The effects of sulfur and selenium on glucoraphanin and seleno-methylselenocysteine concentrations in broccoli *(Brassica oleracea L. italica).*

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Dedication

This thesis is dedicated to Jeff. Thank you for your constant support and encouragement. I couldn't have done this without you.

Abstract

Glucosinolates (GLSs) are sulfur-containing secondary metabolites produced by broccoli (*Brassica oleracea* subsp. *italica*) and other cruciferous vegetables. GSLs exist for use in plant defense, but are gaining research interest for their role in cancer prevention. Glucoraphanin (GR) is a particular glucosinolate found in broccoli that has great health benefit potential. Seleno-methylselenocysteine (SeMSC) is another compound unique to broccoli when it has been exposed to selenium during plant growth, and is also of interest for its chemopreventive potential. To understand the relationship between root fertilization of sulfur and selenium on GR and SeMSC concentrations in a production environment, we exposed a low-GR ('Green Magic') and a high-GR ('Beneforte') broccoli to sulfur (0 – 34 kg•ha^{-1}) and selenium (0 – 3.36 kg•ha^{-1}) fertilization treatments in the field. GR and SeMSC concentrations depended upon cultivar, treatment, and environmental factors. 'Beneforte' consistently delivered the highest GR concentration, and 'Green Magic' the lowest, and 'Beneforte' GR concentrations were less affected by the presence of Se treatments than 'Green Magic'. 'Beneforte' also accumulated higher concentrations of SeMSC overall than 'Green Magic'. A colder, wetter spring in 2013 led to reduced sulfur uptake and lower concentrations of GR overall, while a warmer, drier climate during later Se applications increased Se uptake and subsequent SeMSC concentrations. Contrasting, a warmer, dryer spring the following year gave way to increased sulfur uptake and greater GR concentrations, and wetter, cooler conditions during Se applications negatively impacted SeMSC concentrations overall. To assess the efficacy of foliar Se fertilization as an alternative to root application, the same two varieties were grown in a greenhouse and were subjected to one of four sodium selenate treatments (0 – 93.74 mg Se•plant^{1}). Again, Se significantly affected the concentration of GR in 'Green Magic', but not in 'Beneforte'. Overall, a weak relationship between GR and SeMSC concentrations give a promising outlook to the ability to maximize GR and SeMSC for ultimate benefit upon consumption of broccoli. This is especially true in 'Beneforte', where GR concentrations remain relatively stable in the presence of Se, but still allow Se uptake and SeMSC formation.

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1 Literature Review

1.1 Introduction

Glucosinolates are secondary metabolites synthesized for use in plant defense against fungi, bacteria, and herbivory, and are found in high concentrations in Brassica species. These compounds have recently been gaining research interest for their potential as chemopreventive agents in humans upon consumption. Another budding area of research is in the use of plant-produced selenium compounds for chemoprevention. Secondary accumulator plants like broccoli divert selenium away from incorporation into proteins (a practice that proves toxic in non-accumulator plants) and instead stores it in a safe and beneficial form. Broccoli is hence a natural producer of glucosinolate compounds, as well as beneficial selenium-containing compounds. Glucosinolate compounds require sulfur uptake from the soil via the sulfur assimilation pathway, and because of its chemical similarity to sulfur, selenium enters the plant via the same pathway. Consequently, glucosinolate concentrations seem to be negatively affected by the presence of selenium. Understanding how the application of sulfur and selenium fertilizers may affect the concentrations of these compounds within the broccoli plant could have widespread effects on the chemopreventive potential of broccoli.

1.2 Broccoli, *Brassica oleracea* L. *italica*

Broccoli is the most consumed cruciferous vegetable in America and it becomes more and more prominent as a world crop each year (Schmidt and Bancroft, 2011). Broccoli contains multiple health-promoting compounds, including vitamins A, C and K, flavonoids, selenium and secondary metabolites like glucosinolates (Moreno et al., 2006). With its high national intake levels and emerging health benefits, the study of broccoli can provide important information and lead to significant consumer impact.

Origin and phylogeny

Modern-day, green-sprouting broccoli, also known across the world as calabrese, is in the taxonomic rank *Brassica oleracea* subsp. *italica.* Other plants in the *B. oleracea* species include cabbage, cauliflower, kale, kohlrabi, and brussels sprouts. All of these subspecies stem from the original *B. oleracea* parent, wild mustard, which originated

along the coast of Britain, France, and the Mediterranean around 2500 years ago. Broccoli as we know it is thought to have originated in the eastern Mediterranean centuries ago, and plants were eventually brought to Italy where they diversified intensely (Gray, 1982). It was likely first introduced to the United Sates via east-coast Italian immigrant market gardeners in the early 20th century (Gray, 1982). A multitude of contemporary broccoli varieties have developed over the past 2,000 years, and it is now grown extensively in North America (Buck, 1956).

Broccoli is a diploid member of the Brassicaceae family with chromosome number of 2n=2x=18 and can readily cross to produce fertile hybrids. It also has the ability to cross with other subspecies, including cabbage (*capitata),* brussels sprouts (*gemmifera),* cauliflower (*botrytis)* and multiple kale varieties (including *ramosa, alboglabra, sabellica, medullosa,* and *palmifolia).* While there is a high degree of self-incompatibility among the *Brassica* genus, the fertility of the F_1 hybrid is typically strong enough to assure production of progeny through F_2 and beyond (Schmidt and Bancroft, 2011).

Production

Broccoli is a cool season crop that can be planted in spring or fall, with direct seeding possible as early as three weeks before the last spring frost. Broccoli seeds can germinate at temperatures as low as 40°F, but prefer 50-70°F for optimal growth. Seedlings will develop about four to six weeks after planting, and typically take another 60+ days to mature for harvest. Broccoli prefers eight hours of full sun per day and moist, fertile, loamy, slightly acidic soil. It is a heavy nitrogen feeder that grows 18-36" tall with broad leaves and a thick main stalk. Plants can form single or multiple heads, and are harvested for consumption when flower buds are still green and tight. Broccoli is grown primarily for the fresh market, though a small percentage (2.25%) also goes to processing ("NASS - Statistics By Subject,").

1.3 Glucosinolates

Glucosinolates are sulfur-containing secondary metabolites commonly found in the family Brassicaceae (Halkier and Gershenzon, 2006). These compounds are of particular interest to researchers because once hydrolyzed by the myrosinase enzyme, the products

show a strong inverse relationship with cancer risk (Bjorkman et al., 2011). Table 1.1 contains a list of the major glucosinolates found in cruciferous vegetables. The primary glucosinolate compounds found in broccoli are glucoraphanin (4-methylsulfinylbutyl), glucobrassicin (indol-3-ylmethyl) and neo-glucobrassicin (1-methoxyindol-3-ylmethyl), with glucoraphanin accumulation being predominant (Charron et al., 2005).

Chemistry

Glucosinolates are water-soluble anions belonging to the glucose-containing glucoside group (Halkier and Gershenzon, 2006). The basic glucosinolate structure contains a central carbon atom bound to three parts: a thioglucose group via a sulfur atom, a sulfate group via a nitrogen atom and a side-chain R group derived from methionine, phenylalanine, tryptophan, or branched-chain amino acids, depending on the glucosinolate (Figure 1.1). Different R groups are responsible for variations in plant compound biological activity. There are around 120 known glucosinolate compounds, with brassica plants producing 30-40 different types (Halkier and Gershenzon, 2006). Glucosinolates are categorized into three groups: aliphatic (derived from alanine, leucine, isoleucine, methionine, or valine), aromatic (derived from phenylalanine or tyrosine) and indole (derived from tryptophan) (Halkier and Gershenzon, 2006).

Function in plants

Glucosinolates play numerous roles in plant survival and defense, and are also responsible for the characteristic strong, slightly bitter flavors of cabbage, broccoli and other brassica vegetables (Rosen et al., 2005). There is some dispute about the exact location of glucosinolates and myrosinase within the plant. Earlier research states that glucosinolates are synthesized and stored within each plant organ (Rosa et al., 1996), while myrosinase is sequestered within aqueous vacuoles (Fahey et al., 2001). More recent research places glucosinolates in the vacuoles, and myrosinase within specialized myrosin cells (Grubb and Abel, 2006; Moreno et al., 2006). Either way, it is widely agreed upon that, wherever they may be typically housed, the two reactive compounds are physically separated to prevent hydrolyzation. When plant tissue is damaged, stored glucosinolates are exposed to and react with myrosinase, which cleaves a thioester bond

and releases glucose. The reaction results in three types of unstable hydrolysis products: isothiocyanates, nitriles and thiocyanates (Fahey and Talalay, 1999; Fahey et al., 2001), with the general structure shown in Figure 1.2 and chemical transformation shown in Figure 1.3. The activation of glucosinolates upon damage (e.g. wounding, mastication, bruising, or freeze-thaw), along with the biological properties of the hydrolysis products suggest that the major function of these compounds is defense against pathogens, nematodes, and herbivores, and can be attractants for special feeders (Charron et al., 2005; Halkier and Gershenzon, 2006).

Accumulation

Glucosinolate accumulation in broccoli appears to be most affected by genotype (Giamoustaris and Mithen, 1996; Kushad et al., 1999), as well as climate, nitrogen supply, and sulfur supply (Charron et al., 2005; Schonhof et al., 2007). For instance, Charron (2005) found "Brigadier" broccoli to have a greater concentration of both total glucosinolates and glucoraphanin than "Emperor" broccoli. The same study found the highest concentrations of total glucosinolates in crops harvested during higher temperature, higher photosynthetic photon flux, and longer day length. In a study by Schonhof et al (2007) on the broccoli cultivar "Monaco", the highest concentration of glucosinolates were achieved by providing optimal sulfur and nitrogen nutrition during plant growth.

Recalling the typical glucosinolate structure, it follows that the sulfur-rich nature of these molecules would be affected by available sulfur for adequate glucosinolate production. Nitrogen is also a key component in the molecular makeup of glucosinolates, which is why available nitrogen likely plays a role in accumulation.

Glucosinolates in human health

Glucoraphanin is the specific methionine-derived, sulfur-containing aliphatic glucosinolate of greatest importance in research regarding broccoli and reduced cancer risk. Not only because it accumulates in broccoli in the highest concentration, but because its hydrolysis product, the isothiocyanate sulforaphane, seems to provide the

strongest potential for anti-carcinogenic health benefits (Kushad et al., 1999) (Figure 1.4).

Sulforaphane appears to diminish the effects of carcinogens, toxins and reactive oxygen species in two ways:

1. By inhibiting phase I activation enzymes like cytochrome P_{450} from converting harmless compounds to active carcinogens (Maheo et al., 1997).
2. By inducing phase II antioxidant and detoxification enzymes like quinone reductase, thioredoxin reductase 1 and heme oxygenase 1 that protect against the toxic effects of carcinogens (Talalay et al., 1995; Fahey and Talalay, 1999; James et al., 2012).

Evidence from animal studies shows that sulforaphane is metabolized upon consumption via the mercapturic acid pathway, is then conjugated to glutathione by glutathione *S*-transferase (*GST*), and finally is metabolized sequentially by g-glutamyltranspeptidase (*GTP*), cysteinylglycinase (*CGase*), and acetyltransferase (*AT*) to form mercapturic acids (Shapiro et al., 2001) (Figure 1.5). These isothiocyanates, like sulforaphane, then lead to the induction of protective phase II enzymes (Figure 1.6).

Given the extensive chemopreventive activity of sulforaphane, it can be implied that consuming foods with higher concentrations of isothiocyanates (specifically cruciferous vegetables like broccoli) may provide greater enzyme-inducing power in the diet (James et al., 2012), ultimately reducing the risk for prostate cancer (Kirsh et al., 2007; Richman et al., 2012), breast cancer (Telang et al., 1997), colorectal cancer (Seow et al., 2002; Wu et al., 2013), bladder (Michaud et al., 1999), kidney (Hsu et al., 2007), and lung cancer (Zhao et al., 2001; Lam et al., 2010).

1.4 Selenium

Selenium is a naturally occurring element found in igneous and sedimentary rocks, and has a likely origin in volcanic activity (Bauer, 1997). Selenium enters the soil profile through the slow weathering of these selenium-rich rocks, and is present in the soil at varying levels depending on soil type, alkalinity, and parent material. Selenium is not

essential for plant growth and development, and is even poisonous to many plants. However, it is considered beneficial for some plants.

Chemistry

Selenium can be absorbed by plants in three main forms: inorganic selenate (SeO_4^{2-}), inorganic selenite (SeO_3^{2-}), or organic selenium compounds (Figure 1.7) (Barker and Pilbeam, 2006), with selenate being the main form absorbed (Finley, 2005). Broccoli specifically absorbs ten times more selenate than selenite (Kopsell and Kopsell 2007).

It is proposed that selenate is taken up into roots by high-affinity sulfate transporters, where it is then likely transported to chloroplasts, and then follows the sulfur assimilation pathway (Sors et al., 2005). Once selenate is reduced to elemental selenium it can be inserted into organic molecules as a sulfur substitute, like sulfur-containing amino acids (e.g. selenomethionine, selenocysteine), amino acid derivatives, or methylated forms (e.g. methylselenol and seleno-methylselenocysteine).

Accumulation

Selenium uptake and accumulation depend on the form and concentration of selenium in the soil, the presence of competing ions, and the affinity of a given plant to absorb selenium (Dhillon and Dhillon, 2003; Barker and Pilbeam, 2006). Plants fall into two selenium accumulation categories: non-accumulator or accumulator. In non-accumulator plants, because of its chemical similarity to sulfur, selenium may interchangeably replace sulfur in amino acids. This modification can render the altered amino acids less efficient, or even useless, by sequestering selenium in plant proteins, which can cause toxicity symptoms like mimicked sulfur starvation, stunted growth, withering leaves, plant chlorosis or death (Barker and Pilbeam, 2006).

However, a subset of plants known as selenium accumulators can provide health benefits upon consumption because of their ability to safely store beneficial selenium compounds without risking toxicity. Broccoli has this unique metabolic capability to divert selenium away from incorporation into proteins (Brown and Shrift 1981). Instead, broccoli accumulates selenium in a methylated form and stores it in membrane-bound structures like vacuoles. This ensures the plant has a much smaller amount of total

selenium incorporated into plant proteins (Ip and Lisk 1994), mitigating the risk of plant toxicity.

Selenium in human health

Specific selenium compounds are shown to be particularly effective in reducing the risk of certain cancers, and one of these compounds happens to be the safe, non-toxic, methylated seleno-amino acid found in selenium accumulators in the form of seleno-methylselenocysteine (SeMSC). This form of selenocysteine is diverted away from incorporation into proteins, as mentioned in the above section, and may also prove to be chemopreventive. Epidemiological evidence has shown that high selenium intake~~s~~ may reduce the risk of mammary, prostate, lung, colon, and liver cancer (Zeng et al., 2013), and broccoli happens to contain a large amount of this beneficial compound (Cai et al., 1995; Roberge et al., 2003). SeMSC is believed to be effective as a chemopreventive agent because the methylated form is more bioavailable than other forms upon consumption (Finley, 2000).

Instead of selenium being tied up in amino acids and proteins like they can be in other plants, seleno-methylselenocysteine is metabolized to methylselenol (Ganther and Lawrence, 1997), making it more available for cancer protection (Finley, 2003) (Figure 1.8). While the mechanisms behind the chemopreventive properties of selenium are not fully understood, a study by Zeng et al. (2013) did shed some light on the association between cancer signal pathways and the ability of methylselenol to inhibit tumor cell invasion. Their research demonstrated that exposure to methylselenol inhibited cell growth by affecting genes directly associated to the regulation of cell cycle. Cells treated with methylselenol induced cell cycle arrest in the G1 phase and apoptosis, which led to cell growth inhibition of 50.1-76.5% of the control (Zeng et al., 2013).

1.5 Sulfur and selenium competition

Sulfur and selenium are in the same group on the periodic table, giving them the same number of valence electrons to interact during chemical bonding. They also have similar size, weight and molecular properties, causing further competition in biochemical processes affecting uptake, distribution, and assimilation in plant development (Sors et

al., 2005). Since selenium and sulfur share chemical and physical resemblance, they are believed to be absorbed by plants via the same transporter mechanism in roots (Figure 1.9). When sulfur is absorbed, it is ultimately incorporated into amino acids and beneficial glucosinolates. When selenium is absorbed as it is in broccoli, it has one ultimate fate as part of the chemopreventive agent SeMSC. The unfortunate consequence of this absorption process is that the two anti-carcinogenic compound precursors end up competing for uptake and assimilation.

1.6 Factors influencing glucosinolate and selenium concentration in broccoli

The competition between sulfur and selenium can be impacted by many factors, resulting in plants with varying concentrations of each compound depending on conditions.

Effect of selenium on sulfur uptake and glucosinolate production

Achieving selenium- and glucosinolate-enriched mature broccoli is a challenge because of their competitive nature within the plant. It has been observed that the presence and uptake of selenium in a growth medium causes an increase in sulfate transporters in the roots (Hsu et al., 2011), but this increase in transporters does not seem to lead to increased glucosinolate concentrations (Charron et al., 2001). A study by Toler et al. (2007) had similar results, finding that the presence of selenium in the soil actually increases the uptake of sulfur, but simultaneously inhibits the production of glucosinolates in *Brassicaceous* plants. Kopsell and Randle (1997) suggested that a low concentration of soil selenium may actually enhance sulfur uptake, but that higher concentrations decrease sulfur uptake. Either way, the presence of selenium appears to have a negative impact on production of certain glucosinolates, even when adequate sulfur is present (Toler et al., 2007).

Effect of selenium specifically on glucoraphanin concentration

Glucoraphanin appears to be particularly affected by the presence of selenium. A study by Kim and Juvik (2011) showed that selenium applied to six-week-old broccoli plants fertilized until maturity resulted in a dose-dependent decrease in glucoraphanin concentration, but did not affect indole or aromatic glucosinolate levels. Concentrations

of glucobrassicin and sinigrin in shoot tissue were less affected by selenium fertilization than glucoraphanin in a study by Charron et al. (2001). This implies that glucoraphanin may be more sensitive to selenium fertilization than are other glucosinolates. Since competition between selenium and sulfur decreases available sulfur, less available sulfur means a decrease in concentration of aliphatic glucosinolates like glucoraphanin more than indole glucosinolates. This phenomenon is likely due to inhibition of methionine synthesis, a sulfur-containing amino acid precursor for aliphatic glucosinolate synthesis (Zhao et al., 1994). The presence of selenium within the plant appears to negatively impact production of certain glucosinolates despite increased sulfate transporters and adequate availability of sulfur in the soil (Toler et al., 2007).

Effect of genotype on glucosinolate and selenium concentration

Genotypic selection has a distinct effect on the glucosinolate concentration of broccoli. Charron et al. (2005) found that glucoraphanin concentration in particular varied widely by genotype. Jeffery et al. (2003) had similar findings, citing that the concentration of both glucosinolates and their hydrolysis products varied with genotype, environment and processing.

Selenium accumulation also varies by genotype. Kim and Juvik (2011) found that cultivars with naturally higher concentrations of glucosinolates experienced a greater decrease in glucosinolates when fertilized with selenium than varieties with naturally lower glucosinolate concentrations. Glucosinolate production can be affected by selenium at the site of biosynthesis, but genetic control of glucosinolate production may also be affected by the presence of selenium (Toler et al., 2007). The type of glucosinolates produced are probably determined by genetic factors, but the interaction between genetic and environmental factors likely controls the overall amount of glucosinolates produced in the plant (Bjorkman et al., 2011).

Effect of production practices and environment

Many environmental factors can alter the distribution and concentration of bioactive components in a plant (Robbins et al., 2005). Variation in agronomic conditions, cultivar, developmental stage, plant organ, competition, fertilization, pH, season, climatic factors,

water availability, light intensity/quality/duration and CO_2 are all known to significantly affect content and profile of phytochemicals in plants (Bjorkman et al., 2011). Specifically, glucosinolate concentration can be modified by sulfur content (Zhao et al., 1993; Withers and O'Donnell, 1994), nitrogen content (Zhao et al., 1993) and soil conditions (Josefson, 1997). Jeffery et al. (2003) found that these factors not only affect glucosinolate concentration, but also the concentration of hydrolysis products.

Effect of fertilization timing

The majority of research has explored the interaction of selenium and sulfur on glucosinolates by using selenium fertilization early in plant growth, in hydroponic systems. It appears that early fertilization of selenium leads to decreased glucoraphanin accumulation in edible broccoli harvested at market maturity. Most research seems to conclude that fertilization with selenate early in development (and throughout plant life until harvest) negatively affects glucosinolate and glucoraphanin concentration.

Hsu et al (2011) drew a different conclusion, however, when a foliar application of sodium selenate was applied to three-month-old broccoli plants early in head development. These plants were harvested at market maturity (just before anthesis) and it was determined that ~25% of the selenate (as SeO_4^{2-}) applied was recovered in the broccoli head, indicating efficient leaf-to-head translocation (Hsu et al., 2011). Glucosinolates, including glucoraphanin, were also not significantly affected by this experiment. One way to maximize glucosinolate and selenium concentration may be to apply selenium fertilizer to plants later in development.

1.7 Conclusion

The concentration of glucosinolates and beneficial selenium-containing compounds in broccoli can vary greatly depending on genotype, nutrient availability, timing of amendments, molecular competition, and many environmental factors. Broccoli has the capability to accumulate two specific chemopreventive compounds in its edible florets, namely glucoraphanin and SeMSC, but because of the competitive nature of the compounds' precursors, both are prevented from being maximized. Studying the unique nature of broccoli in order to better understand its biochemistry could provide guidance

regarding fertilization techniques and timing to bolster SeMSC accumulation without sacrificing glucoraphanin concentration, for the ultimate benefit of the human consumer.

Table 1.1 Glucosinolates commonly found in cruciferous vegetables.

Common Name	Chemical Name	R-group
Gluconapin	3-butenyl	Aliphatic
Progoitrin	2-hydroxy-3-butenyl	Aliphatic
Sinigrin	2-propenyl	Aliphatic
Glucobrassicanapin	4-pentenyl	Aliphatic
Glucoraphanin	4(methylsulfinyl)-butyl	Aliphatic
Neoglucobrassicin	1-methoxy-3-ylmethyl	Indole
Glucobrassicin	Indol-3-ylmethyl	Indole
4-methoxy glucobrassicin	4-methoxy-3-ylmethyl	Indole
4-hydroxy glucobrassicin	4-hydroxyindol-3-ylmeth	Indole
Gluconasturtiin	2-phenylethyl	Aromatic
Sinalbin	4-hydroxybenzyl	Aromatic

Figure 1.1 General glucosinolate structure.

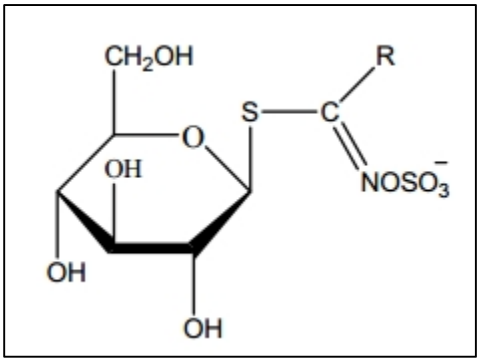
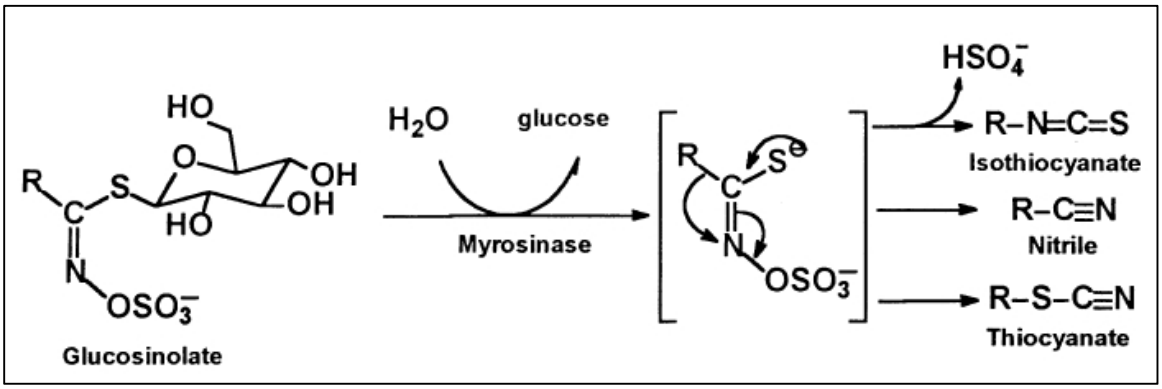
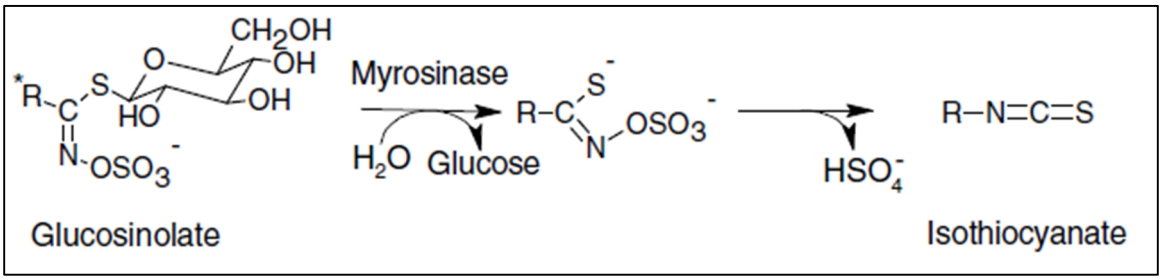


Figure 1.2. Glucosinolate transformation by myrosinase.



(Fahey et al., 2001)

Figure 1.3 Chemical transformation of glucosinolate to isothiocyanate via myrosinase.



(Clarke et al., 2008)

Figure 1.4 Structures of glucoraphanin and sulforaphane.

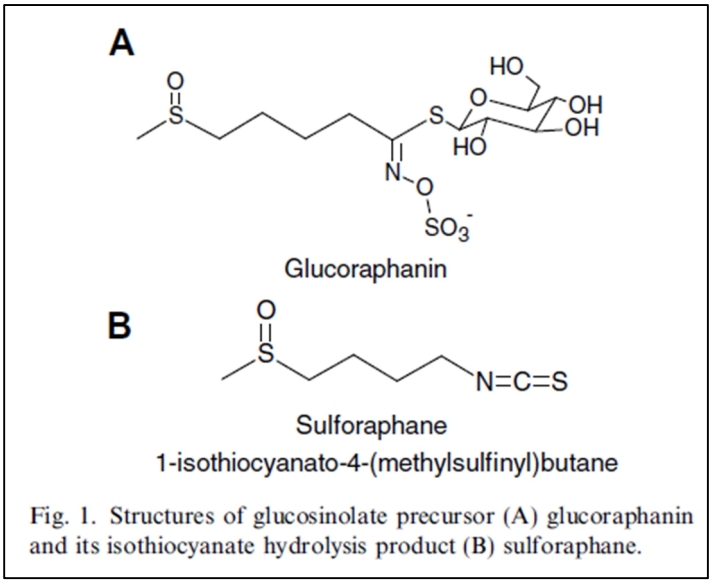
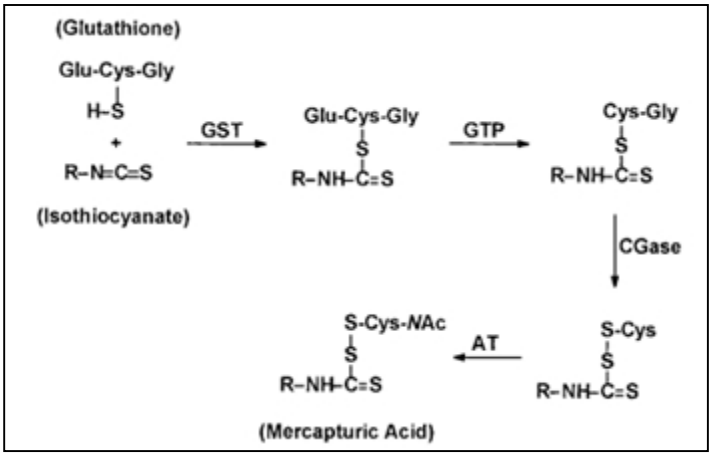


Fig. 1. Structures of glucosinolate precursor (A) glucoraphanin and its isothiocyanate hydrolysis product (B) sulforaphane.

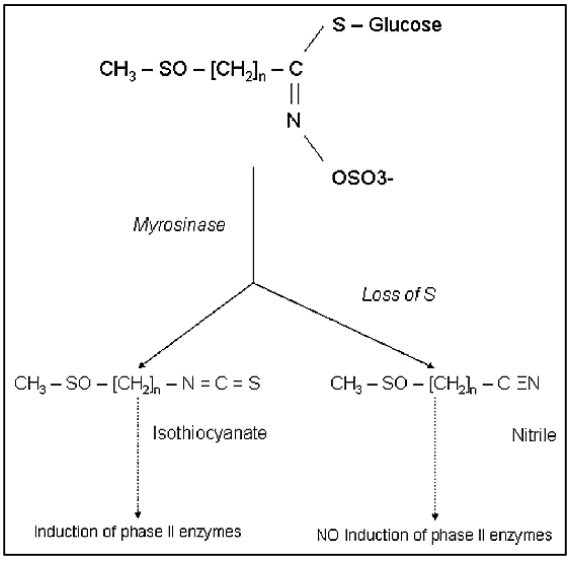
(Clarke et al., 2008)

Figure 1.5 Isothiocyanate transformation to mercapturic acid upon consumption.



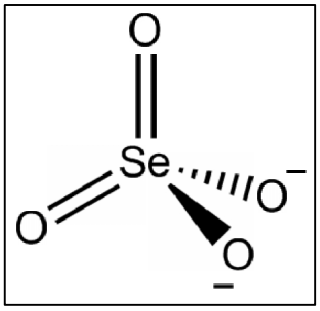
(Shapiro et al., 2001)

Figure 1.6 Hydrolysis of glucosinolates to Phase II enzymes.



(Moreno et al., 2006)

Figure 1.7 Chemical structures of a. Selenate b. Selenite.

a. 

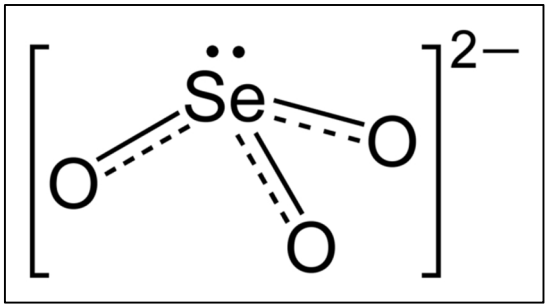
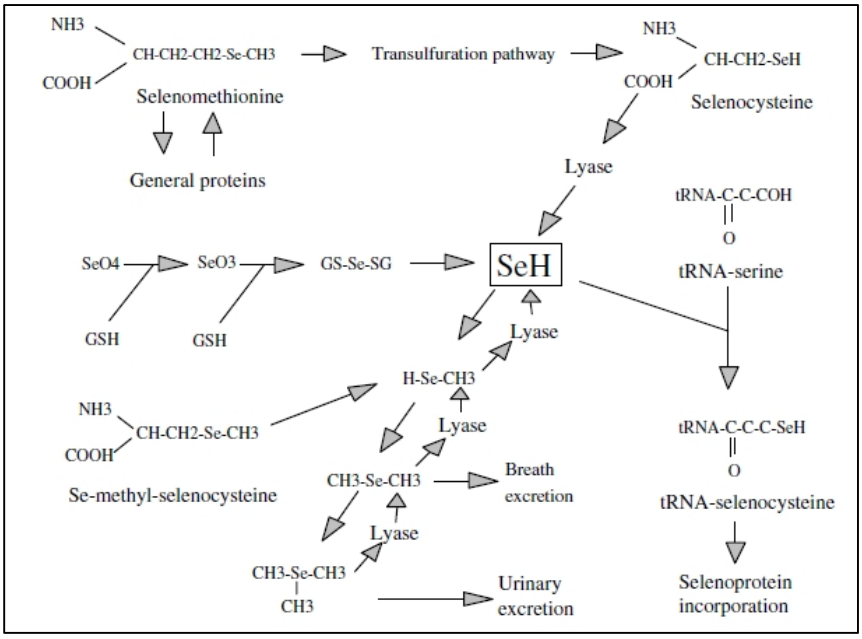
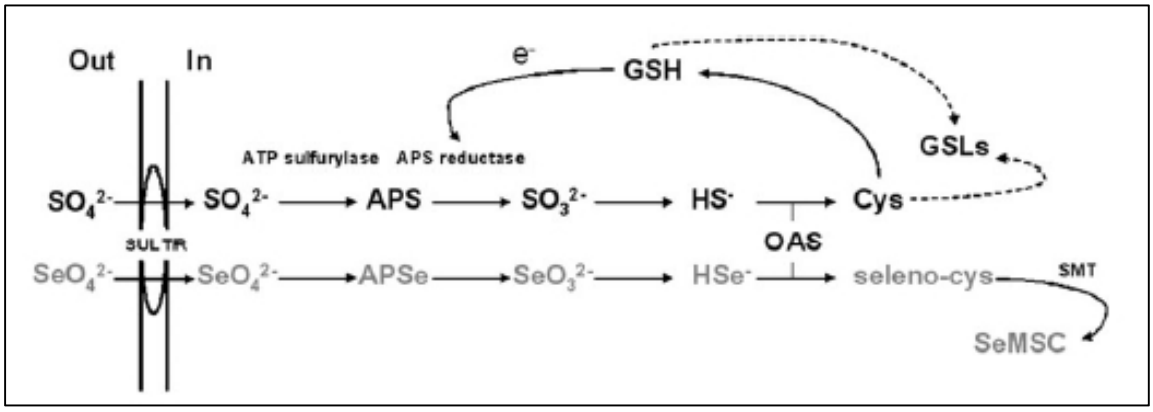
b. 

Figure 1.8 Generalized pathways of selenium metabolism upon consumption.



(Finley, 2000)

Figure 1.9 Current model of sulfate and selenate uptake and assimilation pathways in selenate-fertilized broccoli plants.



(Hsu et al., 2011) SULTR: sulfate transporter; APS: 5'-adenylylsulfate; APSe: 5'-adenylylselenate; OAS: O-acetylserine; Cys: cysteine; GSH: glutathione; GSLs: glucosinolates; SMT: selenocysteine methyltransferase; SeMSC: Se-methylselenocysteine

2 Effect of soil application of sulfur and selenium on glucoraphanin and seleno-methylselenocysteine concentration in field grown broccoli

2.1 Summary

Glucoraphanin (GR) is a specific aliphatic glucosinolate found in large concentrations in broccoli (*Brassica oleracea* subsp. *italica*). GR is a sulfur-containing compound whose concentration in the plant is dependent upon available sulfur (S) in the soil. Once hydrolyzed by myrosinase, GR becomes the isothiocyanate sulforaphane. When consumed, sulforaphane is thought to impart potent chemoprotective qualities by inducing phase II enzymes and inhibiting phase I enzymes (Maheo et al., 1997; Fahey and Talalay, 1999; Kushad et al., 1999).

Seleno-methylselenocysteine (SeMSC) is a methylated seleno-amino acid found in broccoli that has been exposed to selenium (Se). Se is not a required nutrient for normal plant growth and development, and can even be toxic in large enough doses. However, in broccoli Se is diverted away from incorporation into proteins (which leads to toxicity) and is instead integrated into SeMSC. The SeMSC compound is eventually metabolized to methylselenol, CH_3SeH (MeSeH) when consumed, and MeSeH is also documented as having particularly strong chemopreventive properties (Zeng et al., 2009, 2013). Se uptake into the plant occurs via high-affinity sulfate transporters, which is the same route taken by S compounds in the soil, which leads to competition for uptake between Se and S.

The objective of this study was to explore the implications of S and Se fertilization on concentrations of GR and SeMSC in both low-GR and high-GR broccoli, with the ultimate hope of better understanding how rates and timing of S and Se treatments affect the concentrations of these compounds of interest, and how each of these varieties specifically react when exposed to the same treatments. A factorial of 2 x 4 x 2 with split-split-plot design with four replications was used in both 2013 and 2014. ‘Beneforte’ was chosen as a variety with 2-3 times the average GR concentration found in broccoli, while ‘Green Magic’ was selected on its lower-than-average GR concentration.

There was a prevalent effect of year on the data collected during this field experiment, and environmental effects are the likely reason. The field experiment in 2013 yielded 50% less GR than 2014 in both varieties, but the concentration of SeMSC was significantly higher. In contrast, 2014 yielded twice the GR concentration and half the SeMSC. Temperature, soil moisture, and their relationships to the timing of the sodium selenate applications likely influenced the overall results obtained.

2.2 Introduction

Glucosinolates (GSLs) are precursors to isothiocyanates, some of which have particular properties that aid in plant defense while also contributing to human health when they are consumed. Glucoraphanin (GR), the glucosinolate in the largest concentration in broccoli, is of particular importance to researchers because its isothiocyanate, sulforaphane, provides the greatest potential for chemopreventive health benefits upon consumption. Sulforaphane can diminish the effects of carcinogens via two ways: inhibiting phase I activation enzymes (like cytochrome P_{450}) which convert benign compounds into active carcinogens (Maheo et al., 1997), and by inducing phase II enzymes (like quinone reductase) that neutralize carcinogenic compounds (Talalay et al., 1995; Fahey and Talalay, 1999; Kushad et al., 1999). A better understanding of the GR concentrations in broccoli, along with identifying production practices and varieties that can boost GSL production (particularly GR) could provide for vegetables with a stronger impact on improving human health when part of the food supply.

Seleno-methylselenocysteine (SeMSC) is a specific selenium (Se) compound that is also shown to be particularly effective in reducing the risk of certain cancers. In vitro experiments have indicated that methylated Se compounds like SeMSC are more efficient in cancer protection due to their ability to provide a constant production of methylselenol, CH_3SeH (MeSeH) (Tsuji et al., 2009), which is believed to be a key intermediate in chemoprevention (Gammelgaard et al., 2008). SeMSC is metabolized to MeSeH, which has the unique ability to inhibit tumor cell growth by regulating the cell cycle via inducing cell cycle arrest in G1 phase and apoptosis (Zeng et al., 2009, 2013). SeMSC is most efficiently produced in broccoli through fertilization with sodium selenate,

Na_2SeO_4. By studying the variables that most affect SeMSC formation (presence of sulfur, environmental effects, varietal effects, etc.) we can better recommend production practices to maximize SeMSC in broccoli. Ultimately, sodium selenate fertilization rates that maximize SeMSC concentration within the plant without compromising GR concentrations will lend further insight into production practices that could enhance this chemopreventive compound within the edible portions of broccoli.

The 'Green Magic' cultivar was chosen for this study as the low-GR broccoli model. It has lower-than-average GR concentration, but is used with some regularity in commercial broccoli production for its other desirable traits. The 'Beneforte' cultivar was chosen as a high-GR broccoli. 'Beneforte' was developed to enhance assimilation of sulfate and channeling of additional sulfur to GR production (Armah et al., 2013), and as a result, contains 2-3 times the GR concentration of the average commercial broccoli.

GSL (and therefore GR) production is dependent on sulfur (S) uptake, and SeMSC production is dependent on Se uptake. S and Se have similar molecular properties and are both absorbed by the same sulfate transporters in the roots. Since both elements have the potential to be incorporated into beneficial chemopreventive comounds, this study aims to clarify the interactive effects of S and Se within the plant, as well as between cultivars, on the final concentration of GR and SeMSC in broccoli. By temporally separating the fertilizations of S and Se in the field, our hypothesis is that competition for plant uptake between S and Se will be reduced and both compounds will have the opportunity to be incorporated into the beneficial compounds of interest, ultimately boosting the concentrations of both GR and SeMSC. We also believe the concentrations of the target compounds in each cultivar will be differently affected by the treatments administered due to genetic variation. In this study we quantified GR concentration and SeMSC concentration on the fresh weight basis of edible portions of two broccoli cultivars grown with differing rates of S and Se over two years.

2.3 Materials and Methods

This study was conducted at the University of Minnesota Southern Research and Outreach Center (SROC) in Waseca, MN (44.0781° N, 93.5246° W) on a mix of Webster

clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquoll with a pH of 6.4) and Nicollet clay loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll with a pH of 6.4) soils. Seeds were sown in the greenhouse facilities at the University of Minnesota – Twin Cities, St. Paul campus on 3 June 2013 and on 19 May 2014 using 1204 cell tray inserts with 48 cells per tray, and BM2 germination peat moss mix (Berger, Saint-Modeste, Quebec, Canada) as the planting medium. Seedlings were supplemented twice with 125 ppm 15-5-15 Cal-Mag™ fertilizer (Scotts, Marysville, OH). After 3 weeks plants were hardened off outdoors for two weeks prior to transplant.

In 2013, each experimental unit was 1.8 m wide and 5.5 m long, and contained 3 rows of 12 broccoli plants with 45 cm spacing between rows (Figure 2.1). In 2014, spacing was changed to 60 cm between rows to better accommodate tending to the crop, which led to wider experimental units at 2.7 m each, and all other factors the same (Figure 2.2). Plots were fertilized prior to transplant with 90 kg•ha^{-1} nitrogen (N) as urea, $CO(NH_2)_2$, and were side dressed with an additional 67 kg•ha^{-1} N as urea six weeks after transplanting, ultimately providing the recommended total N fertilization for broccoli of 157 kg•ha^{-1} (Rosen and Eliason, 2005). Naturally present sulfate, SO_4^{2-}, concentrations in the plots ranged from 2-6ppm.

The experimental design was a factorial 2 x 4 x 2 split-split plot design with four replications. Each replication was divided into two main plots. Each main plot was comprised one of two sulfur (S) treatments, control (0 kg•ha^{-1} S) or optimal (34 kg•ha^{-1} S) as ammonium sulfate, $(NH_4)_2SO_4$ (AMS) prior to transplant. N fertilization was adjusted as required to account for N present in AMS. Each main plot consisted of four subplots, with each receiving a selenium (Se) treatment: control (0 kg•ha^{-1} Se), low (0.56 kg•ha^{-1} Se), medium (1.68 kg•ha^{-1} Se), or high (3.36 kg•ha^{-1} Se) as sodium selenate (Sigma-Aldrich, St. Louis, MO), Na_2SeO_4. Each plot was then split once more in half, and was randomly assigned one of two cultivars, ‘Beneforte’ (Seminis, St. Louis, MO) or ‘Green Magic’ (Stokes, Buffalo, NY), such that each replication contained one iteration of every possible sulfur/selenium/variety combination, resulting in 16 total experimental units per replication.

The optimal sulfur rate of (34 kg•ha^{-1} S) was chosen based on the recommended S rate for broccoli crops in Minnesota (Rosen and Eliason, 2005). Se rates were selected based on previous studies on the effect of Se on broccoli (Kim and Juvik, 2011; Hsu et al., 2011), as well as through personal consultation (C. Rosen). Genotypes were chosen based on their different concentrations of naturally occurring glucoraphanin (GR). 'Beneforte' contains 2-3 times the GR when compared to other leading commercial broccoli varieties (Charron et al., 2005; Traka et al., 2013), and 'Green Magic' contains 33% the GR of the same leading varieties (Kim and Juvik, 2011).

Planting dates were 2 July 2013 and 26 June 2014. Irrigation drip tape (TSX 508-12-220 tape, T-Systems International, San Diego, CA) was laid between rows prior to transplant, and plants were irrigated as needed throughout the growing seasons. Seedlings were hand-transplanted 45 cm apart within each row, with three rows of the same cultivar per plot. The center row was the only harvested row, and outside rows served only to mitigate border effects. A photo representative of the field trial plots can be found in Figure S1. Conventional management strategies were practiced for pest and weed management, supplemented with hand weeding. Plant growth was monitored regularly, and Se fertilization was administered when heads began to form and were around 3 cm in diameter. Sodium selenate was dissolved in deionized water in concentrations corresponding to the aforementioned Se treatment rates, and was evenly applied to the soil using a Spray N Go™ hand sprayer, model 20200 (Chapin International, Batavia, NY). In 2013, 'Green Magic' received these treatments on 20 August and 'Beneforte' on 23 August. In 2014, sodium selenate was applied to 'Green Magic' on 19 August, and to 'Beneforte' on 3 September. Seasonal temperature and rainfall data are summarized in Table 2.1.

Plants were harvested when heads reached market maturity, just before anthesis. Heads of 'Green Magic' matured faster than 'Beneforte', which lead to earlier harvest of 'Green Magic' overall. In 2013, 'Green Magic' was harvested on 30 August and 'Beneforte' was harvested on 13 September. In 2014, 'Green Magic' was harvested 29 August and 'Beneforte' on 16 September. Three heads within each center row were

harvested and processed for analysis. Heads from each plot were weighed together and recorded for yield measurements (Table S1). On average 'Green Magic' had lower average head weight than 'Beneforte. A composite of the three heads was analyzed for GSL and SeMSC quantification. All samples for GSL analysis were stored at 4°C prior to processing and analysis to mitigate GSL degradation, and were processed within two weeks of harvest. All samples for SeMSC analysis were frozen at -80°C and lyophilized prior to processing and analysis.

Glucoraphanin quantification. Glucoraphanin concentration was determined using the glucosinolate quantification method of Hecht et al. (2004), with modifications from Rosen et al. (2005). Three broccoli heads from each treatment were combined by cutting an equal mass of edible portions (floret and stalk) from each plant (~100 g), such that each aggregated sample weighed 300 g. Samples were then placed into three times the weight per volume of boiling water and boiled for five minutes to deactivate myrosinase activity. Boiled samples were pureed in a blender for two minutes and a 40-mL aliquot of the blended sample was reserved. Samples were then homogenized using a Polytron PT 1300 D homogenizer (Kinematica AG, Lucerne, Switzerland) at 12,000 rpm for two minutes. A 2-ml sample of homogenate was centrifuged for eight minutes at 8,000 g and 4°C.

Extraction of desulfonated glucosinolates (ds-GSL) was achieved using solid phase strong anion exchange (SAX) columns (Sigma-Aldrich, St. Louis, MO). SAX columns were washed with 2-ml of 0.5 M sodium acetate (pH 4.6) buffer, then 2-ml of deionized water, using a vacuum manifold. A 500-ml sample of centrifuged broccoli supernatant was then filtered through the SAX column. Afterward, 1-ml of 0.02 M sodium acetate (pH 4.0) buffer was washed over the column. Following a 1-ml of 0.2 $mg \bullet ml^{-1}$ sulfatase solution (aryl-sulfate sulfohydrolase from *Helix pomatia* – Type H-1, Sigma-Aldrich, St. Louis, MO) was added and vacuumed again. The columns with supernatant and sulfatase were stored at room temperature (~21°C) for ~15 hours, then eluted with 3-ml deionized water, and the eluent volume was determined by weight. The ds-GSL samples were then stored at -20°C until HPLC analysis.

HPLC analysis of GR was conducted on the Agilent 1200 Series Quarternary system (Agilent Technologies, Inc., Santa Clara, CA) with diode array detector set at λ=229 nm using a Luna C18, 5 µm, 250 x 4.6 mm column (Phenomenex, Torrence, CA) set at 30°C. A 50-µl injection of eluent was separated on the machine using flow rates and a gradient as follows: solvent A = water, solvent B = acetonitrile; 0 minutes, 95% A + 5% B, 1.0 ml•min^{-1}; 0-2 minutes, 85%A + 15%B, 1.0 ml•min^{-1}; 2-20 minutes, 53%A + 47%B, 1.0 ml•min^{-1}; 20-22 minutes, 0%A + 100%B, 1.15 ml•min^{-1}; 22-26 minutes, 0%A + 100% B, 1.3 ml•min^{-1}; 26-28 minutes, 0%A + 100%B, 1.0 ml•min^{-1}; 28-34 minutes, 95%A + 5%B, 1.0 ml•min^{-1}. Peaks were displayed using OpenLAB Chromatography Data System with rev. C.01.06 software. The glucoraphanin peak was identified using relative retention times of ds-GSL standard mixes provided by the Hormel Institute in Austin, MN. Ds-GSL concentrations of GR were calculated using relative quantification with an internal standard (sinigrin) and previously published response factors (EU, 1990). Ds-GSL concentrations of GR are reported on a µg•g^{-1} fresh weight (FW) basis. A sample chromatogram of glucosinolate peaks using diode array detection is shown in Figure S2.

Seleno-methylselenocysteine quantification. Seleno-methylselenocysteine concentration was quantified using Sepulveda et al (2013) with slight modifications. Equal portions of edible tissue from the same three harvested heads used in GR quantification were weighed and frozen at -80°C. Frozen samples were lyophilized on a Labconco FreeZone 6 Liter Console freeze dry system (Labconco Corp., Kansas City, MO). Freeze dried samples were pulverized using a mortar and pestal. A 50-mg sample of the broccoli tissue was then placed in a 2-ml centrifuge tube, and 1.5-ml of 50 mM hydrochloric acid (HCl) was added. Samples were vortexed and amino acids were extracted from the broccoli by storing the samples for 18 hours at 4°C. The mixture was then centrifuged at 12,000 g for 10 minutes. Supernatant was recovered and stored in a separate centrifuge tube. Amino acids and modified amino acids, including SeMSC, were then derivatized using the AccQ-Fluor Reagent Kit (Waters Corp., Milford, MA). A 50-µl sample of derivatized amino acid broccoli supernatant was combined with 70-µl of borate buffer and vortexed briefly. A 40-µl sample of AccQ-Fluor reconstituted reagent

was then added and vortexed immediately for 10 seconds. Samples then stood for one minute at room temperature before being moved to the autosampler for separation on the HPLC.

HPLC analysis of SeMSC was conducted on the same system as the GR quantification, but using a 1260 Infinity Fluorescence Detector (Agilent Technologies, Inc., Santa Clara, CA). The same Luna C18 column was used set at 37°C to assist in separation. The fluorescence of derivatized SeMSC was detected using excitation λ=250 and emission λ=395. A 20-µl injection was separated on the HPLC using a 1.0 ml•min^{-1} flow rate and the following gradient: solvent A = 140 mM sodium acetate + 17 mM TEA at pH=5.05 (pH adjusted with phosphoric acid), solvent B = 60% acetonitrile in water; 0 minutes, 100%A + 0%B; 0-0.5 minutes, 98%A + 2%B; 0.5-10 minutes, 80%A + 20%B; 10-20 minutes, 75%A + 25%B; 20-25 minutes, 70%A + 30%B; 25-30 minutes, 60%A + 40%B; 30-35 minutes, 40%A + 60%B; 35-40 minutes, 35%A + 65%B; 40-44 minutes, 98%A + 2%B; 44-45 minutes, 100%A + 0%B. SeMSC peak was identified using co-elution with pure SeMSC (Sigma-Aldrich, Milwaukee, WI), and its concentration was calculated using a calibration curve of known concentrations of pure SeMSC. A sample chromatogram of amino acid peaks using fluorescence detection is shown in Figure S3.

Statistical analysis. Data were analyzed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Due to the strong effect of years, data from 2013 and 2014 were analyzed separately, and in order to best understand how the treatments affected the two varieties individually, 'Beneforte' and 'Green Magic' were also analyzed separately. Significant differences between sulfur and selenium treatments in each year and each variety were determined with a two-factor ANOVA. Dependent variables measured include concentration of glucoraphanin and concentration of seleno-methylselenocysteine. Mean values of response variables were considered significantly different at when the p-value ≤ 0.05, and differences were identified using Tukey's HSD.

2.4 **Results and Discussion**

Glucoraphanin concentration. Variety was significant in the glucoraphanin (GR) response in both 2013 and 2014, and sulfur treatment was significant in 2014 (Tables 2.2

& 2.3). Further analyses were conducted by separating the varieties and looking at the specific effects of each treatment on the chosen cultivars. The overall mean concentration of glucoraphanin (GR) in 2013 for 'Beneforte' was 565.5±175.65 μg GR•g^{-1} fresh weight (FW), and for 'Green Magic' was 115.0±56.97 μg GR•g^{-1} FW (Table 2.4). 'Beneforte' produced a GR concentration almost five times greater than 'Green Magic' during that year. However, there was no significant effect of sulfur (S) nor selenium (Se) treatments on GR concentration in either cultivar in 2013 (Figure 2.3). Overall, the concentration of GR in 2013 plants was about half of the concentration produced in 2014, pointing to strong seasonal effects..

The 2014 overall mean concentration of GR in 'Beneforte' was 1039.0±272.0 μg GR•g^{-1} FW, while 'Green Magic' had an overall mean of 258.9±70.64 μg GR•g^{-1} FW (Table 2.4). The concentration of GR in 'Beneforte' was about four times that of 'Green Magic' in 2014, which is a slightly narrower gap between varieties than found in the previous year, but there were significant effects of S and Se treatments on GR concentration. In 'Green Magic', there was a significant effect of Se on GR, with a significantly higher GR concentration in the 0 kg•ha^{-1} Se treatment than in both the 0.56 kg•ha^{-1} Se and 3.36 kg•ha^{-1} Se treatments (Figure 2.3). In 'Beneforte', the 34 kg•ha^{-1} S treatment was significant in providing higher GR than the 0 kg•ha^{-1} S treatment, across all Se treatments (Figure 2.3).

Armah et al (2013) conducted a study that identified the dose of GR consistent with metabolic change contributing to a reduced risk of cancer to be 400g of high-GR broccoli (average of 21.6 μmol/g dry weight) once a week for 12 weeks. Using this research, we determined the approximate mass of fresh weight broccoli consumption needed to achieve this dose for each variety, treatment, and year (Table 2.5). Depending on the treatment, the ranges for consumption of broccoli were as follows: 2013 Beneforte, 452-671 g•$week^{-1}$ for 12 weeks; 2013 Green Magic, 2144-4291 g•$week^{-1}$ for 12 weeks; 2014 Beneforte, 255-372 g•$week^{-1}$ for 12 weeks; 2014 Green Magic, 944-1520 g•$week^{-1}$ for 12 weeks.

Seleno-methylselenocysteine concentration. In 2013, a full ANOVA analysis indicates a significant effect of variety, as well as significant interactions between sulfur and selenium, and between selenium and variety on the concentration of seleno-methylselenocysteine (SeMSC) (Table 2.6). In 2014, results indicate a significant effect of variety and selenium, as well as a significant interaction between selenium and variety on the concentration of SeMSC (Table 2.7). In order to better understand these results, 'Beneforte' and 'Green Magic' were separated and analyzed individually to determine significant effects for each.

In 2013, the mean concentration of SeMSC was 11.9±4.57 µg SeMSC•g^{-1} FW in 'Beneforte', and 5.5±3.93 µg SeMSC•g^{-1} FW in 'Green Magic'(Table 2.8). These results show 'Beneforte' contained more than double the SeMSC concentration of 'Green Magic' in 2013. There was a significant effect of Se treatments on the level of SeMSC in 'Green Magic', with a significantly lower SeMSC concentration in the 0 kg•ha^{-1} Se than in the 3.36 kg•ha^{-1} Se treatment, as well as a significantly lower SeMSC concentration in the 0.56 kg•ha^{-1} Se than in the 3.36 kg•ha^{-1} Se treatment (Figure 2.4). Significant interactions between S and Se on SeMSC concentration in both 'Green Magic' and 'Beneforte' in 2013 were also observed (Figure 2.4). In 'Beneforte', we observed a significantly higher concentration of SeMSC in the 0 kg•ha^{-1} S: 0.56 kg•ha^{-1} Se treatment than in the 34 kg•ha^{-1} S: 0.56 kg•ha^{-1} Se, 0 kg•ha^{-1} S: 1.68 kg•ha^{-1} Se, or 0 kg•ha^{-1} S: 3.36 kg•ha^{-1} Se treatments. In 'Green Magic' we saw a significantly higher concentration of SeMSC in 34 kg•ha^{-1} S: 3.36 kg•ha^{-1} Se treatment than in 34 kg•ha^{-1} S: 0 kg•ha^{-1} Se or 34 kg•ha^{-1} S: 0.56 kg•ha^{-1} Se.

In 2014, SeMSC concentration in 'Beneforte' was 5.7±2.2 µg SeMSC•g^{-1} FW, and 4.2±2.86 µg SeMSC•g^{-1} FW in'Green Magic' (Table 2.8). This is only about half the concentration of SeMSC that was witnessed in 2013, probably due to the influence of weather conditions and environment. 'Green Magic' SeMSC was highly affected in 2014, both by an interaction between S and Se, as well as a significant effect of Se alone. 'Beneforte' SeMSC concentration, on the other hand, was not affected by S or Se on SeMSC in 2014.

An effective dose of Se to achieve cancer risk reduction was determined by Finley (2003) to be 100-200 µg Se per day. Using this data, we identified the meaningful daily fresh weight dose of broccoli needed to achieve the mid-point recommendation, 150 µg Se, for each cultivar, treatment, and year (Table 2.9). The recommended consumption of broccoli to achieve desired health benefits are as follows: 2013 Beneforte, 8-29 g•day^{-1}; 2013 Green Magic, 13-79 g•day^{-1}; 2014 Beneforte, 23-36 g•day^{-1}; 2014 Green Magic 21-136 g•day^{-1}.

The influence of cultivar. Glucoraphanin and seleno-methylselenocysteine concentrations varied greatly by cultivar. 'Beneforte' was chosen because of its capability to produce some of the highest known GR concentrations in broccoli, while 'Green Magic' was chosen due to its naturally low GR concentrations. There were consistently higher concentrations of both GR and SeMSC in the 'Beneforte' variety. In 2013, 'Beneforte' had almost five times the GR as 'Green Magic' and in 2014, 'Beneforte' had four times the GR concentration of 'Green Magic' (Table 2.4). We were not certain how these genetic traits would translate, however, into SeMSC production. 'Beneforte' ended up with over two times the SeMSC concentration of 'Green Magic' in 2013 and about 1.35 times higher concentration in 2014 (Table 2.8). This shows that not only does 'Beneforte' have a consistently higher concentration of GR, but it is also capable of higher SeMSC concentrations than 'Green Magic' with less fluctuation in GR.

The influence of sulfur. Sulfur alone was not significant in affecting GR or SeMSC concentrations in either variety in 2013, but there was a significant interaction between S and Se affecting concentrations of SeMSC both 'Green Magic' and 'Beneforte' in 2013, which was discussed previously in the *seleno-methylselenocystein concentration* section. In 2014, S was significant in affecting the concentration GR in 'Beneforte' only (Figure 2.3). There was overall a higher concentration of GR in both varieties in 2014, but we didn't see a significant differential between 0 kg•ha^{-1} S and 34 kg•ha^{-1} S treatments for Green Magic. This could be because conditions were optimal for sulfur uptake during 2014, and perhaps the S concentrations naturally present in the soil were enough to maximize GR concentration in 'Green Magic' that year.

The influence of selenium. In 2013, Se was not significant in affecting the GR concentration in either variety, but increasing Se did significantly increase the SeMSC concentration in 'Green Magic', with significantly higher concentrations of SeMSC in the 3.36 kg•ha^{-1} Se treatment than either the 0 kg•ha^{-1} Se or the 0.56 kg•ha^{-1} Se treatments (Figure 2.4). However, in 2014, Se treatments did significantly affect the concentrations of both GR and SeMSC in 'Green Magic' (Figures 2.3 and 2.4). We observed significantly lower GR concentrations in 'Green Magic' with increasing concentrations of Se treatments, and we saw significantly higher SeMSC concentrations in 'Green Magic' as Se treatment concentrations increased. It is especially notable that Se treatments alone were never significant in affecting concentrations of GR or SeMSC in 'Beneforte' in either year.

The influence of environment on glucoraphanin. In 2013, it was a wet early spring, which likely led to reduced S uptake, and ultimately resulted in GR concentrations that were 50% lower than we saw the following year (Table 2.4). The total precipitation over the 2013 growing season was as follows: 'Green Magic', 17.1 cm; 'Beneforte', 17.4 cm (Table 2.1). The wet conditions in the field is likely why there was no significant effect of any S or Se treatments on GR concentrations in either variety in 2013.

In 2014, however, drier conditions prevailed, and GR concentrations were higher overall. Less precipitation, especially early in plant development, may have been more conducive to sulfur uptake, leading to the increased GR concentrations. The average GR concentration in 'Beneforte' in 2014 was almost twice what we saw in the previous year, and we saw more than double the GR concentration in 'Green Magic' when compared to 2013 (Table 2.4). Not only was it dryer earlier in plant development, but total precipitation was less throughout the season. Rainfall totals amounting to 9.2 cm during 'Green Magic' development and 13.2 cm during 'Beneforte' growth likely led to more suitable conditions for sulfur uptake and ultimately increased GR concentrations (Table 2.1).

The influence of environment on seleno-methylselenocysteine. Warmer, drier temperatures around the time of sodium selenate application in 2013 likely contributed to

increased Se uptake and eventual concentrations of SeMSC within the plant. In 2013, from sodium selenate application to harvest,'Green Magic' was grown under an average temperature of 25°C and had less than 2.5 cm precipitation. The same year, 'Beneforte' was grown under a 22°C average temperature with just over 0.25 cm of precipitation between sodium selenate application and harvest (Table 2.1). These conditions resulted in twice the SeMSC concentration as we saw the following year, giving an average of 5.5 µg MSC•g^{-1} FW in 'Green Magic' and 11.9 µg MSC•g^{-1} FW in 'Beneforte' in 2013 (Table 2.8).

The conditions in 2014 were cooler and wetter between Se application and harvest, which may have resulted in the decreased Se uptake and ultimately reduced concentrations of SeMSC. In the days from sodium selenate application until harvest, 'Green Magic' had an average temperature of 21°C, and 4.2 cm precipitation (Table 2.1), which resulted in a SeMSC concentration of 4.2 µg MSC•g^{-1} FW (Table 2.8). 'Beneforte' had an average temperature of 14.4°C and 1.3 cm precipitation from Se fertilization to harvest (Table 2.1), and its SeMSC concentration in 2014 was only 5.7 µg MSC•g^{-1} FW (Table 2.8).

The relationship between glucoraphanin and seleno-methylselenocysteine. The relationship between GR and MSC was weak in both varieties and both years (Figure 2.5), which suggests that utilizing the production strategies outlined in this experiment has the potential to increase both GR and MSC without reducing the concentration of either, especially in 'Beneforte'.

2.5 Conclusion

'Beneforte' consistently produced more glucoraphanin (GR) and seleno-methylselenocysteine (SeMSC) than 'Green Magic' in both years of the study. In 2013 we saw that more rain early in the season led to decreased GR in both varieties. However, warmer and dryer weather later in the season, especially during the application of selenium (Se) treatments, resulted in increased SeMSC concentrations in both varieties. The 2013 conditions led to no significant effect of S or Se on GR in either

variety, but we did see a significant effect of Se on SeMSC in ‘Green Magic’ and a significant S x Se interaction on SeMSC in ‘Beneforte’.

Drier conditions earlier in 2014 led to approximately twice the GR concentration in each variety compared wtih the year prior, while wetter, cooler conditions during Se application exhibited lower SeMSC concentrations when compared to 2013. This environment resulted in more significant effects than the previous year also, with Se having a significant effect on both GR and SeMSC in ‘Green Magic’, and S having a significant effect on GR in ‘Beneforte’.

Both years and both varieties showed weak correlations between GR and SeMSC concentrations, which means the timing of S and Se fertilizations used in this experiment allowed each compound to form relatively independently of each other so that they did not wholly compete for expression within the plant. Overall, we can conclude that warmer, dryer conditions during both the early season S uptake and the later season Se fertilization leads to elevated concentrations of GR and SeMSC, and that ‘Beneforte’ is a better candidate for consistently enhanced SeMSC levels without sacrificing GR.

Table 2.1 Maximum, minimum, and mean daily air temperatures and cumulative precipitation for each month of two growing seasons.

	Year	Maximum Mean Daily Temp (ºC)	Minimum Mean Daily Temp (ºC)	Overall Mean Daily Temp (ºC)	Monthly Cumulative Precipitation (cm)
June	2014	27.0	18.5	22.3	1.60
July	2013	27.7	16.9	22.3	12.27
	2014	25.9	14.9	20.5	2.41
Aug	2013	26.9	15.8	21.3	4.83
	2014	26.9	16.7	21.5	7.75
Sep	2013	26.7	14.7	20.5	0.36
	2014	20.9	9.6	15.0	1.50

Table 2.2 Analysis of variance for 2013 glucoraphanin in field grown broccoli.

Response:	µg GR•g FW^{-1} broccoli				
	df	Sum Sq	Mean Sq	F value	Pr(>F)
s	1	17187	17187	1.0112	0.32
se	3	21952	7317	0.4305	0.7321
variety	**1**	**3067939**	**3067939**	**180.501**	**<2e-16 *****
s:se	3	66848	22283	1.311	0.2825
s:variety	1	4695	4695	0.2762	0.6018
se:variety	3	15294	5098	0.2999	0.8252
s:se:variety	3	92786	30929	1.8197	0.1571
Residuals	45	764856	16997		

Table 2.3 Analysis of variance for 2014 glucoraphanin in field grown broccoli.

Response:	µg GR•g FW^{-1} broccoli				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
s	**1**	**254438**	**254438**	**6.5201**	**0.01390 ***
se	3	123576	41192	1.0556	0.37674
variety	**1**	**9729860**	**9729860**	**249.334**	**< 2e-16 *****
s:se	3	553	184	0.0047	0.99955
s:variety	1	133554	133554	3.4224	0.07048
se:variety	3	61337	20446	0.5239	0.66791
s:se:variety	3	1572	524	0.0134	0.99784
Residuals	48	1873123	39023		

Table 2.4 Glucoraphanin mean concentrations and standard deviations across sulfur and selenium treatments during two growing seasons.

		Mean µg GR • g^{-1} FW broccoli				SD µg GR • g^{-1} FW broccoli			
	Year	Beneforte		Green Magic		Beneforte		Green Magic	
		0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S
0 kg•ha^{-1} Se	2013	682.23	460.09	128.72	112.58	336.34	192.11	70.05	77.66
	2014	985.34	1210.44	316.33	326.91	218.18	318.96	45.77	35.18
0.56 kg•ha^{-1} Se	2013	633.98	511.88	74.69	71.86	133.15	249.22	30.63	29.96
	2014	990.89	1203.34	203.02	238.14	267.24	277.89	50.59	61.39
1.68 kg•ha^{-1} Se	2013	462.65	644.41	147.66	111.13	146.37	112.42	17.98	58.69
	2014	913.84	1127.27	232.07	285.52	299.69	245.53	53.58	108.73
3.36 kg•ha^{-1} Se	2013	590.15	573.84	129.36	143.95	129.03	105.83	54.28	79.43
	2014	829.87	1048.77	214.70	254.52	157.35	346.14	47.76	71.36
		Overall Mean		Overall Mean		Overall SD		Overall SD	
Overall	2013	565.5		115.0		175.65		56.97	
	2014	1039.0		258.9		272		70.64	

Table 2.5 Consumption needed to achieve 21.6 µmol•g^{-1} dry weight broccoli consistent with metabolic change associated with reduced cancer risk, by variety, treatment, and year.

		Approximate g FW broccoli per week for 12 weeks			
	Year	Beneforte		Green Magic	
		0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S
0 kg•ha^{-1} Se	2013	452	671	2397	2740
	2014	313	255	975	944
0.56 kg•ha^{-1} Se	2013	487	603	4130	4291
	2014	311	256	1520	1296
1.68 kg•ha^{-1} Se	2013	667	479	2089	2777
	2014	338	274	1329	1081
3.36 kg•ha^{-1} Se	2013	523	538	2384	2144
	2014	372	294	1437	1212
		Overall Mean		Overall Mean	
Overall Mean	2013	546		2683	
	2014	297		1192	

Table 2.6 Analysis of variance for 2013 seleno-methylselenocysteine in field grown broccoli.

Response	µg SeMSC•g^{-1} FW broccoli				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
s	1	4.31	4.31	0.4031	0.52901
se	3	56.34	18.78	1.7562	0.17065
variety	**1**	**567.94**	**567.94**	**53.1136**	**6.484e-09 *****
s:se	**3**	**227.72**	**75.91**	**7.0987**	**0.0005984 *****
s:variety	1	18.52	18.52	1.7321	0.19545
se:variety	**3**	**164.05**	**54.68**	**5.1141**	**0.0042512 ****
s:se:variety	3	81.81	27.27	2.5502	0.0688295 .
Residuals	41	438.41	10.69		

Table 2.7 Analysis of variance for 2014 seleno-methylselenocysteine in field grown broccoli.

Response:	µg SeMSC•g^{-1} FW broccoli				
	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
s	1	0.64	0.64	0.1262	0.72391
se	**3**	**103.248**	**34.416**	**6.7921**	**0.0006571 *****
variety	**1**	**34.657**	**34.657**	**6.8396**	**0.0118804 ***
s:se	3	4.078	1.359	0.2682	0.84796
s:variety	1	0.973	0.973	0.1921	0.66314
se:variety	**3**	**42.692**	**14.231**	**2.8084**	**0.0494049 ***
s:se:variety	3	9.101	3.034	0.5987	0.61896
Residuals	48	243.221	5.067		

Table 2.8 Seleno-methylselenocysteine mean concentrations and standard deviations across sulfur and selenium treatments during two growing seasons.

		Mean µg SeMSC • g⁻¹ FW broccoli				SD µg SeMSC • g⁻¹ FW broccoli			
	Year	Beneforte		Green Magic		Beneforte		Green Magic	
		0 kg•ha⁻¹ S	34 kg•ha⁻¹ S	0 kg•ha⁻¹ S	34 kg•ha⁻¹ S	0 kg•ha⁻¹ S	34 kg•ha⁻¹ S	0 kg•ha⁻¹ S	34 kg•ha⁻¹ S
0 kg•ha⁻¹ Se	2013	13.55	13.47	4.61	1.93	0.68	1.76	3.53	1.41
	2014	5.82	4.74	1.76	1.11	3.00	2.32	1.19	1.32
0.56 kg•ha⁻¹ Se	2013	18.49	5.23	5.04	3.13	5.45	1.31	3.88	1.87
	2014	4.23	5.27	4.75	2.71	2.68	3.18	2.36	0.75
1.68 kg•ha⁻¹ Se	2013	9.07	9.91	4.79	6.62	0.94	3.57	4.17	2.41
	2014	6.19	6.25	4.11	4.86	1.09	0.83	1.25	1.79
3.36 kg•ha⁻¹ Se	2013	10.04	12.71	6.50	11.12	3.10	3.67	3.62	4.07
	2014	6.31	6.48	7.04	7.18	2.07	2.42	1.32	4.64
		Overall Mean		Overall Mean		Overall SD		Overall SD	
Overall	2013	11.9		5.5		4.57		3.93	
	2014	5.7		4.2		2.2		2.86	

Table 2.9 Broccoli consumption needed to achieve 150 µg Se per day consistent with cancer risk reduction, by variety, treatment, and year.

		Approximate g FW broccoli per day			
	Year	Beneforte		Green Magic	
		0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S
0 kg•ha^{-1} Se	2013	11	11	33	79
	2014	26	32	83	136
0.56 kg•ha^{-1} Se	2013	8	29	30	48
	2014	36	28	32	56
1.68 kg•ha^{-1} Se	2013	16	15	31	23
	2014	24	24	37	31
3.36 kg•ha^{-1} Se	2013	15	12	23	13
	2014	24	23	21	21
		Overall Mean		Overall Mean	
Overall Mean	2013	13		27	
	2014	26		36	

Figure 2.1 Plot plan for 2013 field trial.

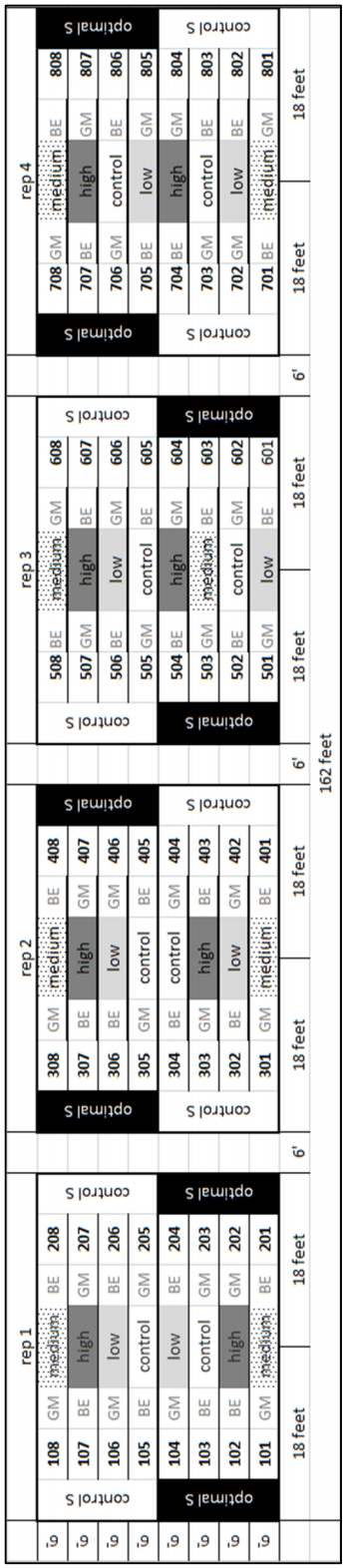


Figure 2.2 Plot plan for 2014 field trial.

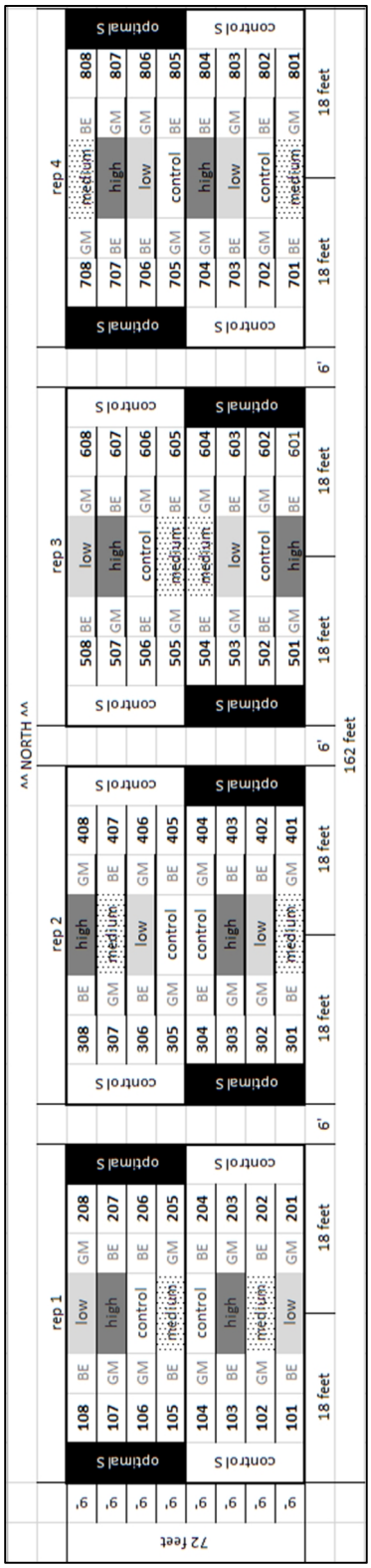


Figure 2.3 Glucoraphanin bar plots across sulfur treatments, selenium treatments, and years.

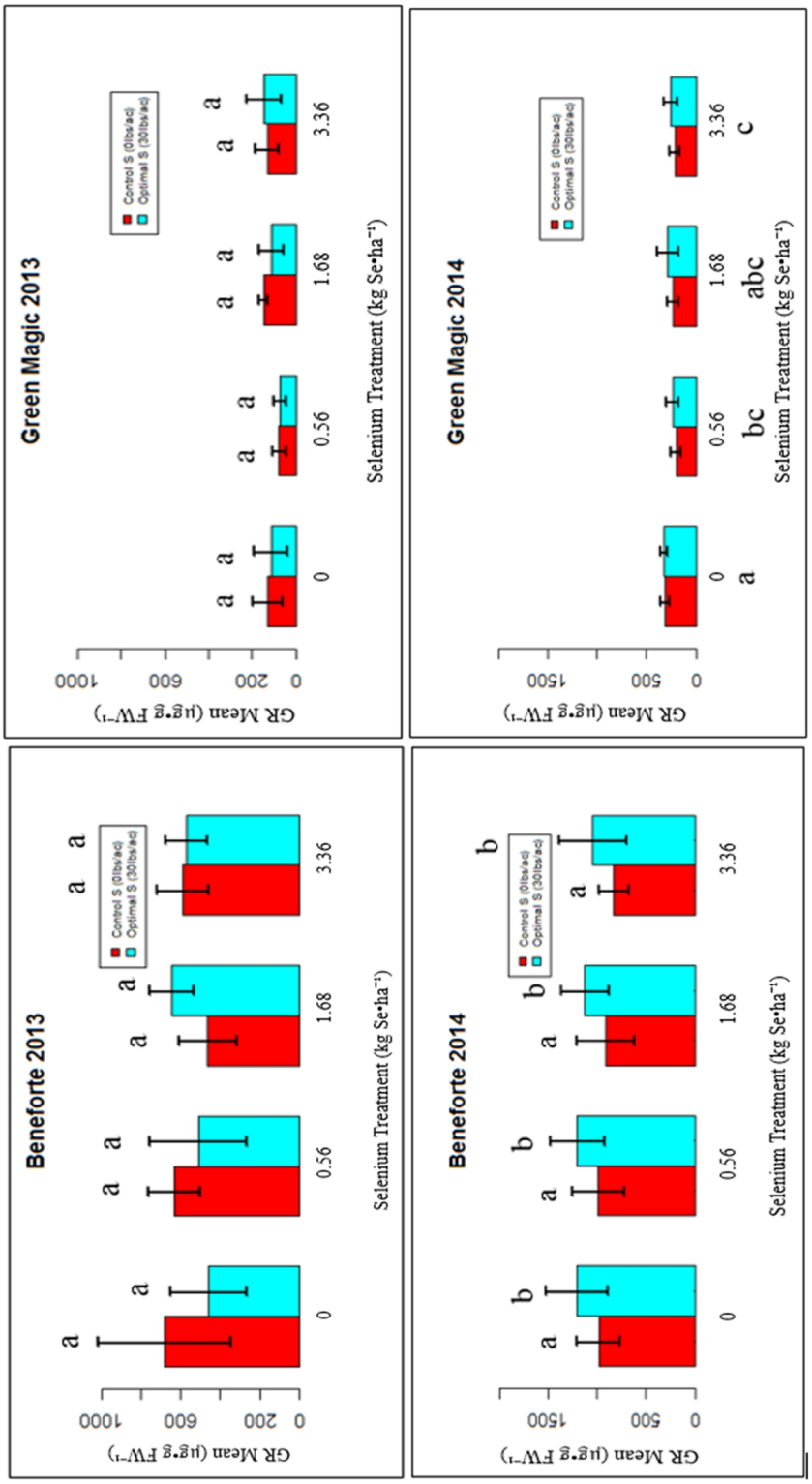


Figure 2.4 Seleno-methylselenocysteine bar plots across sulfur treatments, selenium treatments, and years.

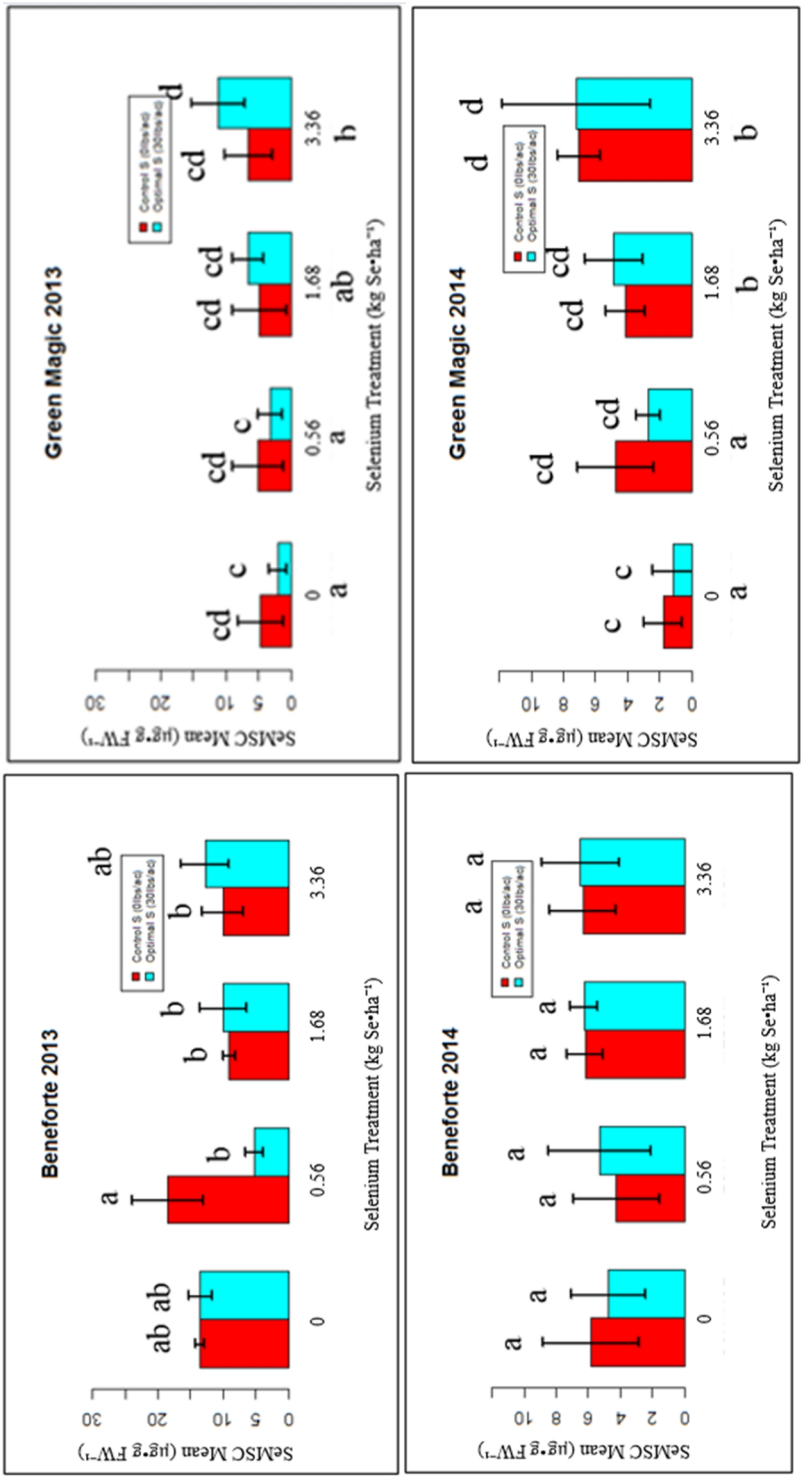
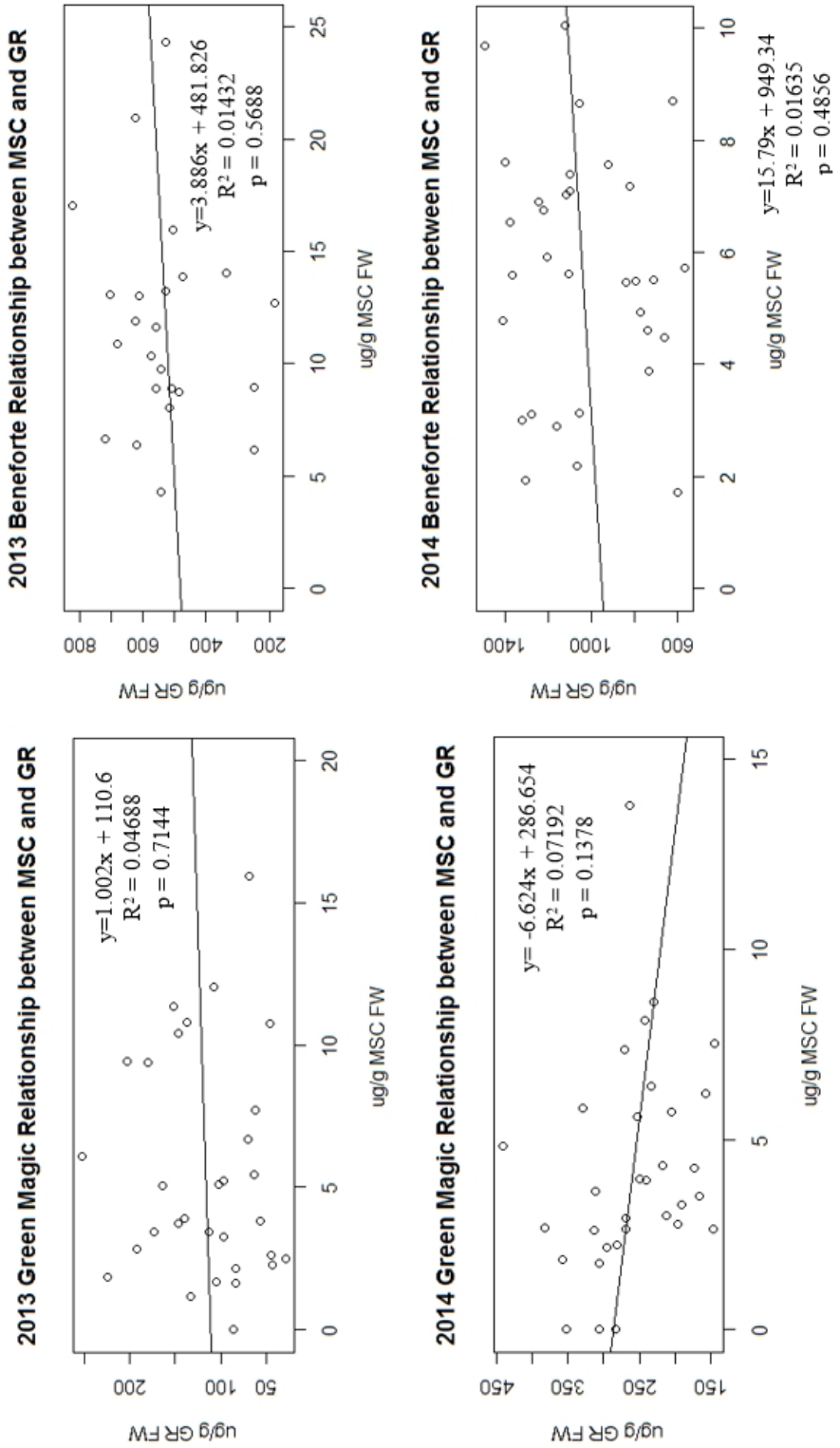


Figure 2.5 Relationships between glucoraphanin and seleno-methylselenocysteine by variety in field grown broccoli.



3 Effect of foliar selenium fertilization on glucoraphanin and seleno-methylselenocysteine concentration in greenhouse grown broccoli

3.1 Summary

Broccoli produces glucosinolates (GSLs), thioglucosides whose hydrolyzed derivatives have been shown to have chemoprotective properties. Glucoraphanin (GR) is a specific glucosinolate that is thought to be especially effective in reducing the risk of cancer. The action of the hydrolyzing enzyme, myrosinase, on GR causes the release of glucose and an unstable intermediate that rearranges to form sulforaphane (Kushad et al., 1999). Sulforaphane is the most potent naturally occurring inducer of phase II enzymes (Fahey and Talalay, 1999; Kushad et al., 1999), making it effective in chemoprevention. It may also inhibit phase I enzymes like cytochrome P_{450}, which could also reduce the risk of cancer (Maheo et al., 1997). Seleno-methylselenocysteine (SeMSC) is a second compound found in broccoli when the plant has been exposed to selenium (Se). SeMSC is a precursor to methylselenol (MeSeH) (Tsuji et al., 2009), another beneficial compound that may play a role in chemoprevention when consumed (Zeng et al., 2009, 2013).

Two cultivars of broccoli ('Beneforte' and 'Green Magic') were grown in the greenhouse and eventually treated with one of four foliar applications of sodium selenate, Na_2SeO_4, to assess the efficiency of foliar uptake of Se and the ultimate concentration of SeMSC in the edible portions of the plant. 'Beneforte' was chosen as a high-GR broccoli, containing a concentration of GR 2-3 times more than the average commercial variety. 'Green Magic' was chosen as a low-GR broccoli, which has a lower-than-average GR concentration. The objective of the study was to determine the efficacy of foliar selenium fertilization on SeMSC production as an alternative to root fertilization, since foliar application would be an easier production practice. We also wanted to determine if foliar applications would differentially affect both the low-GR and high-GR varieties.

Selenium treatments had an overall greater effect on 'Green Magic' when compared to 'Beneforte'. Se treatments significantly affected both GR and SeMSC concentrations

in ‘Green Magic’ while only affecting the SeMSC concentration significantly in ‘Beneforte’, leaving GR concentrations relatively stable.

3.2 Introduction

Glucosinolates (GSLs) are secondary metabolites commonly found in plants of the Brassicaceae family. Degradation products of these GSL compounds help combat plant herbivory, but health benefits like reduced risk for degenerative diseases may also be attributed to these secondary substances (Charron et al., 2005; Bjorkman et al., 2011). The beneficial bioactivity associated with GSLs is understood to stem from GSL byproducts. When cells are ruptured due to cutting, chewing, or other damage, GSLs are hydrolyzed by myrosinase [thioglucosidase (EC 3.2.3.147)] to generate derivatives including isothiocyanates, thiocyanates, and nitriles (Charron et al., 2005). The importance of understanding this hydrolysis system has grown as investigators have discovered possible anticarcinogenic properties of these GSL breakdown products (Fahey et al., 1997). Glucoraphanin (GR) is of particular importance because its hydrolysis product, sulforaphane, has an especially promising role in chemoprevention (Maheo et al., 1997; Fahey and Talalay, 1999; James et al., 2012).

Selenium (Se) is not an essential nutrient for normal plant function, and is even toxic to some plants. However, Se is an essential trace element for humans, and some plants have the ability to absorb certain Se-containing compounds and store them in forms that are non-toxic to the plant. Some of these stored compounds are thought to be useful in chemoprevention. For instance, epidemiological evidence has indicated a reduced risk of cancer when Se levels in the diet are increased (Zeng et al., 2013). Seleno-methylselenocysteine (SeMSC) is a specific form of Se produced and stored safely in broccoli. SeMSC is eventually reduced to methylselenol (MeSeH) when metabolized, and MeSeH likely has chemopreventive benefits. The cancer preventive mechanisms of these Se compounds seems to be via apoptosis in initiated cells (Combs and Gray, 1998; Zeng et al., 2009) and cell cycle arrest in the G1 phase, resulting in reduced proliferation of cancer cells (Zeng and Combs, 2008; Zeng et al., 2009).

Some studies have indicated that the competitive plant uptake between sulfur (GSL precursor) and selenium (SeMSC precursor) may lead to a decreased concentration of one or both of these health-promoting compounds. Few researchers have explored the efficacy of foliar absorption of Se and its interference (or non-interference) with the formation of sulfur-containing GSLs. Foliar Se application may be easier to execute in the field and may also allow for a greater concentration of both GR and SeMSC, due to decreased competition between S and Se in root sulfate transporters. To assess the hypothesis that foliar Se application will result in Se uptake that is at least as effective as root fertilization with Se, and that the chosen cultivars will be differently affected by Se treatments due to genetic variation, we conducted broccoli production utilizing a 4 x 2 split-plot design in a greenhouse setting. We planted 'Beneforte' and 'Green Magic' broccoli seedlings a sulfate-rich potting mix, and applied one of four leaf applications of dissolved sodium selenate, Na_2SeO_4: control (0 mmol Se•L^{-1}), low (0.1 mmol Se•L^{-1}), medium (0.3 mmol Se•L^{-1}), or high (0.6 mmol Se•L^{-1}). Total GR and total SeMSC were then quantified in edible tissue composites across treatments to determine concentrations and significant effects.

3.3 Materials and Methods

This study was conducted at the University of Minnesota – Twin Cities Plant Growth Facilities in St. Paul, MN (44.9886° N, 93.1814° W). Broccoli seedlings of two varieties, 'Beneforte' (BE; Seminis, St. Louis, MO) or 'Green Magic' (GM; Stokes, Buffalo, NY), were sown in the greenhouse facilities at the University of Minnesota – Twin Cities, St. Paul campus in 1204 cell tray inserts with 48 cells per tray, using BM2 germination peat moss mix (Berger, Saint-Modeste, Quebec, Canada) on 24 March 2014. Seedlings were supplemented twice with 125 ppm 15-5-15 Cal-Mag™ fertilizer (Scotts, Marysville, OH), and were transplanted into 5 gallon pots with Sunshine Professional Grow Mix LC8 soilless media (Sun Gro Horticulture, Agawam, MA) on 2 May 2014. Media contained more-than-adequate sulfate levels (150 ppm) for sufficient plant nutrition, as adequate sulfate levels are assumed at 12 ppm. For this reason, the greenhouse study focused only on the effect of foliar selenium (Se) treatments on GSL and SeMSC production.

In order to compare root fertilization of Se with foliar uptake of Se in the greenhouse, we decided to define foliar Se treatments that would be equivalent to the root Se applications. Field trial Se treatment rates were used to determine a an equivalent mmol Se $\bullet L^{-1}$ dose, which were then applied to the point of runoff in a foliar greenhouse trial.

The experimental design was a factorial 4 x 2 split-plot design with four replications (Figure 3.1). Replications were split between two adjacent greenhouses, and each replication contained 64 plants. Plants were first divided into four main plots, each randomly assigned a Se treatment (16 plants per treatment, per rep): control (0 mmol Se$\bullet L^{-1}$), low (0.1 mmol Se$\bullet L^{-1}$), medium (0.3 mmol Se$\bullet L^{-1}$), or high (0.6 mmol Se$\bullet L^{-1}$). Each main plot was divided into two sub-plots of eight plants, each containing one of two broccoli cultivars ('Beneforte' or 'Green Magic'). Plants were watered regularly, and Se treatments were not applied until later in development to allow GSL formation prior to Se exposure.

On 1 July 2015, plants were removed from the greenhouse and grouped by Se treatment. Here, aluminum foil was used to cover the top of each pot to shield the potting medium from Se treatment contamination. Sodium selenate, Na_2SeO_4, (Sigma-Aldrich, St. Louis, MO) was used as the source of Se. Amounts of sodium selenate, Na_2SeO_4, corresponding to the Se treatment rates were dissolved in water with 1% (w/v) Pril Original as surfactant (Henkel AG & Company, Dusseldorf, Germany) to aid absorption. A Spray N Go™ hand sprayer, model 20200 (Chapin International, Batavia, NY) was used to apply the mixture to the leaf surface. Control plants received water plus 1% (w/v) surfactant only.

Plants were harvested at market maturity, just before anthesis. 'Green Magic' matured first, with most heads harvested on 14 July 2014. 'Beneforte' rate of maturity was more variable, with harvests occurring between 21 July 2014 and 21 August 2014. Broccoli heads from each plot were weighed and recorded for yield measurements (Table S2), and aggregated for GSL and SeMSC quantification. Samples for GSL analysis were stored at 4°C prior to processing and analysis to mitigate GSL degradation, and were

processed within one week of harvest. All samples for SeMSC analysis were frozen at -80°C and lyophilized prior to analysis.

Glucoraphanin quantification. Glucoraphanin concentrations were determined using the glucosinolate quantification method of Hecht et al. (2004), with modifications from Rosen et al. (2005). Broccoli heads from each treatment were aggregated by cutting an equal mass of edible portions (floret and stalk) from each plant, with total sample weight dependent upon yield in each treatment group (Table S2). Samples were then placed into three times the weight per volume of boiling water and boiled for five minutes to deactivate myrosinase activity. Boiled samples were pureed in a blender for two minutes and a 40-mL aliquot of the blended sample was reserved. Samples were then homogenized using a Polytron PT 1300 D homogenizer (Kinematica AG, Lucerne, Switzerland) at 12,000 rpm for two minutes. A 2-ml sample of homogenate was measured into a labeled centrifuge tube, and samples were centrifuged for eight minutes at 8,000 g and 4°C.

Extraction of desulfonated glucosinolates (ds-GSL) was achieved using solid phase strong anion exchange (SAX) columns (Sigma-Aldrich, St. Louis, MO). SAX columns were washed with 2-ml of 0.5 M sodium acetate (pH 4.6) buffer, then 2-ml of deionized water, using a vacuum manifold. A 500-ml sample of centrifuged broccoli supernatant was then filtered through the SAX column. Afterward, 1-ml of 0.02 M sodium acetate (pH 4.0) buffer was washed over the column. Following a 1-ml of 0.2 $mg \bullet ml^{-1}$ sulfatase solution (aryl-sulfate sulfohydrolase from *Helix pomatia* – Type H-1, Sigma-Aldrich, St. Louis, MO) was added and vacuumed again. The columns with supernatant and sulfatase were stored at room temperature (~21°C) for ~15 hours, then eluted with 3-ml deionized water, and the eluent volume was determined by weight. The ds-GSL samples were stored at -20°C until high performance liquid chromatography (HPLC) analysis.

HPLC analysis of GR was conducted on the Agilent 1200 Series Quarternary system (Agilent Technologies, Inc., Santa Clara, CA) with diode array detector set at λ=229 nm using a Luna C18, 5 µm, 250 x 4.6 mm column (Phenomenex, Torrence, CA) set at 30°C. A 50-µl injection of eluent was separated on the machine using flow rates and a gradient

as follows: solvent A = water, solvent B = acetonitrile; 0 minutes, 95% A + 5% B, 1.0 ml•min^{-1}; 0-2 minutes, 85%A + 15%B, 1.0 ml•min^{-1}; 2-20 minutes, 53%A + 47%B, 1.0 ml•min^{-1}; 20-22 minutes, 0%A + 100%B, 1.15 ml•min^{-1}; 22-26 minutes, 0%A + 100% B, 1.3 ml•min^{-1}; 26-28 minutes, 0%A + 100%B, 1.0 ml•min^{-1}; 28-34 minutes, 95%A + 5%B, 1.0 ml•min^{-1}. Peaks were displayed using OpenLAB Chromatography Data System with rev. C.01.06 software. The glucoraphanin peak was identified using relative retention times of ds-GSL standard mixes provided by the Hormel Institute in Austin, MN. Ds-GSL concentrations of GR were calculated using relative quantification with an internal standard (sinigrin) and previously published response factors (EU, 1990). Ds-GSL concentrations of GR are reported on a µg•g^{-1} fresh weight (FW) basis. A sample chromatogram of ds-GSL peaks determined with diode array detection is shown in Figure S4.

Seleno-methylselenocysteine quantification. Seleno-methylselenocysteine concentration was quantified using methods described in Sepulveda et al (2013) with slight modifications. Equal portions of edible tissue from the same heads used in GR quantification were weighed and frozen at -80°C. Frozen samples were lyophilized on a Labconco FreeZone 6 Liter Console freeze dry system (Labconco Corp., Kansas City, MO). Freeze dried samples were pulverized using a mortar and pestal. A 50-mg sample of the broccoli tissue was then placed in a 2-ml centrifuge tube, and 1.5-ml of 50 mM hydrochloric acid (HCl) was added. Samples were vortexed and amino acids were extracted from the broccoli by storing the samples for 18 hours at 4°C. The mixture was then centrifuged at 12,000 g for 10 minutes. Supernatant was recovered and stored in a separate centrifuge tube. Amino acids and modified amino acids, including SeMSC, were then derivatized using the AccQ-Fluor Reagent Kit (Waters Corp., Milford, MA). A 50-µl sample of derivatized amino acid broccoli supernatant was combined with 70-µl of borate buffer and vortexed briefly. A 40-µl sample of AccQ-Fluor reconstituted reagent was then added and vortexed immediately for 10 seconds. Samples rested for one minute at room temperature before being moved to the autosampler for separation on the HPLC.

HPLC analysis of SeMSC was conducted on the same system as the GR quantification, but using a 1260 Infinity Fluorescence Detector (Agilent Technologies, Inc., Santa Clara, CA) for SeMSC discovery. The same Luna C18 column was used set at 37°C to assist in separation. The fluorescence of derivatized SeMSC was detected using excitation λ=250 and emission λ=395. A 20-µl injection was separated on the HPLC using a 1.0 ml•min^{-1} flow rate and the following gradient: solvent A = 140 mM sodium acetate + 17 mM TEA at pH=5.05 (pH adjusted with phosphoric acid), solvent B = 60% acetonitrile in water; 0 minutes, 100%A + 0%B; 0-0.5 minutes, 98%A + 2%B; 0.5-10 minutes, 80%A + 20%B; 10-20 minutes, 75%A + 25%B; 20-25 minutes, 70%A + 30%B; 25-30 minutes, 60%A + 40%B; 30-35 minutes, 40%A + 60%B; 35-40 minutes, 35%A + 65%B; 40-44 minutes, 98%A + 2%B; 44-45 minutes, 100%A + 0%B. SeMSC peak was identified using co-elution with pure SeMSC (Sigma-Aldrich, Milwaukee, WI), and its concentration was calculated using a calibration curve of known concentrations of pure SeMSC. A sample chromatogram of amino acid peaks using fluorescence detection is shown in Figure S5.

Statistical analysis. Data were analyzed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Varieties were analyzed separately, and significant differences between Se treatments were determined with a one-factor ANOVA. Dependent variables measured include concentration of glucoraphanin and concentration of seleno-methylselenocysteine. Mean values of response variables were considered significantly different when the p-value ≤ 0.05, and differences were identified using Tukey's HSD.

3.4 **Results and Discussion**

Glucoraphanin concentration. Variety was significant in the ultimate glucoraphanin (GR) response, which is what we hoped and expected (Table 3.1). Therefore in order to parse out the differences in treatments for each variety, data was divided into 'Green Magic' and 'Beneforte' categories and varieties were analyzed separately. The overall mean concentration of GR in 'Beneforte' was 796.4 ± 306.48 µg GR•g^{-1} fresh weight (FW), and in 'Green Magic' was 282.1 ± 84.07 µg GR•g^{-1} FW (Table 3.2).

On average, ‘Beneforte’ contained a GR concentration nearly three times greater than ‘Green Magic’. Selenium (Se) treatments were significant in affecting the GR concentration in ‘Green Magic’, as there was a significantly higher concentration of GR in the 0 mmol Se•L^{-1} treatment than in the 0.3 mmol Se•L^{-1} treatment. ‘Beneforte’ GR concentration, however, was not significantly affected by the Se treatments (Figure 3.2).

Seleno-methylselenocysteine concentration. Variety was significant in the ultimate seleno-methylselenocysteine (SeMSC) response, which is what we hoped and expected (Table 3.3). Therefore in order to parse out the differences in treatments for each variety, data was divided into ‘Green Magic’ and ‘Beneforte’ categories and varieties were analyzed separately. SeMSC mean concentrations were 20.3±21.59 µg SeMSC•g^{-1} FW in ‘Beneforte’, and 14±7.21 µg SeMSC•g^{-1} FW in ‘Green Magic’ (Table 3.4). When compared to root fertilization in the field, foliar absorption of sodium selenate, Na_2SeO_4, was as effective, if not more effective. Se treatments significantly affected the SeMSC concentrations in both ‘Beneforte’ and ‘Green Magic’ varieties (Figure 3.3).

The influence of cultivar. ‘Beneforte’ glucoraphanin concentrations were almost three times greater than ‘Green Magic’ (Table 3.2). It is also notable that GR concentrations in ‘Beneforte’ were not affected by the presence of foliar Se treatments (Figure 3.2). The SeMSC concentration in ‘Beneforte’ was significantly affected by foliar Se treatments, which is expected; and since GR concentrations remained relatively stable across Se treatments (i.e. were not significantly affected), this seems to make it an ideal cultivar for maximization of both GR and SeMSC. ‘Green Magic’ however, had GR concentrations significantly decrease in the presence of foliar Se (Figure 3.2). This is less desirable if we are looking for a cultivar that can maintain its GR status while also producing meaningful concentrations of SeMSC.

The influence of selenium on glucoraphanin. Foliar Se treatments significantly affected the GR concentration in ‘Green Magic’, but not in ‘Beneforte’ (Figure 3.2). The concentration of GR in ‘Green Magic’ decreased significantly with increasing Se treatment rates. When treated foliarly with the 0.3 mmol Se•L^{-1}, GR concentrations was reduced from 351.9 µg GR•g^{-1} FW in control to 261.2 µg GR•g^{-1} FW, a 25% decrease

(Table 3.2). GR levels in 'Beneforte' were not significantly different across all foliar Se treatments. Overall, the Se treatments via foliar application of sodium selenate, Na_2SeO_4, had a greater effect on the GR concentration in 'Green Magic' than in 'Beneforte'.

The influence of selenium on seleno-methylselenocysteine. Foliar Se treatments significantly affected SeMSC concentration in both varieties (Figure 3.3). In 'Green Magic' the control Se treatment plants contained an average concentration of 7.8 µg SeMSC•g^{-1} FW, which was significantly different from both the 0.3 mmol Se•L^{-1} and 0.6 mmol Se•L^{-1} foliar Se treatments, with 16.9 µg SeMSC•g^{-1} FW and 22.6 µg SeMSC•g^{-1} FW respectively. The 0.1 mmol Se•L^{-1} foliar Se treatment plants in 'Green Magic' had 10.1 µg SeMSC•g^{-1} FW, which was also significantly different than the both the 0.3 mmol Se•$L^{-1}$and 0.6 mmol Se•L^{-1}foliar treatments (Table 3.4).

In 'Beneforte', a significantly higher SeMSC concentration was found in the 0.6 mmol Se•L^{-1} foliar treatment when compared to all other Se treatments (Figure 3.3). The 0, 0.1, and 0.3 mmol Se•L^{-1} treatments had concentrations of 20.9, 21.4, and 23.5 µg SeMSC•g^{-1} FW respectively, which was significantly lower than the 0.6 mmol Se•L^{-1} treatment with a concentration of 45.2 µg SeMSC•g^{-1} FW (Table 3.4). Generally, SeMSC concentrations were significantly affected by foliar Se treatments in both varieties, with 'Beneforte' producing 45% higher SeMSC concentrations than 'Green Magic' on average, regardless of foliar Se treatment rate.

Lower Se rates should have been considered for the foliar trial, due to foliar Se sensitivity and an increased risk of toxicity with foliar application. There were some signs of toxicity witnessed (e.g. leaf chlorosis and necrosis) on plants with higher Se doses (Figure S6).

The influence of the greenhouse. Temperature set points for the two greenhouses were 63°F for both day and night. Low, high, and mean temperatures for each are listed in Table 3.5. It is obvious that when compared to average field temperatures, the greenhouse was consistently warmer throughout the day. Average high field temperatures of 27.2 ºC in 2013 and 25.4ºC in 2014, which is substantially lower than the average highs in either greenhouse throughout this experiment. These higher temperatures likely contributed to

poor growing conditions and ultimately affected the yield and quality of heads. Yield was significantly lower in the greenhouse experiment than the field experiments, with overall mean head weights at 0.092 kg for 'Beneforte' and 0.174 kg for 'Green Magic'(Table S2). Not all heads were suitable for analysis due to leafy heads, early bolting, and some rotting (Figure S7), especially in 'Beneforte' since it required more time to mature, which meant more time in the greenhouse.

The relationship between glucoraphanin and seleno-methylselenocysteine production. The relationship between GR and SeMSC was slightly negative in both varieties, such that as SeMSC concentrations increased, GR tended to decrease (Figure 3.4). However, these two variables only explained 13.1% of the relationship between GR and SeMSC in 'Beneforte' and only 17.2% in 'Green Magic'. These low R^2 values show a weak relationship between the compounds analyzed in this experiment, and may indicate the potential to maximize both GR and SeMSC without compromising either.

3.5 Conclusion

Foliar application of sodium selenate, Na_2SeO_4, later in plant development may be a viable option for maximizing both GR and SeMSC compounds in broccoli. The significant effects of selenium (Se) treatments on the concentration of glucoraphanin (GR) and seleno-methylselenocysteine (SeMSC) in 'Green Magic' suggest that it is more significantly influenced by Se than 'Beneforte', making 'Beneforte' a stronger candidate for maximization of GR and SeMSC concentrations.

Table 3.1 Analysis of variance for greenhouse glucoraphanin.

Response:	µg GR•g^{-1} FW broccoli				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
variety	**1**	**4024736**	**4024736**	**83.1769**	**1.903e-12 *****
se	3	365788	121929	2.5198	0.0678
variety:se	3	179319	59773	1.2353	0.3061
Residuals	53	2564547	48388		

Table 3.2 Glucoraphanin mean concentrations and standard deviations across selenium treatments.

	Mean µg GR •g^{-1} FW broccoli		SD µg GR•g^{-1} FW broccoli	
	Beneforte	Green Magic	Beneforte	Green Magic
0 mmol Se•L^{-1}	850.7	351.9	391.12	66.89
0.1 mmol Se•L^{-1}	981.9	293.4	333.50	81.87
0.3 mmol Se•L^{-1}	736.0	208.2	197.01	55.12
0.6 mmol Se•L^{-1}	647.8	261.2	235.77	72.06
Overall Mean	796.4	282.1	306.48	84.07

Table 3.3 Analysis of variance for greenhouse seleno-methylselenocysteine.

Response:	µg SeMSC•g^{-1} FW broccoli				
	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
variety	**1**	**2906.5**	**2906.47**	**13.2032**	**0.0006316 *****
se	**3**	**3736.1**	**1245.35**	**5.6573**	**0.0019421 ****
variety:se	3	501.5	167.18	0.7595	0.5218649
Residuals	53	11667	220.13		

Table 3.4 Seleno-methylselenocysteine concentrations across selenium treatments.

	Mean µg MSC•g^{-1} FW broccoli		SD µg MSC •g^{-1} FW broccoli	
	Beneforte	Green Magic	Beneforte	Green Magic
0 mmol Se•L^{-1}	20.9	7.8	21.72	3.94
0.1 mmol Se•L^{-1}	21.4	10.1	17.91	2.87
0.3 mmol Se•L^{-1}	23.5	16.9	14.79	4.45
0.6 mmol Se•L^{-1}	45.2	22.6	24.52	6.13
Overall Mean	20.3	14.0	21.59	7.21

Table 3.5 Mean temperature data over growing period in each greenhouse.

	Greenhouse	Mean Daily Maximum Air Temperature (ºC)	Mean Daily Minimum Air Temperature (ºC)	Mean Daily Average Air Temperature (ºC)
May	415B5	23.3	17.6	21.1
	415B6	33.2	17.4	21.8
June	415B5	31.3	16.3	22.1
	415B6	32.9	15.3	22.8
July	415B5	32.1	18.9	23.2
	415B6	34.9	18.6	24.3

Figure 3.1 Greenhouse plot plan.

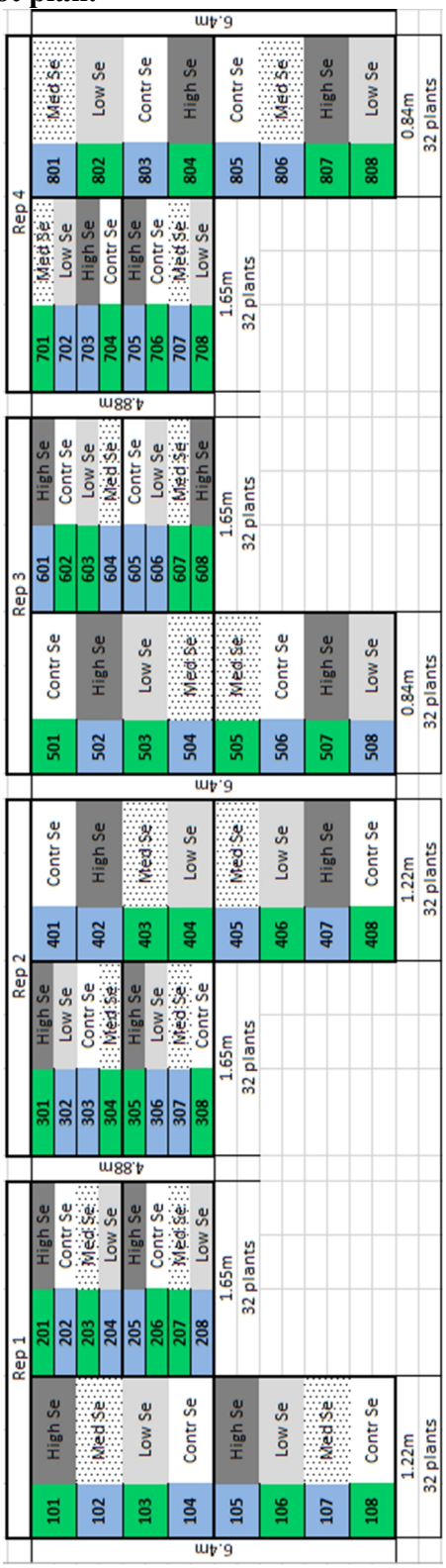


Figure 3.2 Effect of selenium on glucoraphanin by variety in greenhouse grown broccoli.

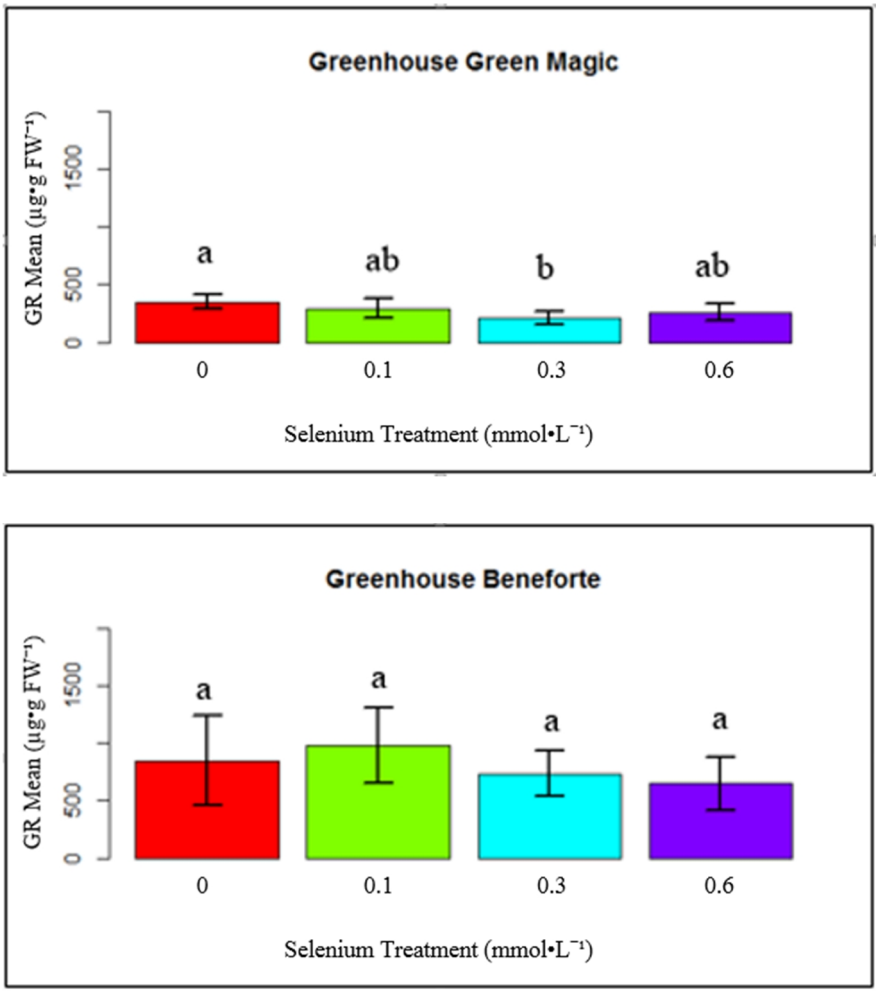


Figure 3.3 Effect of selenium on seleno-methylselenocysteine by variety in greenhouse grown broccoli.

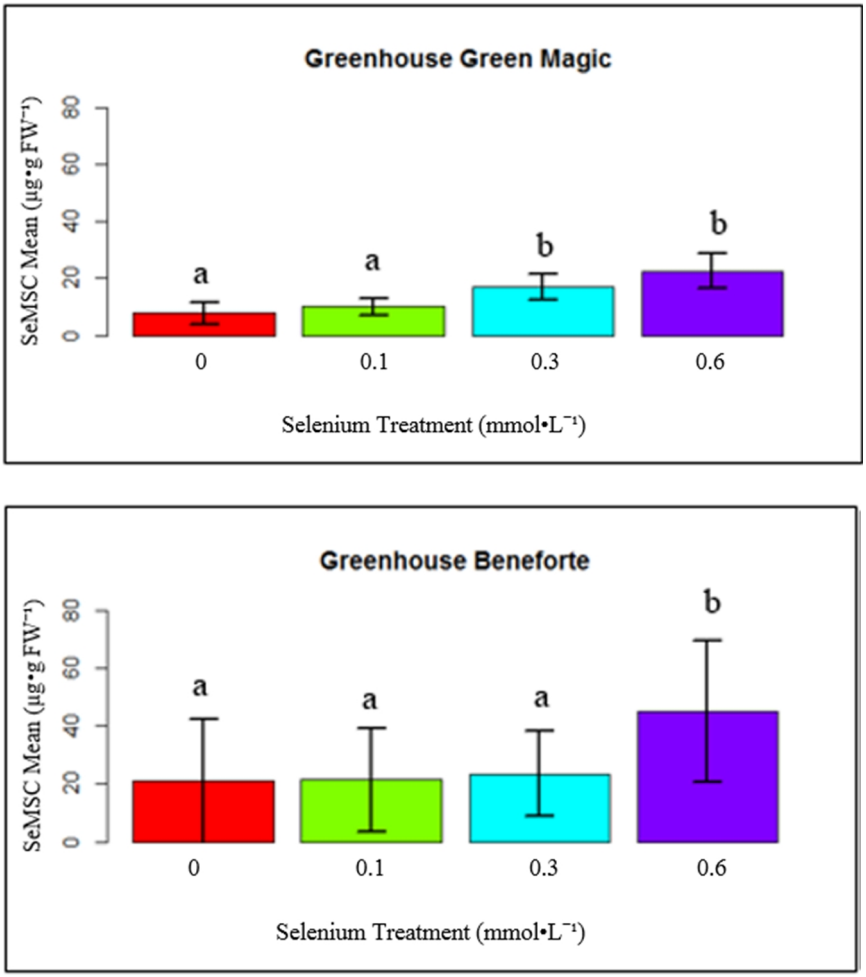
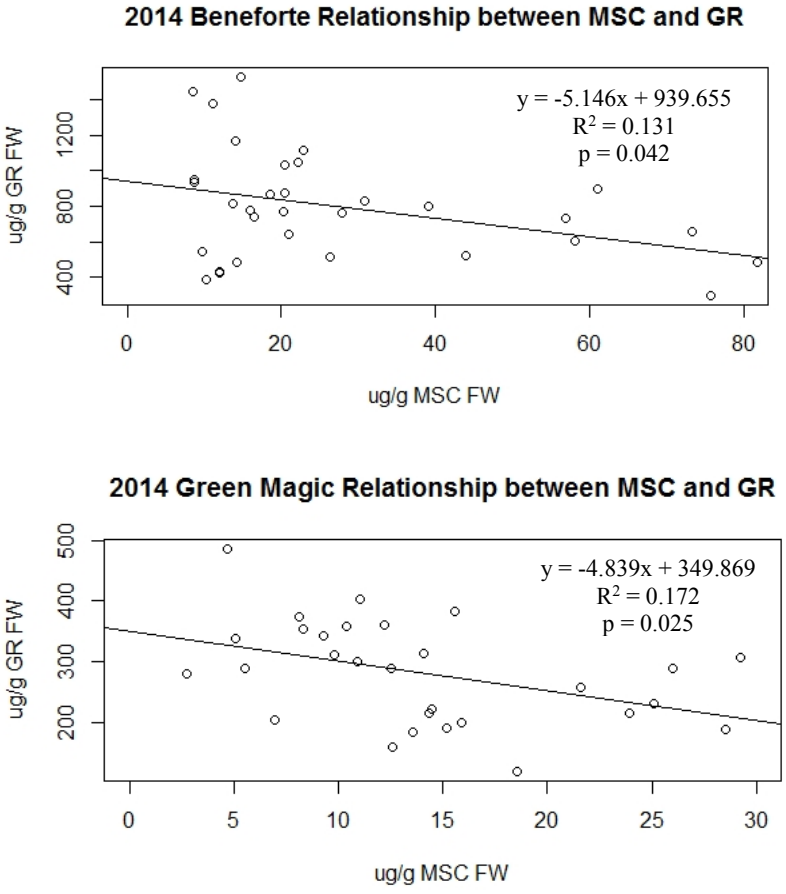


Figure 3.4 Relationships between glucoraphanin and seleno-methylselenocysteine by variety in greenhouse grown broccoli.



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5 Appendix. Supplemental tables and figures.

Table S1. Field grown broccoli yield across sulfur treatments, selenium treatments, variety, and year.

Mean Head Wt (kg)					
		Beneforte		Green Magic	
	Year	0 kg•ha^{-1} S	34 kg•ha^{-1} S	0 kg•ha^{-1} S	34 kg•ha^{-1} S
0 kg•ha^{-1} Se	2013	0.47	0.56	0.34	0.40
	2014	0.70	0.69	0.29	0.32
0.56 kg•ha^{-1} Se	2013	0.47	0.50	0.33	0.38
	2014	0.58	0.59	0.31	0.43
1.68 kg•ha^{-1} Se	2013	0.42	0.43	0.37	0.32
	2014	0.62	0.68	0.35	0.37
3.36 kg•ha^{-1} Se	2013	0.47	0.43	0.30	0.34
	2014	0.59	0.81	0.28	0.37
		Overall Mean		Overall Mean	
Overall Mean	2013	0.47		0.35	
	2014	0.66		0.34	

Table S2. Greenhouse grown broccoli yield by variety across selenium treatments.

	Number of Heads Harvested		Mean Head Weight (kg)	
	Beneforte	Green Magic	Beneforte	Green Magic
0 mmol Se•L^{-1}	27	18	0.09	0.18
0.1 mmol Se•L^{-1}	24	25	0.11	0.17
0.3 mmol Se•L^{-1}	28	12	0.08	0.19
0.6 mmol Se•L^{-1}	24	19	0.08	0.15
Overall Mean	25.75	18.5	0.092	0.174

Figure S1. Image of field trial plot.

Figure S2. Sample field grown broccoli glucosinolate chromatogram with diode array detection.

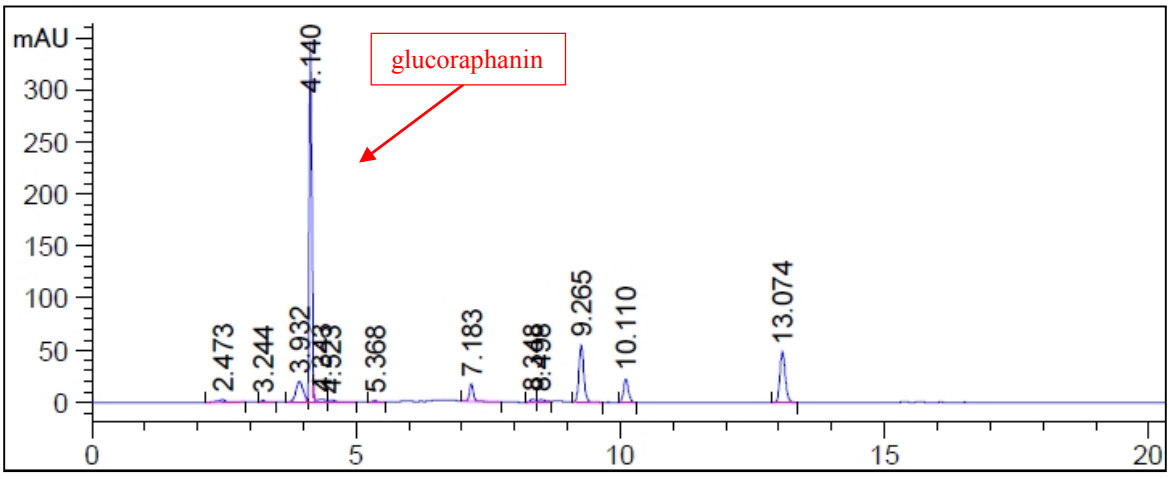

Figure S3. Sample field grown broccoli amino acid chromatogram with fluorescence detection.

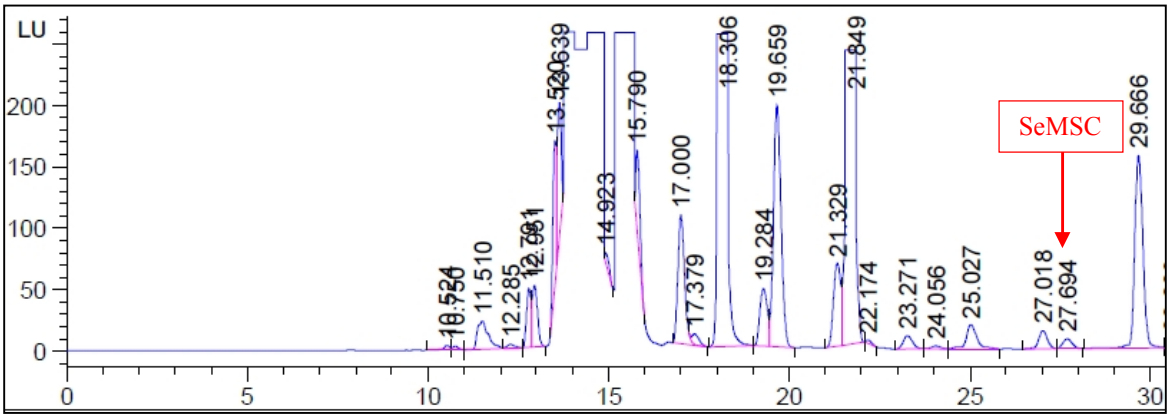

Figure S4. Sample greenhouse grown broccoli glucosinolate chromatogram with diode array detection.

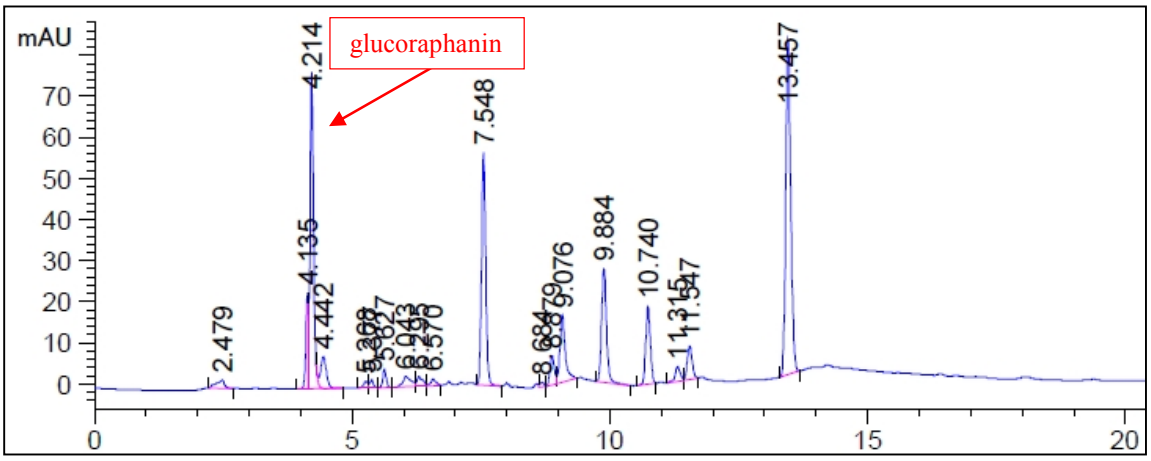

Figure S5. Sample greenhouse amino acid chromatogram with fluorescence detection.

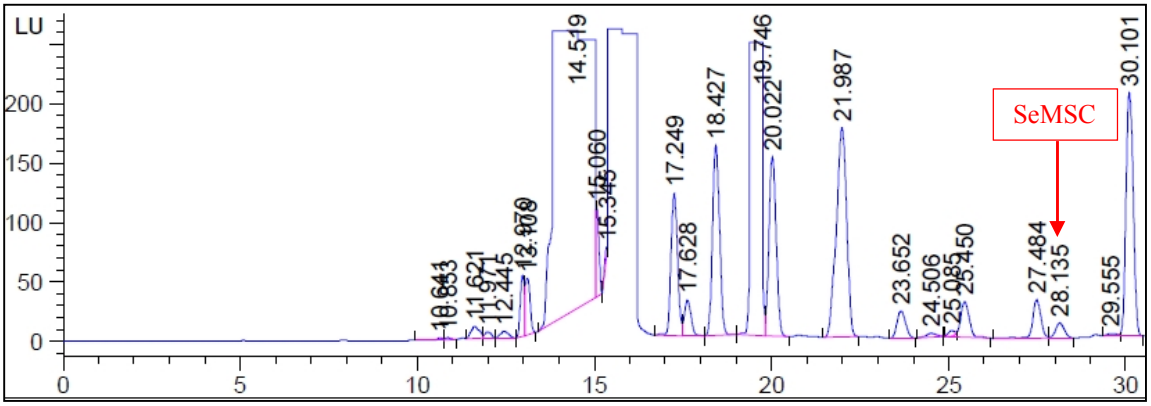

Figure S6. Images indicating possible selenium toxicity.

Figure S7. Images indicating early bolting, rotting, leafy heads.