Kale Carotenoids Remain Stable While Glucosinolates and Flavor Compounds Respond to Changes in Selenium and Sulfur Fertility

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Abstract

Dietary intake of certain carotenoids has been associated with reduced risks of specific cancers and chronic eye diseases. Kale (*Brassica oleracea* L. var. *acephala* D.C.) has been reported to contain the highest levels of the carotenoids lutein and β-carotene among green leafy vegetable crops. *Brassica* vegetables also contain anti-carcinogenic glucosinolates (GS) and S-methyl-cysteine sulfoxide (MCSO) sulfur compounds responsible for flavor. In several experiments, we investigated the influence of S and Se fertility on: 1) elemental accumulation; 2) GS and MCSO production; and 3) the accumulation patterns of carotenoid pigments in the leaf tissues of kale. Plants were greenhouse grown using nutrient solution culture with a range of S and Se concentrations. Increasing S fertility increased S leaf content, but decreased Mg and Ca accumulation. Levels of GS and MSCO increased in response to increasing S in nutrient solution. However, accumulation of lutein and β-carotene were unaffected by S treatment. Decreasing S and increasing Se fertility in kale production will decreases GS and MCSO compounds without affecting carotenoid pigments levels. Understanding the combined impact of fertility on flavor compounds and carotenoid pigments may help improve consumer acceptance of phytonutritionally-enhanced vegetable crops.

INTRODUCTION

Many plants in the Brassicaceae family play integral roles in the diets of the world population. The characteristics flavors of the *Brassica* genus come from breakdown products of glucosinolate compounds, a large group of S-containing glucosides. Glucosinolate breakdown products are potent inducers of Phase II enzymes, and inhibit mitosis and stimulate apoptosis in human tumor cells. The presence of selenium (Se) in soils can impact the uptake of sulfur (S) and influence S metabolism within the plant. Previous research in our group has shown that *Brassica* glucosinolates are reduced in the presence of Se. Vegetables are important in the human diet because they contain many essential vitamins and minerals, but they also contain other compounds that can elicit profound effects on human health. These compounds are called phytonutrients and one important class is the carotenoids. Studies indicate that a high intake of a variety of vegetables providing a mixture of carotenoids is associated with reduced cancer and eye disease risk (Le Marchand et al., 1993). *Brassica* crops contain some of the highest concentrations of important carotenoids among the vegetable crops. Therefore, we have also attempted to understand the impact of S and Se fertility on carotenoid metabolism in the *Brassicas*. An understanding of the impacts of Se and S on biochemical pathways in *Brassicas* may facilitate bioengineering of crops designed to provide a "multi-functional" (Se, glucosinolates and carotenoids) mixture of phytochemcials.

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Selenium and Sulfur in Plant Metabolism

Selenium can be absorbed by plants as inorganic selenate (SeO_4^{-2}), inorganic selenite (SeO_3^{-2}), or as organic Se compounds. Selenate and sulfate (SO_4^{-2}) compete for the same binding sites, and when SeO_4^{-2} is present in high concentrations it can competitively inhibit SO_4^{-2} uptake. Selenium is not essential an essential microelement for plants, however, some researchers have classified Se as beneficial to certain plant species. In many cases, high S-utilizing plants, like the *Brassica* species, are classified as Se accumulators. On a physiological level, Se hyperaccumulators differ from non-accumulators in that they produce a plethora of non-protein selenoamino acids.

The majority of Se taken up by plants supplied SeO_4^{-2} remained as SeO_4^{-2} in plant tissues. Plants supplied with SeO_3^{-2} or Se-Met accumulated Se as Se-Met or similar selenoamino species (Zayed et al., 1998). In most plants, SeO_4^{-2} and SO_4^{-2} exist in an antagonistic relationship with regard to uptake by roots. However, in rapid-cycling *Brassica oleracea*, at low concentrations, increases in Se in solutions results in an increase of Se in plant tissue as well as an increase in S levels in the plant tissue (Kopsell and Randle, 1999; Charron et al., 2001; Toler and Sams, 2001).

Similarities between Se and S also occur for assimilation and metabolism. Selenate is assimilated in the same metabolic pathways as SO_4^{-2} and discrimination between SO_4^{-2} and SeO_4^{-2} was found to occur at the level of amino acid incorporation into proteins (Ferrari and Renosto, 1972). The similarities between Se and S allow for the substitution of Se for S in some plant metabolic pathways, such as the synthesis of the amino acid cysteine. The resulting selenocysteine has different properties from that of its counterpart (Se-Se bonds are longer and weaker than S-S bonds for example) and thus may react differently in biological systems (Brown and Shrift, 1982; Anderson and Scarf, 1983; Charron et al., 2001).

Selenium is an element essential to mammalian nutrition as a component of the enzymes glutathione peroxidase, selenoprotein P, and tetraiodothyronine 5'-deiodinase. Adult humans have a daily requirement of 50–70 µg Se per day, and recent studies have shown a dietary Se supplement of 100–200 µg per day to be associated with a decreased incidence of such cancers as lung and prostate (Ip et al., 1991; Ip and Ganther, 1992). However, there is a fine line between too much and too little Se. Daily intakes in humans exceeding 900 µg Se per day will result in toxicity (Levander, 1982).

Glucosinolates in Plant Metabolism

Many plants in the Brassicaceae family play integral roles in the diets of the world population. Like many *Brassica* species, *B. oleracea* produces a proliferate number of glucosinolates. Glucosinolates are S containing secondary plant metabolites produced in all cruciferous species of plants, as well as 500 non-cruciferous species. In small quantities, the catabolism products of glucosinolates are potent inducers of naturally occurring anti-carcinogens in the human body. These anti-carcinogens are called Phase II enzymes, and include glutathione-S-transferase, quinone reductase, and epoxiel hydrolase (Zhang et al., 1992; Fahey and Stephenson, 1999). When consumed in large quantities, the catabolism products of glucosinolates can cause detrimental effects on the health of the consuming animal (Brown and Morra, 1997). The breakdown products of glucosinolate hydrolysis include nitriles, isothiocyanates, epithionitriles and thiocyanates.

The production of glucosinolates can be influenced by such factors as mechanical stress, environmental stress, the growing season and by plant nutrition. Well-fertilized plants will put out more biomass and thus have greater resources from which to produce glucosinolates. Sulfur incorporation into fertilizers can increase glucosinolate production by 2 to 3 fold, making it indispensable to glucosinolate metabolism. Mailer (1989) showed that S application to *B. napus* and *B. rapa* significantly increased glucosinolate content of seeds. Selenium, which is similar in size and chemistry to S, is another nutrient that can specifically impact glucosinolate production. Charron et al. (2001) found a significant reduction in 4-methylsulphinylbutyl glucosinolate production by *B. oleracea* at a 1 ppm concentration of SeO_4^{-2} in nutrient solution.

Given the importance of Se to mammalian nutrition, the beneficial anti-carcinogen inducing properties of small amounts of glucosinolate by-products, and the negative health impacts that both glucosinolate by-products and Se can induce in excessive quantities, it is essential to understand the unique regulatory mechanisms that guide S and Se uptake and their role in the glucosinolate metabolism of the widely consumed *Brassica* crops.

Carotenoids in Plant Metabolism

Carotenoids are lipid soluble yellow, orange and red pigments that are uniquely synthesized in plants, algae, fungi and bacteria. They are secondary plant compounds which are divided into two groups; the oxygenated xanthophylls such as lutein, zeaxanthin and violaxanthin, and the hydrocarbon carotenes such as β-carotene, α-carotene and lycopene. Within the thylakoid membranes of chlorophyll organelles, carotenoids are found bound to specific protein complexes of photosystem I and photosystem II (PSII). Carotenoids function to help harvest light energy during photosynthesis and dissipate excess energy before damage occurs. When the absorption of light radiation exceeds the capacity of photosynthesis, excess excitation energy can result in the formation triplet excited chlorophyll (^{3}Chl) and reactive singlet oxygen ($^1O_2$). Carotenoid pigments protect photosynthetic structures by quenching excited ^{3}Chl to dissipate excess energy and binding $^1O_2$ to inhibit oxidative damage (Demmig-Adams et al., 1996).

Carotenoid accumulation appears to be shaped by a plant species' physiological, genetic and biochemical attributes, as well as environmental growth factors such as light, temperature and fertility (Goldman et al., 1999; Kurilich et al., 1999). Mercadante and Rodriguez-Amaya (1991) have reported genetic differences in carotenoid content between field grown kale (*Brassica oleracea* L. var. *acephala* D.C.) cultivars. Recently, our work has identified differences in carotenoid content in kale and parsley (*Petroselinum crispum* Nym.) grown under different nitrogen levels (Chenard et al., 2005). Therefore, it is imperative to consider genetic and environmental influences when assessing vegetable carotenoid accumulation.

Carotenoids and Human Health

The nutritional and medicinal importance of the dietary carotenoids is being established. Fruits and vegetables are the primary sources of carotenoids in the human diet and their consumption has been associated with numerous health benefits (Mortensen, et al., 2001). Dietary intake of lutein, β-carotene and other carotenoids has been associated with reduced risk of lung cancer and chronic eye diseases, including cataract and age-related macular degeneration (Le Marchand et al., 1993). Studies indicate that a high intake of a variety of vegetables providing a mixture of carotenoids was more strongly associated with reduced cancer and eye disease risk than intake of individual carotenoid supplements.

In the retina, lutein and zeaxanthin are chiefly responsible for the yellow pigmentation, collectively referred to as macular pigment (MP) (Khachik et al., 1997). The yellow pigments are postulated to participate in photoprotection and diminished MP may be related to retinal damage (Mares-Perlman and Klein, 1999). A direct correlation between MP levels and development of macular disease has not been established (Landrum and Bone, 2001), although strong associative relationships are reported.

Selenium and Rapid-Cycling *Brassica oleracea* Nutrition

The objectives of these studies were: 1) to establish selenate-Se treatments; 2) to establish accumulation patterns of Se in the leaf, stem and root tissues; 3) to determine the effect of increasing Se on the nutrient content and 4) to examine effects on growth and dry matter production under increasing selenate-Se for Rapid-cycling *Brassica oleracea*.

Plants of rapid-cycling *B. oleracea* population were grown in nutrient solutions amended with Na_2SeO_4 at 0.0, 3.0, 6.0 and 9.0 mg·L^{-1}. All treatments contained an $MgSO_4·7H_2O$ concentration of 246.48 mg L^{-1} (96 mg L$^{-1}SO_4^{-2}$). Plant tissues were divided and analyzed for total Se using GFAA, total S using a Leco Sulfur Determinator, and total N was measured using an autoanalyzer. Macronutrient and micronutrient levels were determined by ICAP (Inductively Coupled Argon Plasma Spectrophotometry).

No differences were detected among Se treatments for either leaf FW or DW (Kopsell and Randle, 1999). Selenium concentration in the leaf, stem and root tissues differed in response to the Na_2SeO_4 treatments. A significant linear increase in leaf S with increasing Na_2SeO_4 treatment concentrations was found. Leaf tissue B, Fe and P content decreased, while Se, S and K content increased with increasing selenate-Se treatments. Significant quadratic responses were found for Mg and Mo (Table 1; Kopsell and Randle, 1999). Changes in plant nutrient content can be expected when *Brassicas* are enhanced for delivery of beneficial organic Se.

Selenium and Rapid-Cycling *Brassica oleracea* Glucosinolates

Since *Brassicas* can accumulate high levels of Se and Se can interfere with S metabolism, the objectives of this study were to measure the effects of Se fertility on glucosinolate compounds in a rapid-cycling *B. oleracea*.

In the first experiment, plants of rapid-cycling *B. oleracea* population were grown in nutrient solutions amended with Na_2SeO_4 at 0.0, 3.0, 6.0 and 9.0 $mg{\cdot}L^{-1}$. All treatments contained an $MgSO_4{\cdot}7H_2O$ concentration of 246.48 mg L^{-1} (96 mg $L^{-1}SO_4^{-2}$). In the second experiments, rapid-cycling *B. oleracea* were grown in nutrient solutions amended with 1.0, 2.0 and 3.0 mg Na_2SeO_4 L^{-1}. Mineral elements in freeze dried leaves were analyzed using ICAP. Aliphatic and indole glucosinolates were separated with a HPLC using a C-18 ODS reverse-phase column and UV detector at a wavelength of 230 nm.

Selenium accumulation increased with increasing levels of $NaSeO_4$ in nutrient solutions in each of the experiments. Levels of total, aliphatic and indole glucosinolates in the leaf tissues decreased in response to increases in $NaSeO_4$. Linear decreases were found for 3-butenyl, 4-methylsulfinylbutyl, 2-hydroxy-3-butenyl, 2-propenyl and 3-indolylmethyl glucosinolates (Table 2; Charron et al., 2001).

Sulfur and *Brassica oleracea* Flavor Compounds and Carotenoid Pigments

The goal of this study was to investigate the influence of different S fertility levels on: 1) elemental accumulation; 2) GS and MCSO production; and 3) the accumulation patterns of carotenoid pigments in the leaf tissues kale.

Three different kale cultivars (‘Winterbor’, ‘Redbor’ and ‘Toscano’) were cultured in nutrient solutions. Plants were grown under increasing S treatment concentrations at 4, 8, 16, 32 and 64 mg $S{\cdot}L^{-1}$ supplied as $MgSO_4{\cdot}7H_2O$. Dried leaves were analyzed for elemental concentrations by ICAP. Glucosinolate and flavor compounds were extracted from freeze dried leaf tissues and separated with a HPLC Carotenoid pigments were extracted and analyzed using HPLC.

Leaf tissue percent Mg responded significantly to S treatment and cultivar. Accumulation of percent Ca in leaf tissues responded significantly to S treatment and cultivar. Accumulation of percent S in leaf tissues responded significantly to S treatment concentration, cultivar, and the interaction of S treatment and cultivar. Glucosinolate and methyl-cysteine sulfoxide compounds were affected by increasing S concentrations in nutrient solution (Table 3; Kopsell et al., 2003).

Lutein and β-carotene carotenoids responded significantly to kale cultivar. However, no significant trends for leaf tissue accumulation were found for any of the pigments in response to increasing S treatment concentrations in nutrient solution from 4 to 64 mg $S{\cdot}L^{-1}$ (Kopsell et al., 2003).

Selenium and *Brassica oleracea* Carotenoids and Elemental Concentrations

The objectives of this study were to determine the influence of increased Se (as SeO_4^{-2} or SeO_3^{-2}) fertility levels on (1) the accumulation patterns of carotenoid pigments, and (2) elemental accumulation in the leaves of kale, a member of the *Brassica* family.

‘Winterbor’ kale was grown in nutrient solutions. Plants were grown in separate studies under increasing Se treatment concentrations at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g Se/L as Na_2SeO_4 and at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg Se/L as Na_2SeO_3. Elemental concentrations were measured using ICAP, and Se analysis was carried out using GFAA. Freeze dried tissues were measured for carotenoid pigments using HPLC

None of the pigments measured responded to increases in SeO_4^{-2} or SeO_3^{-2} concentrations. Elemental nutrient levels in the kale leaves were within reported ranges for mature, greenhouse-grown plants (Mills and Jones, 1996). Leaf tissue K, Mg, P, S, B, Cu, Mo and Se were affected by selenium treatments. Tissue Se results from the current study are within the ranges of reported Se accumulation in the leaves of rapid-cycling *B. oleracea* grown under increasing levels of SeO_4^{-2} in nutrient solutions (Kopsell and Randle, 1999; Kopsell et al, 2000; Charron et al., 2001; Kopsell et al., 2003).

CONCLUSIONS

Brassica crops have the ability to accumulate high concentrations of Se. Incorporation of Se into plant pathways will interfere with normal S metabolism. Glucosinolates are S-compounds in the *Brassicas* that confer their distinctive flavors. Members of *B. oleracea*, such as kale, can accumulate high levels of lutein and β-carotene carotenoids. Results show that kale can bio-accumulate high levels of Se, which increases tissue Se levels and influences flavor intensity by reducing some of the glucosinolate compounds. With the important health benefits associated with increased consumption of plant-derived lutein, β-carotene and Se in the diet, production practices may be modified in order to maximize the nutritional properties of kale and other *Brassica* crops. An understanding of the impacts of Se and S on biochemical pathways in *Brassicas* may facilitate bioengineering of crops designed to provide a “multi-functional” (Se, glucosinolates and carotenoids) mixture of phytochemicals.

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Tables

Table 1. Tissue accumulation of Se and S in a rapid-cycling *Brassica oleracea* grown under 0.0, 3.0, 6.0 and 9.0 mg $NaSeO_4$ (adapted from Kopsell and Randle, 1999).

Tissue	mg Na_2SeO_4 per L				Contrasts
	0.0	3.0	6.0	9.0	
		µg Se per g dry weight			
Leaf	nd	552	1275	1916	L
Stem	nd	267	721	1165	L
Root	nd	338	857	1636	L
		mg S per g dry weight			
Leaf	13.4	21.6	24.1	26.5	L
Stem	8.0	6.0	8.5	11.2	^{ns}
Root	9.3	8.7	10.4	7.3	^{ns}

nd = Nondetectable; ^{ns} = Nonsignificant; L = linear orthogonal contrast significant at $P \leq 0.05$.

Table 2. Glucosinolate (GS) concentrations in the leaf tissue of rapid-cycling *Brassica oleracea* grown under increasing concentrations of Na_2SeO_4 (adapted from Charron et al., 2001).

GS	mg Na_2SeO_4 per L						Contrasts
$\mu mol \cdot g^{-1}$	0.0	1.0	2.0	3.0	6.0	9.0	
Total GSs	5.84	4.36	4.49	2.79	2.26	1.90	L
Aliphatic GSs	5.39	3.99	3.98	2.56	2.10	1.76	L
Indole GSs	0.45	0.37	0.50	0.23	0.16	0.14	L
3-Butenyl	1.66	0.84	0.59	0.84	0.36	0.49	L
4-Methyl-sulfinylbutyl	0.40	0.04	nd	0.01	0.02	0.02	L
2-Hydroxy-3-butenyl	1.41	1.60	0.62	0.58	0.65	0.29	L
2-Propenyl	1.54	1.43	1.77	1.04	0.88	0.76	L
4-Methoxy-3-indolylmethyl	0.10	0.12	0.14	0.07	0.01	0.01	L

nd = nondetectable; L = linear orthogonal contrast significant at $P \leq 0.05$.

Table 3. Glucosinolate and methylcysteine sulfoxide (MCSO) concentrations for leaf tissue of 'Winterbor' kale grown under increasing sulfur (S) concentrations in nutrient solutions (adapted from Kopsell et al., 2003).

mg·S per L	Glucosinolates ($mg \cdot 100\ g^{-1}$)				MCSO ($mg \cdot g^{-1}$)
	glucoinerin	sinigrin	glucobrassicin	4-hydroxygluco-brasscicin	
4	nd	2.1	45.3	3.9	0.6
8	5.6	14.3	104.4	4.4	2.0
16	22.9	22.5	274.3	6.1	4.4
32	25.6	26.1	302.4	9.9	4.9
64	23.1	29.8	247.5	7.3	4.6
Contrasts					
Linear	$P<0.001$	$P=0.013$	$P<0.001$	$P=0.087$	$P<0.001$

nd = nondetectable; ^{ns} = nonsignificant.