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Kale Carotenoids Remain Stable while Flavor Compounds Respond to Changes in Sulfur Fertility†

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Dietary intake of certain carotenoids has been associated with a reduced risk of disease. Kale (*Brassica oleracea* L. Acephala Group) has the highest levels of carotenoids lutein and β-carotene, and is an excellent source of minerals among the green leafy vegetable crops. However, *Brassica* vegetables contain glucosinolate (GS) and *S*-methylcysteine sulfoxide (MCSO). While these sulfur compounds have medicinal value, they are also responsible for the bitter, acrid flavors that are often regarded as objectionable by consumers. Therefore, the objectives of this study were to investigate the influence of increased S fertility levels on (1) elemental accumulation, (2) GS and MCSO production, and (3) the accumulation patterns of carotenoid pigments in the leaves of three kale cultivars. Winterbor, Redbor, and Toscano kale were greenhouse-grown using nutrient solution culture with S treatment concentrations of 4, 8, 16, 32, and 64 mg of S/L. Decreasing S fertility decreased S leaf content, but increased the levels of Mg and Ca accumulation, two important minerals for human health. Levels of GS and MSCO decreased in response to a decreasing S level in nutrient solution. However, accumulation of lutein and β-carotene was unaffected by S treatment. Lowering the S fertility in the production of kale should decrease the levels of negative flavors associated with high levels of GS and MCSO without affecting carotenoid pigment levels. Understanding the combined impact of fertility on flavor compounds and carotenoid pigments may help improve consumer acceptance of phytonutritionally enhanced vegetable crops.

KEYWORDS: ***Brassica oleracea*; glucosinolates; *S*-methyl-L-cysteine sulfoxide; macronutrients; micronutrients; lutein; β-carotene; HPLC**

INTRODUCTION

Foods have long been considered beneficial for human health maintenance. Among those thought to have high nutritional and medicinal value are the *Brassica* vegetables, which contain essential dietary minerals and sulfur and carotenoid compounds. Understanding how these compounds are metabolized in plants and how they can be manipulated to enhance their medicinal value has become increasingly important.

In plants, sulfur (S) is actively transported into roots as sulfate (SO_4^{2-}) and translocated to shoots and leaves. Sulfate can be stored in cellular vacuoles, but reduction to S^{2-} is required prior to incorporation into cysteine (*1*). Many plant species also incorporate S into a wide range of secondary compounds responsible for characteristic odors and flavors. S deficiencies can appear as mild chlorosis and reductions in the rates of plant growth. Conversely, some plant species have been shown to be

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comparatively insensitive to high SO_4^{2-} concentrations in the growing environment (*2*).

Glucosinolates [β-thioglucoside *N*-hydroxysulfates (GS)] and *S*-methylcysteine sulfoxide (MCSO) are secondary S-containing compounds present in *Brassica oleracea* L. (*3*). Glucosinolates and MCSO have no identifiable primary function in plants, but have been theorized to protect against predation and pathogens, as well as to act as S storage reserves (*4*). Following cellular disruption, GS and MSCO are enzymatically decomposed to produce compounds responsible for the characteristic flavor of *Brassica*. Breakdown products of GS have also been shown to possess anticarcinogenic activity and may be useful as chemopreventative agents in humans (*3*).

More than 100 different GS compounds have been identified in plants, but only approximately 12 are found in *Brassica* (*3*). When GS compounds are decomposed by the enzyme myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1), glucose, bisulfate, and aglucones, which can then fragment or rearrange to form mixtures of volatile and nonvolatile compounds, including isothiocyanates, thiocyanates, and nitriles, are produced (*5*, *6*). Myrosinase and GS compounds are differentially sequestered in plant cells; however, upon tissue disruption during insect feeding, harvesting, processing, food preparation, mastication, or digestion, they are brought into contact, and decomposition occurs (*7*). The identity of the side chains of GS, which include aliphatic, aromatic, or heteroaromatic groups, determines the nature of the isothiocyanate, thiocyanate, or nitrile formed. Isothiocyanates are a group of hot and bitter compounds commonly termed "mustard oils" (*8*).

Carotenoids are C_{40} isoprenoid polyene secondary plant compounds that form lipid soluble yellow, orange, and red pigments. Examples of carotenoids include the oxygenated xanthophyll lutein [(3*R*,3′*R*,6′*R*)-β,ϵ-carotene-3,3′-diol] and the hydrocarbon carotene β-carotene (β,β-carotene; *9*). Carotenoids span the thylakoid membranes of chlorophyll complexes and function in accessory roles of light harvesting, photoprotection, and structural stabilization (*10*, *11*). Carotenoid pigments protect photosynthetic structures by quenching excited triplet chlorophyll (^{3}Chl) to dissipate excess energy (*12*) and binding singlet oxygen ($^1O_2$) to inhibit oxidative damage (*10*, *11*). The nutritional and medicinal importance of the dietary carotenoids is being established (*13*, *14*). Plants are the primary sources of carotenoids in the diet, and leafy members of the Cruciferae, including subspecies of *B. oleracea* L., contain abundant amounts relative to other vegetables (*15*). Carotenoids exhibit antioxidant and anticarcinogenic activity (*13*, *14*). Dietary intake of lutein, β-carotene, and other carotenoids has been associated with a reduced risk of lung cancer and chronic eye diseases, including cataract and age-related macular degeneration (*16*, *17*). Studies have indicated that consumption of a variety of vegetables providing a mixture of carotenoids was more strongly associated with disease reduction than individual carotenoid supplements (*16*). Although these associative epidemiological relationships indicate carotenoids may serve photoprotective and antioxidant functions in humans, direct evidence of these actions is still lacking (*18*, *19*).

Green leafy vegetables are rich in dietary carotenoids, and kale (*B. oleracea* L. Acephala Group) ranks the highest among all vegetable crops for reported lutein and β-carotene content (*20*). The *Brassica* vegetables are also good dietary sources of Ca and Mg (*21*). Glucosinolates and MCSO are S-containing compounds in *B. oleracea* responsible for flavor and potential health benefits. However, because these S compounds can impart negative flavor attributes at high tissue concentrations, understanding how their levels can be lowered is important for consumer preference and fresh consumption. Therefore, the goal of this study was to investigate the influence of different S fertility levels on (1) mineral accumulation, (2) GS and MCSO production, and (3) the accumulation patterns of carotenoid pigments in the leaf tissues of three kale cultivars. Three kale cultivars were evaluated to assess the genetic variability previously reported for GS and carotenoid accumulation (*22*, *23*).

MATERIALS AND METHODS

Plant Culture. On January 31, 2002, three kale cultivars [Winterbor, Redbor, and Toscano (Johnny's Selected Seed, Albion, ME)] were seeded into rockwool cubes (Grodan A/S, Dk-2640 Hedehusene, Denmark). The medium was supplied with bottom heat (23 °C) and grown in a greenhouse (22 °C during the day and 14 °C at night) for 2 weeks under natural photoperiods (latitude, 43° 09′ N). Nutrients were applied as needed using 200 mg/L Peter's 20N-6.9P-16.6K water-soluble fertilizer (Grace-Sierra, Milpitas, CA).

On February 15, 2002, plants were transferred to 37.9 L containers (Rubbermaid Inc., Wooster, OH) having 30 L of a half-strength modified Hoagland's nutrient solution (*24*). Five plants of each of the three kale cultivars were placed into 2.22 cm holes with 10.6 cm × 9.5 cm spacing on each container lid. Elemental concentrations of the nutrient solutions were as follows: 434.0 mg/L NO_3-N, 9.0 mg/L NH_4-N, 15.3 mg/L P, 117.1 mg/L K, 80.2 mg/L Ca, 24.6 mg/L Mg, 0.5 mg/L Fe, 0.25 mg/L B, 0.005 mg/L Mo, 0.01 mg/L Cu, 0.25 mg/L Mn, and 0.025 mg/L Zn. Plants were grown under increasing S treatment concentrations at 4, 8, 16, 32, and 64 mg of S/L supplied as $MgSO_4 \cdot 7H_2O$. Sulfur was partially supplied as Na_2SO_4 during the treatment with 64 mg of S/L, and Mg was partially supplied as $MgCl_2$ during the treatments with 4, 8, and 16 mg of S/L to maintain a level of 24.6 mg of Mg/L across all the treatments. Solutions were aerated with an air blower (model VB-007S, Sweetwater, Ft. Collins, CO) connected to air stones. The experimental design was a split plot, with S treatment as the main plot and kale cultivar as the subplot. Each treatment consisted of five plants per cultivar and was replicated four times. Each solution was replaced every 2 weeks throughout the experiment to refresh the solution to the initial nutrient concentrations.

Plants were harvested on March 29, 2002. Once they were harvested, shoot and root tissue were separated, and shoot tissue from five plants in each treatment/cultivar combination was bulked and weighed for fresh mass accumulation. Plant tissues were washed with soap (Aquet, Bel-art Products, Pequannock, NJ), rinsed, and blotted dry with paper towels. One shoot tissue group was dried at 45 °C for no less than 72 h, at which time the dry weight was calculated. One shoot tissue group was placed in a −80 °C freezer prior to lyophilization. Tissues were lyophilized for 48 h (model 6 L FreeZone, LabConCo, Kansas City, MO) prior to extraction.

Elemental Determination. Dried leaves were ground so they would pass through a 0.5 mm screen in a sample mill grinder (model 1093, Cyclotec-Tector, Höganäs, Sweden). A 0.3 g tissue sample was combined with 10 mL of concentrated nitric acid (70% HNO_3) and digested in a microwave-accelerated reaction system (MARS5, CEM Corp., Matthews, NC). Digestion solutions were allowed to cool to room temperature (∼26 °C) and adjusted to a final volume of 40 mL with deionized water. Elemental concentrations were determined by inductively coupled argon plasma atomic emission spectrometry (ICP-AES; model Vista AX, Varian, Inc., Palo Alto, CA).

Glucosinolate and Methylcysteine Sulfoxide Determination. *Glucosinolates. (1) Tissue Extraction*. Glucoiberin (3-methylsulfinylpropyl), sinigrin [prop-2-enyl(allyl)], glucobrassicin (3-indolylmethyl), neoglucobrassicin (1-methoxy-3-indolylmethyl), 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), and 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl) were extracted from freeze-dried kale leaf tissue. For GS analysis, 0.1 mg samples were combined with 1 mL of benzyl GS solution (1 mM) as an internal standard, 2.0 mL of methanol, and 0.3 mL of barium-lead acetate (0.6 M) in a 16 mm × 100 mm culture tube and shaken at 60 rpm for 1 h. Each tube was then centrifuged at

$2000g_n$ for 10 min. A 0.5 mL aliquot of supernatant was then added to a 1 mL column containing 0.3 mL of DEAE-Sephadex A-25. The sample was desulfated by the procedure of Raney and McGregor (*25*).

(2) High-Performance Liquid Chromatography (HPLC) Analysis. Extracted desulfoglucosinolates were separated with a Hewlett-Packard (Palo Alto, CA) HPLC system using a C-18 ODS reverse-phase column [250 mm × 4.6 mm (inside diameter), 5 μm] and a UV detector at a wavelength of 230 nm. The column temperature was set at 35 °C. A flow rate of 1.5 mL/min was used. For 1 min, the solvent was 100% water. This was followed by a 15 min linear gradient to 75% water and 25% acetonitrile. Solvent levels were then held constant for 5 min, and over the final 5 min, a linear gradient to 100% water was used. Desulfoglucosinolates were identified by comparison with retention times of authentic standards or previously reported results (*22*, *26*).

Methylcysteine Sulfoxide. *(1) Tissue Extraction*. Ground sample leaf tissue was redried at 65 °C in a forced air oven (Linberg Blue, Asheville, NC). Methylcysteine sulfoxide (MCSO) was extracted from 0.2 g of dried tissue by adding 30 mL of a 12:5:3 methanol/chlorform/water mixture (MCW) and allowing it to incubate overnight at −20 °C (*27*). Sixty milliliters of chloroform was then added to the MCW extract, and the mixture was allowed to separate in a separatory funnel. A 2.5 mL subsample of the polar fraction was dried using forced ambient air (Evap-O-Rac, Cole Parmer, Vernon Hills, IL). The dried sample was rehydrated in 1 mL of deionized and distilled water, and a 0.25 mL aliquot was taken and combined with 0.5 mL of an internal standard, (±)-ethylcysteine sulfoxide (ECSO) (1 mg/mL), prepared using a modification of the method of Lancaster and Kelly (*28*). The sample solution was then dried using forced ambient air and redissolved with 1 mL of an aqueous hydrochloric acid [HCl (pH 2.5)] solution prior to HPLC analysis.

(2) HPLC Analysis. Sample MCSO concentrations were determined using the method of Edwards et al. (*29*). A Waters 2690 series HPLC unit with a photodiode array detector (PDA) (model 996, Waters Corp., Milford, MA) was used for sample separation. Fifty microliters of the sample was injected into two C18 4.6 mm × 250 mm, 5 μm [ODS(2) SphereClone, Phenomenex] columns with one 4 mm × 3 mm C18 guard column insert (Security Guard, Phenomenex). A dilute HCl (pH 2.5) isocratic eluent was used with a flow rate of 0.9 mL/min. The eluent was filtered with 0.45 μm nylon filters (Micron Separations, Inc., Westboro, MA), and MCSO was detected at 210.2 nm. Peak assignment was performed by comparing retention times and line spectra obtained from PDA with an MCSO standard prepared according to the method of Lancaster and Kelly (*28*). Quantification was achieved by comparing the relative areas of the MCSO peak with those of the internal standard (ECSO) using Millenium Chromatography Software (Waters Corp.).

Carotenoid and Pigment Determination. *Tissue Extraction*. Freeze-dried tissues were combined with ~50 g of dry ice in a household food chopper (Handy Chopper Plus, Black & Decker, Towson, MD). Macerated tissues were placed in 20 mL scintillation vials, and CO_2 gas was vented prior to storage at −20 °C. Carotenoids and pigments were extracted and separated according to the method of Beecher and Howard (U.S. Department of Agriculture Food Composition Laboratory, Beltsville, MD), which is based on the method of Khachik et al. (*30*). A 0.10 g subsample was rehydrated with 0.8 mL of ddH_2O at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl β-8-apocarotenoate (Sigma Chemical Co., St. Louis, MO) and 2.5 mL of tetrahydrofuran (THF) stabilized with 25 ppm 2,6-di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenized in a Potter-Elvehjem (Kontes, Vineland, NJ) tissue grinding tube using ~25 insertions with a pestle attached to a drill press (Craftsman 15 in. drill press, Sears, Roebuck and Co., Hoffman Estates, IL) set at 540 rpm. During homogenation, the tube was immersed in ice to dissipate heat. The tube was then placed in a clinical centrifuge for 3 min at $500g_n$. The supernatant was removed, and the sample pellet was resuspended in 2.0 mL of THF and homogenized again with the same extraction technique. The extraction procedure was repeated two more times until the supernatant was colorless. The volumes of the combined supernatants were reduced to 0.5 mL using nitrogen (model N-EVAP 111, Organomation Inc., Berlin, MA) at 40 °C, and 2.5 mL of MeOH and 2.0 mL of THF were added to the sample prior to HPLC analysis.

HPLC Analysis. An Agilent 1100 series HPLC unit with a PDA detector (Agilent Technologies) was used for sample separation. All samples were analyzed for carotenoid and chlorophyll compounds using a Vydac RP-18 5.0 μm, 250 mm × 4.6 mm column (model 201TP54, Phenomenex, Torrance, CA) fitted with a 4 mm × 3.0 mm, 7.0 μm guard column (RP-18, Phenomenex). The column was maintained at 16 °C using a thermostated column compartment. Eluents were (A) 75% acetonitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethylamine (TEA) in water (v/v) and (B) 50% acetonitrile, 25% THF, 25% hexane, and 0.013% TEA in water (v/v). The flow rate was 0.7 mL/min, and the gradient is as follows: 100% A for 30 min, 50% A and 50% B for 2 min, 100% B for 2 min, and 50% A and 50% B for 2 min. The eluent composition was returned to 100% A, and the column was equilibrated for 10 min prior to the next injection. Eluted carotenoid and chlorophyll compounds from a 20 μL injection were detected at 452, 652, and 665 nm, and data were collected, recorded, and integrated using 1100 HPLC ChemStation Software (Agilent Technologies). Peak assignment was performed by comparing retention times and line spectra obtained from the PDA with those of authentic standards purchased from commercial vendors.

Statistical Analysis. Data were analyzed by the GLM procedure of SAS (Cary, NC). The relationships between experimental dependent variables and S treatments were determined by regression analysis. Orthogonal polynomials were also used to study changes associated with decreasing S levels by partitioning the sums of squares into components associated with linear and quadratic terms (*31*).

RESULTS AND DISCUSSION

Plant Growth. Differences in shoot fresh weight (SFW; $F = 5.64$, $P < 0.001$) and dry weight (SDW; $F = 4.89$, $P < 0.001$) were found among the cultivars (data not shown). However, no differences in SFW or SDW were measured for S treatments or the interaction of S and cultivar. The nonsignificance of SFW and SDW among the kale cultivars in response to increasing S treatments follows previously reported trends in other *Brassica* crops. Matula and Zukalová (*32*) reported that dry matter yield of oilseed rape (*Brassica napus* L.) was not affected by increasing $MgSO_4$ fertility in potted soil culture. Hara and Sonoda (*33*) also observed no differences in SFW and SDW in cabbage (*Brassica oleracea* L. Capitata Group) grown in nutrient solution culture at 10 and 100 mg of S/L. Yield considerations, therefore, should not be noteworthy when manipulating S among the range provided in this study.

Mineral Elements. Accumulation of S (%S) in leaves responded significantly to S treatment concentration ($F = 113.25$, $P < 0.001$), cultivar ($F = 22.07$, $P < 0.001$), and the interaction of S treatment and cultivar ($F = 2.33$, $P < 0.044$). Leaf %S increased linearly for all cultivars [%S = 0.20 + 0.40(trt) for Winterbor, %S = 0.15 + 0.27(trt) for Redbor, and %S = 0.17 + 0.30(trt) for Toscano] in response to increasing S content in nutrient solution (**Table 1**). S levels in the leaves ranged from 0.18% for Redbor at 4 mg of S/L to 1.79% for Winterbor in response to 64 mg of S/L. The reported leaf S sufficiency range of most plants is between 0.15 and 0.50% (*2*). Kastori et al. (*34*) reported that when the S level was increased from 0 to 96 mg of S/L in nutrient solution culture, the level of leaf tissue S of sugar beet increased 1100%. Increasing the concentration of S in the nutrient solution from 4 to 64 mg of S/L increased leaf tissue S levels of kale 716% in Winterbor, 622% in Redbor, and 638% in Toscano.

Concentrations of other mineral elements were slightly above sufficiency ranges previously reported in leaves of mature, field-grown kale (data not shown) (*35*). Only levels of Mg and Ca, however, were significantly affected by S availability in the nutrient solutions. The level of leaf Mg (%Mg) responded significantly to S treatment ($F = 11.69$, $P < 0.001$) and cultivar

Table 1. Percent Macronutrient Content[a] of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

mg of S/L	%S	%Mg	%Ca
	Winterbor		
4	0.25 ± 0.03	0.82 ± 0.04	5.25 ± 0.29
8	0.46 ± 0.09	0.76 ± 0.05	4.86 ± 0.43
16	1.04 ± 0.06	0.76 ± 0.05	5.00 ± 0.06
32	1.37 ± 0.12	0.70 ± 0.06	4.30 ± 0.33
64	1.79 ± 0.06	0.72 ± 0.03	4.45 ± 0.38
contrasts			
linear	$P < 0.001$	$P = 0.016$	$P = 0.001$
quadratic	ns[b]	ns[b]	ns[b]
	Redbor		
4	0.18 ± 0.02	0.85 ± 0.02	4.90 ± 0.38
8	0.29 ± 0.03	0.75 ± 0.02	4.35 ± 0.21
16	0.54 ± 0.04	0.69 ± 0.05	4.01 ± 0.22
32	1.09 ± 0.05	0.66 ± 0.06	3.60 ± 0.25
64	1.12 ± 0.06	0.69 ± 0.02	3.85 ± 0.33
contrasts			
linear	$P < 0.001$	$P < 0.001$	$P < 0.001$
quadratic	ns[b]	$P = 0.001$	$P = 0.010$
	Toscano		
4	0.21 ± 0.03	1.03 ± 0.06	4.13 ± 0.26
8	0.36 ± 0.05	0.71 ± 0.05	3.70 ± 0.43
16	0.68 ± 0.09	0.80 ± 0.06	3.59 ± 0.12
32	1.13 ± 0.20	0.80 ± 0.08	3.36 ± 0.53
64	1.34 ± 0.19	0.74 ± 0.05	3.27 ± 0.51
contrasts			
linear	$P < 0.001$	ns[b]	$P = 0.004$
quadratic	ns[b]	ns[b]	ns[b]

[a] Percent composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. [b] Nonsignificant.

($F = 7.23$, $P = 0.002$), but not for the interaction of S treatment and cultivar. As S availability decreased, %Mg increased from 0.72 to 0.82% for Winterbor [%Mg = 0.81 − 0.02(trt)] and from 0.66 to 0.85% for Redbor [%Mg = 0.85 − 0.04(trt)] (**Table 1**). Although the Toscano leaf tissue Mg concentration increased from 0.74 to 1.03%, no trend was apparent. Accumulation of Ca (%Ca) in kale leaves responded significantly to S treatment ($F = 21.26$, $P < 0.001$) and cultivar ($F = 75.22$, $P < 0.001$), but not to the interaction of S and cultivar. As S availability decreased, leaf %Ca increased linearly for all the cultivars that were tested [%Ca = 5.52 − 0.24(trt) for Winterbor, %Ca = 4.99 − 0.28(trt) for Redbor, and %Ca = 4.13 − 0.20(trt) for Toscano] (**Table 1**). Lowering S availability, therefore, may improve the leaf content of these two important mineral elements.

The *Brassica* vegetables do not accumulate oxalate, a C_2 dicarboxylic acid, to detoxify excess Ca to protect against cell death. When consumed, oxalic acid in plants can bind with both Ca and Mg in the human intestinal track to form insoluble salts, lowering the rate of uptake (*36*). In fact, diets high in oxalate-rich vegetables, low in Ca-rich foods such as milk and cheese, and low in vitamin D may lead to Ca deficiency (*37*). Therefore, kale can be an excellent source of Ca and Mg in the diet. Interestingly, the level of intestinal absorption of Ca from members of the *Brassica* genus can equal or exceed the level of Ca absorption from milk (*38*). Because high S availability would lower the value of kale as a dietary Ca and Mg source, S levels should be considered in production strategies aimed at Ca and Mg enhancement of kale.

Sulfur Compounds. Most GS compounds were affected by decreasing S availability in the nutrient solutions. Glucoiberin accumulation responded significantly to S treatment ($F = 7.27$, $P < 0.001$), cultivar ($F = 17.75$, $P < 0.001$), and the interaction of S with cultivar ($F = 2.42$, $P = 0.03$). As S availability decreased, significant linear decreases in glucoiberin content were measured [glucoiberin = −3.1 + 6.3(trt) for Winterbor, glucoiberin = −4.4 + 3.0(trt) for Redbor, and glucoiberin = −1.0 + 0.8(trt) for Toscano] (**Table 2**). Glucoiberin was undetectable at the lowest S concentration for all cultivars.

Sinigrin, an aliphatic glucosinolate, accumulation responded significantly to S treatment ($F = 2.59$, $P = 0.05$) and kale cultivar ($F = 27.87$, $P < 0.001$). Sinigrin was undetectable at a concentration of 4 mg of S/L for Winterbor and Redbor, while the sinigrin content in Toscano was unaffected by S treatment (**Table 2**). Significant linear decreases in sinigrin content in leaf tissues were measured for Winterbor [sinigrin = −1.2 + 6.7(trt)] and Redbor [sinigrin = −1.3 + 0.9(trt)] in response to decreasing S availability. Similarly, Davik and Bakken (*39*) reported minimal detection of aliphatic GS in seeds of oilseed rape (*B. napus* L.) at low S fertility, but measured significant increases in concentration in response to increasing S levels in inbred and hybrid lines.

Glucobrassicin accumulation responded significantly to only S treatment and was the most abundant GS in all cultivars ($F = 14.86$, $P < 0.001$) (**Table 2**). Significant linear decreases in glucobrassicin content in leaf tissues were measured in response to decreased S availability [glucobrassicin = 14.1 + 60.2(trt) for Winterbor, glucobrassicin = −34.7 + 75.6(trt) for Redbor, and glucobrassicin = 51.1 + 43.7(trt) for Toscano]. Neoglucobrassicin accumulation responded significantly only to kale cultivar ($F = 24.36$, $P < 0.001$) (**Table 2**). However, the trends in response to S availability were cultivar-dependent. Neoglucobrassicin content in leaf tissue linearly increased for Redbor [neoglucobrassicin = 0.9 + 0.9(trt)], increased and then decreased quadratically for Winterbor [neoglucobrassicin = −4.3 + 14.2(trt) − 2.2(trt^2)], and decreased and then increased quadratically for Toscano [neoglucobrassicin = 12.4 − 7.3(trt) + 1.4(trt^2)].

4-Hydroxyglucobrassicin accumulation responded significantly to only S treatment ($F = 3.62$, $P = 0.01$) (**Table 2**). Redbor was the only cultivar whose level decreased in response to decreasing S availability [4-hydroxyglucobrassicin = 1.3 + 1.6(trt)]. Davik and Bakken (*39*) reported that S supply and inbred or hybrid lines influenced the concentration of 4-hydroxyglucobrassicin in seeds of oilseed rape. 4-Methoxyglucobrassicin accumulation was only significant among kale cultivar ($F = 31.09$, $P < 0.001$).

Glucosinolate content and accumulation in vegetable Brassicas appears to be determined by S fertility, cultivar or accession, harvest time and date, and growing season. Total GS and leaf S levels in vegetable turnip rape (*Brassica rapa* L.) increased in response to increasing S fertility from 0.5 to 2.0 mM in pot culture (*40*). Kushad et al. (*22*) reported significant differences in individual and total GS accumulation among 65 different cultivars and accessions of *B. oleracea*. Rosa et al. (*41*) observed significant diurnal differences in the GS content in the leaves of *B. oleracea* Acephala and Capitata Groups, the lowest concentrations of which occurred between 10:00 a.m. and 2:00 p.m. Significant differences in both individual and total GS among nine harvest dates and two growing seasons in *B. oleracea* and *B. napus* have also been reported (*42*). In one growing season, Kushad et al. (*22*) reported levels of sinigrin were 5−190 times higher than levels of glucobrassicin in field-grown Vates and Winterborne kale. In contrast, our results demonstrate glucobrassicin at concentrations much higher than

Table 2. Mean Glucosinolate (Milligrams per 100 g of Dry Mass) and Methylcysteine Sulfoxide (MCSO; Milligrams per Gram of Dry Mass) Content[a] of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

	glucosinolates						
mg of S/L	glucoiberin	sinigrin	glucobrassicin	neoglucobrassicin	4-methoxygluco-brassicin	4-hydroxygluco-brassicin	MCSO
				Winterbor			
4	nd[b]	2.1 ± 3.0	45.3 ± 7.8	9.2 ± 2.3	12.9 ± 2.9	3.9 ± 4.6	0.6 ± 0.7
8	5.6 ± 3.9	14.3 ± 8.6	104.4 ± 51.2	11.3 ± 4.4	9.9 ± 7.6	4.4 ± 4.4	2.0 ± 0.5
16	22.9 ± 8.5	22.5 ± 31.1	274.3 ± 92.9	21.8 ± 10.9	14.4 ± 2.9	6.1 ± 5.7	4.4 ± 1.1
32	25.6 ± 14.3	26.1 ± 15.6	302.4 ± 79.5	17.3 ± 9.9	13.4 ± 5.7	9.9 ± 4.1	4.9 ± 0.9
64	23.1 ± 4.9	29.8 ± 9.5	247.5 ± 78.8	11.6 ± 4.9	11.8 ± 4.6	7.3 ± 2.8	4.6 ± 1.0
contrasts							
linear	$P < 0.001$	$P = 0.013$	$P < 0.001$	ns[c]	ns[c]	$P = 0.087$	$P < 0.001$
quadratic	$P = 0.021$	ns[c]	$P = 0.016$	$P = 0.039$	ns[c]	ns[c]	$P = 0.006$
				Redbor			
4	nd[b]	nd[b]	39.6 ± 22.1	1.2 ± 1.4	16.1 ± 10.9	2.4 ± 3.7	nd[b]
8	nd[b]	0.2 ± 0.6	94.3 ± 63.8	3.0 ± 0.9	35.2 ± 15.8	4.2 ± 3.4	1.5 ± 0.4
16	2.7 ± 3.5	0.9 ± 1.1	232.3 ± 90.4	3.9 ± 1.9	36.3 ± 6.3	7.5 ± 2.8	2.0 ± 0.2
32	10.9 ± 9.5	3.1 ± 2.9	258.9 ± 89.9	5.1 ± 3.2	37.3 ± 5.4	8.0 ± 4.8	2.0 ± 0.1
64	9.7 ± 10.3	3.3 ± 1.7	335.2 ± 268.4	4.6 ± 3.4	36.8 ± 10.8	7.1 ± 2.4	2.8 ± 0.7
contrasts							
linear	$P = 0.007$	$P = 0.001$	$P = 0.001$	$P = 0.022$	$P = 0.024$	$P = 0.009$	$P < 0.001$
quadratic	ns[c]	ns[c]	ns[c]	ns[c]	$P = 0.056$	ns[c]	$P = 0.032$
				Toscano			
4	nd[b]	nd[b]	56.54 ± 24.7	6.5 ± 1.7	24.6 ± 7.5	1.7 ± 2.2	2.5 ± 0.3
8	0.5 ± 0.8	nd[b]	127.9 ± 22.1	3.5 ± 2.5	31.2 ± 14.3	6.6 ± 6.3	3.3 ± 0.1
16	1.3 ± 2.5	nd[b]	256.8 ± 104.5	3.1 ± 2.7	28.2 ± 8.2	5.0 ± 0.9	5.3 ± 0.2
32	2.3 ± 3.1	nd[b]	261.5 ± 84.7	5.3 ± 4.6	29.8 ± 13.9	4.8 ± 3.2	4.6 ± 0.3
64	3.2 ± 4.0	nd[b]	208.1 ± 100.4	10.6 ± 7.7	25.6 ± 18.4	7.1 ± 2.4	5.0 ± 0.8
contrasts							
linear	$P = 0.042$	nd[b]	$P = 0.005$	ns[c]	ns[c]	ns[c]	$P < 0.001$
quadratic	ns[c]	nd[b]	$P = 0.017$	$P = 0.023$	ns[c]	ns[c]	$P = 0.006$

[a] Composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. [b] Nondetectable. [c] Nonsignificant.

that of sinigrin among all the S treatment levels among the kale cultivars (**Table 3**). Ciska et al. (*43*) reported levels of glucobrassicin 4 and 20 times higher than that of sinigrin in field-grown Srednio Wysoki Zielony kale in growing seasons 1 and 2, respectively. In that same study, sinigrin levels increased from 2.21 to 22.72 mg/100 g of dry mass and glucobrassicin levels increased from 43.44 to 92.13 mg/100 g of dry mass from season 1 to season 2 in response to the decreased rainfall and increased growing temperatures experienced in season 2 (*43*). Therefore, many environmental and cultural factors need to be considered in plant improvement strategies focusing on glucosinolate accumulation.

Methylcysteine sulfoxide was affected by decreasing S concentrations in nutrient solution ($F = 75.17$, $P < 0.001$), by cultivar ($F = 111.69$, $P < 0.001$), and by the interaction of S with cultivar ($F = 5.06$, $P = 0.002$). MCSO content decreased linearly in all the cultivars in response to decreasing S availability [MCSO = 0.01 + 1.09(trt) for Winterbor, MCSO = 0.16 + 0.61(trt) for Redbor, and MCSO = 2.24 + 0.64(trt) for Toscano] (**Table 2**). Decreases in levels of GS and MCSO compounds paralleled decreases in leaf tissue %S.

Cellular disruption in *Brassica* results in the release of a cysteine lyase enzyme (EC 4.4.1.6) and a subsequent α,β-elimination of the *S*-oxide from MCSO, giving rise to volatile and odorous thiosulfinates and low molecular weight disulfide compounds (*3*). The thiosulfinates give rise to creamy, sulfury, and cabbage-like flavors with a 0.1 ppm taste panel threshold level (*44*). Increases in MCSO levels in response to increasing S treatment levels would be expected to enhance the flavor attributes associated with consuming raw kale. Increasing S fertility has been reported to increase the total amount of S-substituted cysteine sulfoxides in onion bulbs (*Allium cepa* L.) and their flavor potential (*27*).

Carotenoid Compounds. Lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* pigments differed among kale cultivars only ($F = 39.6$ and $P < 0.0001$, $F = 21.4$ and $P < 0.0001$, $F = 32.9$ and $P < 0.0001$, and $F = 37.7$ and $P < 0.0001$, respectively). However, no responses to decreasing S concentrations in nutrient solutions were noted for any carotenoid (**Table 3**). Values found for lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* content were within previously reported ranges for field-grown kale (*23*). In previous research, sugar beet (*Beta vulgaris* L.) leaf chlorophyll content (milligrams per gram of fresh mass) showed no response to increasing S levels when supplied at concentrations of 24−48 mg of S/L in perlite media (*45*). In a similar study, sugar beet leaf chlorophyll and carotenoid accumulations did not respond to treatments with 32 and 96 mg of S/L (*34*).

Lutein is only one of two dietary carotenoids selectively deposited in the human retina and lens (*46*). In the retina, lutein is responsible for the yellow pigmentation termed macular pigment (MP; *47*). Macular pigment is postulated to participate in photoprotection, and diminished MP levels may be related to retinal damage (*48*, *49*). Increases in MP levels can be achieved through diet (*50*) and supplementation (*51*). However, studies have indicated that consumption of a variety of vegetables providing a mixture of carotenoids was more strongly associated with reduced eye disease and cancer risk than individual carotenoid supplements (*16*, *52*). Consumption of vegetable carotenoids may prove to be effective at delaying the

Table 3. Mean Pigment Content[a] (Milligrams per 100 GFW) of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

	pigment (mg/100 GFW)			
mg of S/L	lutein	β-carotene	chlorophyll *a*	chlorophyll *b*
		Winterbor		
4	10.1 ± 0.6	8.8 ± 1.5	214.1 ± 30.9	65.3 ± 9.3
8	10.1 ± 1.3	8.7 ± 0.9	233.1 ± 25.8	65.0 ± 8.3
16	9.6 ± 1.7	8.6 ± 1.2	220.8 ± 36.1	63.5 ± 6.9
32	10.1 ± 1.4	8.9 ± 0.6	221.5 ± 27.6	65.1 ± 4.9
64	10.1 ± 0.7	8.5 ± 0.6	223.1 ± 18.7	63.8 ± 6.6
contrasts				
linear	ns[b]	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]	ns[b]
		Redbor		
4	10.8 ± 1.7	9.8 ± 1.4	206.5 ± 44.2	72.3 ± 12.8
8	11.4 ± 1.8	9.7 ± 1.7	233.3 ± 41.5	75.3 ± 12.9
16	11.5 ± 2.2	10.4 ± 1.7	252.0 ± 48.9	80.6 ± 14.9
32	10.5 ± 1.7	8.7 ± 0.8	216.9 ± 26.6	68.9 ± 7.7
64	11.7 ± 2.4	9.6 ± 1.8	244.5 ± 34.1	78.4 ± 14.2
contrasts				
linear	ns[b]	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]	ns[b]
		Toscano		
4	16.1 ± 2.9	12.8 ± 3.3	328.4 ± 74.1	105.7 ± 27.5
8	16.2 ± 3.3	12.0 ± 1.5	311.1 ± 49.7	98.8 ± 13.2
16	16.9 ± 2.6	13.6 ± 3.4	376.8 ± 95.0	111.4 ± 20.3
32	14.0 ± 3.2	11.1 ± 3.4	292.6 ± 76.7	91.5 ± 20.1
64	14.9 ± 1.6	11.8 ± 2.3	317.3 ± 63.1	101.3 ± 23.8
contrasts				
linear	ns[b]	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]	ns[b]

[a] Composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. [b] Nonsignificant.

normal effects of aging on retinal function and, in some cases, the progression of retinal changes resulting in age-related macular degeneration. β-Carotene is biologically cleaved to produce retinol (vitamin A), required for vision, epithelia maintenance, secretion of mucus, and reproduction (*53*). Lower S fertility in kale production would provide more palatable raw produce, while still providing beneficial dietary lutein and β-carotene.

Consumers often cite the bitter, astringent flavor of raw *Brassica* vegetables as unpleasant or objectionable (*8*, *54*). To foster consumer acceptance, it is important to consider the sensory response to foods when developing strategies aimed at improving dietary quality (*54*). The lower S treatment levels in this study reduced GC and MCSO content in kale which should weaken the bitter and unpleasant flavors associated with eating raw *Brassica*. Lowering the levels of GS compounds in kale, however, would be expected to decrease the health benefits associated with the isothiocyanates produced from GS decomposition. Lowering S fertility in this study did not significantly reduce lutein and β-carotene levels, thereby preserving the health benefits of carotenoid consumption. These results suggest lowering S fertility in kale production will provide more palatable raw produce, with enhanced Ca and Mg content, but will not affect the levels of beneficial dietary lutein and β-carotene. Understanding the combined impact of fertility on flavor compounds, carotenoid pigments, and elemental content may help improve consumer acceptance of phytonutritionally enhanced vegetable crops.

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