (weighted mean) for relative yield; 9.1 (A1), 17.4 (B), and 8.0 (weighted mean) for relative S yield; 5.3 (A1), NS (B), and 3.2 (weighted mean) for yield response; and NS (A1, B, and weighted mean) for S response. The critical x values

for acid CaP extractable S were: 8.3 (A1), 27.8 (B), and 14.7 (weighted mean) for relative yield; 8.3 (A1), 27.8 (B), and 13.9 (weighted mean) for relative S yield; 2.8 (A1), 2.7 (B), and 3.1 (weighted mean) for yield response; and 1.8 (A1) , 1.5 (B), and 19.3 (weighted mean) for S response. The critical x values for Mehlich3 extractable S were; 18.1 (A1), 29.6 (B), and 16.2 (weighted mean) for relative yield; 18.1 (A1), 17.3 (B), and 16.2 (weighted mean) for relative S yield; 13.1 (A1), 8.6 (B), and 14.0 (weighted mean) for yield response; and NS (A1), 8.9 (B), and NS (weighted mean) for S response.

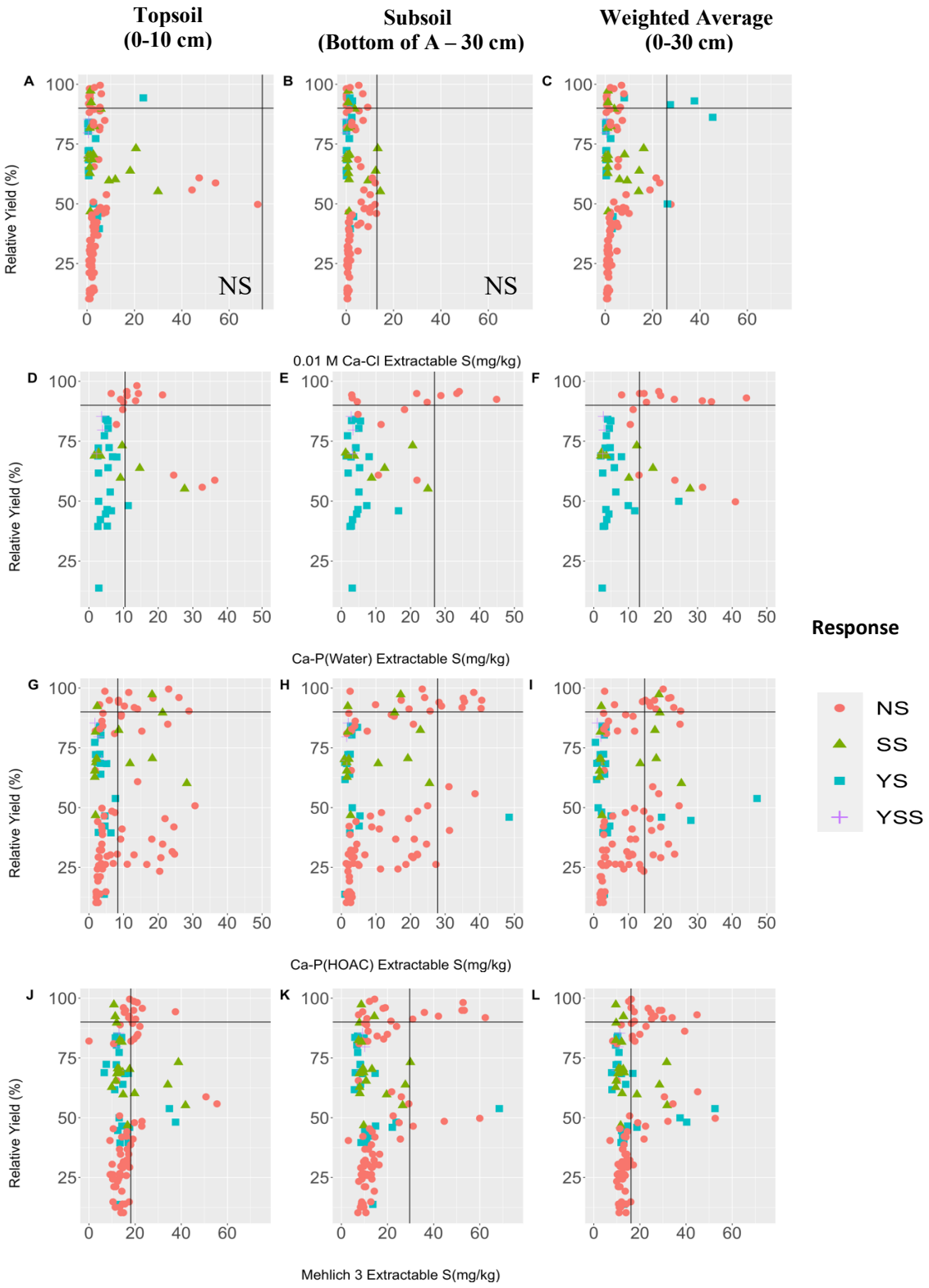


Figure 9. Cate Nelson graphs identifying critical level for extractable S assuming a critical relative yield of 90% and Relative Yield (%) Calculated as the yield of the no S treatment divided by the highest yielding plot for that crop x year for four different soil extractants (1) 0.01 M $CaCl_2$, (2) 0.002M $Ca(H_2PO_4)_2$ in water (3) 0.002M $Ca(H_2PO_4)_2$ in 2M HOAC, and (4) Mehlich3, for 0-10 cm and subsoil (bottom of A or 20 cm-30cm) horizons and the weighted average for the full 0-30cm soil sample. NS=Individual plots within fields that did not have a significant yield or S response, SS=Individual plots within fields with a significant S response, YS=Individual plots within fields with significant yield response, and YSS =Individual plots within fields with significant yield and S response based off the ANOVA model presented in Chapter 3. NS=not significant, all unmarked plots significant at $p<0.05$

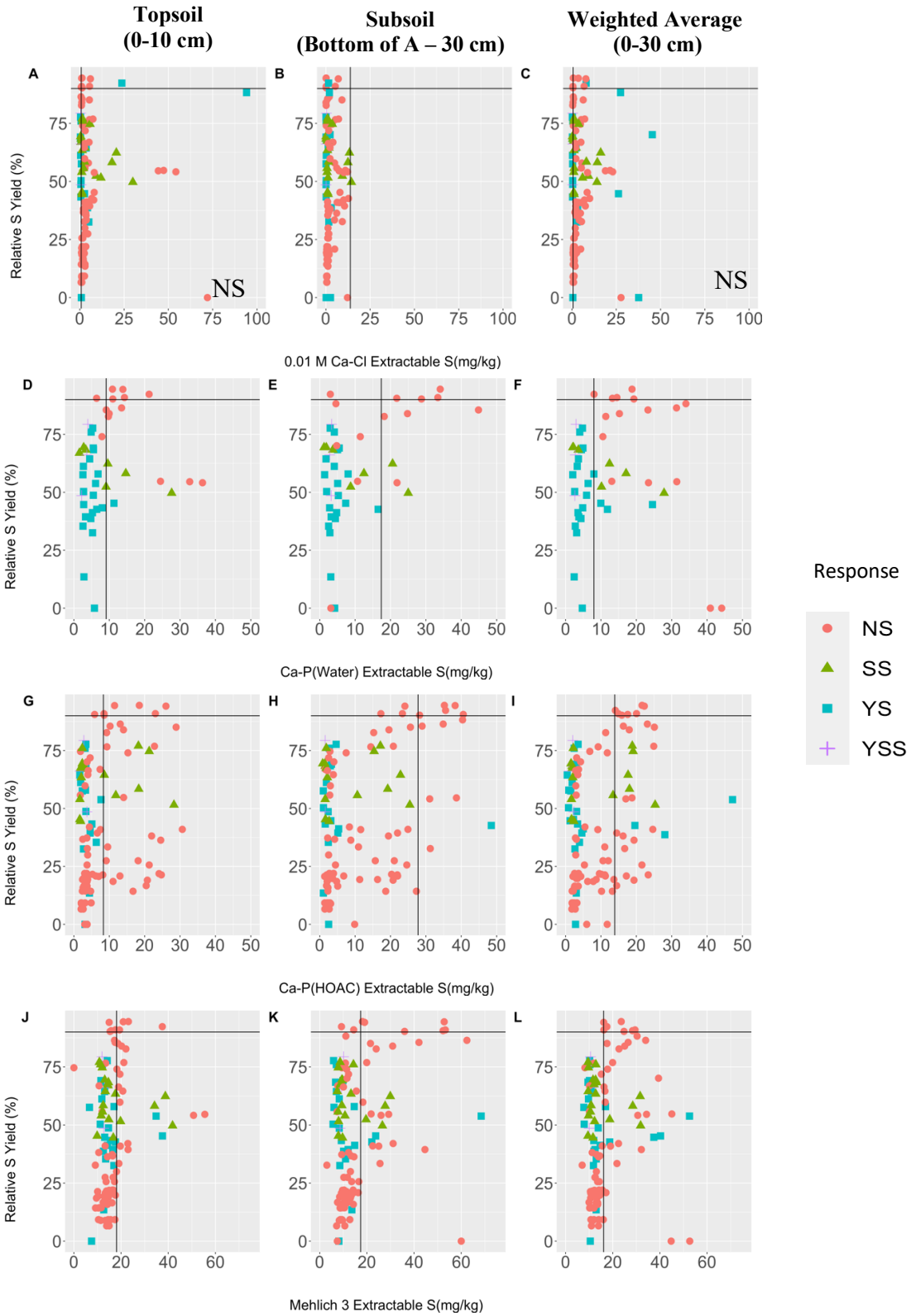


Figure 10 Cate Nelson graphs identifying critical level for extractable S assuming a critical relative S yield of 90%. Relative S yield (%) calculated as the S content of the no S treatment x yield of the no S treatment divided by the highest S yield for that crop x year for four different soil extractants ((1) 0.01 M $CaCl_2$, (2) 0.002M $Ca(H_2PO_4)_2$ in water (3) 0.002M $Ca(H_2PO_4)_2$ in 2M HOAC, and (4) Mehlich3, for 0-10 cm and subsoil (bottom of A or 20 cm-30cm) horizons and the weighted average for the full 0-30cm soil sample. NS=Individual plots within fields that did not have a significant yield or S response, SS=Individual plots within fields with a significant S response, YS=Individual plots within fields with significant yield response, and YSS =Individual plots within fields with significant yield and S response as determined by a split plot ANOVA. NS=not significant, all unmarked plots significant at $p<0.05$

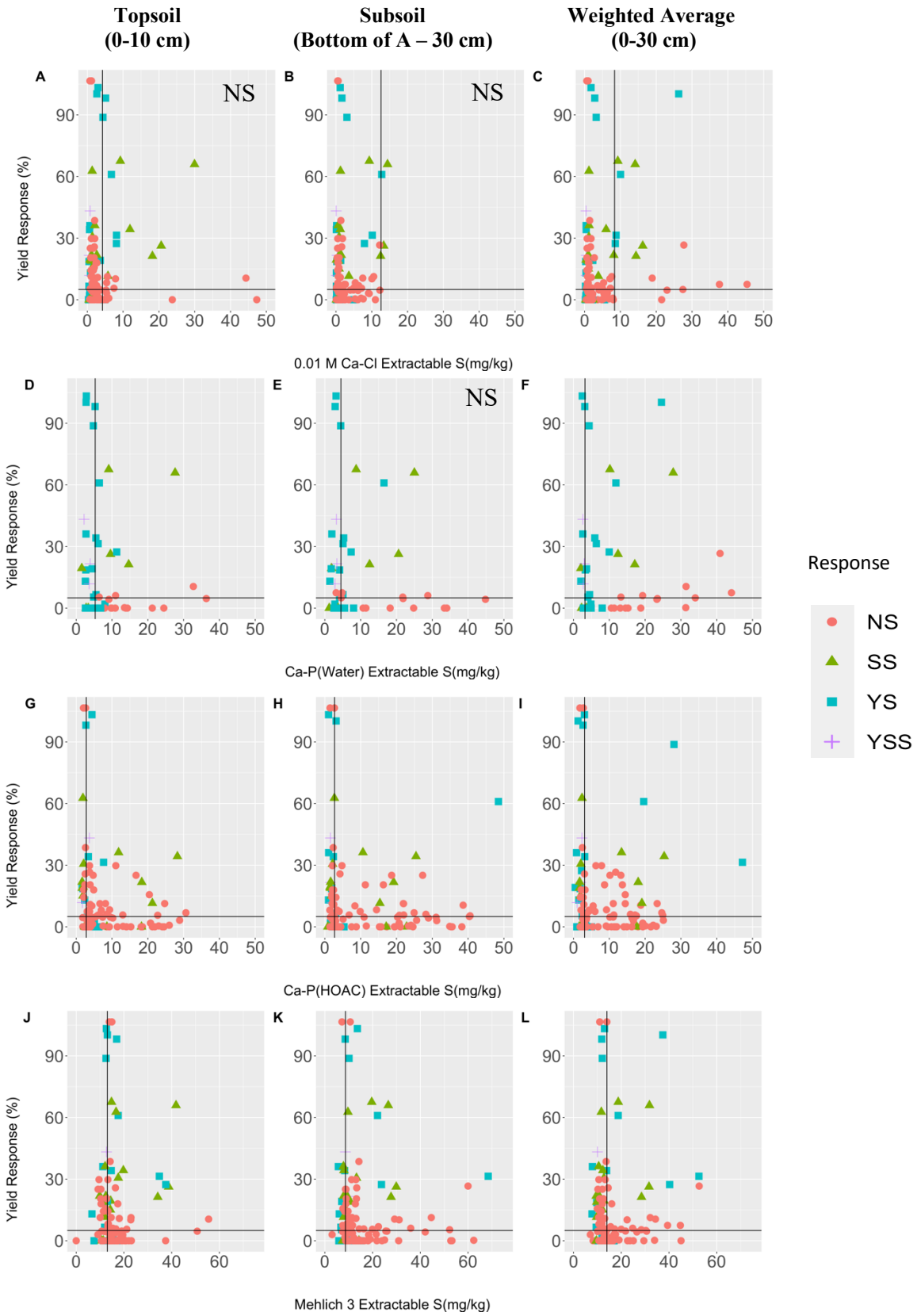


Figure 11 Cate Nelson graphs identifying critical level for extractable S assuming a critical yield response level of 5%. Yield Response (%) calculated as (the highest yielding S fertilized plot – the yield of the control plot)/ yield of the control plot presented for four different soil extractants((1) 0.01 M $CaCl_2$, (2) 0.002M $Ca(H_2PO_4)_2$ in water (3) 0.002M $Ca(H_2PO_4)_2$ in 2M HOAC, and (4) Mehlich3,) for 0-10 cm and subsoil (bottom of A or 20 cm-30cm) horizons and the weighted average for the full 0-30cm soil sample. NS=Individual plots within fields that did not have a significant yield or S response, SS=Individual plots within fields with a significant S response, YS=Individual plots within fields with significant yield response, and YSS =Individual plots within fields with significant yield and S response determined by a split plot ANOVA model presented in Chapter 3. NS=not significant, all unmarked plots significant at p<0.05

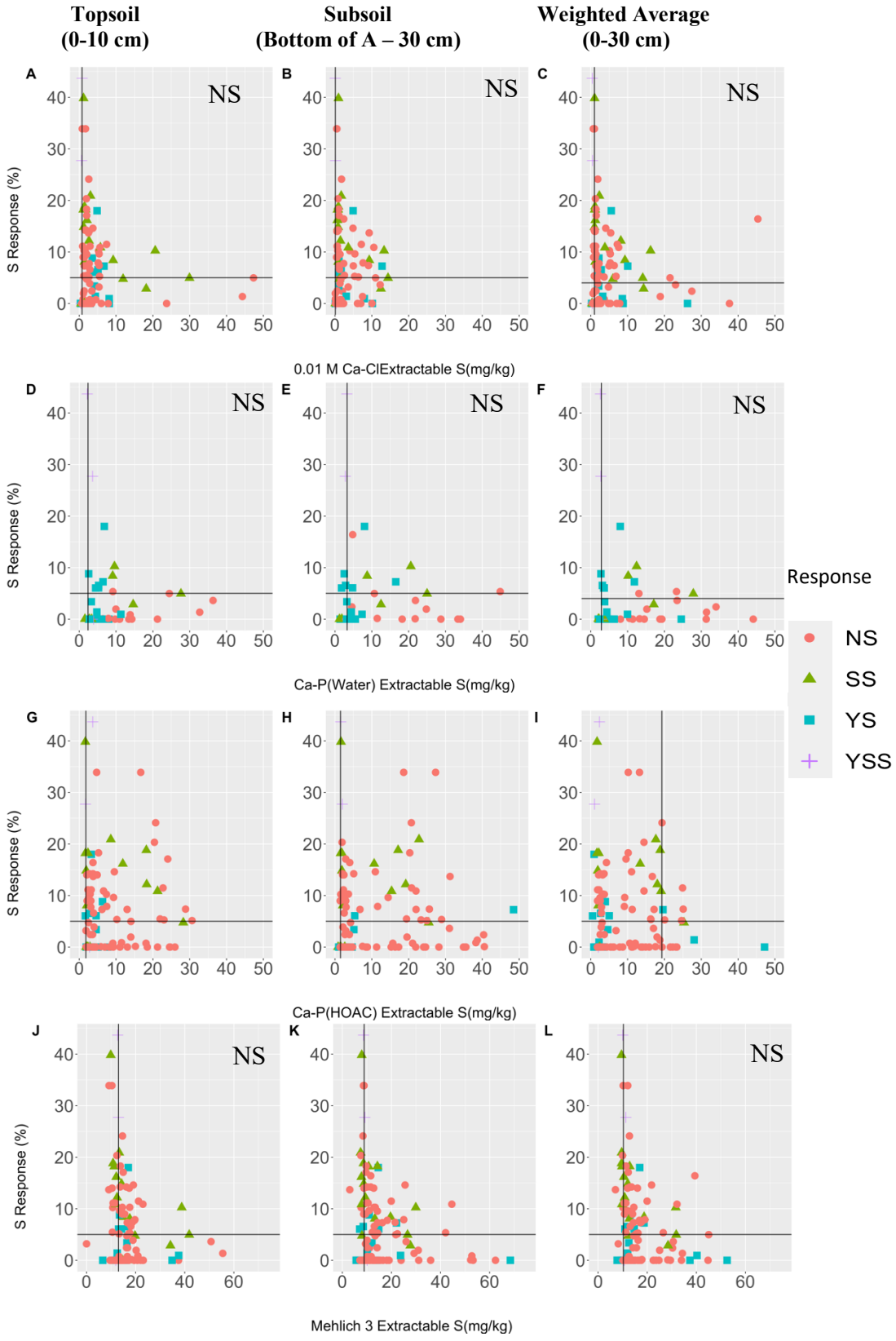


Figure 12 Cate Nelson graphs identifying critical level for extractable S assuming a critical S response level of 5%. S Response (%) calculated as (the highest S yield plot- S yield of the control plot)/ S yield of the control plot for four different soil extractants (1) 0.01 M $CaCl_2$, (2) 0.002M $Ca(H_2PO_4)_2$ in water (3) 0.002M $Ca(H_2PO_4)_2$ in 2M HOAC, and (4) Mehlich3 for 0-10 cm and subsoil (bottom of A or 20 cm-30cm) horizons and the weighted average for the full 0-30cm soil sample. NS=Individual plots within fields that did not have a significant yield or S response, SS=Individual plots within fields with a significant S response, YS=Individual plots within fields with significant yield response, and YSS =Individual plots within fields with significant yield and S determined by the split plot ANOVA model presented in Chapter 3. NS=not significant, all unmarked plots significant at p<0.05

Table 16. Yield, Relative Yield, Relative Sulfur Yield, % Yield Response, and % S response for 23 fields grown throughout the 2017, 2018 and 2019 growing seasons. Values represent the mean value and standard error for the field. FS= Full season soybean, DC= Double crop soybean grown after a cereal grain, CB= Common dry bean.

Field	Year	Crop	N	Yield (kg/ha)	Relative Yield (%)	Relative S Yield (%)	Yield Response (%)	S Response (%)
5-18O	2017	FS	12	3913±34	92.4±0.8	87.3±1.1	1.6±0.5	0.8±0.3
5-39B	2017	DC	24	1892±6	69.3±0.2	68.5±0.3	6.5±3.2	0.0±0.0
5-43A	2017	DC	24	2136±62	78.3±2.3	64.7±4.5	25.6±4.7	23.8±6.4
5-43B	2017	FS	12	3131±63	73.9±1.5	55.2±4.0	11.6±2.4	0.0±0.0
5-17C	2018	DC	30	2087±66	62.9±2	55.7±1.5	45.2±6.5	6.6±0.9
5-18	2018	FS	12	2661±60	56.3±1.3	40.9±7.1	10.4±3.0	3.3±0.5
5-25A	2018	DC	12	1794±88	54.1±2.6	49.9±1.9	29.9±6.5	6.5±2.2
5-39B	2018	CB	18	1570±130	52.9±4.4	45.1±3.6	76.6±10.1	3.5±0.9
5-39C	2018	FS	12	4314±44	91.3±0.9	62.7±11.2	5.0±0.9	6.3±2.6
UMBB	2018	CB	9	1054±115	35.5±3.9	32.4±3.4	25.8±13.5	4.6±1.0
5-17Ca	2019	FS	12	2226±66	29.5±0.9	21.0±0.8	12.6±2.2	5.2±2.3
5-17Cb	2019	FS	12	2226±66	29.5±0.9	21.0±0.8	12.6±2.2	5.2±2.3
5-25C	2019	FS	24	3371±90	44.6±1.2	37.0±0.9	2.5±0.9	6.1±1.5
5-39	2019	CB	24	2026±81	78.1±3.1	63.6±2.3	17.3±3.6	14.0±1.3
5-39A	2019	DC	9	1410±111	36.1±3.0	29.9±2.6	20.2±3.9	5.2±1.0
5-40	2019	FS	12	1510±123	20.0±1.6	13.3±1.1	17.8±7.1	7.6±1.2
5-40b	2019	FS	30	1510±123	20.0±1.6	13.3±1.1	17.8±7.1	7.6±1.2
5-43A	2019	FS	18	2146±70	28.4±0.9	19.8±0.6	5.5±1.8	20.0±0.8
5-7A	2019	CB	12	1813±91	69.9±3.5	58.5±3.0	24.9±4.7	16.5±3.0
5-7F	2019	DC	12	1798±35	50.0±0.9	38.93±1.0	6.6±1.3	8.3±1.6
DS1	2019	DC	12	3102±101	79.4±2.6	68.5±2.5	4.4±0.9	5.8±1.3
JL1	2019	FS	12	6706±141	88.8±1.9	65.7±1.3	1.1±0.4	2.9±1.0
SK1	2019	DC	12	3625±66	92.7±1.7	86.8±2.0	2.5±0.6	6.0±1.3

Table 17. Average Soil S and SE determined by ICP for four extractants (1) CaCl2 (2) CaP, (3) Acid CaP, and (4) Mehlich3 for three horizons and the weighted average for the full 0-30cm soil sample. A1=0-10 cm, A2=10-20 cm or bottom of A horizon, B = bottom of A horizon or 20 cm – 30 cm, Mean = weighted average for the 0-30 cm sample based on the depth of the sample and the bulk density of a representative "Sandy" and "Silty" field at CMREC Beltsville. FS=Full season soybean, DC=Double crop soybean planted after winter cereal grain harvest (typically wheat), CB = Common dry bean. n.d=no data available

Field ID	Year	Crop	Horizon	N	CaCl2	CaP	Acid CaP	Mehlich3
					----------mg S/kg----------			
5-18O	2017	FS	A1	10	1.12±0.08	10.6±0.82	11.5±1.19	19±0.82
			A2	10	0.54±0.08	10.3±1.09	11.9±2.04	20.8±1.52
			B	10	0.42±0.19	34.2±5.85	26.6±3.52	38.3±5.39
			Mean		0.66±0.05	17.6±1.97	16.5±1.19	25.7±1.37
5-39B	2017	DC	A1	3	0.69±0.08	2.56±0.53	2.41±0.27	14±0.49
			A2	3	0.35±0.05	2.83±0.45	1.37±0.02	13.2±0.86
			B	3	0.16±0.02	2.27±0.75	1.28±0.22	9.3±0.19
			Mean		0.4±0.04	2.64±0.49	1.66±0.1	12.5±0.45
5-43A	2017	DC	A1	3	0.67±0.07	3.25±0.53	2.73±0.57	12.5±0.33
			A2	3	0.47±0.06	2.23±0.15	1.96±0.05	10.3±0.68
			B	3	0.18±0.07	3.14±0.17	1.7±0.15	9.3±0.43
			Mean		0.44±0.02	2.79±0.1	1.81±0.4	10.6±0.37
5-43B	2017	FS	A1	10	0.61±0.06	4.85±0.55	3.21±0.29	11.7±0.9
			A2	10	0.32±0.03	3.88±0.41	2.38±0.24	10.6±0.67
			B	10	0.15±0.02	3.74±0.45	2.41±0.35	7.5±0.46
			Mean		0.37±0.03	4.17±0.38	2.54±0.28	10.2±0.56
5-18	2018	FS	A1	4	19.5±4.26	15.2±4.32	8.9±5.15	32.4±6.07
			A2	4	9.8±3.43	18.5±5.93	33.6±15.17	25.7±1.9
			B	4	12.4±1.1	16.7±3.71	42.5±7.94	26±2.22
			Mean		13.5±1.48	16.9±3.93	24.8±9.01	27.7±3.07
5-17C	2018	DC	A1	4	54.5±6.21	36.2±5.62	n.d.	66.4±7.75
			A2	4	10.2±0.85	11.2±3.01	n.d.	26.1±6.25
			B	4	10.5±1.12	33.5±10.23	n.d.	34.2±8.71
			Mean		22.8±1.85	27.2±5.92	n.d.	40.7±5.03
5-25A	2018	DC	A1	4	6.97±0.78	7.68±1.22	5.46±2.18	26.7±5.48
			A2	4	8.46±0.67	10.0±0.86	n.d.	36.7±11.54
			B	4	8.95±1.67	9.23±2.5	80±31.47	32.2±12.24
			Mean		8.24±0.95	9.05±1.19	17.5±10.77	32.1±8.63
5-39B	2018	CB	A1	4	3.94±0.52	4.32±0.55	2.19±0.52	13.9±1.02
			A2	4	2.03±0.21	2.84±0.65	2.51±2.16	13.2±1.36
			B	4	18.5±16.47	18.1±15.04	37.9±34.71	25.3±16.78
			Mean		8.66±5.88	8.93±5.22	8.12±6.66	18.1±6.46
5-39C	2018	FS	A1	4	100.5±28.45	120±36.96	55.3±51.85	88.1±21.02
			A2	4	2.87±0.62	4.14±0.96	5.38±1.06	14.4±2.71
			B	4	2.14±0.23	3.88±0.47	22.5±9.09	9.8±0.94
			Mean		29.6±8.08	36.1±10.58	21.7±13.75	33±5.96
UMBB	2018	CB	A1	4	3.64±0.25	3.48±0.61	4.98±0.45	14.2±0.82
			A2	4	1.73±0.11	2.68±0.8	4.27±0.39	12.8±1.14
			B	4	1.06±0.05	3.41±0.48	3.49±1.05	12.9±0.85
			Mean		2.06±0.09	3.12±0.31	4.15±0.43	13.3±0.48
5-17Ca	2019	FS	A1	8	1.47±0.23	10.7±0.82	9.78±2.08	12.9±1.02
			A2	8	2.22±0.92	n.d.	9.79±2.21	11.6±0.51
			B	8	1.37±0.53	n.d.	7.8±2.36	11.5±0.74

		Mean		1.72±0.53	n.d.	8.75±1.37	12±0.61
5-17Cb	2019 FS	A1	8	2.08±0.38	n.d.	9.9±2.96	13.4±0.94
		A2	8	0.92±0.09	n.d.	14.8±3.17	13.3±0.92
		B	8	0.96±0.2	n.d.	17.7±3.16	11.9±1.11
		Mean		1.27±0.18	n.d.	13.6±2.36	12.8±0.69
5-25C	2019 FS	A1	4	2.65±0.29	n.d.	20.2±5.78	11.8±1.19
		A2	4	2.74±0.55	n.d.	15.5±5.83	11.6±0.64
		B	4	6.08±1.37	n.d.	21.1±4.76	13.1±3.99
		Mean		4.01±0.71	n.d.	19.3±1.93	12±1.86
5-39A	2019 CB	A1	6	4.48±1.61	n.d.	17.8±2.84	13.4±1.31
		A2	6	2.46±0.26	n.d.	19.8±1.05	10.5±0.55
		B	6	5.94±3.46	n.d.	18.4±2.17	8.2±0.29
		Mean		1.49±0.15	n.d.	18.7±1.56	10.4±0.43
5-39A	2019 DC	A1	4	1.59±0.43	n.d.	3.16±0.36	16.2±0.78
		A2	4	1.4±0.08	n.d.	2.61±0.11	12.5±1.02
		B	4	3.79±1.15	n.d.	2.83±0.47	12.5±1.09
		Mean		1.73±0.12	n.d.	2.8±0.13	13.6±0.33
5-40a	2019 FS	A1	8	1.3±0.19	n.d.	3.26±0.3	14.6±0.71
		A2	8	0.74±0.07	n.d.	2.92±0.18	14.9±0.62
		B	8	0.56±0.04	n.d.	3.4±0.34	11.4±0.64
		Mean		0.84±0.06	n.d.	2.97±0.22	13.8±0.59
5-40b	2019 FS	A1	8	2.11±0.29	n.d.	2.44±0.15	14±0.95
		A2	8	0.94±0.11	n.d.	2.21±0.19	12.4±0.73
		B	8	0.67±0.05	n.d.	2.12±0.19	9.7±0.66
		Mean		1.19±0.05	n.d.	2.26±0.16	12.1±0.56
5-43A	2019 FS	A1	4	2.15±0.15	n.d.	17.6±4.2	13.9±0.58
		A2	4	1.38±0.08	n.d.	11.4±5.35	12±0.6
		B	4	1.17±0.21	n.d.	11.5±5.23	8.8±0.52
		Mean		1.51±0.14	n.d.	13.3±2.27	11.6±0.62
5-7A	2019 CB	A1	6	1.34±0.09	n.d.	1.93±0.11	13.6±1.28
		A2	6	1.13±0.06	n.d.	2.06±0.13	10.4±0.8
		B	6	0.91±0.08	n.d.	1.95±0.17	10.8±1.03
		Mean		1.12±0.04	n.d.	1.98±0.11	11.4±0.6
5-7F	2019 DC	A1	4	5.59±0.86	n.d.	7.01±1.07	21.1±1.03
		A2	3	3.41±0.25	n.d.	8.27±1.93	15.5±0.82
		B	4	8.27±1.3	n.d.	13.2±3	31.6±4.55
		Mean		5.99±0.81	n.d.	10.0±1.88	23.9±2.85
DS1	2019 DC	A1	4	4.74±0.6	n.d.	5.1±1.93	9±3.06
		A2	4	5.7±0.27	n.d.	5.79±2.85	11.9±0.47
		B	4	4.78±0.6	n.d.	5.43±3.02	8.8±0.83
		Mean		5.13±0.12	n.d.	5.69±1.44	10.1±0.75
JL1	2019 FS	A1	4	2.82±0.1	n.d.	3.93±0.3	19.7±0.38
		A2	4	1.73±0.1	n.d.	2.56±0.05	15.8±1.69
		B	4	1.93±0.25	n.d.	2.89±0.37	14±1.82
		Mean		2.13±0.07	n.d.	3.09±0.15	16.3±0.38
SK1	2019 DC	A1	4	6.09±0.47	n.d.	25.2±1.44	18.1±1.29
		A2	4	7.69±0.7	n.d.	21.9±2.58	16.9±0.79
		B	4	7.15±0.77	n.d.	23.5±1	18.7±1.53
		Mean		7.07±0.32	n.d.	23.08±1.2	17.5±0.88

Sites correctly identified by the Cate-Nelson analysis were non-responsive with extractable S above the critical level (NR) and responsive sites (YS, SS, or YSS) with extractable S below the critical level (Table 18). In almost every case the A1 had a higher probability of correctly identifying sites that responded to S application than the B horizon alone. Additionally, in almost every case soil tests were able to correctly identify sites a higher percentage of times based on yield and relative S yield than yield response or S response. Critical x values were similar for relative yield and relative S yield, but overall using relative yield as the response variable identified a higher percentage of sites than relative S yield. Based on relative yield the CaCl2 extract weighted mean correctly identified 43.5% of all sites and 100% of responsive sites. The CaP extract correctly identified 90% (A1, B, and weighted mean) of all sites and 85.7% (A1), 100% (B), and 85.7% (weighted mean) of the responsive sites. The acid CaP extract correctly identified 68.2% (A1), 31.8% (B), and 50% (weighted mean) of all sites and 87.5% (A1), 75.0% (B), and 75% (weighted mean) of responsive sites. The Mehlich3 extract correctly identified 52.2 % (A1), 47.8% (B), and 52.2% (weighted mean) of all sites and 77.8 (A1), 88.9 (B), and 66.7 (weighted mean) of responsive sites.

There were no significant responses to applied S on any of the soybeans grown in 2019 (DC or FS). The CaP test was not completed on the 2019 samples. In order to confirm the results observed on all 23 fields, we re-ran the comparisons for just the 2017 -2018 fields (10 fields) so that all four extracts would be equally represented (Table 19). Based on relative yield the CaCl2 extract correctly identified 90% (weighted mean) of all sites and 100% (weighted mean) of responsive sites. The acid CaP extract correctly identified 100% (A1), 55.6% (B), and 88.9% (weighted mean) of all sites and 100 %

(A1), 66.7% (B), and 83.3% (weighted mean) of responsive sites. The Mehlich3 extract correctly identified 80% (A1 and B), and 50% (weighted mean) of all sites, and 71.4% (A1), 85.7% (B), and 57.1% (weighted mean) of responsive sites. In agreement with the results from all 23 fields, the A1 soil test results for the 2017-2018 fields were able to correctly identify responsive sites an equal or higher percentage of times than the weighted mean. In almost every case, the A1 identified a higher percentage of responsive sites than the B horizon alone.

The linear relationships between the S levels analyzed by each extract vs. Mehlich3 S levels (Figure 12) show that the extractable S in the A1 horizon for CaCl2 and Mehlich3 are highly correlated (R^2=0.87) but poorly correlated for the B horizon (R^2=0.3). Mehlich3 is well correlated with CaP (water) in the A1, B, and weighted mean with R^2 values of 0.76, 0.65, and 0.74 respectively. Mehlich3 was not well correlated with Acid CaP in any of the horizons.

Table 18 Summary of four soil extractions ability to predict response to S application on 23 fields grown during 2017-2019. The four soil extractions used were (1) 0.01 M CaCl2, (2) 0.002M $Ca(H_2PO_4)_2$ in water, (3) 0.002M $Ca(H_2PO_4)_2$ in 2N HOAC, and (4) Mehlich3. The critical x value was determined by Cate-Nelson analysis from 122 individual blocks within the 23 fields. Fields that had a significant yield, seed S content, or both yield and seed S content response to applied S were designated as having a significant response. Fields were grouped into four categories (I) No Response and above critical level (II) No response and below critical level, (III) significant response and below critical level, and (IV) significant response and above critical level. The number of fields within each category was used to determine the % of sites correctly identified by a soil test (100(I fields + +III Fields)/total fields) and the % of correctly identified responsive sites (100*III Fields/ (III Fields + IV Fields)A1= 0-10 cm, B = Bottom of A or 20 cm – 30 cm, mean=Weighted average of 0-30 cm based on the bulk density of a field of similar soil type.*

Soil Test	Horizon	Critical X Value	p-value	N	No Response Above Critical Level	No Response Below Critical level	Significant Response below Critical Level	Significant Response Above Critical Level	Correctly Identified by Soil test	Responsive Sites Identified
									---------%----------	
Relative Yield										
CaCl2	A1	74.0	NS	23	-	-	-	-	-	-
	B	13.8	NS	23	-	-	-	-	-	-
	Mean	26.0	0.035	23	1	13	9	0	43.5	100.0
CaP	A1	10.4	0.001	10	3	0	6	1	90.0	85.7
	B	26.9	0.000	10	2	1	7	0	90.0	100.0
	Mean	13.2	0.000	10	3	0	6	1	90.0	85.7
Acid CaP	A1	8.3	0.001	22	8	6	7	1	68.2	87.5
	B	27.8	0.000	22	1	13	6	2	31.8	75.0
	Mean	14.7	0.001	22	5	9	6	2	50.0	75.0
Mehlich3	A1	18.1	0.011	23	5	9	7	2	52.2	77.8
	B	29.6	0.000	23	3	11	8	1	47.8	88.9
	Mean	16.2	0.000	23	6	8	6	3	52.2	66.7
Relative S Yield										
CaCl2	A1	0.8	NS	23	-	-	-	-	-	-
	B	13.8	0.06	23	0	14	8	1	34.0	88.9
	Mean	0.6	NS	23	-	-	-	-	-	-
CaP	A1	9.1	0.020	10	3	0	6	1	90.0	85.7

	B	17.4	0.020	10	2	1	6	1	80.0	85.7
	Mean	8.0	0.020	10	3	0	4	3	70.0	57.1
Acid CaP	A1	8.3	0.032	22	8	6	7	1	68.2	87.5
	B	27.8	0.000	22	1	13	6	2	31.8	75.0
	Mean	13.9	0.000	22	9	6	5	2	63.6	71.4
Mehlich3	A1	18.1	0.000	23	5	9	7	2	52.2	77.8
	B	17.3	0.003	23	4	10	6	3	43.5	66.7
	Mean	16.2	0.000	23	6	8	6	3	52.2	66.7
S Response (%)										
CaCl2	A1	0.8	NS	23	-	-	-	-	-	-
	B	0.1	NS	23	-	-	-	-	-	-
	Mean	1.0	NS	23	-	-	-	-	-	-
CaP	A1	2.4	NS	10	-	-	-	-	-	-
	B	3.3	NS	10	-	-	-	-	-	-
	Mean	2.9	NS	10	-	-	-	-	-	-
Acid CaP	A1	1.8	0.043	22	14	0	0	8	63.6	0.0
	B	1.5	0.085	22	14	0	1	7	68.2	12.5
	Mean	19.3	0.007	22	4	10	8	0	54.5	100.0
Mehlich3	A1	13.0	NS	23	-	-	-	-	-	-
	B	8.9	0.040	23	14	0	2	7	69.6	22.2
	Mean	10.3	NS	23	-	-	-	-	-	-
Yield Response (%)										
CaCl2	A1	4.2	NS	23	-	-	-	-	-	-
	B	12.6	NS	23	-	-	-	-	-	-
	Mean	8.3	0.085	23	2	12	7	2	39.1	77.8
CaHPO4 (Water)	A1	5.3	0.070	10	3	0	5	2	80.0	71.4
	B	4.5	ns	10	-	-	-	-	-	-
	Mean	3.2	0.011	10	3	0	3	4	60.0	42.9
	A1	2.8	0.000	22	13	1	4	4	77.3	50.0

CaHPO4 (HOAc)	B	2.7	0.002	22	13	1	4	4	77.3	50.0
	Mean	3.1	0.011	22	10	4	4	4	63.6	50.0
Mehlich 3	A1	13.1	0.007	23	11	3	2	7	56.5	22.2
	B	8.6	0.014	23	14	0	2	7	69.6	22.2
	Mean	14.0	0.013	23	6	8	6	3	52.2	66.7

Table 19 Summary of four soil extractions ability to predict response to S application on 10 fields grown during 2017-2018. The four soil extractions used were (1) 0.01 M $CaCl_2$, (2) 0.002M $Ca(H_2PO_4)_2$ in water, (3) 0.002 M $Ca(H_2PO_4)_2$ in 2N HOAC, and (4) Mehlich3. The critical x value was determined by Cate-Nelson analysis from 122 individual blocks within the 23 fields. Fields that had a significant yield, seed S content, or both yield and seed S content response to applied S were designated as having a significant response. Fields were grouped into four categories (I) No Response and above critical level (II) No response and below critical level, (III) significant response and below critical level, and (IV) significant response and above critical level. The number of fields within each category was used to determine the % of sites correctly identified by a soil test (100(I fields + +III Fields)/total fields) and the % of correctly identified responsive sites (100*III Fields/ (III Fields + IV Fields)A1= 0-10 cm, B = Bottom of A or 20 cm – 30 cm, mean=Weighted average of 0-30 cm based on the bulk density of a field of similar soil type.*

Soil Test	**Horizon**	**Critical X Value**	**p-value**	**N**	**No Response Above Critical Level**	**No Response Below Critical level**	**Significant Response below Critical Level**	**Significant Response Above Critical Level**	**Correctly Identified by Soil test**	**Responsive Sites Identified**
		Relative Yield							**------%----------**	
	A1	74.0	NS	10	-	-	-	-	-	-
	B	13.8	NS	10	-	-	-	-	-	-
CaCl2	Mean	26.0	0.035	10	1	2	7	0	90.0	100.0
	A1	10.4	0.001	10	3.0	0.0	6	1	90.0	85.7
	B	26.9	0.000	10	2.0	1.0	7.0	0	90.0	100.0
CaP	Mean	13.2	0.000	10	3	0	6	1	90.0	85.7
	A1	8.3	0.001	9	3	0	6	0	100.0	100.0
	B	27.8	0.000	9	1	2	4	2	55.6	66.7
Acid CaP	Mean	14.7	0.001	9	3	0	5	1	88.9	83.3

	A1	18.1	0.011	10	3	0	5	2	80.0	71.4
	B	29.6	0.000	10	2	1	6	1	80.0	85.7
Mehlich 3	Mean	16.2	0.000	10	3	0	4	3	50.0	57.1
		Relative S Yield								
	A1	0.8	NS	0	-	-	-	-	-	-
	B	13.8	0.06	0	0	3	6	1	60	85.7
CaCl2	Mean	0.6	NS	0	-	-	-	-	-	-
	A1	9.1	0.020	10	3	0	6	1	90.0	85.7
	B	17.4	0.020	10	2	1	6	1	80.0	85.7
CaP	Mean	8.0	0.020	10	3	0	4	3	70.0	57.1
	A1	8.3	0.032	9	3	0	6	0	100.0	100.0
	B	27.8	0.000	9	1	2	4	2	55.6	66.7
Acid CaP	Mean	13.9	0.000	9	3	0	5	1	88.9	83.3
	A1	18.1	0.000	10	3	0	5	2	80.0	71.4
	B	17.3	0.003	10	2	1	4	3	60.0	57.1
Mehlich 3	Mean	16.2	0.000	10	3	0	4	3	70.0	57.1
		S Response								
	A1	0.8	NS	0	-	-	-	-	-	-
	B	0.1	NS	0	-	-	-	-	-	-
CaCl2	Mean	1.0	NS	0	-	-	-	-	-	-
	A1	2.4	NS	0	-	-	-	-	-	-
	B	3.3	NS	0	-	-	-	-	-	-
CaP	Mean	2.9	NS	0	-	-	-	-	-	-
	A1	1.8	0.043	9	3	0	0	6	33.3	0.0
	B	1.5	0.085	9	3	0	1	5	33.3	0.0
Acid CaP	Mean	19.3	NS	9	3	0	6	0	-	-
Mehlich 3	A1	13.0	NS	0	-	-	-	-	-	-

	B	8.9	0.040	10	3	0	1	6	40.0	14.3
	Mean	10.3	NS	0	-	-	-	-	-	-
		Yield Response								
	A1	4.2	NS	10	-	-	-	-	-	-
	B	12.6	NS	10	-	-	-	-	-	-
CaCl2	Mean	8.3	0.085	10	2	1	5	2	70.0	71.4
	A1	5.3	0.070	10	3	0	5	2	80.0	71.4
	B	4.5	NS	10	-	-	-	-	-	-
CaP	Mean	3.2	0.011	10	3	0	3	4	60.0	42.9
	A1	2.8	0.000	9	3	0	3	3	66.7	50.0
	B	2.7	0.002	9	3	0	3	3	66.7	50.0
Acid CaP	Mean	3.1	0.011	9	3	0	3	3	66.7	50.0
	A1	13.1	0.007	10	3	0	2	5	50.0	28.6
	B	8.6	0.014	10	3	0	1	6	40.0	14.3
Mehlich 3	Mean	14.0	0.013	10	3	0	4	3	70.0	57.1

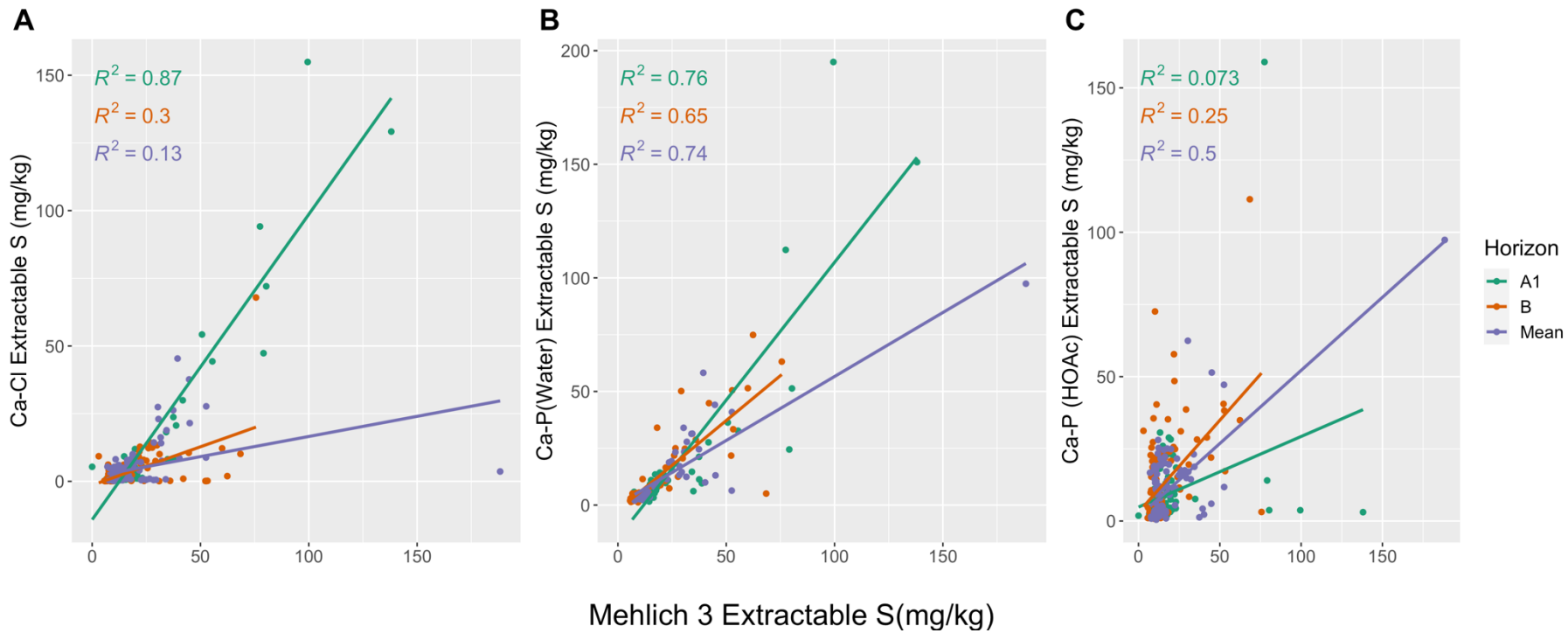


Figure 13 Linear relationship between Mehlich extractable S (mg/kg) vs 0.01 M Calcium Chloride (A), 500 ppm Calcium Phosphate in water (B) and 500 ppm Calcium Phosphate in 2 N HOAc (C) for the A1 horizon (0-10 cm), B horizon (Bottom of A – 20 cm) and the weighted average of the whole 0-30 cm sample. All R^2 values were significant at $p<0.05$ level.

Discussion

The results of this study suggest that the critical levels for the four extracts based on relative yield are 26 mg/kg, 10.4 mg/kg, 8.3 mg/kg, and 18.1 mg.kg for CaCl2, CaP, acid CaP and Mehlich3 respectively and the CaP extracts (both in water and acid) performed better than CaCl2 or the commonly used Mehlich 3. Contrary to our expectations, extractable S in the subsoil correctly identified a lower percentage of sites than the A1 alone or the weighted mean. In fact, the A1 horizon extractable S was better able to predict where applied S would be needed than either the B horizon or the weighted average for the whole 0-30 cm sample. The only times that the weighted average improved the accuracy of the soil test was for the number of responsive sites predicted correctly by CaCl2 for relative yield, relative S yield, and yield response.

The CaCl2 extract performed the worst out of all the extracts. The CaCl2 extraction, a weak neutral salt solution, extracts mainly SO_4^{2-} already in easily soluble form along with some relatively soluble low molecular weight S-containing organic molecules such as amino acids (Kowalenko and Lowe, 1975; Reisenauer, 1975; Ketterings et al., 2011). The CaCl2 extract is unable to extract the adsorbed fraction of SO_4^{2-} and less soluble forms that may become dissolved or plant available over time (Reisenauer, 1975; Ketterings et al., 2011). It would follow that on sandy coastal plain soils in the mid-Atlantic, such as those used in this study, the CaCl2 extraction was not able to accurately predict soils that would respond to S fertilization because these soils are often low in readily available SO_4^{2-} that is easily detected by CaCl2.

These results are in contrast to the results from Ketterings et al. (2011) that suggested CaCl2 was most sensitive to changes in soil S content and least effected by differences in soil type. Another study done by Ketterings et al. (2012) looking at S

application on alfalfa identified the critical level for CaCl2 extractable S to be around 8 mg-S/kg for a 20 cm deep soil sample, however, our dataset indicated that the critical level for CaCl2 extractable S was NS for A1 samples (10cm) and around 26 mg/kg for the whole 30 cm sample in sandy coastal plain soils based on relative yield. The divergence in critical values may be a result of the very different soil mineralogy and texture in the present study compared to the New York study. The New York soils studied were much higher in pH, soil organic matter, total clay and 2:1 clay minerals than the low organic matter, low clay, and relatively high iron-aluminum oxide sandy coastal plain soils used in this study (Ketterings et al., 2011, 2012). Anion adsorption capacity generally decreases with increasing pH and 2:1 type clay mineralogy (Reisenauer, 1975; Ketterings et al., 2011, Ensminger, 1954). The pH of soils used in this study ranged from 4.9 – 7.0, with a mean of 5.99 in the A1 and 5.89 in the B horizon. At these pH levels, adsorbed SO_4^{2-}, which is not easily measured using CaCl2, would play a more significant role in determining the supply of S to plants during the growing season.

Extracting solutions that include a strongly sorbed anion, such as PO_4^{3-}, that can easily replace adsorbed SO_4^{2-} were better able to predict plant available S than CaCl2. This would suggest that CaCl2 extracts a smaller proportion of available S in soils that have a higher portion of S adsorbed onto subsurface clays and Fe/Al oxides as is characteristic of sandy coastal plain soils. These results are consistent with Sahrawat et al. (2009) that found the CaP extracts to be able to better detect adsorbed sulfate in lower pH soils than CaCl2. The results of this study found the critical level for CaP and acid CaP to be 10.4 and 8.3 mg/kg respectively for the A1 horizon based on relative yield. These results are in agreement with prior research that found the critical level for CaP to

be between 7-13 mg/kg (Fox et al., 1964; Hoeft et al., 1973a; Nguyen et al., 1989; Blair et al., 1993).

The Mehlich3 extractant has not been extensively evaluated as a test for S. The limited research done on Mehlich3 found the critical level to be around 20-22 mg/kg which is similar to the critical value determined by this study which was 18.1 for the A1 horizon based on relative yield (Soil Fertility Management, 2010; Seth et al., 2018). The strong correlation between Mehlich3 and CaCl2 in the A1 horizon suggests that both are able to extract readily available SO_4^{2-} present in the A1 horizon as dissolved inorganic SO_4^{2-} that is part of the soil solution, SO_4^{2-} that is weakly held by soil organic matter and small soluble organic compounds. However, the weak relationship in the B horizon with CaCl2 and stronger relationship between Mehlich3 and CaP in the B horizon suggests that Mehlich3 is better able to displace adsorbed SO_4^{2-} held on the surface of clays and Fe/Al oxides. This agrees with prior research that found Mehlich3 extracted higher amounts of S than CaCl2 likely due to the acetate and nitrate anions present in the extracting solution (Seth et al., 2018). Despite the correlation between Mehlich3 and CaP, the Mehlich3 extraction correctly identified a lower percentage of sites than the CaP extracts and thus was worse at predicting the S needs of the 23 sites used in the study. These results are in agreement with prior research that found Mehlich3 to be a poor predictor of crop response to applied S (Hoeft et al., 1973a; Sahrawat et al., 2009; Ketterings et al., 2011).

Soil properties are an important aspect to take into consideration when evaluating the effectiveness of a soil test. However, other external factors are also important to consider when evaluating the need to apply additional fertilizer. One important aspect to

consider when trying to predict a crop response to applied S is any history of applied manure or other S containing fertilizers. None of the fields used in this study at CMREC Beltsville had recent manure applications, but all had had some application of S containing fertilizers in the prior five years to the study, most notably the four fields that received application of 1120 kg/ha gypsum (190kg-S/ha) in 2017 (Table 2). Gypsum is often used as a soil amendment for its soil flocculation properties, however the S from gypsum (which has a solubility rate of ~2.0–2.5 g/l at 25 °C) could remain in the soil for several years, therefore satisfying any crop demand for S. Although the residual effects of gypsum are thought to be negligible on sandy soils, they can be significant on finer textured soils and there is little research confirming the legacy impact of gypsum (Camberato et al., 2012; Camberato and Casteel, 2017).

Another major factor that the soil test does not take into account is the weather throughout the growing season. The weather patterns varied dramatically from year to year during the study period and likely had major impacts on crop yields and S uptake observed. The 2017 and 2019 growing seasons overall had much lower than average rainfall, and the 2018 growing season had much higher than average rainfall. Additionally, in 2019 there was a six week drought period that occurred from mid-August to mid-October that coincided with the pod filling stage of the soybean growth and caused low yields across the entire state of Maryland (Whetstone, 2019). When plants are drought stressed, water is likely the most limiting factor for crop growth making a response to nutrient unlikely until the water limitation is removed. Thus, the lack of yield response to S in 2019 may have been more related to limiting factors other than S such as late planting or water stress. Double crop soybeans are especially susceptible to limiting

conditions of low temperature, excessive crop residue interfering with stand establishment, early frost date, and soil nutrient deficiencies (Hansel et al., 2019). The CaP extracts (which did a better job at predicting responsive sites than CaCl2 or Mehlich3) incorrectly identified 6-9 sites that tested low but gave no response. These sites may have been S deficient but did not respond due to other limiting factors such as low rainfall, other nutrient deficiencies, heat stress, etc. These results necessitate more research on a wider range of soils, climate zones, and crops to further evaluate the potential of the extracting solutions to predict crop response to applied S.

When interpreting the soil test results in order to make a recommendation to farmers it is important to understand the results within the context of reducing the risk to the farmer of either applying fertilizer and having no crop response (a low soil test but no response) or not applying fertilizer to a field where there would be a response (high soil test with response). Due to the fact that the CaP extracts identified the most sites correctly, these tests offer the lowest risk of applying S where it is not needed. However, more research is needed on a wider range of soils in order to further calibrate the critical values to make recommendations to farmers across a wide range of soil types and climate regimes.

Conclusions

Results of this study indicate that CaP and Acid CaP do a better job than CaCl2 or Mehlich3 at identifying soils that will be responsive to applied S for sandy coastal plain soils in the mid-Atlantic region of the United States. The results of this study suggest that the top 10 cm extractable S levels were able to accurately identify responsive sites more often than the weighted average of a 30 cm sample or the subsoil alone. Based on the A1

horizon alone results for relative yield the critical level for CaP and acid CaP extractable S were 10.4 and 8.3 mg/kg respectively. Further research on soil S test calibration on a wider range of soils and crops is warranted in order to confirm the critical values and create appropriate recommendations for farmer's S management. Results of this study indicate that 15 cm samples are likely sufficient to provide recommendations for S fertility however more research is needed to understand the role that subsoil S plays in S soil fertility management in order to further inform recommendations for soil sampling depth.

Appendix 1: Site History Information

Table 20 Summary of site cropping history and agronomic treatments for each field used over the 2017, 2018, 2019 growing season. Table includes Field ID, Crop, Variety, Sulfur treatments applied in field trial, Tillage History, S application to field prior to use in study, prior crop rotation, herbicide application during study, plant data, harvest date, Epsom Salt Spray date, Gypsum application date. DC= double crop Soybean planted after wheat harvest; FS = full season soybean; CB=common dry bean;B0F0=No S control,B0F1 = Epsom applied at a rate of 86 kg/ha as a foliar spray at first flower; B1F0=Gypsum applied at a rate of 560 kg/ha at the time of planting and; B1F1 = combination of gypsum and Epsom

Year	Field ID	Crop	Cultivar	Treatments	Tillage (past 5 years)	Prior S Application (kg-S/ha)[1]	Crop Rotation	Herbicide Application	Plant Date	Harvest Date	Epsom Applied	Gypsum Applied
2017	5-43A	DC	TA3959R2S	B0F0 B0F1 B1F0 B1F1	No-Till	2017-32 2016-20 2015-0	2017 Wheat/DC Soybean 2016 FS Soybean 2015 Sorghum/FS Soybean 2014 Wheat/DC Soybean 2013 FS Soybean 2012 Corn	glufosinate @ 0.85 L Ammonium Sulfate @1.3 kg glyphosate	7/11/17	10/31/17	8/31/17	4/11/17
2017	5-39B	DC	TA3959R2S	B0F0 B0F1 B1F0 B1F1	No-Till	2017-20 2016-0 2015-20	2017 Wheat/DC Soybeans 2016 FS Soybeans 2015 Wheat/DC Soybeans 2014 FS Soybeans 2013 Corn 2012 Wheat/Barley DC Soybeans	glufosinate @ 0.85 L 1.3 kg Ammonium Sulfate, glyphosate	7/11/17	10/31/17	8/31/17	4/11/17
2017	5-43B	FS	Pioneer 40T84X	B0F0 B0F1 B1F0 B1F1	No-Till	2017-190 kg-S/ha as gypsum 2016-32 2015-20	2017 FS Soybeans 2016 Corn 2015 Wheat DC Soybeans 2014 FS Soybeans 2013 Corn	0.95L glyphosate,0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide, Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/10/17	10/18/17	8/10/17	4/12/17

2017	5-18O	FS	Pioneer 40T84X	B0F0 B0F1 B1F0 B1F1	No-Till	2017-0/32 2016-0/32 2015-0/32	2017 FS Soybeans 2016 Corn 2015 FS Soybeans 2014 Corn 2013 Wheat/DC Soybeans 2012 FS Soybeans	0.95L glyphosate,0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide, Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/10/17	10/26/17	8/26/17	4/12/17
2018	5-17C	DC	Asgrow 4135	B0F0 B0F1 B1F0 B1F1	No-Till	2018- 20 2017-32 2016-20 2015-32, +190 kg-S/ha as gypsum	2018 Wheat/DC Soybeans 2017 Corn 2016 Wheat/DC Soybeans 2015 Corn 2014 Wheat/DC Soybeans 2013 Corn	0.85L glufosinate, 0.17 L Clethodim, 1.3 kg Ammonium Sulfate,1.4L Glyphosate,	7/10/18	12/6/18	9/4/18	7/1/18
2018	5-25A	DC	Asgrow 4135	B0F0 B0F1 B1F0 B1F1	No-Till	2018 - 0/32 2017-0/32+ 190 kg-S/ha as gypsum 2016-0/32 2015-0/32	2018 Wheat/DC Soybeans 2017 FS Soybeans 2016 Corn 2015 Wheat/ DC Soybeans 2014 FS Soybeans 2013 Corn	0.85L glufosinate, 0.17 L Clethodim, 1.3 kg Ammonium Sulfate,1.4L Glyphosate,	7/10/18	11/29/18	9/4/2018	7/1/18
2018	5-39C	FS	Pioneer 31A22	B0F0 B0F1 B1F0 B1F1	No-Till	2018-0 2017-32 2016-32 2015-32	2018 FS Soybeans 2017 Corn 2016 Corn 2015 Corn 2014 Wheat/DC Soybeans 2013 Corn	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/18/18	10/9/18	8/5/18	5/25/18
2018	5-18E	FS	Pioneer 31A22	B0F0 B0F1 B1F0 B1F1	No-Till	2018 - 0/32 2017-0/32 2016-0/32 2015-0/32	2018 FS Soybeans 2017 Corn 2016 FS Soybeans 2015 Corn	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide	6/18/18	12/10/18	8/17/18	5/25/18

							2014 Wheat/DC Soybeans 2013 FS Soybeans	Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate				
2018	UMBB	CB	Midnight Black Turtle	B0F0 B0F1 B1F0 B1F1	2018[1]	None	2018 CB bean 2017 No Crop 2016 Soybeans 2015 Soybeans 2014 corn 2013 Soybeans	none	6/19/18	9/11/18	8/8/18	6/19/18
2018	5-39B	CB	Midnight Black Turtle	B0F0 B0F1 B1F0 B1F1	2018[1]	2018 - 0 2017-20 2016-0 2015-20	2018 CB bean 2017 Barley/DC Soybeans 2016 Wheat/DC Soybeans 2015 Corn 2014 Wheat/DC Soybeans 2013 Corn	0.94 L Paraquat, 0.02 L halosulfuron methyl, 0.75 L Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide, 0.4 L Clethodim	5/30/18	9/11/18	8/8/19	6/1/19
2019	5-39A	DC	Asgrow 43X7	B0F0 B0F1	No-Till	2019-20 2018 – 32 2017-0 2016-32 2015-20	2019 Wheat/DC Soybeans 2018 Wheat/DC Soybeans 2017 FS Soybeans 2016 Corn 2015 Wheat/DC Soybeans 2014 FS Soybeans	0.85 L glufosinate, 0.35 L Fluazifop-P-butyl, 1.3 kg Ammonium sulfate8-1-2018, 1.4 L glyphosate	7/14/19	11/4/19	8/26/19	NA
2019	5-7F	DC	Asgrow 43X7	B0F0 B0F1	No-Till	2019-NA 2018 – 32 2017-0 2016-32 2015-NA	2019 Oats/DC Soybeans 2018 Corn 2017 Wheat/DC Soybeans 2016 Corn 2015 Wheat/DC Soybeans 2014 Corn 2013 Wheat/DC Soybeans	0.85 L glufosinate, 0.35 L Fluazifop-P-butyl, 1.3 kg Ammonium sulfate8-1-2018, 1.4 L glyphosate	7/15/19	11/6/19	8/26/19	NA

2019	5-43A	FS	Pioneer P29A25X	B0F0 B0F1	No-Till	2019-0 2018 - 20 2017-32 + 190 kg-S/ha gypsum 2016-20 2015-0	2019 FS Soybeans 2018 Wheat/DC Soybeans 2017 Corn 2016 Wheat/DC Soybeans 2015 FS Soybeans 2014 Wheat/DC Soybeans	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	5/31/19	10/3/19	7/28/19	NA
2019	5-25C	FS	Pioneer P29A25X	B0F0 B0F1	No-Till		2019 FS Soybeans 2018 Wheat/DC Soybeans 2017 Corn 2016 Corn 2015 Wheat/DC Soybeans 2014 FS Soybeans	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/4/19	10/3/19	7/28/19	NA
2019	5-40	FS	Pioneer P42A96X	B0F0 B0F1	No-Till		2019 FS Soybeans 2018 Corn 2017 Wheat/DC Soybeans 2016 FS Soybeans 2015 Corn 2014 Wheat/DC Soybeans	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-anisic acid), 0.65 L glyphosate	6/8/19	10/14/19	7/28/19	NA
2019	5-17A	FS	Pioneer P42A96X	B0F0 B0F1	No-Till	2019-0 2018 - 32 2017-20 2016-32 2015-32	2019 FS Soybeans 2018 Corn 2017 Wheat/DC Soybeans 2016 Corn 2015 Wheat/DC Soybeans 2014 FS Soybeans	0.95L glyphosate, 0.13 Sulfentrazone 0.75 L pt. Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide Diglycolamine salt of dicamba (3,6-dichloro-o-	6/8/19	10/11/19	7/28/19	NA

								anisic acid), 0.65 L glyphosate				
2019	5-39A	CB	Eclipse	B0F0 B0F1 B1F0 B1F1	2019[2]	2019-20 2018 - 32 2017-0 2016-32 2015-20	2019 Black Bean 2018 Wheat/DC Soybeans 2017 FS Soybeans 2016 Corn 2015 Wheat/DC Soybeans 2014 FS Soybeans	0.01 L Halosulfuron-Methyl, 0.56 L Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide	6/6/19	10/4/19	7/20/19	6/6/19
2019	5-7A	CB	Eclipse	B0F0 B0F1 B1F0 B1F1	2019[2]	2019-NA 2018 - 32 2017-NA 2016-32 2015-0	2019 Black Bean 2018 FS Soybeans 2017 Vegetables 2016 Vegetables 2015 Sweet Corn 2014 Vegetables	0.01 L Halosulfuron-Methyl, 0.56 L Metolachlor: 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide	6/6/19	10/19/19	7/20/19	6/6/19
2019	SK1	DC	Pioneer P41T65PR	B0F0 B0F1	No Till		corn, wheat/DC Soybeans	0.95 L glyphosate	6/17/19	10/18/19		
2019	JL1	FS	Pioneer 33A24X	B0F0 B0F1	2016[3]		2019 -Soybeans 2018-Corn 2017-Soybeans 2016-Corn 2015-Soybeans	0.95 L glyphosate	4/11/19	9/27/19		
2019	DS1	FS	HS44X80	B0F0 B0F1	2016 2018[4]		2019 – Wheat/DC Soybeans 2018- Potatoes (rye covercrop) 2017- Watermelons (rye covercrop) 2016- FS Soybeans 2015- Milo 2014- Wheat/DC Soybeans		6/28/19	11/5/19	8/6/19	

[1]All prior S treatments applies as 22-0-0-5S analysis fertilizer, except gypsum where indicated.
[2]Surface Till, No Till prior five years
[3]Sludge Application with Conservation tillage, prior five years no till
[4]Moldboard plowed every three before 2016, Chisel plowed in 2018

Appendix 2: Comparison of soil test measurements between two soil test labs

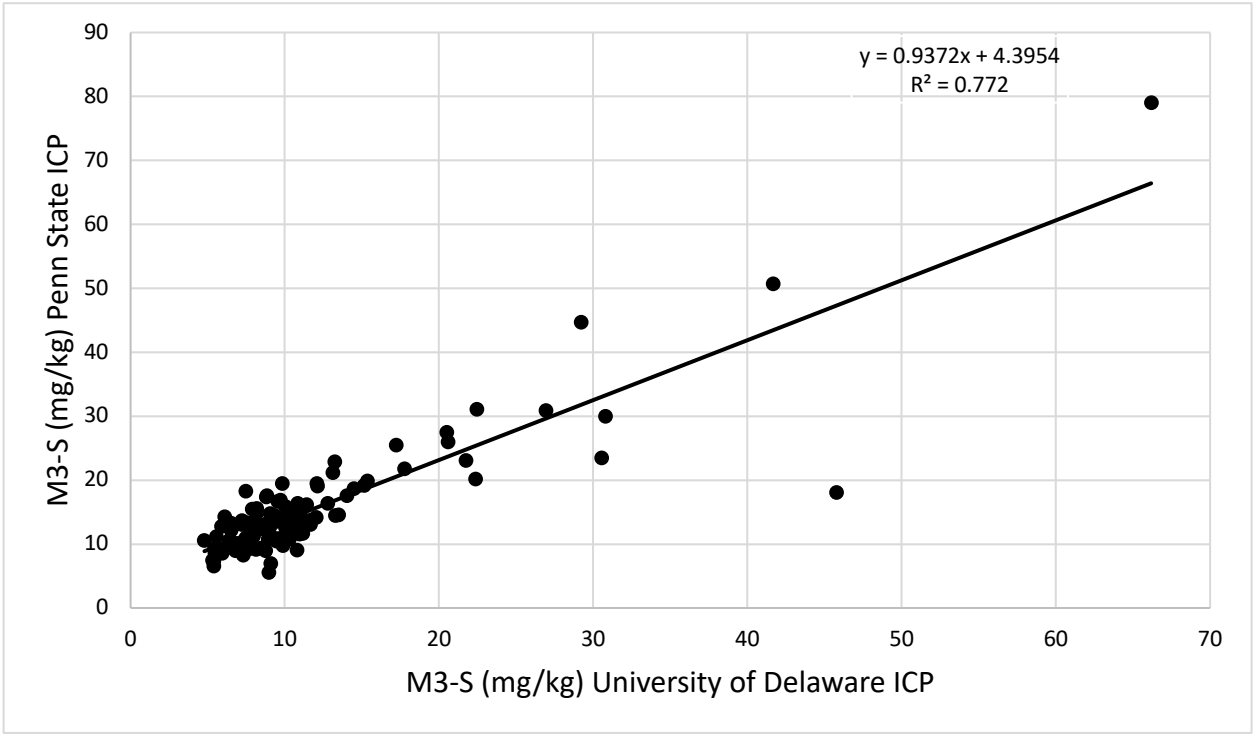


Figure 14. Linear relationship between Mehlich3 extractable S mg/kg analyzed by ICP at the Agricultural Analytical Services lab at Penn State University and Mehlich3 Extractable S mg/kg analyzed by ICP at University of Delaware Soil test lab.

Chapter 6: Conclusion

The goal of this thesis project was to advance the literature on S fertility management for grain legumes. Despite challenges from several non-treatment environmental factors, this study showed that on S deficient soils S application can significantly increase grain legume yields, seed S content, S yield, and seed CYS + MET content. This study also confirmed that XRF can be used as a rapid tool for measuring seed S content. This eliminates one of the major barriers for evaluating S content of crops, that has relied on expensive and time-consuming analysis methods.

Growing interest in organic foods and increasing demand for plant-based proteins, has increased the importance of enhanced MET +CYS content of grain legumes. Currently MET+CYS are synthetically added to animal feed rations, however this practice is strictly regulated under organic regulations. Agronomic management of S for enhanced feed value of legumes is a cost-effective way to enhance protein quality.

The current state of our commodity food system only compensates farmers for yield and thus incentives farmers to continually increase yields. This project could help pave the way for a food system that compensates farmers for crop quality in addition to quantity. With more research to calibrate the correlation between seed S content and MET+CYS content of grain legumes as well as other crops, the XRF could be used to rapidly test incoming crops to a wholesale buyer, such as a grain elevator, and allow farmers to receive a premium for higher protein crops.

More research is needed to further evaluate soil S tests in order to make accurate fertilizer recommendations for farmers however the CaP extractions are promising alternatives to Mehlich3, which can often provide misleading results. The results of this

project merit further research on S fertility management in the mid-Atlantic as occurrence of S deficiency will likely increase as the current soil reserves are depleted.

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