

REGULATION OF SULFATE UPTAKE, EXPRESSION OF SULFATE  
TRANSPORTER GENES IN RAPID-CYCLING BASE POPULATIONS OF THE  
BRASSICA (BRASSICA OLERACEA) AS AFFECTED BY TEMPERATURE,  $\text{SeO}_4^{-2}$   
AND  $\text{SO}_4^{-2}$  NUTRITION.

by

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(Under the Direction of David Knauft)

ABSTRACT

A rapid-cycling base population of *Brassica* was used as a model system for investigating the effects of temperature on Se and S metabolism in an initial study. *B. oleracea*, plants were grown in growth chambers using nutrient solutions with temperatures ranging from 10 to 30 °C. The responses of total Se and S accumulation were similar but temperature dependent and tissue specific.

In a second study, different ratios of  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  were applied to investigate Se effects on S metabolism. Plant fresh weight, S and  $\text{SO}_4^{-2}$  accumulation were highest when plants were grown in medium with  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  ratio of 1:250. The accumulation of Se was highest found when low amounts  $\text{SeO}_4^{-2}$  were added to nutrient solutions without  $\text{SO}_4^{-2}$ . Glutathione peroxidase activity (GPx) and glucosinolates were measured and affected by Se status, as well.

In the literature,  $\text{SeO}_4^{-2}$  was suggested to regulate  $\text{SO}_4^{-2}$  uptake and affect the expression of sulfate transporters. In a third study, cDNAs associated with 12 different

sulfate transporter genes were isolated from *B. oleracea* grown under various  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  nutritional regimes. A semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) method was used to show how different ratios of  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  impacted the expression of sulfate transporter genes. Gene expression from these different transporters showed tissue specificity and regulation dependent on  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  status.

INDEX WORDS: *Brassica oleracea*, temperature, sodium selenate, magnesium sulfate, glutathione peroxidase activity, glucosinolates, sulfate transporter, cDNA, gene expression, semi-quantitative PCR, RT-PCR.

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## CHAPTER 1

### INTRODUCTION

Selenium (Se) is an element that is wide spread in trace amounts in soils and in natural feeds. Selenium has similar chemical properties to sulfur (S) and exists naturally as selenide ( $\text{Se}^{2-}$ ), element Se ( $\text{Se}^0$ ), thioselenate ( $\text{Se}_2\text{O}_3^{2-}$ ), selenite ( $\text{SeO}_3^{2-}$ ) and selenate ( $\text{SeO}_4^{2-}$ ). At very low levels, Se is an essential micronutrient and has important health benefits for humans and animals (Levander, 1982). However, at higher concentrations it may become toxic (Rosenfeld and Beath, 1964; Wilber, 1980; Lemly, 1997).

Sulfur metabolism and accumulation has been well investigated. A clear biosynthetic pathway and genetic control was established for  $\text{SO}_4^{2-}$  uptake, transport, reduction and incorporation into cysteine (Heiss et al., 1999; Leustek et al., 2000). Because of their chemical similarities, Se can theoretically be taken up by plants and replace S in its metabolic pathways (Läuchli, 1993). Bailey et al. (1995) found different rates of  $\text{SeO}_4^{2-}$  in *Ruppia maritima* under high/low  $\text{SO}_4^{2-}$  conditions, indicating  $\text{SO}_4^{2-}$  and  $\text{SeO}_4^{2-}$  competition. Similarly, Se concentration was proposed to interfere with S metabolism in plants (White et al., 2004).

Since Se is a central component of the antioxidant, glutathione peroxidase, the accumulation of Se and the compounds it forms in plants has attracted research attention (Rotruck et al., 1973). Moreover, Se was implicated in cancer suppression (Jansson, 1980) and to relieve symptoms associated with AIDS (Hori et al., 1997). Absorption, digestion, and metabolism, bioavailability of selenoproteins, and toxicity are affected by the chemical forms of

Se in foods (Thomson, 1998). Selenium enriched vegetables have been suggested a means for dietary Se supplementation (Ip and Lisk, 1994a).

*Brassica* species are important food crops, which provide substantial health benefit when consumed (Verhoeven et al, 1997). *Brassica* and *Allium* species are excellent candidates for delivering Se to human diets because of their S accumulating properties (Block et al., 1996). In *Brassica*, glucosinolates (GLs) are associated with anti-carcinogenic activities (Wattenberg et al., 1986; Verhoeven et al., 1997), and are the dominant flavor precursors produced from S compounds (Fenwick et al., 1983a, 1983b; Halkier and Du, 1997; Rosa et al., 1997).

Environmental factors, such as temperature, water, and  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$  ratio, can change S uptake, metabolism and flavor intensity associated with S-compounds. Increasing temperature increased S concentration and to a lesser extent, increased total  $\text{SO}_4^{-2}$  in onion bulbs (Coolong and Randle, 2004). Furthermore, increasing  $\text{SO}_4^{-2}$  concentration increased glucosinolates (Mailer, 1989; Booth et al., 1991). Our work in Chapter 3 show that Se and S accumulation was temperature dependent and tissue specific, although the patterns of accumulation were similar between Se and S.

Glucosinolates, one of the dominant flavor precursor groups in *Brassicac*s, are hydrolyzed by myrosinase (E.C. 3.2.3.1) to produce a unique flavor (Gilbert and Nusrten 1972; MacLeod, 1976; Fenwick et al., 1983a). When *Brassica* plants were exposed to different  $\text{Na}_2\text{SeO}_4^2$  concentrations, high  $\text{SeO}_4^{-2}$  levels decreased glucosinolate content (Charron et al., 2001). Similar effects on onion flavor intensity when were reported in onion grown under different  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  nutrient concentrations (Kopsell and Randle, 1997). Since Se and glucosinolates produce health benefits and flavor intensity, changes in glucosinolate content because of Se nutrient availability may be of interest if *Brassicac*s are also consumed for

supplying Se to the diet. In Chapter 4  $\text{SO}_4^{-2}$  /  $\text{SeO}_4^{-2}$  ratios were tested in a rapid-cycling *B. oleracea* population for their effects on plant growth, S,  $\text{SO}_4^{-2}$  and Se accumulation; changes in glucosinolate content, and selenium bioavailability via glutathione peroxidase.

Previously, competition between  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$  was shown for absorption in roots (Ulrich and Shrift, 1968). Se and S were reported to share the same transport pathway in uptake and translocation within plants (Abrams et al., 1990; Arvy, 1993; Anderson, 1993). However, how Se affects transporter regulation has not been completely understood in higher plants. Several sulfate transporter genes were cloned and characterized from *Arabidopsis* (Takahashi et al., 1996, 1997, 1999, 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2003; Maruyama-Nakashita et al., 2003; Buchner et al., 2004b; Kataoka et al., 2004a, 2004b) and *Brassica* (Hawkesford et al., 2003; Buchner et al., 2004a). Therefore, through genetic manipulation: a semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) method was used to show how different ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  impacted on expression of sulfate transporter genes in a rapid-cycling *B. oleracea* in Chapter 5.

Overall, the *Brassicac*s are important as food in daily diets because of their flavor and health benefits. Selenium, however, is also necessary for humans and plants exposed to a Se-enriched environment results in enhanced Se accumulation, changes of flavors and Se bioavailability. Understanding the effects of Se on plant nutritional values may be a useful indication for human dietary and for agricultural utilization.

## CHAPTER 2

### LITERATURE REVIEW

#### Selenium biochemistry

Selenium (Se) was first identified when Jon Jacob Berzelius oxidized sulfur dioxide from copper pyrites in 1818 (McNeal and Balistrieri, 1989).

Selenium is an element, which is wide spread in trace amounts in soils and in natural feeds. The forms of selenium naturally exist as selenide ( $\text{Se}^{-2}$ ), element Se ( $\text{Se}^0$ ), thioselenate ( $\text{Se}_2\text{O}_3^{-2}$ ), selenite ( $\text{SeO}_3^{-2}$ ) and selenate ( $\text{SeO}_4^{-2}$ ), which are similar to the oxidation of sulfur (Combs and Combs, 1986). Selenium can be absorbed by plants as inorganic forms, such as selenate and selenite, and organic compounds, such as selenocysteine and selenomethionine (Mikkelsen et al., 1989). There are some differences in chemical properties between selenate and selenite; therefore, it is accepted that uptake and translocation of selenate and selenite in plants are the result of different metabolic processes.

The concentration and speciation of selenium in the environment depends on the pH and redox conditions, biological interactions, salt solubility, and reaction kinetics (McNeal and Balistrieri, 1989). The (+6) selenium is stable in oxidized environments, such as in well-aerated or semiarid soils. Selenium exists as selenic acid ( $\text{H}_2\text{SeO}_4$ ) or selenate salts ( $\text{SeO}_4^{-2}$ ) under these conditions. Selenate salts are highly soluble in water (McNeal and Balistrieri, 1989). Most of the water-soluble Se in soil solution is selenate (Yamada et al., 1998); therefore, it is absorbed easily by plants (Mikkelsen et al., 1989).

On the other hand, the (+4) selenium may become a good oxidizing agent and tends to be reduced to  $\text{Se}^0$  when pH is between acidic to neutral (McNeal and Balistrieri, 1989). The (+4) selenium can present as selenium dioxide ( $\text{SeO}_2$ ), selenious acid ( $\text{H}_2\text{SeO}_3$ ), and selenite salts ( $\text{SeO}_3^{-2}$ ). Selenite salts have less solubility than selenate salts because selenite may easily form insoluble complexes with  $\text{Fe}^{+3}$ ,  $\text{Mn}^{+2}$ , and  $\text{Al}^{+3}$  in acidic soil (McNeal and Balistrieri, 1989). Selenite is seldom used as the Se fertilizer for plants absorption because of its availability in soils (Bange, 1973).

### **Selenium in the soil**

Even though selenate and selenite are the two dominant forms of inorganic selenium in agricultural area (Combs and Combs, 1986), other inorganic Se, such as  $\text{Se}^{-2}$  and  $\text{Se}^0$  also exist in the soil depending on environmental pH and oxidation-reduction status (Elrashidi et al., 1989). In addition, organic forms of Se, selenomethionine, can be found in soils (Abrams et al., 1990). Besides soil pH and redox status, the distribution of Se in soils is affected by its adsorption and affinity (Elrashidi et al., 1989). Furthermore, the abundance of organic matter (Adriano, 1986), original concentration of Se in parent material, deposition by Se accumulated plants, and erosion and redistribution from Se containing rocks (Boon, 1989) all contribute the various distribution and content of Se in the soil.

*Mobility of Se in the Soil.* The mobility of Se in the soil depends on its chemical forms. Since soil pH and redox potential affect Se speciation, they are strongly related to mobile Se in soils. When soil is more acidic to neutral, a large fraction of Se is present as selenite. Selenite has a strong affinity with iron oxides; therefore, in low pH, insoluble ferric oxide-selenite complex are found (Mikkelsen et al., 1989). To raise soil pH, adding lime will increase the availability of Se to plants (Adriano, 1986) because liming of soils reduces the formation of Fe-

Se complex (Neal et al., 1987). In contrast, (+6) Se as selenate is usually found in well-aerated or in alkaline conditions and is more mobile than selenite in soils.

### **Selenium metabolism in plants**

Plant species can uptake and accumulate selenium depending on their capacity to tolerate selenium concentrations and their affinity of absorption in the rooting medium. Basically, plants can be classified to three groups according to their ability to accumulate Se (Rosenfeld and Beath, 1964). The species which can grow in seleniferous soils and accumulate selenium from 100 to 10000 mg Kg<sup>-1</sup> dry weight are called “selenium-accumulators” or “primary indicators”, such as *Astragalus*, *Stanleya*, and *Haplopappus* (Brown and Shrift, 1982). Bañuelos et al. (1997) identified that fast growing *Brassica* species, Indian mustard and canola, can also accumulate several hundred milligrams of Se Kg<sup>-1</sup> dry weight in their shoot tissues when grown in levels of Se. Another group is classified as “secondary indicators” which may grow in areas with low to medium selenium and accumulate selenium from 25-100 mg Kg<sup>-1</sup> dry weight, including *Atriplex*, *Castilleja*, *Machaeranthera*, and *Mentzelia* (Mikkelsen et al., 1989). The third group is classified as “non-accumulators” and includes plants sensitive to selenium in the environment and normally have selenium content < 25 mg Kg<sup>-1</sup> dry weight. Most plants are non-accumulators crops species, such as grains, grasses and weeds (Mikkelsen et al., 1989).

*Uptake.* Plants can uptake inorganic selenate, selenite, or organic Se compounds from the environment for further metabolism. It has been proposed that selenate is taken up by the same active transport process as sulfate in the root plasma membrane because of similarities between S and Se (Abrams et al., 1990; Arvy, 1993; Anderson, 1993). Therefore, the putative absorption of selenate via high-affinity sulfate transporters is suggested (Figure 1) (Terry et al., 2000). Shibagaki et al. (2002) reported that lesions found in a sulfate transporter gene *Sultr1;2*

were associated with selenate-resistant mutants. Another study showed that overexpression of *Stylosanthes hamata* sulfate transporter (*SHST1*) gene increases accumulation of selenate in transgenic plants than in wild type (Smith et al., 1997). This evidence supports the theory that accumulation of selenate is related to high-affinity sulfate transporters. However, low-affinity sulfate transporter did not significantly affect selenate accumulation (Terry et al., 2000).

The first sulfate transporter genes were isolated from the tropical legume *Stylosanthes hamata* (Smith et al., 1995). Thereafter, sulfate transporter genes have been cloned and characterized from *Arabidopsis* (Takahashi et al., 1996, 1997, 1999, 2000; Yamaguchi et al., 1997; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2003; Maruyama-Nakashita et al., 2003; Buchner et al., 2004b; Kataoka et al., 2004a, 2004b), Indian mustard (Heiss et al., 1999), corn (Bolchi et al., 1999), barley (Smith et al., 1997), and *Brassica* species (Hawkesford et al., 2003; Buchner et al., 2004a). The expression of sulfate transporter genes is related to S status. High concentrations of sulfate and glutathione (GSH) have been proved to down-regulate gene expression (Smith et al., 1997; Lappartient et al., 1999). Generally, selenate is readily absorbed by plants (Asher et al., 1977; Mikkelsen et al., 1989) and accumulates more in tissues than selenite (Gissel-Nielsen, 1973; Läuchli, 1993; Gupta et al., 1993). Läuchli (1993) indicated that selenate was detectable in xylem and easily transported to the shoot (Gissel-Nielsen, 1987; Wu et al., 1988; Zayed et al., 1998; Zhang et al., 2003). After selenate is absorbed by roots, it still exists in inorganic forms and can be transported to leaf tissue for further metabolism (Shrift and Ulrich, 1969).

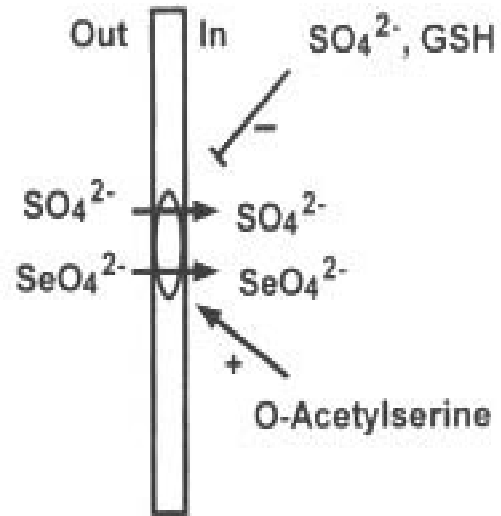


Figure 1. Selenite uptake across the root plasma membrane is mediated by the high-affinity sulfate transporter. The expression of the high-affinity sulfate transporter is regulated positively by O-acetyls erine and negatively by sulfate and glutathione. GSH, glutathione. (adapted from Terry et al. 2000).

*The competition between S and Se.* Since sulfate and selenate compete for absorption by roots (Ulrich and Shrift, 1968), high concentrations of selenate can inhibit sulfate uptake (Leggett and Epstein, 1956; Hawkesford et al., 1993). When plants are grown with a mix of sulfate and selenate, the ratio of Se: S in the medium is not the same as the Se and S ratio found in plant tissues (Bell et al., 1992; Ellis and Salt, 2003). It suggests that transporters in plants for uptake and translocation are selective for either selenate or sulfate (White et al., 2004). Previous studies showed the antagonistic relationship between selenate and sulfate: as the  $\text{SeO}_4^{-2}$ :  $\text{SO}_4^{-2}$  ratio is 1:1 in medium, a decrease of sulfate uptake and accumulation is found in onion (Barak and Goldman, 1997); however, when  $\text{SeO}_4^{-2}$ :  $\text{SO}_4^{-2}$  ratio drops between 1:125 to 1:500, low levels of selenate increases sulfate concentrations in onion (Kopsell and Randle, 1997).

*Metabolism.* Similarities also exist in assimilation and metabolism of Se and S. Sulfate ( $\text{SO}_4^{-2}$ ) enters root cells and it can be bound to different secondary metabolites or reduced and assimilated immediately (Leustek, 1996). Selenate ( $\text{SeO}_4^{-2}$ ) as the analogue of sulfate can be assimilated in the same metabolic pathway. Selenate is absorbed by roots and activated by ATP sulfurylase to form APSe, which is reduced to selenite ( $\text{SeO}_3^{-2}$ ). It was reported that ATP sulfurylase mediates  $\text{SeO}_4^{-2}$  reduction and is the rate-limiting step to assimilate organic Se compounds (Pilon-Smits et al., 1999). When  $\text{SeO}_4^{-2}$  is reduced to  $\text{SeO}_3^{-2}$ , it is coupled to glutathione (GSH) to form a selenotrisulfide, which is reduced to selenogluthathione and then to selenide ( $\text{Se}^{-2}$ ). Selenide reacts with *O*-acetylserine to form selenocysteine (SeCys), which can be further converted to selenomethionine (SeMet) via selenocystathionine and selenohomocysteine (Figure 2) (Läuchli, 1993).

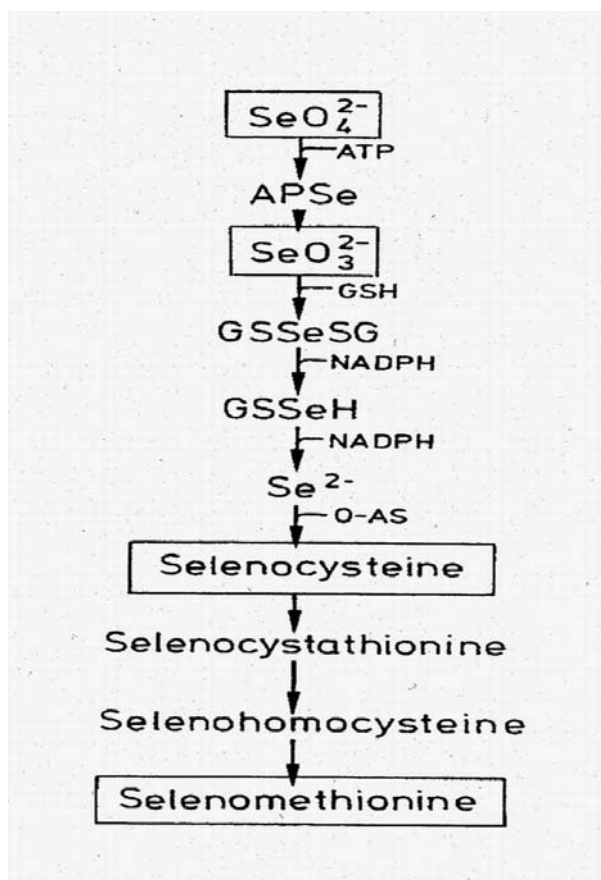


Figure 2. Proposed pathway for formation of the Se-cysteine and Se-methionine in plants. APSe, adenosine 5'-selenophosphate; GSH, glutathione; GSSeSG, selenotrisulphide; GSSeH, selenogluthathione; O-AS, acetyls erine (adapted from Lauchli, 1993).

*Toxicity.* Plants may show the symptoms of Se toxicity, such as chlorosis on leaves, stunting of growth, pink color on roots (personal observation), and premature death of the plant when they are grown in high concentration of Se (Mengel and Kirkby, 1987). Selenium toxicity is mainly from the interaction/substitution of S with Se (Mikkelsen et al., 1989). However, there are differences between Se-accumulators and non-accumulators. Previous studies demonstrated that selenate entered into Indian mustard via sulfate pathway (Pilon-Smits et al., 1999; Terry et al., 2000), and it was proposed that metabolism of  $\text{SeCN}^{-1}$  was analogous to  $\text{SCN}^{-1}$  in plants (de Souza et al., 2002). Generally, in non-accumulators, SeCys and SeMet would replace Cys and Met, respectively to incorporate into proteins. Therefore, the nonspecific substitutions result in non-functional proteins. The abnormal substitutions are considered as the mechanism of Se toxicity in plants (Ng and Anderson, 1979; Eustice et al., 1981). On the other hand, in Se-accumulators, either Se may be accumulated as nonprotein seleno-amion acids or SeCys can be methylated by selenocysteine methyltransferase to avoid Se toxicity (Shrift, 1969; Brown and Shrift, 1981, 1982).

### **Selenium and human health**

Selenium is an essential micronutrient in human health and is associated with immune system enhancement and anticarcinogenesis (Levander, 1982). An optimal Se level is necessary to avoid some viral diseases and decrease the risk of cancer. Selenium-dependent glutathione peroxidase (GPx), an antioxidant, protects oxidative stress by reducing free radical from injury to cells. In addition, it can repair damaged DNA, remove hydrogen peroxide and lipid hydroperoxides (Lawson and Birt, 1983).

*Immune System Enhancement.* Selenium has been demonstrated in response to lower viral diseases (McKenzie et al., 1998). Evidence has shown that HIV-infected population has

lower concentrations of selenium in blood plasma and a decrease in the glutathione peroxidase (Allavena et al., 1995). Furthermore, Se deficiency impairs the ability of phagocyte to destroy antigen in immune system which results in increasing viral infection during the progression of HIV-1 to AIDS (Baum et al., 1997; Baum and Shor-Posner, 1998). Therefore, appropriate level of Se is related to immune system protection.

*Anticarcinogenic Effects of Se.* The relationship between Se status and anticarcinogenic activity has been found from epidemiological studies (Combs and Gray, 1998; Combs and Clark, 1999). It has been demonstrated that supranutritional levels of Se can prevent tumourgenesis (Ip et al., 1992; Ip and Lisk, 1994b; Ip, 1998). Both inorganic and organic Se compounds are related to anticarcinogenic potential (Combs and Gray, 1998). Selenite and selenate were found to inhibit mammalian tumor growth (Spyrou et al., 1996) and SeCys and SeMet had strong inhibitory effects on tumorigenesis in rats, as well (Ip and White, 1987). Previous studies also indicated that lower level of Se was found in cancer patients than control (Clark et al., 1993; Nomura et al., 2000). It is likely that lack of Se may limit the expression of Se-dependent enzymes, such as glutathione peroxidase, which are involved in antioxidant protection. Clark et al. (1993) showed an association between low blood Se and possible of cancer induction. Furthermore, when patient treated with selenized yeast, it was found to lower the cancer mortality (Clark et al., 1996). Therefore, it is suggested that Se supplementation in diets is associated with anticarcinogenic activity.

In contrast, selenium toxicity is also a concern. Selenium toxicity is found in animals when the amounts of dietary Se are excessive from grazing on Se-accumulator plants. The symptoms of Se toxicity in animals are acute poisoning, blind staggers, and alkali disease (Rosenfeld and Beath, 1964). In humans, too much selenium in diets causes hair and teeth loss,

morphological changes in fingernails, and garlic breath odor (American Medical Association, 1989).

### **Using Plants to Deliver Selenium to Human Diets**

People absorb essential minerals and organic compounds directly by eating plants or indirectly when plants are fed to animals and then consumed by people. In addition to nutrients, fruits and vegetables also provide health-related compounds, e.g. antioxidants, carotenoids, and phytochemicals (Grusak and DellaPenna, 1999). Therefore, dietary absorption of essential nutrients and those health phytochemical compounds from plants depends on their ability of nutrients accumulation.

Adding Se to low-Se fertility regimes is able to improve dietary Se level through food chain (Reilly, 1996). In addition, using plants to deliver selenium to human diets is an efficient way to improve intake of selenium. Thus, those crops with great potential to accumulate Se will be good candidates for Se supplementation to human diets, such as *Allium* and *Brassica* species (Whanger, 1989). It has been shown that *Allium* is an anticarcinogenic genus because of highly Se compound accumulation, especially in garlic (Ip et al., 1992; Ip and Lisk, 1994a; Cai et al., 1994). *Brassica* species are also known to possess highly active phytochemicals with anticarcinogenic potential (Irion, 1999). Brassicas grown in Se-rich environments are able to convert selenium into Se-containing amino acids (Zayed and Terry, 1992).

#### **The *Brassica oleracea***

There are several closely related *Brassica oleracea* species of economic importance. They are cabbage, broccoli, cauliflower, kale, kohlrabi, and Brussels sprouts are polymorphic forms of *Brassica oleracea*. It is believed that these variations originated from the wild species

through mutation, selection, and adaptation. Different variety has its own morphology, growing conditions, nutrition and health value, and taste and flavor.

*Flavor Chemistry.* In *Brassicac*s, almost their volatiles as flavor are derived from the degradation of amino acids. Glucosinolates, which are predominant flavor precursors in *Brassicac*s are amino acid-derived secondary compounds containing a sulfur element (Fenwick et al., 1983a, 1983b; Halkier and Du, 1997; Rosa et al., 1997). In *Brassicac* plants, Mailer (1989) and Booth et al. (1991) showed that increasing sulfate concentrations enhanced glucosinolates content. It is thought that cysteine is used as the sulfur source in the biosynthesis of glucosinolates in plants (Figure 3). When plant tissue is damaged the glucosinolates are hydrolyzed by the endogenous enzyme myrosinase (E.C. 3.2.3.1) to produce breakdown products (Figure 4). One of the breakdown products, isothiocyanate, is volatile and considered as a flavor indicator (MacLeod, 1976) because of its pungent, lachrymatory, garlic-like, and bitter features (Fenwick et al., 1983a). Thus, to modify flavor profile to satisfy consumer preferences is important for plant breeders.

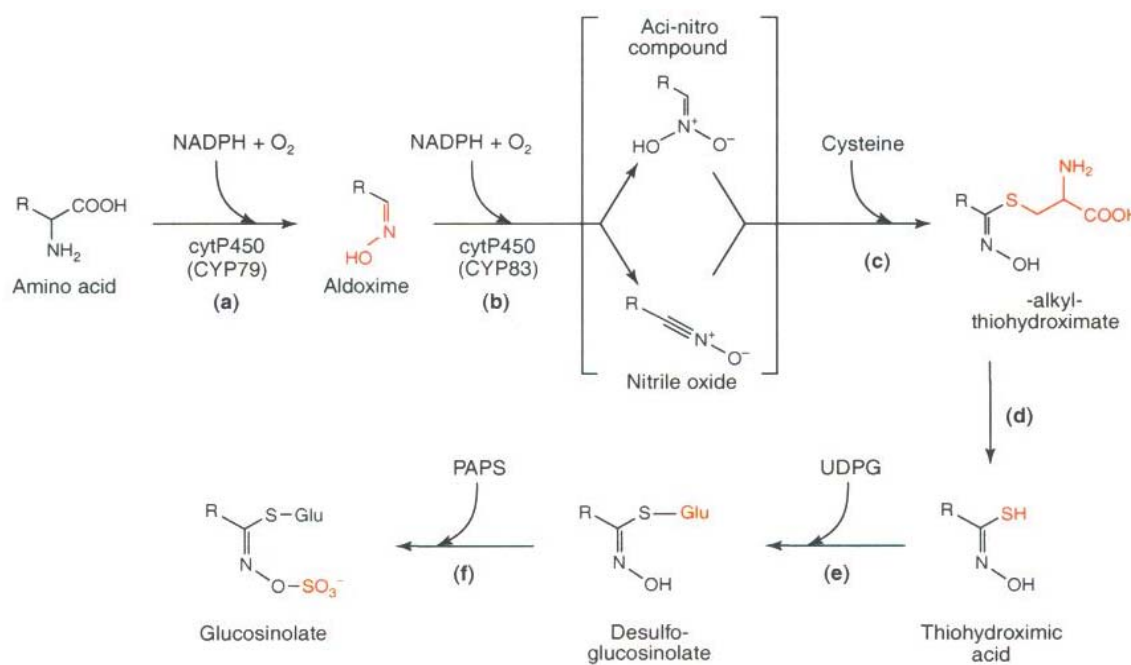


Figure 3. Glucosinolate biosynthesis. The central steps are: conversion of the amino acid to an oxime, synthesis of the glucone moiety, and chain modification (adapted from Wittstock and Halkier, 2002).

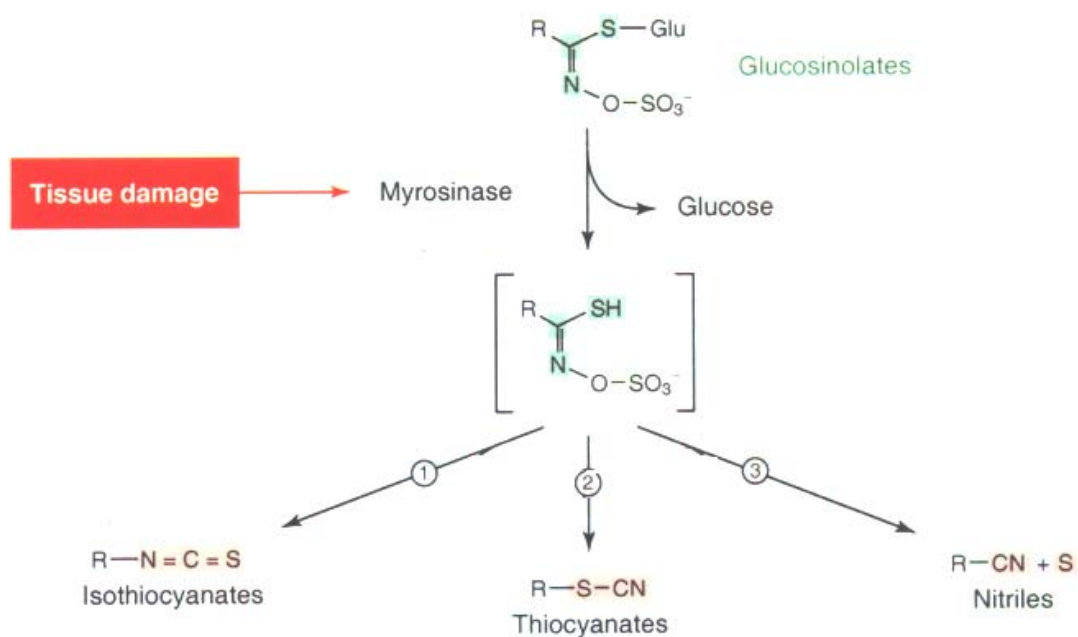


Figure 4. General structure and enzymatic hydrolysis of glucosinolates through myrosinase activity: 1, isothiocyanates. 2, thiocyanates. 3, nitriles. (adapted from Wittstock and Halkier, 2002).

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## CHAPTER 3

### INFLUENCE OF TEMPERATURE ON THE SELENIUM AND SULFUR ACCUMULATION IN *BRASSICA OLERACEA*<sup>1</sup>

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<sup>1</sup> Chang, P. and W.M. Randle. 2006. J. Hort. Sci. Biotech. 81(4): 754-758.

**ABSTRACT**

Selenium (Se) and sulfur (S) accumulation can be affected by many factors in plants. To understand how growing temperature affected Se and S uptake and accumulation in *Brassica oleracea*, plants were grown at three different temperatures: 10, 20, and 30 °C, respectively. Plant growth, as reflected in fresh and dry wt., increased with increasing temperature. Se and S accumulation was temperature dependent and tissue specific. Se in plant leaf tissues increased linearly with increasing temperatures and ranged from 1734 to 2541  $\mu\text{g Se g}^{-1}$  dry wt. Conversely, Se accumulation in root tissues decreased linearly with increasing temperatures and ranged from 2870 to 2167  $\mu\text{g Se g}^{-1}$  dry wt. Growing temperature significantly affected S accumulation in leaves, but not in shoots or roots. Sulfur ranged from 20.828 to 30.983  $\text{mg S g}^{-1}$  dry wt. in the leaves and increased with higher temperatures like with Se. Sulfate accumulation was similar to S, except that accumulation decreased at the highest growing temperature. Vegetable crops high in S are an efficient means of delivering Se in human diets. To ensure that optimal Se levels are achieved in plants without negatively affects plant productivity, factors that affect uptake and accumulation, like growing temperature, need to be better understood.

## INTRODUCTION

Though selenium (Se) is not an essential plant micronutrient, Se has been widely investigated in plants because of its beneficial and toxic effects in mammals (Rosenfeld and Beath, 1964; Wilber, 1980; Lemly, 1997; Rayman, 2000). Health benefits associated with proper Se ingestion include immune system enhancement, cancer suppression, and reduce cardiovascular disease (Levander, 1982). Areas of the world with low Se can have high incidence of Se-deficiency related diseases in animals and humans (Gissel-Nielsen et al., 1984). To avoid low Se in the diet, applying Se fertilizer to crops and pastures has been used to supplement Se in the diet.

Selenium (Se) and sulfur (S) can compete for uptake and accumulation in plants because of their chemical similarities. While Se is not an essential plant nutrient, it can substitute in the S metabolic pathway, and at high concentrations, cause problems in some plants. Alternatively, moderate levels of Se accumulation in plants can be a beneficial source of this essential micro-element when consumed in mammalian diets (Ip et al., 2000). Selenium is present in the environment as selenide ( $\text{Se}^{-2}$ ), element Se ( $\text{Se}^0$ ), thioselenate ( $\text{Se}_2\text{O}_3^{-2}$ ), selenite ( $\text{SeO}_3^{-2}$ ) and selenate ( $\text{SeO}_4^{-2}$ ) (Mikkelsen et al., 1989). Selenium uptake and accumulation is influenced by soil pH and redox status, and other anions such as sulfate and phosphate (Blaylock and James, 1994; Dhillon and Dhillon 2003). Selenate utilizes the same active absorption process as sulfate in the root plasma membrane (Abrams et al., 1990; Arvy, 1993; Anderson, 1993) and is facilitated by high-affinity transporters (Terry et al., 2000).

Plants can absorb inorganic  $\text{SeO}_4^{-2}$  and  $\text{SeO}_3^{-2}$  as well as organic compounds like selenocysteine and selenomethionine (Mikkelsen et al., 1989). Generally,  $\text{SeO}_4^{-2}$  is absorbed more readily with greater accumulation in plant tissues compared to  $\text{SeO}_3^{-2}$  (Gissel-Nielsen,

1973; Läuchli, 1993), although much of the  $\text{SeO}_4^{-2}$  absorbed by plants remains inorganic (Shrift and Ulrich, 1969).  $\text{SeO}_4^{-2}$  can be measured in the xylem (Läuchli, 1993) and is easily transported to the shoot (Gissel-Nielsen, 1987; Wu et al., 1988; Zayed et al., 1998; Zhang et al., 2003). Conversely, Arvy (1989; 1993) showed that absorbed  $\text{SeO}_3^{-2}$  was metabolized in the roots and that Se concentrations in the xylem were low even though high  $\text{SeO}_3^{-2}$  was supplied in the medium. It was suggested that much of the  $\text{SeO}_3^{-2}$  remained in the roots (Asher et al., 1977). Other studies have confirmed that  $\text{SeO}_3^{-2}$  remains in the root and is not as readily transported to shoot (Gissel-Nielsen, 1973; de Souza et al., 1998). Zayed et al. (1998) reported that  $\text{SeO}_3^{-2}$  was converted to organic compounds and retained in the roots after being absorbed.

Plant species can be divided into three groups according to their ability to accumulate Se (Rosenfeld and Beath, 1964; Brown and Shrift, 1982; Wu, 1998; Ellis and Salt, 2003). Plants that accumulate less than 25 mg Se  $\text{kg}^{-1}$  dry wt. are “non-accumulator” species, and include many agronomic and horticultural crops (Shrift, 1969). “Se-indicator” species accumulate 25 to 100 mg Se  $\text{kg}^{-1}$  dry wt. The third group is “Se-accumulators” and these amass >100 mg Se  $\text{kg}^{-1}$  dry wt. in high Se environments. Examples of Se-accumulators would include *Astragalus*, *Morinda*, *Neptunia* (Rosenfeld and Beath, 1964; Shrift, 1969), and some *Cruciferae* (Mayland et al., 1989).

Absorbed  $\text{SeO}_4^{-2}$  is incorporated into organic compounds via its reduction to  $\text{SeO}_3^{-2}$  (Brown and Shrift, 1982). Läuchli (1993) found that selenocysteine was further converted to selenomethionine via the  $\text{SO}_4^{-2}$  metabolic pathway. In some plant species these Se-amino acids can substitute for their S-amino acid analogs and rearrange the S-containing proteins. This substitution can cause Se-toxicity in “non-accumulator” species because S-protein function is affected (Wu, 1998; Terry et al., 2000; Ellis and Salt, 2003). However, “Se-indicator” and “Se-

accumulator” species can methylate Se-amino acids to form Se-methylselenocysteine and selenocystathionine, which prevent their incorporation into S-containing proteins, thereby, avoiding Se-toxicity (Brown and Shrift, 1982).

To further our understanding of those factors that influence Se uptake and accumulation in plants, the effect of growing temperature was investigated. The objective of this study was to determine how increasing temperatures affected Se accumulations of in leaf, stem and root tissues in *Brassica oleracea*. Because Se and S can compete each other, and temperature was shown to influence S uptake and accumulation in plants (Coolong and Randle, 2003), we simultaneously measured S and  $\text{SO}_4^{2-}$  accumulation in the same tissues.

## MATERIAL AND METHODS

Seeds of a rapid cycling *Brassica oleracea* population (Crucifer Genetics Cooperative, Department of Plant Pathology, University of Wisconsin, Madison, WI) were sown into growing cubes (Dk-2640. Hedehusene, Denmark) on 9 January 2003. The cubes were covered in fine vermiculite and watered. Seeds were germinated at day/night set point temperatures of 20/12 °C in a greenhouse. Seedlings were fertilized weekly with 1 L of a 200 mg·L<sup>-1</sup> Peter’s 20N-20P-20K (Scotts-Sierra Co., Marysville, OH). The first true leaves were observed on 21 January 2003 and plants were transferred to 40-L containers (Rubbermaid, Inc., Wooster, OH) on 27 January 2003. For each container, ten plants were placed in evenly spaced 2.2-cm holes on each lid. The containers were filled with 30 L of a modified half-strength Hoagland’s solution (Hoagland and Arnon, 1950). Each solution contained 472.4 mg·L<sup>-1</sup>  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 303.3 mg·L<sup>-1</sup>  $\text{KNO}_3$ , 57.5 mg·L<sup>-1</sup>  $\text{NH}_4\text{H}_2\text{PO}_4$ , 246.5 mg·L<sup>-1</sup>  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (96 mg  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ), 2.86 mg·L<sup>-1</sup>  $\text{H}_3\text{BO}_3$ , 1.81 mg·L<sup>-1</sup>  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.22 mg·L<sup>-1</sup>  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.08 mg·L<sup>-1</sup>  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.02 mg·L<sup>-1</sup>

$\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ ,  $10 \text{ mg} \cdot \text{L}^{-1}$  Fe (EDTA) and  $5.0 \text{ mg} \cdot \text{L}^{-1}$   $\text{Na}_2\text{SeO}_4$  ( $3.78 \text{ mg SeO}_4^{-2} \cdot \text{L}^{-1}$ ) which provided  $\text{SeO}_4^{-2}:\text{SO}_4^{-2}$  ratios of 1:25. The solutions were aerated using aquarium air stones and a compressor.

The containers were moved into growth chambers with a 24-h photoperiod and temperature treatments were set at a constant 10, 20, and 30 °C. Four reps and ten plants per rep were used in each temperature treatment. Solutions were refreshed every two weeks and deionized water was added daily to maintain the liquid volume. On 21 February 2003 (~ four weeks), ten plants were harvested from each container. Each 10-plant combination was separated into leaves, shoots, and roots, and their fresh wt. were recorded. The plant tissues were washed using deionized water and then placed into a force-air oven at 70 °C for 48 hr. Tissue dry weights were recorded, and then ground to pass a 0.5 mm screen with a Cyclotec Mill (model 1093, Tector, Höganäs, Sweden).

To measure total S, 0.25 g of dried tissue was mixed with 0.1 g vanadium pentoxide accelerant (Leco Corp., St. Joseph, MI) and combusted at 1300 °C with  $\text{O}_2$  in a Leco 232 S machine (Leco Corp.). To determine  $\text{SO}_4^{-2}$ , another 0.25 g dried tissue was placed into 125 mL flask with 50 ml HPLC grade water. Suspensions were shaken for 30 min at 150 rpm and filtered through a 0.22  $\mu\text{m}$  nylon syringe filter (Fisher Scientific, Pittsburg, PA) into 1.0 mL plastic vials (National Scientific Company, Lawrenceville, GA). Samples were run on a Waters 2690 Separations Module using a Waters 432 Conductivity Detector (Waters Corp., Milford, Mass). Forty  $\mu\text{L}$  of sample solution were injected into an IC-PAK Anion HR column coupled with an IC-PAK Anion Guard Pak (Waters Corp.). Column temperature was set at 30 °C and an isocratic sodium borate-gluconate eluent was used at a flow rate of  $1 \text{ mL} \cdot \text{min}^{-1}$ . The peak was

quantified and integrated by Millennium Chromatography Software (version 3.05, Waters Corp.) against a ten ppm  $\text{Na}_2\text{SO}_4$  standard.

For total Se analysis, a wet acid digestion was used to prepare the samples. Ground tissues (0.25g) were placed into 125-mL flask with 4 mL concentrated nitric acid (70%  $\text{HNO}_3$ ) and incubated over night. The next day, the flasks were put on a 120°C hot plate (Model 2200; Thermolyne, Dubuque, IA) for 1 h. Flasks were removed and cooled to room temperature and four mL 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was added, and the flasks were placed back on the hot plate. The 4 mL  $\text{H}_2\text{O}_2$  was repeated until the digest was colorless. The flasks were cooled to room temperature and 10 mL deionized water was added. The solutions were filtered (Whatman #1 filter paper) and Se was measured by atomic absorption spectrophotometry (GFAA; Model 4100ZL; Perkin-Elmer Corporation, Norwalk, CT).

Data were analyzed by the GLM procedure and linear and polynomial regression using SAS statistical software (Version 8.2, SAS, Cary, NC).

## RESULTS AND DISCUSSION

As temperature increased, plants became visibly larger. Increasing temperatures significantly increased plant fresh wt. ( $P \leq 0.001$ ) in leaves, stems and roots (Figure 1). The response was linear with increasing temperature (Leaf FW =  $2.889 + 0.290 \text{ }^\circ\text{C}$ ,  $R^2 = 0.84$ ; Shoot FW =  $0.328 + 0.313 \text{ }^\circ\text{C}$ ,  $R^2 = 0.89$ ; Root FW =  $0.577 + 0.052 \text{ }^\circ\text{C}$ ,  $R^2 = 0.75$ ). Mean fresh wt. ranged from 10.37 g per plant to 23.46 g per plant. Significant increases in plant dry wt. ( $P \leq 0.001$ ) were also found with increasing temperature (1.06 g per plant to 2.60 g per plant; Figure 1). Dry wts. from leaf, shoot, and roots increased linearly with temperature (Leaf DW =  $0.129 + 0.420 \text{ }^\circ\text{C}$ ,  $R^2 = 0.82$ ; Shoot DW =  $0.060 + 0.031 \text{ }^\circ\text{C}$ ,  $R^2 = 0.88$ ; Root DW =  $0.049 + 0.004 \text{ }^\circ\text{C}$ ,  $R^2$

= 0.82). Increasing temperature over a wide range was reported to increase plant yield in crops such as onion (Coolong and Randle, 2003), potato (Baghour et al., 2002), and peanut (Awal et al., 2003).

Selenium accumulation in the plant was tissue specific and dependant on the growing temperatures (Figure 2). As temperatures increased, Se accumulation increased linearly in leaves ( $P \leq 0.05$ ; Leaf Se =  $1385.1 + 41.462 \text{ } ^\circ\text{C}$ ,  $R^2 = 0.81$ ), whereas it significantly decreased in roots ( $P \leq 0.05$ ; Root Se =  $3280.6 - 35.35 \text{ } ^\circ\text{C}$ ,  $R^2 = 0.69$ ). Accumulation in the shoots did not significantly respond to changes in growing temperatures. Mean Se accumulation in leaves ranged from 1734 to 2541  $\mu\text{g Se g}^{-1}$  dry wt. while Se decreased from 2870 to 2167  $\mu\text{g Se g}^{-1}$  dry wt. in roots. According to Läubli, (1993) lower Se concentrations are expected in the roots because they are the means of absorption and transport, and not accumulation. In our study the lower growing temperature caused Se accumulated in the roots at higher levels than in the leaves. Se was shown to increase in potato tubers with increasing temperature (Baghour et al., 2002).

Total S accumulation also varied among tissues and was temperature dependent, but the trend was different from Se (Fig. 3). Overall S accumulation in the leaves, roots, and shoots were 27.03, 12.27 and 10.09  $\text{mg S g}^{-1}$  dry wt., respectively. However, the effect of temperature was only significant for S accumulation in the leaves ( $P \leq 0.05$ ) which increased linearly with increasing temperature (Leaf S =  $16.873 + 0.508 \text{ } ^\circ\text{C}$ ,  $R^2 = 0.46$ ). Mean leaf S ranged from 20.828 to 30.983  $\text{mg S g}^{-1}$  dry weights. It appears that S was readily transported to the leaves where S is stored, reduced, or incorporated into amino acids. Temperature also affected S uptake and accumulation in tomato (Tindall et al., 1990), potato (Baghour et al., 2002), and onion (Coolong and Randle, 2003).

Similar to total S, increasing temperature significantly influenced  $\text{SO}_4^{-2}$  accumulation in the leaves ( $P \leq 0.01$ ), but not in shoots and roots (Fig. 4). A quadratic increase then decrease was found for leaf  $\text{SO}_4^{-2}$  responding to increasing temperature (Leaf  $\text{SO}_4^{-2} = 1.86 + 1.058 \text{ }^\circ\text{C} - 0.0249 \text{ }^\circ\text{C}^2$ ,  $R^2 = 0.68$ ). In potato, higher temperatures resulted in more  $\text{SO}_4^{-2}$  accumulation in leaflets (Baghour et al., 2002). When plants are grow with minimal S-fertility, most of the absorbed  $\text{SO}_4^{-2}$  is reduced and enters the organic pathways, but when S-fertility is luxuriant, much of the absorbed  $\text{SO}_4^{-2}$  is stored in the vacuoles of the plant's cells (Randle et al., 1999).

When applied as fertilizers, the ratio of Se to S supplied is very important in both Se and S uptake and accumulation by plants (White et al., 2004). Se and S compete for absorption into plants (Gissel-Nielsen, 1973; Barak and Goldman, 1997; Bell et al., 1992; Hopper and Parker, 1999; Mikkelsen and Wan, 1990; Pezzarossa et al., 1999) and evidence suggests that Se and S share the same plant transport pathway (Abrams et al., 1990; Lauchli, 1993). Initial reports indicated Se suppressed S uptake when Se was made available to plants at levels nearly equal to S (Ferrari and Renosto, 1972). However, when  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  were supplied at ratios between 1:125 to 1:500, S uptake and accumulation was actually enhanced when compared to plants that did not receive Se (Kopsell and Randle, 1997; 1999). Therefore, the amount and ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  made available to the plant will affect S and Se uptake and accumulation, as well as S partitioning within the plant. Interpreting S and Se uptake and accumulation in response to increasing temperature must consequently take into account relative and absolute S and Se fertility levels. In our study, the ratio of  $\text{SeO}_4^{-2} : \text{SO}_4^{-2}$  made available in the nutrient solutions was 1:25.

However, excess Se can cause phytotoxicity because of substitution that can occur in S-proteins. In *Brassica*, however, much of the selenium is converted to selenocysteine, selenomethionine, and Se-methylselenocysteine (Zayed and Terry, 1992).

## CONCLUSIONS

Vegetable crops high in Se are an efficient means of delivering Se in human diets (Ip et al., 1992; Ip and Lisk, 1994, 1995; Lu et al., 1996). The benefits of Se have been associated with antioxidant activity (Sreekala et al., 1999; Tapiero et al., 2003), immune system enhancement (Campa et al., 1999), and cancer suppression (Ip et al., 2000, Raich et al., 2001). *Brassicas* plants grown in hydroponic solutions and exposed to different temperature treatments accumulated increasing levels of Se and S in leaves as temperatures increased. However, the accumulation pattern of Se and S differed in response to temperature. To ensure that optimal Se levels are achieved in plants, factors that affect uptake and accumulation, like growing temperature, need to be better understood.

Fig. 1.

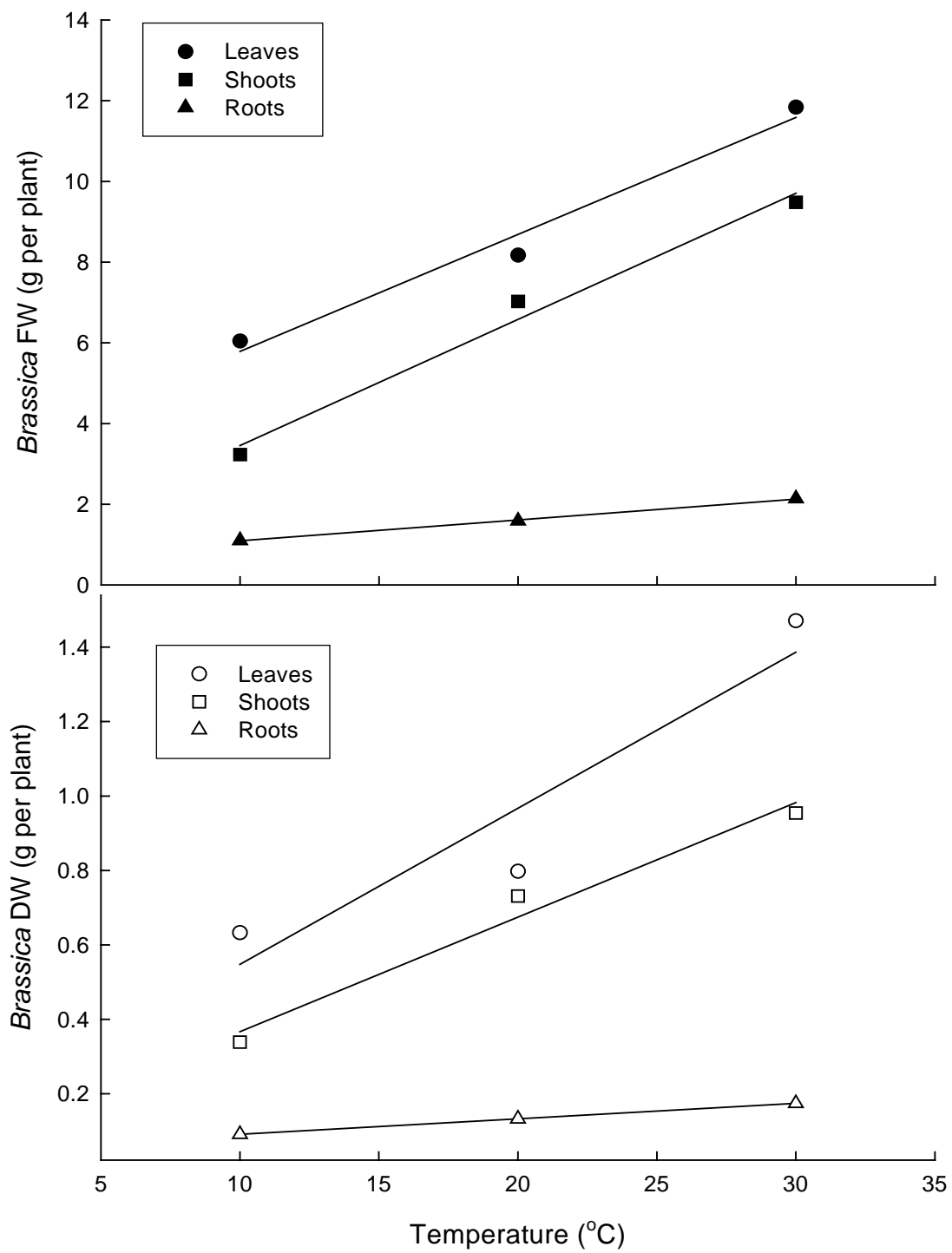


Fig. 2.

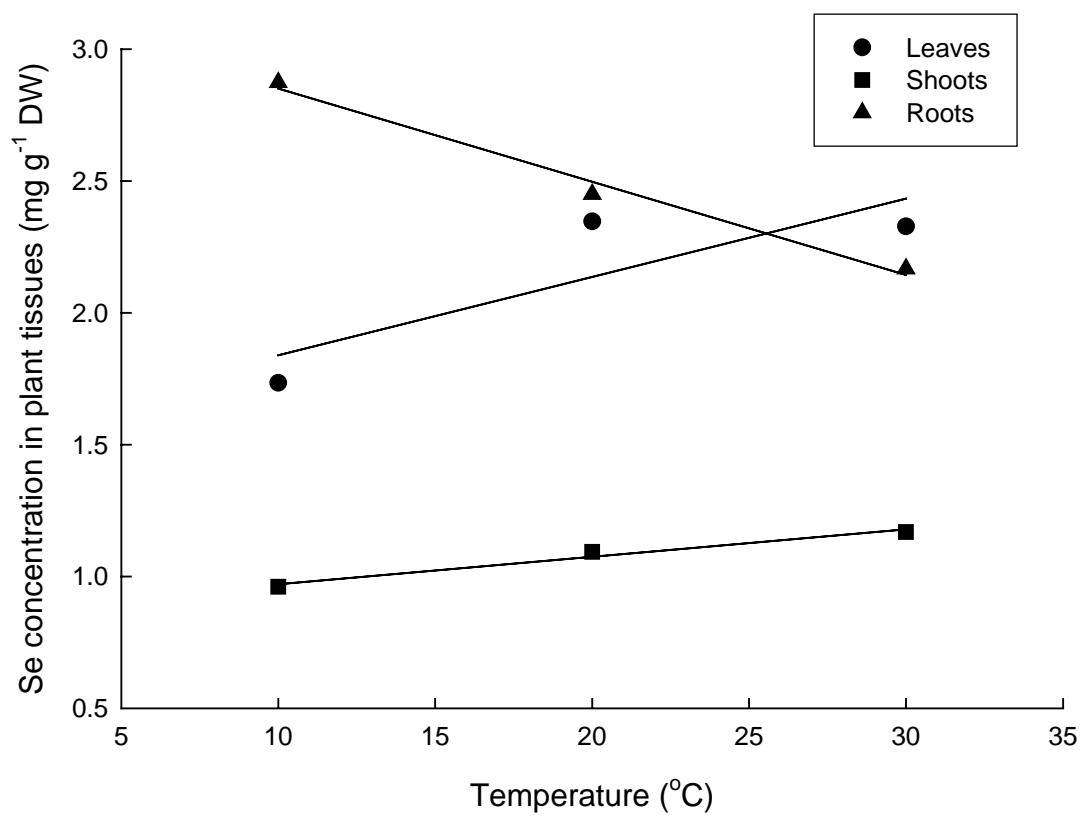


Fig. 3.

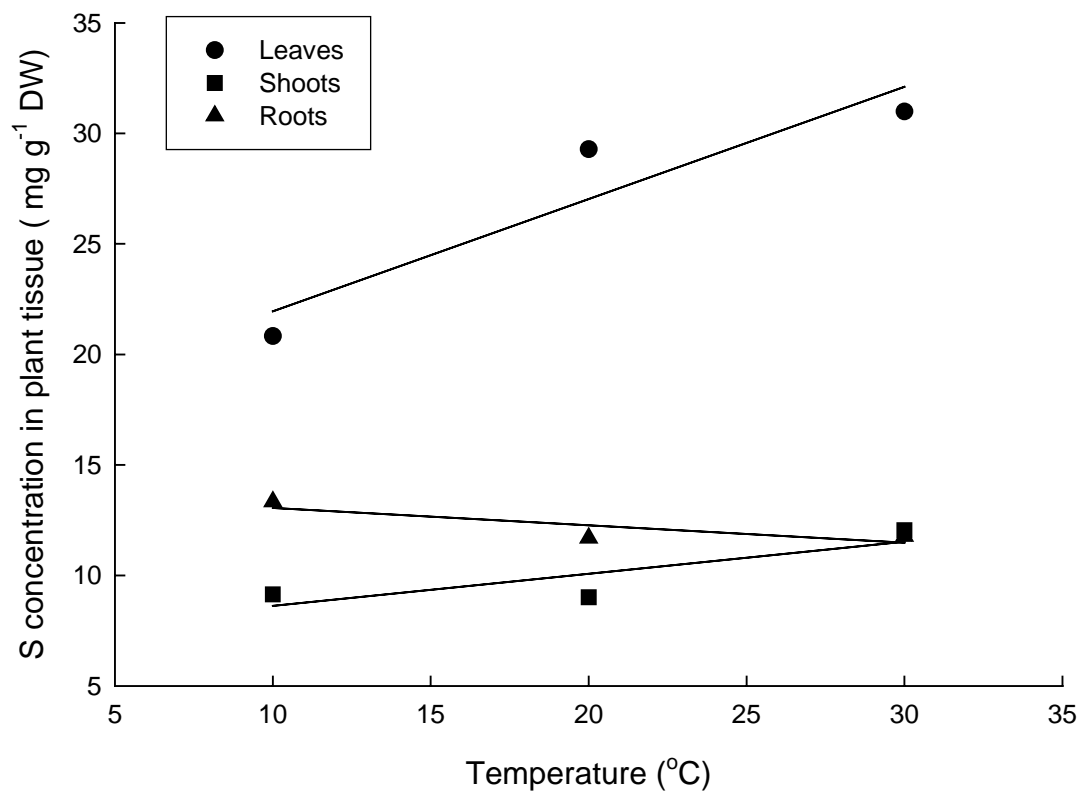


Fig. 4.

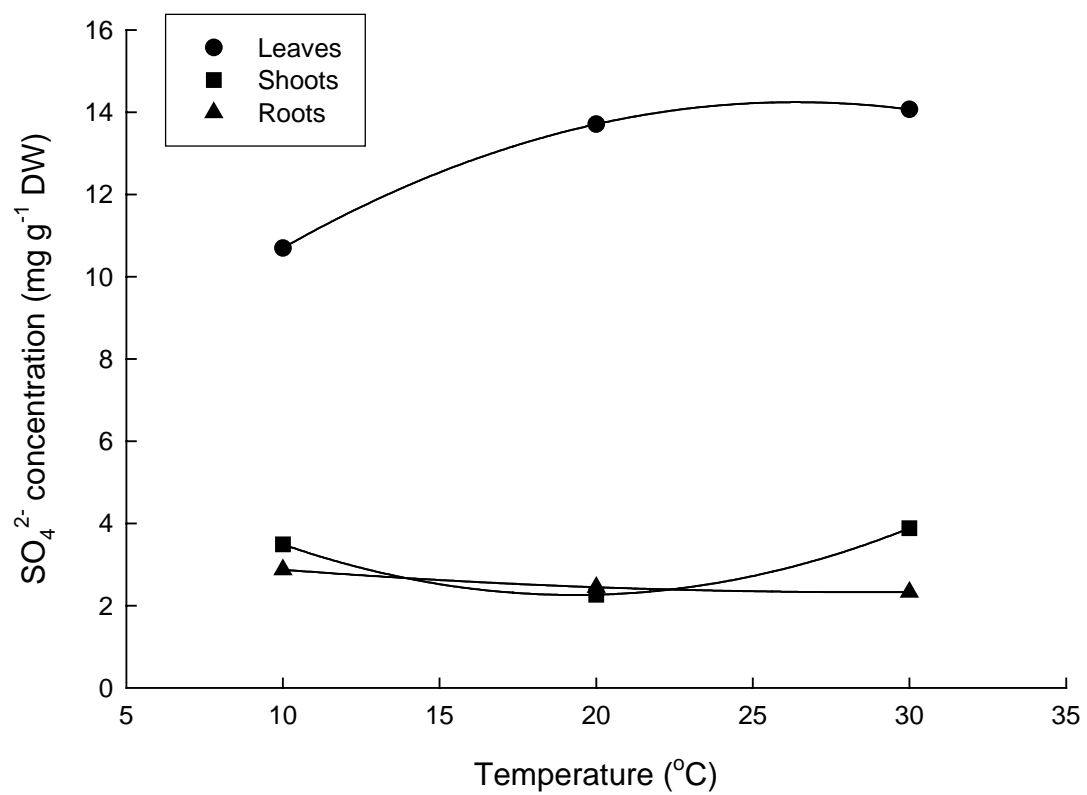


Fig. 1. Different tissue fresh weights of *Brassica oleracea* increased linearly (Leaf FW =  $2.889 + 0.290 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.84$ ; Shoot FW =  $0.328 + 0.313 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.89$ ; Root FW =  $0.577 + 0.052 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.75$ ) with increasing temperature.

Different tissue dry weights of *Brassica oleracea* increased linearly (Leaf DW =  $0.129 + 0.420 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.82$ ; Shoot DW =  $0.060 + 0.031 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.88$ ; Root DW =  $0.049 + 0.004 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.82$ ) with increasing temperature.

Fig. 2. Se accumulation in leaves (Leaf Se =  $1385.1 + 41.462 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.81$ ) and in roots (Root Se =  $3280.6 - 35.35 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.69$ ) showed contrary results with increasing temperature, and non-significant difference was shown on shoot tissues.

Fig. 3. S accumulation in leaves was linear increased with increasing temperature (Leaf S =  $16.873 + 0.508 \text{ }^{\circ}\text{C}$ ,  $R^2 = 0.46$ ) and non-significant differences were found in shoot and root tissues.

Fig. 4. Leaf sulfate was significantly influenced by increasing temperature ( $P \leq 0.01$ ), and a quadratic increase was found for leaf  $\text{SO}_4^{-2}$  responding to increasing temperature (Leaf  $\text{SO}_4^{-2} = 1.86 + 1.058 \text{ }^{\circ}\text{C} - 0.0249 \text{ }^{\circ}\text{C}^2$ ,  $R^2 = 0.68$ ). Temperature did not significant affect the  $\text{SO}_4^{-2}$  concentration in shoots and roots.

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**CHAPTER 4****HIGH AND LOW CONCENTRATIONS OF  $\text{Na}_2\text{SeO}_4$  IN NUTRIENT SOLUTIONS  
AFFECT THE ACCUMULATION OF SULFATE AND SELENATE IN *BRASSICA*  
*OLERACEA*<sup>1</sup>**

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<sup>1</sup> Chang, P.T., W.M. Randle, and C.E.Sams. For submission to HortScience.

### Abstract

Selenium is associated to human health benefits, and *Brassica* species are good candidates to deliver Se to human diet. Se and S compete for absorption and accumulation in plant tissue; to establish treatments responses for a *Brassica oleracea* two levels of  $\text{Na}_2\text{SeO}_4$  ( $96.0 \text{ mg L}^{-1} \text{ SeO}_4^{-2}$  and  $0.384 \text{ mg L}^{-1} \text{ SeO}_4^{-2}$ ) were applied to nutrient solutions with or without  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $96.0 \text{ mg L}^{-1} \text{ SO}_4^{-2}$ ). Different ratios of Se and S affected *Brassica oleracea* vegetative growth and mineral accumulations in plant tissues. The highest plant fresh weight and S and  $\text{SO}_4^{-2}$  accumulation were found when plants were grown in the medium with a  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  ratios of 1: 250. However, the highest accumulation of Se was found when a low level of selenate ( $0.384 \text{ mg L}^{-1} \text{ SeO}_4^{-2}$ ) was added into nutrient solutions without S. In addition, varying concentrations changed in glutathione peroxidase (GPx) activity and glucosinolates (GLs) levels in plants. The activity of GPx was regulated by Se status; the highest GPx activity was measured when a high level of selenate ( $96.0 \text{ mg L}^{-1} \text{ SeO}_4^{-2}$ ) was supplied to medium without S supplementation. The lowest concentration of total GLs was found when adding selenate into nutrient solutions without S. Se can be delivered through plants to human diet, and S and Se compete for absorption and accumulation in plants; therefore, the proper ratios of Se/S as fertilizer need to be considered to achieve optimal Se levels in plants.

### Introduction

It has been proposed that Se can be metabolized via the S assimilation pathway (Läuchli, 1993; Pilon-Smits et al., 1999; Terry et al., 2000) and subsequently can be incorporated into Se-amino acids or proteins in higher plants (de Souza et al., 2000; Terry et al., 2000) because these two elements are in the same Group VI in the periodic table and have similar chemical

properties. Several studies showed in accumulators either Se is incorporated into nonprotein Se-amino acids or SeCys can be methylated by selenocysteine methyltransferase to avoid Se toxicity (Shrift, 1969; Brown and Shrift, 1981, 1982). On the other hand, in non-accumulators Se-amino acids have been shown to substitute for S-amino acids nonspecifically in metabolic pathways and could be incorporated into non-functional proteins (Ng and Anderson, 1979; Eustice et al., 1981). Such abnormal substitutions are considered as the cause of Se toxicity in plants.

Selenium is an essential trace nutrient in the human diet. There is a narrow range between Se sufficiency and toxicity (Rosenfeld and Beath, 1964; Wilber, 1980; Lemly, 1997). Se can be delivered to the human daily diet through standard food uptake (Gissel-Nielsen et al., 1984; Ip et al., 1992; Ip and Lisk, 1994; Reilly, 1996). Therefore, the amount of human Se intake depends on the amount of Se in their food, and the amount of Se in plant material depends on the availability of Se in the soil and the ability of plants to take it up. In vegetables, *Brassica* species are good Se deliverers because of their high accumulation of Se; e.g., Indian mustard (*Brassica juncea* L.) (Bañuelos et al., 1997b) and canola (*Brassica napus* L.) (Bañuelos, 2002) are able to accumulate several hundred milligrams of Se per kilogram dry weight in their shoot tissues when grown with high levels of Se.

*Brassica* crops are consumed because of their nutritional benefits (Stoewsand et al., 1989; Finley, 2003), while many people are attracted to their bitter flavor and distinctive odor (Fenwick et al., 1983a, 1983b). The accumulation of Se and its compounds in *Brassica* species has drawn attention because Se was found as a component of the antioxidant enzyme, glutathione peroxidase (GPx), which may have human health benefits (Rotruck et al., 1973). The Se-dependent GPx was first found in green alga, *Chlamydomonas reinhardtii*, (Fu et al., 2002) but its presence has not been proved in higher plants. Furthermore, in *Brassic*as, glucosinolates

(GLs) are not only related to anticarcinogenic effects but also are the dominant flavor precursors. Glucosinolates are hydrolyzed by myrosinase (E.C. 3.2.3.1) when plant tissue is broken to produce their unique flavor (MacLeod, 1976; Fenwick et al., 1983a, 1983b). Glucosinolates in *Brassicaceae* are amino acid-derived secondary products containing a sulfur element (Fenwick et al., 1983a; Fenwick et al., 1983b; Halkier and Du, 1997; Rosa et al., 1997). In *Brassica* plants, Mailer (1989) and Booth et al. (1991) showed that increasing sulfate concentrations not only influenced sulfur concentrations in plants but also enhanced glucosinolates content. However, when *Brassica* plants were exposed to different sodium selenate concentrations, high selenium levels decreased glucosinolates content (Charron et al., 2001). Bailey et al. (1995) pointed out different selenate uptake rates under high and /-low sulfate conditions in *Ruppia maritime* L., indicating sulfate and selenate competition. When the ratio of  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  was below 1: 125; a slight amount of Se enhanced  $\text{SO}_4^{-2}$  uptake and accumulation in onions (Kopsell and Randle, 1997).

Selenium has been associated with antioxidant activity when present at appropriate levels; however, it can become toxic when present in higher levels (Raisbeck, 2000; Vinceti et al, 2001). Because *Brassica* species are good candidates to supply both Se and GLs to the human diet (Sigrid-Keck and Finley, 2004; Finley et al., 2005), changes in Se accumulation and glucosinolates amounts in *Brassica* species needs more evaluation when plants are grown in areas contaminated with selenium. Therefore, this study was conducted to examine the effects of Se/S ratios on plant growth; to measure the accumulation patterns of S,  $\text{SO}_4^{-2}$ , and selenate-Se in plant tissues (i.e. roots, stems, and leaves); and to determine glutathione peroxidase (GPx) activity and flavor intensity (GLs) in *B. oleracea* under different ratios of selenate and sulfate.

## Materials and Methods

*Plant material.* On 19 June 2006, seeds of a rapid cycling *Brassica oleracea* L. population (RCBP) (Crucifer Genetics Cooperative, Department of Plant Pathology, University of Wisconsin, Madison, Wis.) were sown in growing cubes (Smithers-Oasis, Ohio, U.S.), covered with vermiculite and watered as needed, and put in a growth chamber (Model E15; Conviron, Asheville, NC) at temperatures of 24°C and a 24-h photoperiod with average fluorescent flux at  $250 \mu\text{mol}^{-1} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (Basic Quantum Meter, Spectrum Tech. Plainfield, Ill.). Seeds germinated in 7 days and seedlings were fertilized with 100 mL of a 200 mg·L<sup>-1</sup> Peter's 20N-20P-20K (Scotts-Sierra Co., Marysville, Ohio). On 1 July 2006, the first true leaves emerged and plants were transferred to 270 mL crystallizing dishes (89000-288, VWR, Atlanta, Ga.) containing 100 mL modified half-strength Hoagland's solution (Hoagland and Arnon, 1950) for a week. Thereafter, plants were rinsed in deionized water and groups of 10 seedlings were subsequently transferred to a 270 mL crystallizing dishes with fresh half-strength nutrient solution and different selenate-Se treatments were supplied. The dishes were placed back into a growth chamber at temperatures 24°C and 16-h photoperiod for 72 hr with average fluorescent flux at  $250 \mu\text{mol}^{-1} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ .

*Treatments.* Two levels of Na<sub>2</sub>SeO<sub>4</sub> were applied in this study as follows, each solution contained 1.0 mM MgSO<sub>4</sub>·7H<sub>2</sub>O (96 mg SO<sub>4</sub><sup>-2</sup>·L<sup>-1</sup>) as control, 0.6 mM Na<sub>2</sub>SeO<sub>4</sub> (96 mg SeO<sub>4</sub><sup>-2</sup>·L<sup>-1</sup>) + 1.0 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.68 μM Na<sub>2</sub>SeO<sub>4</sub> (0.384 mg SeO<sub>4</sub><sup>-2</sup>·L<sup>-1</sup>) + 1.0 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.6 mM Na<sub>2</sub>SeO<sub>4</sub> + 0 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 2.68 μM Na<sub>2</sub>SeO<sub>4</sub> + 0 mM MgSO<sub>4</sub>·7H<sub>2</sub>O. The two treatments that included both Se and S provided SeO<sub>4</sub><sup>-2</sup>: SO<sub>4</sub><sup>-2</sup> ratios of 1:1 and 1:250. After 72 hr, each ten-plant combination was harvested and separated into leaves, stems, and roots. Plant fresh weights were recorded. The plant tissues were washed using

deionized water and frozen in liquid N<sub>2</sub>, placed in plastic bags and put into a freeze drier (Model 79480; Labconco corporation, Kansas, Mich) at -40°C for 72 hr. Tissues dry weights were recorded, and tissue was stored at -80°C for later analysis.

*Mineral analysis.* Total S in each plant tissue part was measured using a Leco Sulfur Determinator (Model SC-232; Leco Corp., St. Joseph, Mo). Twenty mg of dried tissue were combined with a vanadium pentoxide accelerator (Leco Corp., St. Joseph, Mo) and combusted at 1300°C with O<sub>2</sub>. Total S was quantified against S standards. To determine total SO<sub>4</sub><sup>-2</sup>, another 20.0 mg of dried tissue were put into a 125.0 mL flask with 50.0 mL HPLC grade (18Ω) water. Suspensions were shaken at 100 rpm overnight and injected into 1.0 mL plastic vials (National Scientific Company, Lawrenceville, Ga) through a 0.22 μm nylon syringe filter (Fisher Scientific, Pittsburgh, Pa). Forty μL of sample solution were carried out on a Waters 2690 Separations using a Waters 432 Conductivity Detector (Waters Corp., Milford, Mass). The peak, representing SO<sub>4</sub><sup>-2</sup> was quantified and integrated by Millennium Chromatography software (version 3.05, Waters Corp., Milford, Mass) against a 30.0 ppm SO<sub>4</sub><sup>-2</sup> standard.

A wet-acid digestion was applied to measure total Se from each tissue part. Twenty milligram of dried tissue was placed into a 125.0 mL flask with 4.0 mL concentrated nitric acid (70% HNO<sub>3</sub>) and incubated overnight. Then, flasks were set on a hot plate (Model 2200; Thermolyne, Dubuque, Iowa) at 120°C for 1h. A funnel test tube was put into each flask filled with water as a condenser to reduce sample loss during digestion. Flasks were removed and allowed to cool to room temperature, and 4.0 mL of 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added. Flasks were placed back on the hot plate with additional 4.0 mL H<sub>2</sub>O<sub>2</sub>. The flasks were cooled to room temperature and adjusted to a final volume of 10.0 mL with deionized water. The solutions were filtered through a 0.22 μm nylon syringe filter (Fisher Scientific, Pittsburgh, Pa). Total Se

was measured by graphite furnace atomic absorption spectrophotometry (GFAA; Model 300; Perkin Elmer Corp., Norwalk, Conn).

*Glutathione peroxidase activity.* Total glutathione peroxidase (GPx) activity was measured from 100.0 mg of fresh leaves using Total Glutathione Peroxidase Assay Kits (ZeptoMetrix Corp., Buffalo, N.Y.). Changes in absorbance were recorded every 15 sec by spectrophotometer at 340 nm for 1 min. The first 40 seconds were not taken into consideration. The GPx activity was calculated by subtracting a blank from the measurements of each sample.

*Glucosinolates analysis.* To measure glucosinolates, 20.0 mg of dried leaf tissues were extracted with 0.25 mL of benzyl GLs solution (1.0 mM), 0.5 mL of methanol and 0.075 mL of barium lead acetate (0.6 M) in a 16 mm x 100 mm tube, and shaken at 60 rpm for an hour. Samples were centrifuged at 2000 g for 10 min. Two hundred  $\mu$ L of supernatant were desulfated on a 1.0 mL column containing 0.3 mL preswollen DEAE Sephadex A-25 by the procedure of Raney and McGregor (1990). The extraction of desulphoglucosinolates was carried out on a Hewlett-Packard (Palo Alto, Calif) high performance liquid chromatograph (HPLC) using a C-18 ODS reverse-phase column (250  $\times$  4.6 mm ID, 5- $\Phi$ m) and detected by a UV detector at a wavelength of 230 nm. A water-acetonitrile mobile phase gradient was used for separation of desulfoglucosinolates. Desulphoglucosinolates were identified and quantified by comparison with retention times of standards. Desulfonated forms of glucoiberin (GI, 3-methylsulfinylpropyl GS), glucobrassicin (GB, indol-3-ylmethyl GS), gluconapin (GNP, 3-butenyl GS), 4-methoxyglucobrassicin (4MGB, 4-methoxyindol-3-ylmethyl GS), neoglucobrassicin (NGB, 1-methoxyindol-3-ylmethyl GS), progoitrin (PRO, 2-hydroxybut-3-enyl GS), and sinigrin (SN, 2-propenyl GS) were used as internal standards (Sandro Palmieri of the Istituto Sperimentale Industriali, Bologna, Italy). Gluconasturtiin (GNS, 2-phenylethyl GS)

was purchased from LKT Laboratories (LKT Inc., St. Paul, Minn). Response factors used were from the International Organization for Standardization Method 9167-1.

*Statistical analysis.* The experimental design was a randomized complete design with three replications and ten plants per replication. To test the effects of various treatments on plant growth, mineral accumulation, and GPx activity and GLs amount, data were analyzed by the GLM procedure using SAS statistical software (version 9.1.3, SAS, Cary, NC).

## Results

*Plant growth observation.* Some *Brassica* species are classified as a Se-accumulator species. In our study, Se toxicity was observed when  $\text{Na}_2\text{SeO}_4$  was applied to the nutrient solutions. *B. oleracea* grown at the highest  $\text{Na}_2\text{SeO}_4$  showed chlorotic spots on old leaves. In addition, a pinkish-orangey color appearing on roots occurred. As increasing the ratios of  $\text{SeO}_4^-$ :  $\text{SO}_4^{2-}$ , the reductions of plant growth were more obvious. However, low level of Se stimulated plant growth.

*Brassica oleracea fresh weight and dry weight.* The fresh and dry weights of *Brassica oleracea* plants were impacted significantly by adding Se in growing medium ( $P \leq 0.001$ ). Significant decreases were found both in fresh weight and dry weight in leaves, stems, and roots ( $P \leq 0.001$ ) when plants were grown in the medium under high concentration of selenate ( $96 \text{ mg L}^{-1}$ ) without sulfate. When *Brassica oleracea* plants were grown in the nutrient solutions under a low concentration of selenate ( $0.384 \text{ mg L}^{-1}$ ), there were no statistically significant differences in fresh weight and dry weight in different parts of plant tissue (Table 1).

*Total sulfur, sulfate and selenium.* The accumulation of S in *B. oleracea* varied depending on plant tissues. Adding sodium selenate to nutrient solutions affected S

accumulation. Sulfur concentration in leaf ( $P \leq 0.001$ ), stem ( $P \leq 0.001$ ), and root ( $P \leq 0.001$ ) differed in response to different treatments. The highest leaf, stem, and root S concentrations were found when *B. oleracea* plants were grown in S- sufficient nutrient solutions with a low concentration of selenate ( $0.384 \text{ mg L}^{-1}$ ) (Table 2). A significant decrease of S accumulation was observed in both leaf and stem tissues ( $P \leq 0.001$ ) but not in root tissues when plants were transferred to a nutrient solution with low concentrations of selenate ( $0.384 \text{ mg L}^{-1}$ ) without S supplement, as compared to control plants. In addition, a decrease ( $P \leq 0.001$ ) was found in leaf, stem, and root S when plants were grown with a 1:1 ratio of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the solutions. Furthermore, S concentrations in stem and root tissues were significantly lower than in control plants when *B. oleracea* plants were grown with high concentrations of selenate ( $96.0 \text{ mg L}^{-1}$ ) while no significant difference was seen in leaf tissues when *B. oleracea* plants were grown with high concentration of selenate ( $96.0 \text{ mg L}^{-1}$ ) without sulfur supplementation (Table 2).

Tissue sulfate concentrations also differed among treatments. The accumulation of  $\text{SO}_4^{-2}$  in leaves, stems, and roots increased ( $P \leq 0.001$ ) when plants were grown in a S-sufficient solution with a low concentration of selenate ( $\text{SeO}_4^{-2}$ :  $\text{SO}_4^{-2}$  was 1: 250). As expected, a high concentration of  $\text{SeO}_4^{-2}$  decreased  $\text{SO}_4^{-2}$  accumulation in leaves, stems, and roots. When a low concentration of  $\text{SeO}_4^{-2}$  was added to nutrient solution without S, it decreased  $\text{SO}_4^{-2}$  concentration in plant tissues significantly as well (Table 2).

When *B. oleracea* plants were grown in the Se-containing nutrient solutions, tissue Se concentrations increased ( $P \leq 0.001$ ). The concentration of Se in different plant tissues differed depending on the  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  concentrations in the nutrient solution (Table 2). Concentrations of Se in control plants, which were grown without  $\text{Na}_2\text{SeO}_4$ , were below the limits of GFAA detection. Adding various levels of selenate resulted in significant Se

accumulation in plant tissues; however, the concentration of Se in leaf and root tissues were not statistically different between 1:1 and 1:250 ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$ . Additionally, there was no difference in Se accumulation in stem tissues between 1  $\text{SeO}_4^{-2}$ : 1  $\text{SO}_4^{-2}$  ratios and high Se addition without S.

*Glutathione peroxidase activity.* The various treatments resulted in differences in glutathione peroxidase (GPx) activity (Table 3). All treatments containing Se had higher GPx activity than the control treatment, with the highest activity in the treatment with 96 mg Se and no S. The highest level of GPx activity was found in *B. oleracea* plants when the solution contained 96 mg L<sup>-1</sup> Se and no S. A low level (0.384 mg L<sup>-1</sup>) of Se also induced an increase of GPx activity. However, no statistical discrimination existed for the comparison between adding low concentration of Se in nutrient solutions with and without S (Table 3).

*Flavor intensity.* Adding a high level (96 mg L<sup>-1</sup>) of Se in the nutrient solution resulted in the lowest concentration of total glucosinolates (GLs). When a high level of Se, with or without S was used there was no statistical difference in total GLs concentration (Table 4). In contrast, when 0.384 mg L<sup>-1</sup>  $\text{SeO}_4^{-2}$  was applied, a difference was found in total GLs between nutrient solutions with S and without S ( $P \leq 0.05$ ). *B. oleracea* was grown in the S-sufficient medium with a low concentration of selenate had higher total GLs than plants were grown in the same level of Se without S (Table 4). The highest total GLs concentration was found when plants were grown in the S-sufficient with a low concentration of selenate, and there was no difference between this treatment and the control.

## Discussion

There were variations in plant growth, mineral accumulation, GPx activity, and total GLs in response to Se supplementation. Decreases in plant fresh weight and dry weight were found in alfalfa (*Medicago sativa* L.) and clover (*Trifolium repens*) (Broyer et al., 1966), *Brassica juncea* (Bañuelos et al., 1997b), rice (*Oryza sativa* L.) (Zhou, 1990), and wheat (*Triticum* spp) (Peng et al., 2000) in response to Se addition. In this study, a high concentration of  $\text{SeO}_4^{-2}$  ( $96.0 \text{ mg L}^{-1}$ ) reduced the growth of *B. oleracea*. This is considered to be Se toxicity because retardation of plant growth is one of the symptoms noted when plants were grown with high levels of Se (Bañuelos et al., 1997a). Even though Brassica species are able to accumulate abundant Se, the threshold of Se accumulation before growth reductions occur may differ based on plant age and sulfate concentrations in the medium. Therefore, in the early vegetative stage, an overdose of Se resulted in growth inhibition.

When plants are grown in a medium with a mixture of selenate and sulfate, the ratios of  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  play a role in the regulation of sulfate uptake (Ferrari and Renosto, 1972; Mikkelsen and Wan 1990; Bell et al., 1992; Kopsell and Randle 1997, 1999; White et al., 2004). Differences in preference of sulfate and selenate were found between accumulators and non-accumulators. Ferrari and Renosto (1972) reported that under high ratios (1.4 to 1.0) of  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$ ,  $\text{SO}_4^{-2}$  is taken up more than  $\text{SeO}_4^{-2}$  in non-accumulators. On the other hand, Se accumulators are able to take up Se preferentially even when high  $\text{SO}_4^{-2}$  is supplied (Bell et al., 1992). Onions and brassicas are Se-enriched vegetables (Ip et al., 1992; Ip and Lisk, 1994). Kopsell and Randle (1997) reported that when the  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  ratios lowered to 1:125 or 1:500, Se increased  $\text{SO}_4^{-2}$  uptake and accumulation in onions. Moreover, S concentrations increased in leaf tissues of *B. oleracea* when the  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  ratio was increased from 1:42 to

1:14 (Kopsell and Randle, 1999). In this study, the highest accumulation of  $\text{SO}_4^{-2}$  is in response to the ratio of  $\text{SeO}_4^{-2}$ :  $\text{SO}_4^{-2}$  (1:250). Additionally,  $\text{SO}_4^{-2}$  concentration decreased dramatically when plants were transferred to both high and low levels of Se supplementation without sulfate addition in nutrient solutions (Table 2). Other studies have shown that application of  $\text{Na}_2\text{SeO}_4$  leads to a S-starvation condition, which triggers sulfate reduction to keep plants alive (Chen and Leustek, 1995; Leustek et al., 2000). In the current study, the accumulation of S in leaf tissues was reduced under S deprivation (Table 2).

The accumulation of Se in different parts of plant tissue varies depending on plant species, growth stage, and fertilizer, especially sulfate. In this experiment, substantial amounts of Se accumulated in young leaves when no further sulfate was applied (Table 2). It is possible that S-deficiency results in up-regulation of sulfate transporters, which leads to increasing  $\text{SeO}_4^{-2}$  uptake and further accumulation in plants (Zayed and Terry, 1992; Bolchi et al., 1999). This hypothesis is based on the assumption that  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$  share the same transporters (Abrams et al., 1990; Arvy, 1993). Competition between sulfate and selenate uptake was found in this study. High concentrations of  $\text{SO}_4^{-2}$  directly inhibit  $\text{SeO}_4^{-2}$  uptake by plants (Wu and Huang, 1991; Bell et al., 1992; Barak and Goldman 1997). In this study the amounts of Se were twice as high in the solution in the leaf tissue when the ratio of  $\text{SeO}_4^{-2}$  to  $\text{SO}_4^{-2}$  is 1:250 than when it is 1:1. The accumulation of Se under high levels of sulfate in the nutrient solution was reported when organic Se compounds, such as selenomethionine (SeMet) were applied to wheat seedlings (Abrams et al., 1990).

Glutathione peroxidase (GPx), which reduces hydroperoxides, lipid peroxides, and protects cells from oxidative damages, was discovered to contain Se as an essential component (Rotruck et al., 1973). The functions and protective mechanisms of GPx have been reported

mostly in relation to mammals. Selenocysteine (SeCys) has been shown to incorporate into GPx in green alga, *Chlamydomonas reinhardtii*, which directly indicates that a Se-dependent GPx has been found in the plant kingdom (Fu et al., 2002). In this study, we did not prove whether Se-dependent GPx is found in higher plants; however, GPx activity increased significantly with increasing Se concentration, especially when Se was added to nutrient solutions without containing S (Table 2). It is possible that addition of Se caused other isozymes activity, such as phospholipid hydroperoxide glutathione peroxidase which was found in citrus (Eshdat et al., 1997).

It was reported that addition of selenium decreased glucosinolates concentrations in *B. oleracea* (Charron et al., 2001). Similar results were found in our study especially when a high concentration of Se was added to the medium whether with S or without S. Interestingly, in the same study it was also reported that an increase in S uptake resulted in lower GLs concentrations (Charron et al., 2001). However, in this experiment Se not only enhanced  $\text{SO}_4^{2-}$  uptake, but also increased GLs concentrations at low ratios of Se to /S (1:250).

### **Conclusion**

When *Brassica oleracea* was grown in a nutrient solution with a 1:250 ratio of  $\text{SeO}_4^{2-}$  and  $\text{SO}_4^{2-}$ , S accumulation increased as well as Se accumulation in plant tissues. Se is important because it is related to antioxidant activity; therefore, the brassica vegetables could be used as a system to provide Se to the mammalian diet. Se application also affected the concentrations of glucosinolates. The breakdown products of glucosinolates include hot and bitter flavor compounds. Thus, it is important to find out a proper relationship among Se content, glucosinolates concentrations, and flavor intensity to satisfy consumer acceptance.

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Table 1. Fresh and dry weight of 10-plant tissue combination of *B. oleracea* grown with different ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the nutrient solutions.

Treatment		Leaf	Stem	Root
$\text{SeO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ ) : $\text{SO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ )				
Fresh weight (g)				
(0)	(96)	$1.48 \pm 0.07$ <sup>ab</sup>	$1.22 \pm 0.05$ <sup>b</sup>	$0.77 \pm 0.02$ <sup>a</sup>
(96)	(96)	$1.30 \pm 0.08$ <sup>b</sup>	$0.82 \pm 0.04$ <sup>c</sup>	$0.58 \pm 0.01$ <sup>b</sup>
(0.384)	(96)	$1.55 \pm 0.03$ <sup>a</sup>	$1.51 \pm 0.03$ <sup>a</sup>	$0.78 \pm 0.03$ <sup>a</sup>
(96)	(0)	$1.06 \pm 0.04$ <sup>c</sup>	$0.72 \pm 0.03$ <sup>c</sup>	$0.51 \pm 0.01$ <sup>c</sup>
(0.384)	(0)	$1.40 \pm 0.05$ <sup>ab</sup>	$1.45 \pm 0.04$ <sup>a</sup>	$0.78 \pm 0.01$ <sup>a</sup>

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Dry weight (g)				
(0)	(96)	$0.15 \pm 0.01$ <sup>a</sup>	$0.12 \pm 0.01$ <sup>b</sup>	$0.07 \pm 0.00$ <sup>ab</sup>
(96)	(96)	$0.12 \pm 0.01$ <sup>b</sup>	$0.08 \pm 0.00$ <sup>c</sup>	$0.05 \pm 0.00$ <sup>c</sup>
(0.384)	(96)	$0.14 \pm 0.00$ <sup>ab</sup>	$0.14 \pm 0.00$ <sup>a</sup>	$0.07 \pm 0.00$ <sup>b</sup>
(96)	(0)	$0.09 \pm 0.00$ <sup>c</sup>	$0.06 \pm 0.00$ <sup>c</sup>	$0.05 \pm 0.00$ <sup>c</sup>
(0.384)	(0)	$0.13 \pm 0.00$ <sup>ab</sup>	$0.14 \pm 0.00$ <sup>a</sup>	$0.08 \pm 0.00$ <sup>a</sup>

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Table 2. Sulfur, sulfate, and selenium accumulation in 10-plant tissue combination of *B. oleracea* grown with different ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the nutrient solutions.

Treatment		Leaf	Stem	Root
$\text{SeO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ ) : $\text{SO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ )				
Sulfur ( $\text{mg g}^{-1}$ dry weight)				
(0)	(96)	$5.77 \pm 0.05^b$	$4.04 \pm 0.03^b$	$4.37 \pm 0.04^b$
(96)	(96)	$4.11 \pm 0.02^d$	$2.38 \pm 0.13^d$	$4.09 \pm 0.02^d$
(0.384)	(96)	$6.22 \pm 0.21^a$	$4.69 \pm 0.06^a$	$5.38 \pm 0.04^a$
(96)	(0)	$5.47 \pm 0.03^b$	$2.78 \pm 0.06^c$	$4.11 \pm 0.02^d$
(0.384)	(0)	$4.85 \pm 0.04^c$	$2.24 \pm 0.05^d$	$4.66 \pm 0.04^b$

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Sulfate ( $\text{mg g}^{-1}$ dry weight)				
(0)	(96)	$2.86 \pm 0.07^b$	$1.97 \pm 0.06^b$	$1.79 \pm 0.09^b$
(96)	(96)	$1.39 \pm 0.01^d$	$1.01 \pm 0.01^c$	$0.89 \pm 0.05^c$
(0.384)	(96)	$4.21 \pm 0.09^a$	$3.74 \pm 0.06^a$	$2.74 \pm 0.08^a$
(96)	(0)	$0.44 \pm 0.01^e$	$0.41 \pm 0.01^d$	$0.24 \pm 0.01^d$
(0.384)	(0)	$1.89 \pm 0.07^c$	$1.24 \pm 0.17^c$	$0.78 \pm 0.02^c$

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Selenium ( $\mu\text{g g}^{-1}$ dry weight)				
(0)	(96)	nd	nd	nd
(96)	(96)	$4.90 \pm 1.52^c$	$9.18 \pm 3.24^c$	$2.44 \pm 0.31^c$
(0.384)	(96)	$10.26 \pm 3.30^c$	$21.49 \pm 2.41^b$	$4.67 \pm 0.34^c$
(96)	(0)	$26.39 \pm 2.31^b$	$11.72 \pm 3.93^c$	$25.79 \pm 1.08^a$
(0.384)	(0)	$40.42 \pm 2.10^a$	$33.56 \pm 1.57^a$	$18.49 \pm 1.09^b$

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Table 3. Glutathione peroxidase (GPx) activity in leaves of *B. oleracea* grown with different ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the nutrient solutions.

Treatment		Glutathione peroxidase (GPx) activity ( $\mu\text{mol}\cdot\text{min}^{-1}\text{L}^{-1}$ )
$\text{SeO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ ) : $\text{SO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ )		
(0)	(96)	$196.14 \pm 1.24^{\text{d}}$
(96)	(96)	$380.47 \pm 14.99^{\text{b}}$
(0.384)	(96)	$329.35 \pm 13.92^{\text{c}}$
(96)	(0)	$604.95 \pm 12.19^{\text{a}}$
(0.384)	(0)	$358.37 \pm 6.20^{\text{bc}}$

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

Table 4. Glucosinolates (GLs) concentrations in leaves of *B. oleracea* grown with different ratios of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the nutrient solutions.

Treatment		Glucosinolates concentration ( $\mu\text{mol g}^{-1}$ )		
$\text{SeO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ ) : $\text{SO}_4^{-2}$ ( $\text{mg}\cdot\text{L}^{-1}$ )		Aliphatic GLs	Indole GLs	Total GLs
(0)	(96)	$29.56 \pm 1.38^a$	$1.46 \pm 0.05^{ab}$	$31.01 \pm 1.33^a$
(96)	(96)	$19.05 \pm 1.34^b$	$0.89 \pm 0.16^{cd}$	$19.94 \pm 1.50^b$
(0.384)	(96)	$29.42 \pm 1.66^a$	$1.67 \pm 0.17^a$	$31.09 \pm 1.49^a$
(96)	(0)	$18.98 \pm 2.31^b$	$0.54 \pm 0.12^d$	$19.51 \pm 2.18^b$
(0.384)	(0)	$18.74 \pm 0.50^b$	$1.08 \pm 0.07^{bc}$	$19.82 \pm 0.57^b$

\* Significant at  $P < 0.05$ , Means within column with the same letter are not significantly different.

## **CHAPTER 5**

### **EXPRESSION LEVELS OF SULFATE TRANSPORTER GENES IN BRASSICA OLERACEA AS AFFECTED BY SODIUM SELENATE NUTRITION<sup>1</sup>**

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## ABSTRACT

Sulfate transporter genes are regulated in response to sulfur (S) status. To investigate the effects of selenate on sulfate transporter genes expression, *B. oleraceae* plants were grown under both S-deficient and –sufficient conditions at two levels of selenate concentration. Semi-quantitative reverse transcriptase - polymerase chain reaction (RT-PCR) method was used to quantify expression of the twelve sulfate transporter genes. The twelve sulfate transporters showed a complicated pattern of expression in both leaf and root tissues under the two selenate concentrations, but could be divided to roughly four groups. Under high selenate conditions, *BSultr1;2* and *BSultr2;1* were up-regulated in roots and leaves, respectively. The other sulfur transporters showed either down-regulation or no affect under these growth conditions. In contrast, under low selenate growth conditions, *BSultr1;2* and *BSultr4;1* were highly expressed in root tissues, while only *BSultr3;2* was found to increase activity in leaf tissues. The other sulfate transporters were either down-regulated or unaffected under these conditions. The variations of expression of the sulfate transporters in different parts of tissue may implicate to optimize sulfate requirement within plants.

## INTRODUCTION

Sulfate taken up by roots is the major source of sulfur (S) for plant growth (Marschner, 1995). Sulfate ( $\text{SO}_4^{-2}$ ) enters root cells and is transported to leaves where it can be stored in the vacuoles as a sulfate pool, or it can be reduced and assimilated into organic compounds, and secondary metabolites (Leustek, 1996; Leustek and Saito, 1999; Saito, 2000). The uptake of sulfate by roots and its long distance transport to aerial tissues during plant growth requires sulfate-specific transporters. Sulfate transporters are not only found in roots but also in leaves

(Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002, 2003; Maruyama-Nakashita et al., 2003; Buchner et al., 2004a, 2004b; Kataoka et al., 2004a, 2004b). The specific localization and activities of these transporters are considered to be responsible for sulfur availability and regulation within plants (Smith et al., 1995a, 1995b, 1997; Takahashi et al., 1997, 1999, 2000; Vidmar et al., 2000; Yoshimoto et al., 2002, 2003; Maruyama-Nakashita et al., 2003; Buchner et al., 2004a, 2004b; Kataoka et al., 2004a, 2004b).

Several sulfate transporter genes have been isolated and characterized with respect to uptake and transport mechanisms in various plant species e.g. *Stylosanthes hamata* (Smith et al., 1995b), *Arabidopsis* (Takahashi et al., 1996, 1997, 1999, 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2003; Maruyama-Nakashita et al., 2003; Buchner et al., 2004b; Kataoka et al., 2004a, 2004b), barley (Smith et al., 1997), corn (Bolchi et al., 1999), Indian mustard (Heiss et al., 1999), tomato (Howarth et al., 2003), and *Brassica oleracea* (Hawkesford et al., 2003; Buchner et al., 2004a). Twelve sulfate transporter genes involved in sulfate uptake and transport have been cloned and identified as a gene family in *Arabidopsis* (The Arabidopsis Genome Initiative, 2000). These sulfate transporter genes are divided into four groups according to their phylogenetic relationships and patterns of tissue-specific localization (Grossman and Takahashi, 2001). Each group contains multiple sulfate transporters and their functions and localizations have been described (Takahashi et al., 1997, 1999a, 1999b, 2000; Vidmar et al., 2000; Yoshimoto et al., 2002, 2003; Shibagaki et al., 2002; Maruyama-Nakashita et al., 2003; Kataoka et al., 2004a, 2004b). Sulfate transporters in Group 1 have high affinity for sulfate and are mainly expressed in root tissues (Takahashi et al., 2000). Group 1 transporter gene expression is typically when sulfur is limiting (Hawkesford and Wray, 2000; Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002; Buchner et al., 2004a). Therefore, this

group is thought to be responsible for the initial sulfate uptake into roots (Takahashi et al., 2000; Maruyama-Nakashita et al., 2003). Yoshimoto et al., (2003) reported that one specific isoform in *Arabidopsis* Group 1, *AtSultr1;3*, localized to the phloem where it might control redistribution of sulfur between source and sink tissues.

Group 2 sulfate transporters have a lower affinity for sulfate and are expressed in vascular tissues of roots and shoots. This expression pattern suggests involvement of translocation of sulfate between roots and leaves (Takahashi et al., 1997, 2000).

Although sulfate transporters in Group 3 are expressed preferentially in *Arabidopsis* leaves (Takahashi et al., 1999b; Hawkesford, 2003), *AtSultr3;5* was found to be expressed in *Arabidopsis* root tissues (Kataoka et al., 2004b). Similarly, Group 3 sulfate transporters were also expressed in *B. oleracea* root tissues (Buchner et al., 2004a). There is some controversy about whether this group has redundant function or is just highly specialized because expression of Group 3 transporters is not affected by sulfur availability (Takahashi et al., 1999b; Hawkesford, 2003; Buchner et al., 2004a).

Sulfate transporters in Group 4 have been found to localize in the tonoplast of cells in roots and shoots using green fluorescent protein (GFP) protein fusion constructs (Kataoka et al., 2004b). In *Arabidopsis* root tissues, *AtSultr4;1* was not affected by changes in sulfate levels, but *AtSultr4;2* accumulated under sulfate limitation (Kataoka et al., 2004b). However, both *BSultr4;1* and *BSultr4;2* showed high expressions in *B. oleracea* roots in response to sulfate starvation (Buchner et al., 2004a). Moreover, the expression of these two transporters increased in shoots when sulfate was limited (Kataoka et al., 2004b).

Competition between Se and S has been seen both in plant uptake (White et al., 2004) and in incorporation into proteins (Ng and Anderson, 1979; Eustice et al., 1981; de Souza et al.,

2002) when plants were grown in media containing various proportions of selenate and sulfate. Selenate ( $\text{SeO}_4^{-2}$ ), as analogue of sulfate, can be assimilated by similar metabolic pathways, and it has been proposed that selenate utilizes the same active absorption processes as sulfate at the root plasma membrane (Abrams et al., 1990; Arvy, 1993; Anderson, 1993), including the high-affinity Group 1 sulfate transporters (Terry et al., 2000). Previous studies have shown that Se and S ratios in the growth medium are not reflected by the ratios in plant tissues (Bell et al., 1992; Ellis and Salt, 2003). Consequently, it has been suggested that the plant transporters for uptake and translocation may selectively discriminate between selenate and sulfate (White et al., 2004). In addition, S uptake and accumulation was actually enhanced when  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  were supplied at ratios between 1: 125 to 1: 500 (Kopsell and Randle, 1997). Thus, the amount and ratio of  $\text{SeO}_4^{-2}$  and  $\text{SO}_4^{-2}$  in the external supply can affect S and Se uptake and accumulation, as well as S partitioning within the plant.

Because sulfate uptake, transport, and regulation is dependent on S availability, we examined the effect of different concentrations of sodium selenate under S-sufficient and – deficient growth conditions for *Brassica oleracea*. In this study, sulfate transporters gene expression was assessed using a semi-quantitative RT-PCR method. Gene expression was differently by tissue and S status.

## **MATERIALS AND METHODS**

### **Plant Material**

Seeds of a rapid cycling *Brassica oleracea* population (RCBP) (Crucifer Genetics Cooperative, Department of Plant Pathology, University of Wisconsin, Madison, WI) were germinated in growing cubes (one seedling/ cube) (Dk-2640. Hedehusene, Denmark) covered

with vermiculite and watered daily starting on 19 June 2006. Seeds were set at temperatures of 24 °C and 24-h photoperiod in a growth chamber (Model E15; Conviron, Asheville, NC). One hundred milliliter of a 200 mg·L<sup>-1</sup> Peter's 20N-20P-20K (Scotts-Sierra Co., Marysville, OH) was applied 5 days after germination. The first true leaves were seen on 1 July 2006 and plants were transferred to a 270 ml crystallizing dish (VWR 89000-288) containing a modified 50% Hoagland's solution (Hoagland and Arnon, 1950) for one week. Seedlings were subsequently rinsed with deionized water and ten-plant as a group was transferred to a dish containing fresh nutrient solution with or/ without magnesium sulfate (MgSO<sub>4</sub>·7 H<sub>2</sub>O) as the source of nutritional sulfur. The dishes were moved to a chamber for further growth at 24°C and a 16-h photoperiod.

At harvest, each 10-plant group was separated into leaves and roots, and plant tissues were frozen in liquid N<sub>2</sub> prior to storage at -80°C for future analysis.

### **Experimental Design**

The experimental design was a randomized complete design with three replications and ten plants per replication. For control and S-sufficient plants, the medium SO<sub>4</sub><sup>-2</sup> concentration was 96 mg/L, while SO<sub>4</sub><sup>-2</sup> was omitted from medium for S-deficient plants. For high- and low-selenate treatments, 96 mg/L and 0.384 mg/L SeO<sub>4</sub><sup>-2</sup> were added, respectively, to S-sufficient or S-deficient plants. The resultant S: Se ratios for these combinations were 1:1 and 1:250.

### **RNA Extraction and Purification**

Total RNA was extracted from 100 mg aliquots of root or leaf tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). RNA content of each sample was quantified by spectrophotometry at 260 nm. All RNA samples had OD<sub>260</sub>:OD<sub>280</sub> above 1.8 indicating the RNA

isolates to be relatively free of contaminating proteins or phenolic compounds. All RNA samples were treated with DNase I (RNase-free) (Promega Corporation, Madison, WI) to remove residual contaminating genomic DNA. The quality of DNase-treated RNA was verified by gel electrophoresis in 1.0% agarose containing 0.05  $\mu$ l/ml ethidium bromide in 1 X Tris-Acetate-EDTA (TAE) buffer (0.04M Tris base, 0.02 M Acetic acid, and 0.01 M EDTA adjust pH to 8.0).

### **cDNA Synthesis**

First strand cDNA synthesis was carried out using the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen). DNase-treated RNA (0.5  $\mu$ g) from root or leaf tissue was mixed with 1  $\mu$ l oligo-dTVN (0.5  $\mu$ g/ $\mu$ l) primer (Integrated DNA Technologies (IDT) Inc., Coralville, IA) in DEPC-treated H<sub>2</sub>O, and heated at 65°C for 10 min. The mixture was snap-cooled on ice prior to addition of 1  $\mu$ l 10 mM dNTPmix, 2  $\mu$ l 10X RT buffer(100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM MgCl<sub>2</sub>), 2  $\mu$ l 25 mM MgCl<sub>2</sub>, and 2  $\mu$ l 0.1 M DTT (Invitrogen). The resultant mix was incubated at 42°C for 3 min. One  $\mu$ l SuperScript Reverse Transcriptase (50 units/ $\mu$ l) (Invitrogen) was added, and the mixture was first incubated at 42°C for 50 min and then at 70°C for 15 min. The RT reaction was stopped by adding 1  $\mu$ l RNase H (2 units/ $\mu$ l) (Invitrogen) and heated at 37°C for 30 min. The resultant cDNA stock was diluted 10-fold and stored at -20°C.

### **Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**

#### *Primer selection*

Buchner et al. (2004a) deposited partial sequence data for 12 different sulfate transporter genes from *B. oleracea* in GenBank (National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov/>). Partial cDNAs corresponding to the 12 different sulfate transporter genes and the actin as internal control gene were synthesized using first strand cDNAs and specific sense and/-antisense primer sets. Primer combinations specific for each sulfate transporter gene and actin were identified using on-line software provided by IDT (<http://www.idtdna.com>) to analyze the sulfate transporter gene sequences from *B. oleracea* and *B. napus* in GenBank (Table I).

Sequence data for the 12 different *B. oleracea* and *B. napus* sulfate transporter genes and actin were deposited in GenBank under the accession numbers AJ 416460, AJ 311388, AJ 633707, AJ 633705, AJ 223495, AJ 581745, AJ 601439, AJ 704373, AJ 704374, AJ 633706, AJ 416461, AJ 555124, and AF 044573.

#### *PCR manipulation*

Semi-quantitative RT-PCR was used to measure gene expression (McCaig et al, 2005). To insure that no false positive PCR signals were generated by primer dimer formation, all primer pairs were checked using water as a negative control. Polymerase chain reactions were carried out using the same model thermocycler (Gene Amp PCR System 9700; Perkin Elmer, Norwalk, CT.) and consistent reaction conditions with 3  $\mu$ l (150 ng) of cDNA, 1  $\mu$ l each of the sense and/-antisense primers (10  $\mu$  M), and 5  $\mu$ l 2X PCR Master Mix buffer (Promega Corporation, Madison, WI) in a total reaction volume of 10  $\mu$ l. The cycling conditions were program as follows: denaturation at 94°C for 3 min, followed by 22 to 34 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2.5min. PCR products were subsequently allowed to stabilize at 72 °C for 10 min, before storage at 4 °C. Multiple amplifications for each gene were

tested to identify the appropriate number of cycles to yield a linear response when the RT-PCR products were quantified on agarose gels. For purposes of quantification, it was important that amplification products fell on the linear portion of the exponential curve and not on the reaction plateau. Ideally, the optimal number of amplification cycles should be the same for genes of interest and the internal controls so that the samples may be directly compared and quantified on the same gel.

### **Gel Electrophoresis**

The PCR products were loaded on 1% agarose gels containing 0.05  $\mu$  l/ml ethidium bromide in 1 X Tris-Acetate-EDTA (TAE) buffer (0.04M Tris base, 0.02 M Acetic acid, and 0.01 M EDTA adjust pH to 8.0). A 200 bp DNA fragment size ladder (Promega) was included on every gel to confirm expected size of each gene amplimer.

### **Gel Images and Quantitative Analysis**

Images of agarose gel stained with ethidium bromide were collected using a MultiImage Light Cabinet with a CCD camera (AlphaImager; Alpha Innotech, San Leandro, CA). DNA bands were quantified using AlphaImager Spot Densitometry software (Version 4.1.0.2). Band intensity was represented as absorbance area. The relative quantitative values were calculated as the ratio between target genes and the actin internal control gene, which was used to normalize variations in sample concentration and loading efficiency.

### **Statistical Analysis**

RT-PCR signals were quantified and averaged for two replications of each sample. The relative RT-PCR ratio values were generated using the GLM procedure in SAS statistical software (version 9.1.3, SAS, Cary, NC).

## **RESULTS AND DISCUSSION**

### **Determination of the Exponential Range of Amplification for Sulfate Transporters**

Semi-quantitative RT-PCR may be used to obtain a rough determination of gene expression levels, but the exponential nature of the amplification reaction requires care that reactions be terminated in the linear portion of the reaction so that product levels do not plateau. To get uniform results, the internal control and target genes must be quantified in the same gel and reactions must be performed with the same number of amplification cycles. For this study, the (22-34 cycles) delimited the linear range of the amplification curve for the various sulfate transporter genes as well as the internal actin control. The intensity of sulfate transporter PCR products continued to increase at 34 cycles, but internal actin control reached a plateau at 31 cycles (Fig. 1). This appeared to be consistent with the fact that actin gene expression is typically high in most tissues. From this study, the optimal number of PCR cycles for subsequent analyses was chosen as 29.

### **Expression of Sulfate Transporters under High Selenium Growth Condition**

Not all sulfate transporters were sensitive to high concentration of selenate in *B. oleracea* roots. For the Group 1 transporters, while *BSultr1;2* expression was detectable under high selenate conditions, no signal was detected for either *BSultr1;1* or *BSultr1;3* (Fig. 2). This contrasts with the report of Yoshimoto et al. (2002) who noted that *AtSultr1;1* transcripts, but not

*AtSultr1;2* was affected by selenate treatment. The increasing expression of *BSultr1;2* suggested that this high-affinity transporter was induced because high concentrations of Se resulted in S deprivation. Similar results were reported for *Arabidopsis* (Takahashi et al., 2000) and *B. oleracea* (Buchner et al., 2004a). Because *BSultr1;2* was the only Group 1 transporter up-regulated in this study, it may be the gene with primary responsible for high-affinity sulfate uptake in *B. oleracea* roots in the presence of Se. The *Arabidopsis Sultr1;3* has been shown to mediate the transport of sulfate from source to sink tissue in plants under sulfate stress (Yoshimoto et al., 2003). While the expression of *BSultr1;3* was reported to be low in *B. oleracea* roots grown under conditions of S deficiency (Buchner et al., 2004a), in our study, *BSultr1;3* expression could not be detected, even when high concentrations of selenate were present in nutrient solutions.

With respect to Group 2 transporters, the expression levels of both *BSultr2;1* and *BSultr2;2* in roots were both decreased by high concentrations of  $\text{Na}_2\text{SeO}_4$  (Fig. 3). In contrast, the expressions levels of the *AtSultr2;1* and *AtSultr2;2* genes in *Arabidopsis* root tissues increased when 0.1mM selenate was added to nutrient medium containing 1.7 mM sulfate nutrition (Takahashi et al., 2000). Since the Group 2 sulfate transporters are considered to have relatively low-affinity for sulfate (Takahashi et al, 2000), it seems possible that high concentrations of selenate used in our study caused sulfate starvation, which resulted in depression of Group 2 transporters.

The expression pattern described for the two Group 2 sulfate transporters in *Arabidopsis* suggested that they were involved in vascular transport of sulfate in plants under sulfur stress (Takahashi et al., 2000). When *B. oleracea* growth medium was supplemented with high concentrations of selenate, the sulfate concentration in roots and leaves decreased threefold (data

not shown). In contrast to the what was seen in *Arabidopsis*, *BSultr2;1* and *BSultr2;2* expression was reduced under these conditions.

There are five members of sulfate transporter genes in Group 3, of which *BSultr3;3* showed the strongest expression, *BSultr3;1*, *BSultr3;2*, and *BSultr3;4* showed significantly reduced expression in *B. oleracea* root tissues exposed to high levels of selenate (Fig. 4). Expression of Group 3 sulfate transporters *AtSultr3;1*, *AtSultr3;2*, and *AtSultr3;3* were reported to be expressed only in leaves of *Arabidopsis* (Takahashi et al., 1999b; Hawkesford, 2000). While only *AtSultr3;5* was reportedly expressed in *Arabidopsis* root tissues (Kataoka et al., 2004a), all of entire Group 3 sulfate transporters examined in *B. oleracea* were expressed in roots (Buchner et al., 2004a). It was previously suggested that the Group 3 genes in *Arabidopsis* had diverged to perform physiological functions other than sulfate transport because their expression was not affected by S status (Kataoka et al., 2004a; Buchner et al., 2004a). However, Kataoka et al (2004a) showed that *AtSultr3;5* expression in *Arabidopsis* roots was stimulated when *AtSultr2;1* was expressed suggesting that *AtSultr3;5* might actually be responding to sulfate transported by *AtSultr2;1*. In our study, decreased of expression was seen for both *BSultr3;1* and *BSultr3;4* in selenate exposed roots. As was seen in *Arabidopsis*, perhaps this down-regulation was associated with the decreased expression of Group 2 transporters in roots. However, further study will be required to test this possibility.

The expression of Group 4 sulfate transporters *BSultr4;1* and *BSultr4;2* was decreased in roots under high Se treatment (Fig. 5). Group 4 transporters are supposed to be localized to the tonoplast where they control release of sulfate from vacuolar storage (Kataoka et al., 2004b). Elevated expression of *BSultr4;1* and *BSultr4;2* was seen in *B. oleracea* roots grown under S deficient conditions (Buchner et al., 2004a). Decreased of sulfate concentration in *Brassica* roots

was previously associated with up-regulation of *BSultr4;1* in plants grown under sulfate limitation (Hawkesford et al., 2003). However, we found a decreased expression in both *BSultr4;1* and *BSultr4;2*, as well as in reduced sulfate content in *B. oleracea* roots grown under S-deficient conditions (data not shown). At high selenate concentrations, *BSultr4;1* transporter expression was affected more than was expression of *BSultr4;2* (Fig. 5).

Since leaves are the main tissue for sulfate assimilation, it is also necessary to investigate how selenium affects these transporters in leaves. When *B. oleracea* were grown with high levels of selenate present in the growth medium, only *BSultr1;2*, could be detected in leaves. This is consistent with the observed expression of *AtSultr1;2* detected in *Arabidopsis* leaves (Shibagaki et al., 2002; Yoshimoto et al., 2002). A previous study showed that the Group 1 transporters were expressed in *B. oleracea* leaf tissues under S limitation (Buchner et al., 2004a); however, we were unable to detect expression of either *BSultr1;1* or *BSultr1;3*, under control or high selenate growth condition (Fig. 6). Although expression of *BSultr1;2* was detected in leaves under high selenate conditions, expression levels were not significantly different from those in controls ( $P \geq 0.14$ ) (Fig. 6). It is possible that *B. oleracea* the *BSultr1;1* and *BSultr1;3* transporters are expressed mainly in root tissues, and do not function in sulfate mobilization in leaf tissues. Because the *BSultr1;2* transporter was not affected by sulfate status in leaves, it is possible that this transporter plays some independent of sulfate condition in leaves.

The expression of *BSultr2;1* was up-regulated in leaf tissues of plants grown in the presence of  $96 \text{ mg L}^{-1} \text{ SeO}_4^{2-}$  (Fig. 7). However, there was no statistically significant difference in expression of *BSultr2;2* between high selenate and control conditions ( $P \geq 0.11$ ).

Group 3 sulfate transporters were described as having leaf-specific expression in *Arabidopsis* (Takahashi et al., 1999b; Hawkesford, 2000), and that this expression was not

affected by S status (Buchner et al., 2004a). We found *BSultr3;3* to have the highest expression in leaf tissues; *BSultr3;1* and *BSultr3;2* expression in *B. oleracea* leaf tissues were most affected by growth in the presence high selenate levels. *BSultr3;1* expression decreased and *BSultr3;2* expression was not detected under these conditions (Fig. 8). The other transporter genes in Group 3 showed no response to high levels of  $\text{Na}_2\text{SeO}_4$ .

The expression of *BSultr4;1* and *BSultr4;2* was detected in *B. oleracea* leaf tissues. High selenate treatment decreased *BSultr4;1* expression, but did not influence expression of *BSultr4;2* (Fig. 9). It is possible that *BSultr4;1* is more affected than *BSultr4;2* in leaf tissues.

### **Expression of Sulfate Transporters under Low Selenium Growth Condition**

When *B. oleracea* were grown in the presence of low concentrations of selenate ( $0.384 \text{ mg L}^{-1} \text{ SeO}_4^{-2}$ ), the amount of sulfate accumulated in root tissues was unaffected (data not shown). Both *BSultr1;1* and *BSultr1;2* were expressed but no detectable signal was found in *BSultr1;3* (Fig. 2). At it was under high selenate conditions, expression of the *BSultr1;2* transporter was up-regulated under low selenate growth conditions. However, no statistically significant difference in expression was found for *BSultr1;1* under low selenate and control conditions. The results suggested that *BSultr1;2* is the primary transporter for initial uptake of sulfate regardless of external S status, and that *BSultr1;1* may provide backup for absorption of sulfate when plants are grown in a low Se environment.

Expression of both *BSultr2;1* and *BSultr2;2* in roots was affected by low concentrations of selenate. The decreased expression was statistically significant for *BSultr2;1* but not *BSultr2;2* (Fig. 3). Overall, expression of the Group 2 sulfate transporters in root tissues was down-regulated under both high and low concentrations selenate growth conditions.

Expression of the Group 3 sulfate transporters in root tissues was detected under low levels of selenate addition. However, there was no significant difference in expression between low Se and control conditions (Fig. 4), similar to a previous report that expression of these genes was not affected by S status (Buchner et al., 2004a).

*BSultr4;1* expression was up-regulated and *BSultr4;2* expression was down-regulated in *B. oleracea* roots grown under selenate conditions (Fig. 5). A similar result was found in *B. oleracea* roots grown under sulfur-deficient conditions (Buchner et al., 2004a). The *Arabidopsis AtSultr4;1* transporter is responsible for rapid release of sulfate from root vacuoles and *AtSultr4;2* has been suggested to reinforced the efflux of sulfate from root vacuoles (Kataoka et al., 2004b). When the supply of sulfate is sufficient, the vacuolar sulfate transporters are up-regulated to release extra sulfate from this storage location (Kataoka et al., 2004b). The released sulfate is subsequently transported to aerial portions on the plant where it can be assimilated into S-containing compounds and contribute to growth. When grown in the nutrient solutions having  $\text{SeO}_4^{-2} : \text{SO}_4^{-2}$  ratios below 1:125, even a slight amount of Se enhanced uptake and accumulation of sulfate in onions (Kopsell and Randle, 1997). In this study, sulfate was similarly found to accumulate in root tissues when *B. oleracea* plants grown in nutrient solutions where the  $\text{SeO}_4^{-2} : \text{SO}_4^{-2}$  ratios were low (1:250, data not shown). The upregulation of *BSultr4;1* transporter supported that more sulfate was accumulated and had to be released from root vacuoles when plants grown in low selenate conditions.

Substantial levels of sulfate were found in *B. oleracea* leaf tissues from plants grown in the presence of low levels of selenate suggesting that sulfate assimilation and transport was not greatly affected by these conditions (data not shown). Group 1 sulfate transporter genes showed the same pattern of expression under low selenate conditions as they did under high selenate

conditions. Only *BSultr1;2*, expression was detected in leaf tissues (Fig. 6), and expression levels were not affected by selenate.

*BSultr2;1* and *BSultr2;2* gene expression was detected in leaves; however, there was no statistically significant difference ( $P \geq 0.1$ ) between expression levels in tissues from low Se treatments and controls (Fig. 7). Group 2 sulfate transporters are generally thought to be involved in vascular transport of sulfate (Takahashi et al., 2000).

Even *BSultr3;3* is by far the most strongly expressed gene, *BSultr3;1* and *BSultr3;2* expression in leaf tissues was affected by low levels of selenate. Decreased expression was detected for *BSultr3;1* while slightly higher expression was found for *BSultr3;2* (Fig. 8). Although expression could be detected for other sulfate transporters in Group 3, none were significantly changed from controls ( $P \geq 0.16$ ) and low selenate conditions. Comparing leaf expression of Group 3 transporters between low and high Se treatments, both *BSultr3;1* and *BSultr3;2* were affected by S status; but the others were not (Fig. 8). A strong expression of *BSultr3;3* transporter and weak expression of *BSultr3;1* and *BSultr3;2* may suggest that only these three transporters are responsible for sulfate transport in leaf tissues, and others may be a support of redundant hypothesis (Kataoka et al., 2004a; Buchner et al., 2004a).

Expression of *BSultr4;1* and *BSultr4;2* was seen in *B. oleracea* leaf tissues when plants were grown under low Se conditions. The pattern of expression for *BSultr4;1* and *BSultr4;2* in leaves did not vary between low Se and high Se treatments. Decreased expression of *BSultr4;1* but not *BSultr4;2* was seen in response to low concentrations of Se (Fig. 9).

## COUCLUSION

This study showed that multiple and complex patterns of expression of sulfate transporters were induced when *B. oleracea* seedlings were grown in S-sufficient or –deficient environments with varying levels of selenate. The twelve *B. oleracea* sulfate transporters showed tissue-specific expression which is consistent with a role in regulating sulfate uptake and transport within plants. *BSultr1;2* appeared to be the primary Group 1 transporter in root tissues where it may be responsible for initial uptake of sulfate. The Group 2 transporter, *BSultr2;1* demonstrated an expression pattern suggesting it to play a role in transport of sulfate between roots and shoots under conditions of severe sulfate limitation. The function of Group 3 sulfate transporters remains unclear, although the expression patterns for *BSultr3;1*, *BSultr3;2*, and *BSultr3;3* suggest that they may carry out their functions in root and leaf tissues, respectively. *BSultr4;1* is the main Group 4 transporter affected by S status, and may be responsible for vacuolar efflux when plants are grown with adequate sulfur nutrition. However, substantial additional research will be necessary to more thoroughly investigate the relationship between sulfate uptake and transport, and the expression of *B. oleracae* sulfate transporters in response to signals conducted between the roots to shoots.

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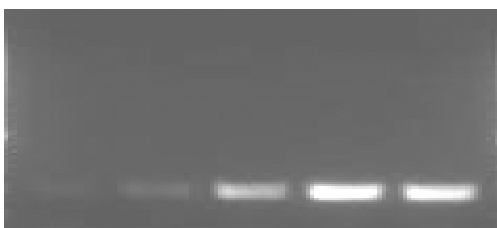
Table 1. General primers used to amplify *B. oleracea* sulfate transporters and actin in RT-PCR assays..

Gene Name	Accession Number	Primer Sequence
BSultr1;1	AJ 416460	Forward 5'-TCACGGGTGGAATTGGCAGACTAT-3' Reverse 5'-CGGCATGTCTGCTGCTTTAGCTTT-3'
BSultr1;2	AJ 311388	Forward 5'-TGGTGCATCCTTCTTGACCTTCCT-3' Reverse 5'-CAACGGAAGCAAAGATGACGCCAA-3'
BSultr1;3	AJ 633707	Forward 5'-AGCAGCCATAACCATAGCCCTTCA-3' Reverse 5'-ATGATCGCAGCAAGAATCGCGTTG-3'
BSultr2;1	AJ 633705	Forward 5'-AAGAACGACCTCATGGCTGGCTTA-3' Reverse 5'-AAACCCTTAAGCCGTTGGAGTCCT-3'
BSultr2;2	AJ 223495	Forward 5'-ACGGAAGCAATTGCAGTAGGGAGA-3' Reverse 5'-CTTCCAAATGTGCAAAGCGCCAGA-3'
BSultr3;1	AJ 581745	Forward 5'-TTCTCGAAGCCTCTCTCGGCATTT-3' Reverse 5'-TTCCCACAGCTATCCCTTCTGCAA-3'
BSultr3;2	AJ 601439	Forward 5'-TCTTGGAATGGGCTCGTGGTTACA-3' Reverse 5'-TGCTCCAGCCATGAACCCTATGAT-3'
BSultr3;3	AJ 704373	Forward 5'-ACTTCTTGCTACGTCACAACCGGA-3' Reverse 5'-TTGAACAACGATAGTCCCACCGCT-3'
BSultr3;4	AJ 704374	Forward 5'-TGGGAAACTATCGTGATGGGCCTT-3' Reverse 5'-AGCCAAGAGGCACAGAGACAAAGA-3'
BSultr3;5	AJ 633706	Forward 5'-TGGTCAAAGGGACAGAACATGGGA-3' Reverse 5'-AGCACAAGCATCATGCAAAGACCC-3'
BSultr4;1	AJ 416461	Forward 5'-GTGCTTCGGCCATTGTCATTGGAT-3' Reverse 5'-TTTCTGTACTGTGGTGCCTGGA-3'
BSultr4;2	AJ 555124	Forward 5'-TTTGTGCCGGTGATTGTGTATGCC-3' Reverse 5'-AATCTCTTGTCCACACGCCACA-3'
Actin	AF 044573	Forward 5'-AGAGGTCTTGTTCCAGCCATCGTT-3' Reverse 5'- TCTTCATGCTGCTTGGTGCAAGTG-3'

Figure 1. PCR cycle optimization for actin and sulfate transporter transcript assays. Gel images of actin bands (A), and *BSultr1;2* bands (B) after 22-34 PCR cycles.

Number of cycles

(A) 22 25 28 31 34



(B)

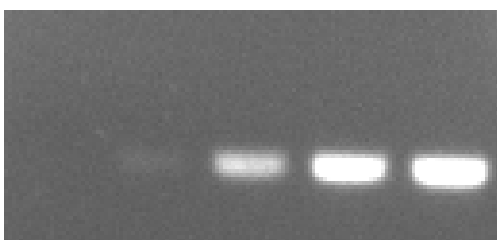


Figure 2. The expression Group 1 sulfate transporters in *B. oleracea* roots grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

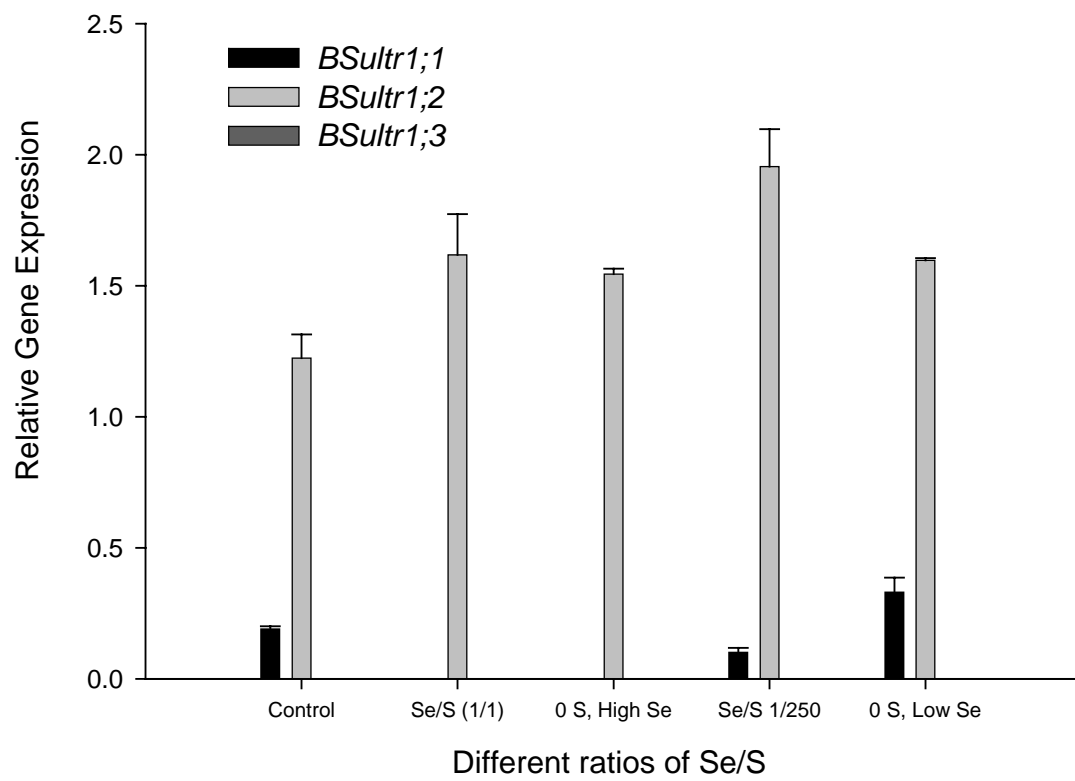


Figure 3. The expression Group 2 sulfate transporters in *B. oleracea* roots grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

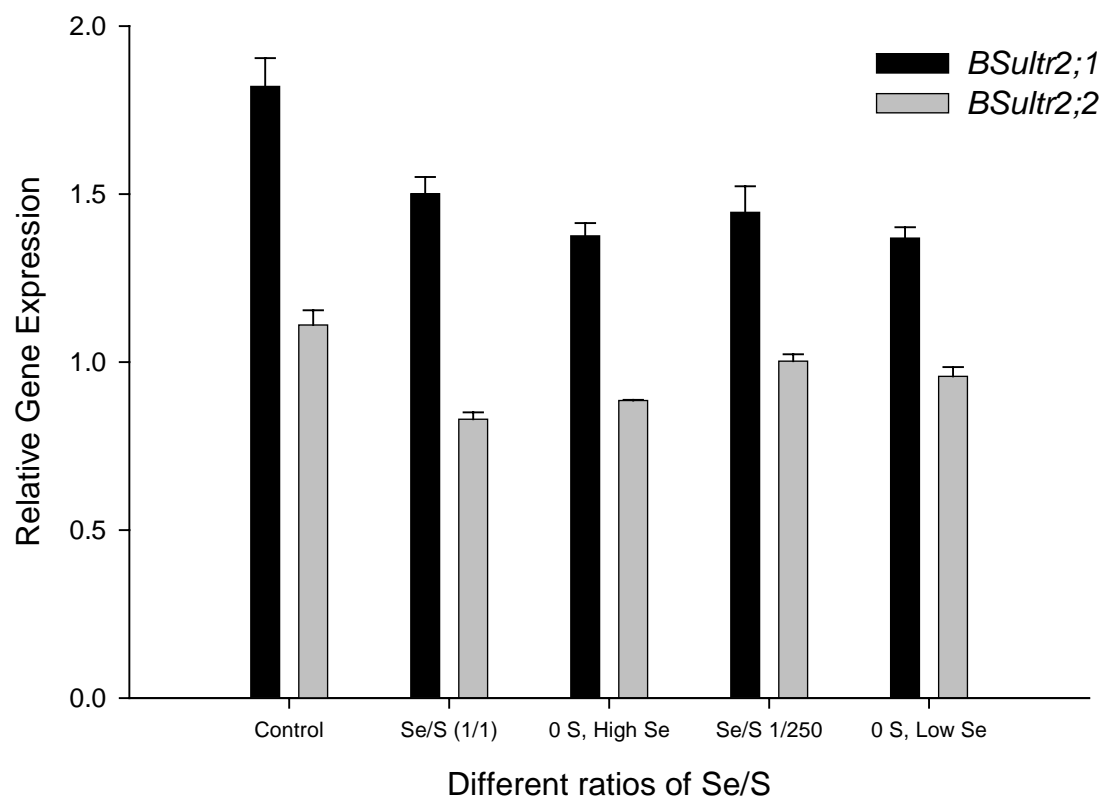


Figure 4. The expression Group 3 sulfate transporters in *B. oleracea* roots grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

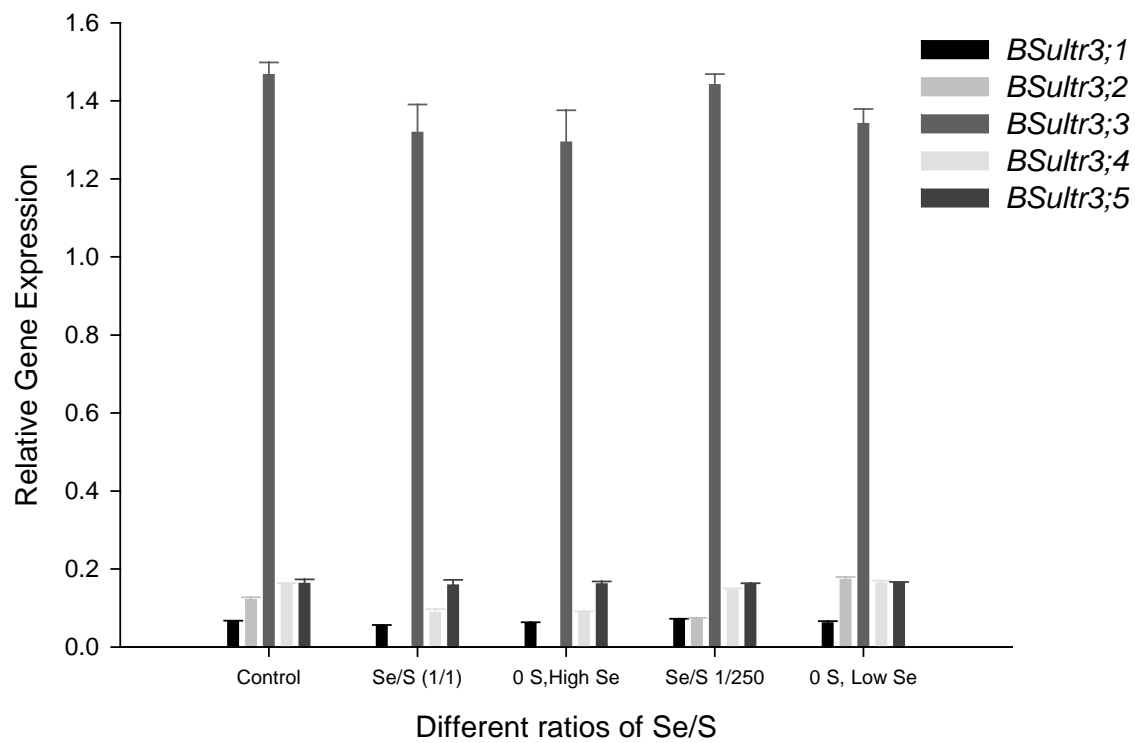


Figure 5. The expression Group 4 sulfate transporters in *B. oleracea* roots grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

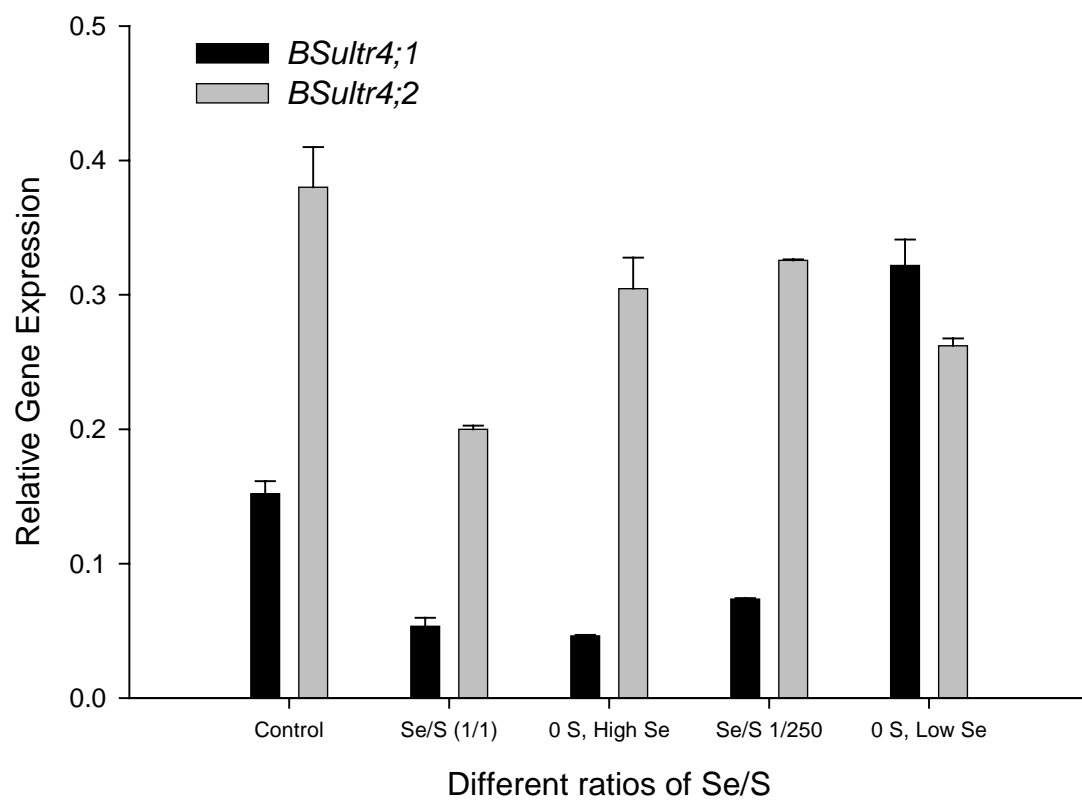


Figure 6. The expression Group 1 sulfate transporters in *B. oleracea* leaves grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

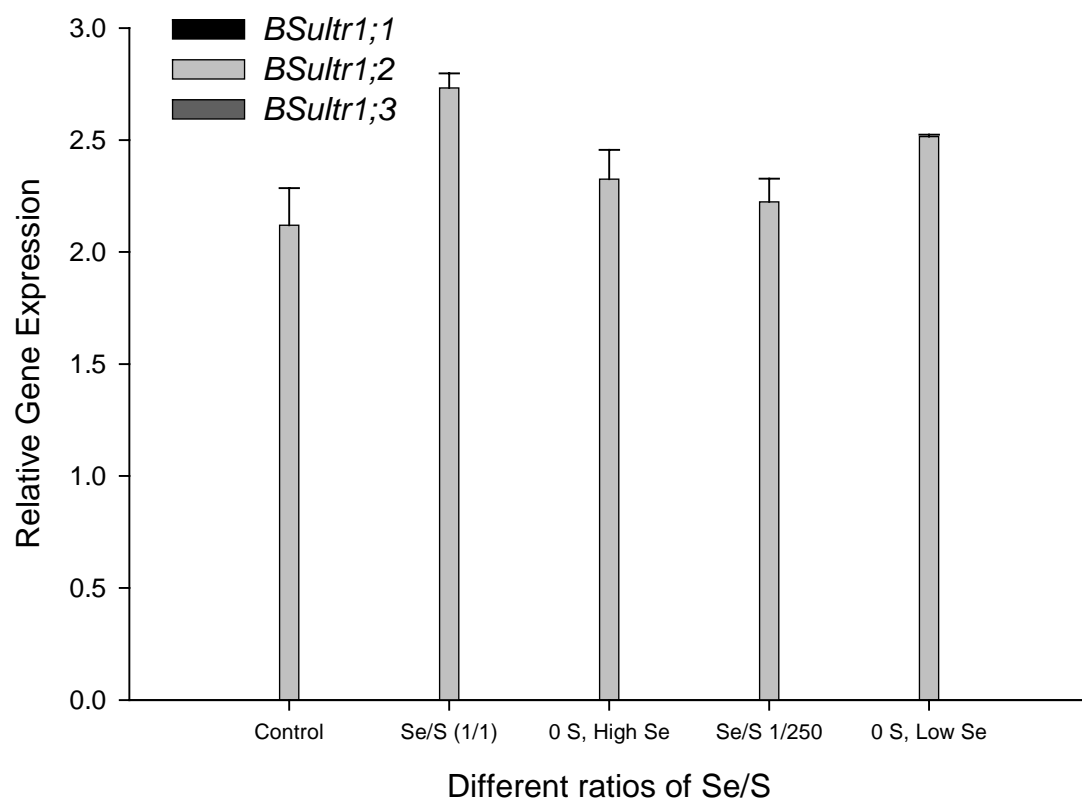


Figure 7. The expression Group 2 sulfate transporters in *B. oleracea* leaves grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

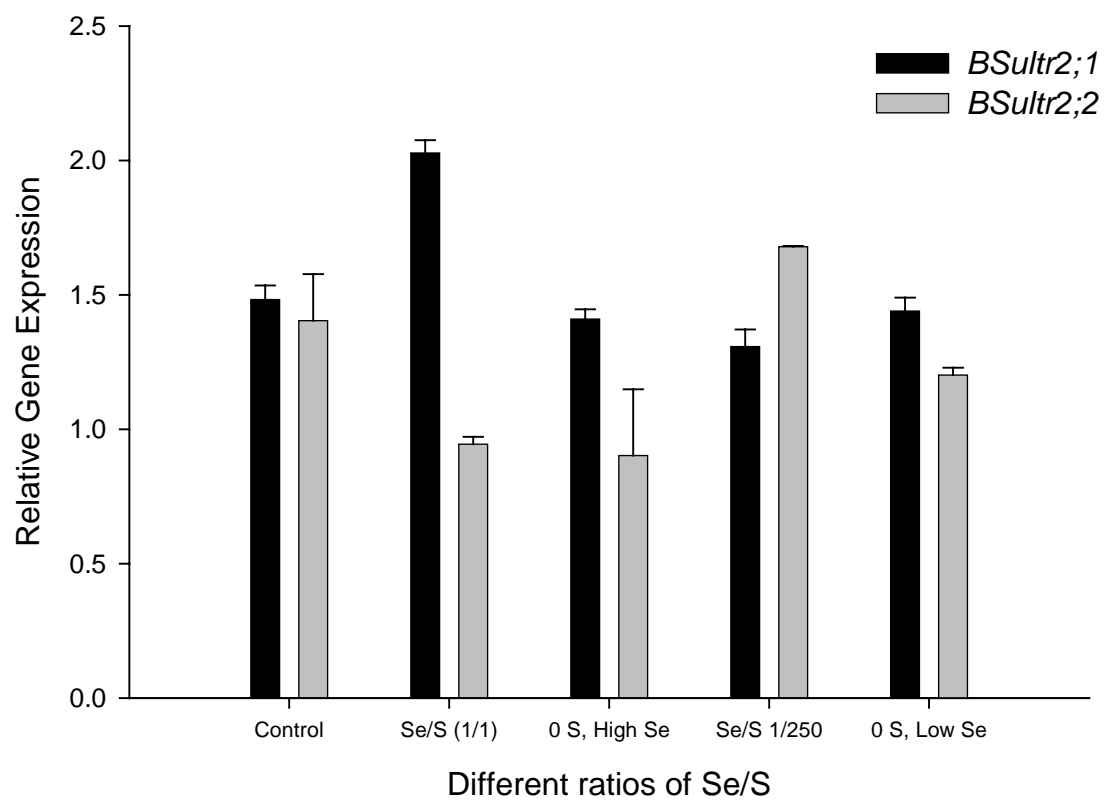


Figure 8. The expression Group 3 sulfate transporters in *B. oleracea* leaves grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).

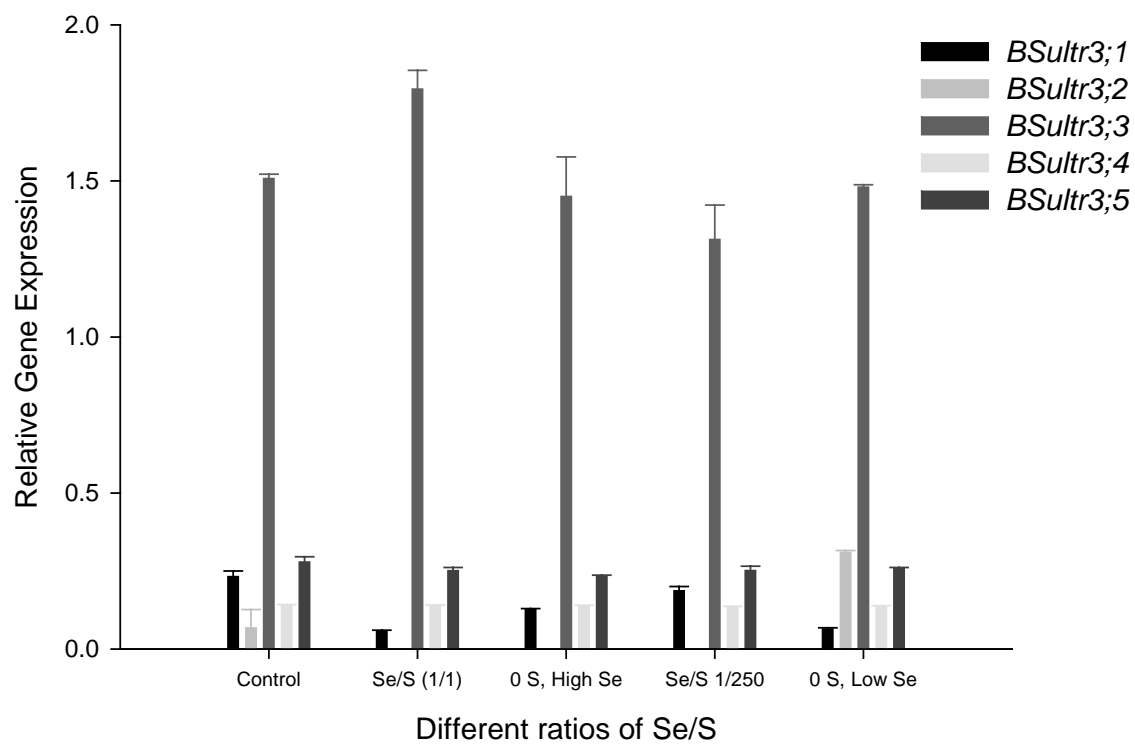
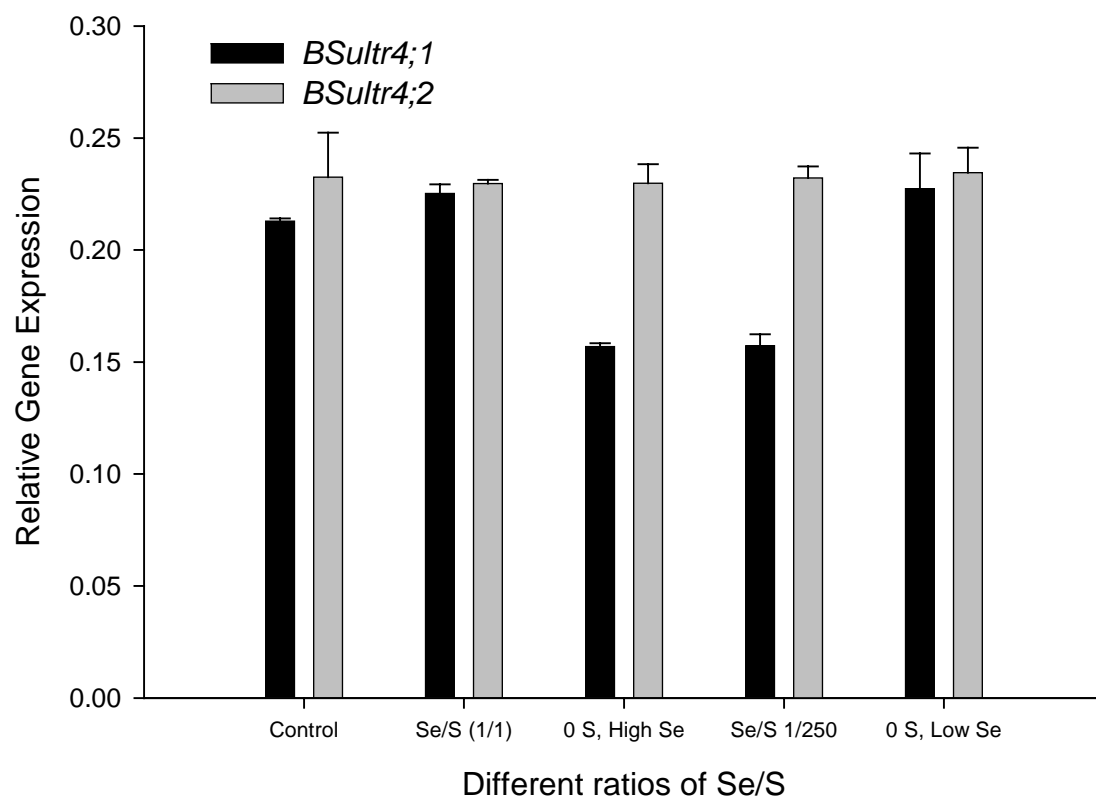


Figure 9. The expression Group 4 sulfate transporters in *B. oleracea* leaves grown in the presence of various levels of  $\text{SO}_4^{-2}$  and  $\text{SeO}_4^{-2}$ . *B. oleracea* seedling controls were grown in 50% Hoagland solution.  $\text{Na}_2\text{SeO}_4$  was added to 50% Hoagland solution to final concentrations of  $126.89 \text{ mg}\cdot\text{L}^{-1}$  and  $0.507 \text{ mg}\cdot\text{L}^{-1}$  to yield  $\text{SeO}_4^{-2}/\text{SO}_4^{-2}$  of 1/1 and 1/250, respectively. The same concentrations of  $\text{Na}_2\text{SeO}_4$  were added to 50% Hoagland solution lacking  $\text{SO}_4^{-2}$  to create the 0 S, High Se and 0 S, Low Se nutrient solutions. Histogram bars represent the means of two measurements of transcripts recovered from pooled tissue of 10 plants for each treatment ( $\pm$  SD).



## CHAPTER 6

### CONCLUSIONS

Chapter 3 determined the effect of temperature on selenium and sulfur accumulation. We were able to find out that accumulation of Se and S in *B. oleracea* is temperature-dependent and tissue-specific via mineral analysis. Se and S accumulation in plants increased with increasing temperature. The highest concentration of Se and S were found in leaf tissues. Changes in both Se and S levels can be expected when brassica are grown in different temperature regions.

*Brassicacae* have been proposed to be excellent candidates to deliver Se to human diets.

Therefore, to keep an optimal level of Se in plants for human intake, the factors that affect uptake and accumulation of Se are necessary considered.

Previous studies shown the ratio of Se/S resulted in different accumulation of S and Se in plant tissues. Therefore, the effect of high and low ratio of Se/S on plant growth was studied in chapter 4. Data analysis indicated that when Se/S ratio was 1:250, it induced the highest amount of plant growth (e.g. fresh weight and dry weight) and mineral accumulation (e.g. sulfur and sulfate). However, the highest concentration of Se was found when only  $0.507 \text{ mg}\cdot\text{L}^{-1}$  of sodium selenate was applied without S supplementation. In addition, Brassica plants are consumed for their flavor and their health values. Se is associated with glutathione peroxidase (GPx) activity. The GPx activity increased with increasing Se treatments suggested that Se may activate the protective mechanism. Flavor intensity of Brassica species was affected by Se treatments. High

concentration of Se decreased GLs. It does not matter whether growing medium containing S or not. However, when *B. oleracea* were grown in the nutrient solutions with the ratios of Se/S was 1:250, the highest concentrations of total GLs was found. Therefore, data could be used as a prediction for Se fertilizer used in the future.

Since selenate and sulfate are proposed to be taken up by roots through the same transporters and share the similar metabolic pathways within plants, in chapter 5 the effect of selenate on expression of sulfate transporter genes in *B. oleracea* is tissue-specific via semi-quantitative RT-PCR. The competition between selenate and sulfate was shown in some sulfate transporter genes. The variations of gene expression may indicate that not all of these sulfate transporters are selective for either sulfate or selenate.

Our studies tried to provide information about the relationship between Se and S according to biological measurements and gene expression. Previous research suggested that Se uptake, metabolism, and assimilation are closely related to S. However, a fully understanding of Se and S metabolism in plants needs more studies in biochemical interaction and molecular analysis. Once responsible genes encoding proteins are proved to be involved in uptake, transport, and assimilation, genetic engineering may be applied for improving Se accumulation. Thus, it will be possible to use transgenic plants as a means to restore environmental contamination and to provide sufficient Se to humans.

It is getting popular that more and more people are attracted to functionality of plants. Nowadays, it is possible to identify and extract the specific components from agricultural and horticultural products which are directly associated with human health. Therefore, as a plant scientist, it will be the mission to increase nutritional value in dietary crops for human consumption.