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Influence of Nitrogen and Sulfur on Biomass Production and Carotenoid and Glucosinolate Concentrations in Watercress (*Nasturtium officinale* R. Br.)

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Watercress (*Nasturtium officinale* R. Br.) is a perennial herb rich in the secondary metabolites of glucosinolates and carotenoids. 2-Phenethyl isothiocyanate, the predominate isothiocyanate hydrolysis product in watercress, can reduce carcinogen activation through inhibition of phase I enzymes and induction of phase II enzymes. Sulfur (S) and nitrogen (N) have been shown to influence concentrations of both glucosinolates and carotenoids in a variety of vegetable crops. Our research objectives were to determine how several levels of N and S fertility interact to affect watercress plant tissue biomass production, tissue C/N ratios, concentrations of plant pigments, and glucosinolate concentrations. Watercress was grown using nutrient solution culture under a three by three factorial arrangement, with three S (8, 16, and 32 mg/L) and three N (6, 56, and 106 mg/L) fertility concentrations. Watercress shoot tissue biomass, tissue %N, and tissue C/N ratios were influenced by N but were unaffected by changes in S concentrations or by the interaction of N × S. Tissue pigment concentrations of β-carotene, lutein, 5,6-epoxylutein, neoxanthin, zeaxanthin, and the chlorophyll pigments responded to changes in N treatment concentrations but were unaffected by S concentrations or through N × S interactions. Watercress tissue concentrations of aromatic, indole, and total glucosinolate concentrations responded to changes in N treatments; whereas aliphatic, aromatic, and total glucosinolates responded to changes in S treatment concentrations. Individual glucosinolates of glucobrassicin, 4-methoxyglucobrassicin, and gluconasturriin responded to N fertility treatments, while gluconapin, glucobrassicin, and gluconasturiin responded to changes in S fertility concentrations. Increases in carotenoid and glucosinolate concentrations through fertility management would be expected to influence the nutritional value of watercress in human diets.

KEYWORDS: β-Carotene; gluconasturtiin; HPLC; human health; lutein; zeaxanthin

INTRODUCTION

Watercress (*Nasturtium officinale* R. Br.) is a perennial herb found growing in natural waterways in many parts of Europe and the Americas. It is commercially produced in annual production systems as a raw salad crop either in natural water systems or in greenhouse hydroponic culture. Watercress is rich in the glucosinolate compound gluconasturtiin (2-phenethyl glucosinolate). Upon cellular disruption (chewing or chopping), gluconasturtiin is hydrolyzed by the enzyme myrosinase (β-thioglucoside glucohydrolase; EC 3.2.3.1), or by intestinal microbial thioglucosidases to produce 2-phenethyl isothiocyanate (*1, 2*).

2-Phenethyl isothiocyanate, the predominate glucosinolate hydrolysis product in watercress, has received attention for its role in the reduction of carcinogen activation through inhibition of phase I enzymes (such as cytochrome P450s) and its potential to induce phase II enzymes (*3, 4*). Most notably, 2-phenethyl isothiocyanate can effectively inhibit tumorigenesis by increasing metabolism and enhancing excretion of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, as demonstrated in both animal and human studies (*5–7*). When human subjects consumed 30 g of raw watercress containing 21.6 mg gluconasturtiin, in vivo urinary excretion studies revealed the potential conversion to 2.3–5.0 mg of 2-phenethyl isothiocyanate (*8*).

Carotenoids are lipid-soluble pigments found in all photosynthetic organisms. They are divided into oxygenated xanthophylls, such as lutein, zeaxanthin, and violaxanthin, and hydrocarbon carotenes, such as β-carotene, α-carotene, and lycopene (*9*). There are over 600 carotenoids found in nature, with 40 dietary carotenoids regularly consumed in the human diet (*10*). Pro-vitamin A activity is the classical biological function of carotenoids in mammalian systems. Health benefits attributed to carotenoids

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include prevention of certain cancers (*11–13*), cardiovascular diseases (*14*), and aging-eye diseases (*15, 16*), as well as enhanced immune system functions (*17, 18*). Watercress can be an excellent source of dietary carotenoids and concentrations can range from 5.8 to 10.7 mg lutein/100 g fresh mass (FM) and from 2.8 to 5.9 mg β-carotene/100 g FM (*19–21*).

The growing environment can have dramatic influences on secondary metabolite production in vegetable crops. Previously, watercress gluconasturtiin concentrations increased under conditions of lower temperatures, longer photoperiods (16 h), and exposure to red light (*22*). Similarly, after endogenous gluconasturiin hydrolysis, watercress 2-phenethyl isothiocyanate concentrations were found to increase under longer photoperiods (12 h) and higher light intensity (*23*). However, Palaniswamy et al. (*23*) reported higher 2-phenethyl isothiocyanate watercress concentrations, after endogenous gluconasturiin hydrolysis, at higher growing temperatures under a 12-h photoperiod. As sulfur-based compounds, glucosinolate concentrations are influenced by sulfur (S) fertility levels (*24–27*). Increasing S concentrations in hydroponic culture can increase glucosinolate compounds in kale (*Brassica oleracea* L. Acephala Group) and 2-phenethyl isothiocyanate concentrations in watercress after endogenous gluconasturiin hydrolysis (*24, 28, 29*). Fluctuations in nutrient solution nitrogen/sulfur (N/S) ratios have increased 2-phenethyl isothiocyanate concentrations in watercress after endogenous gluconasturiin hydrolysis (*29*). However, increases in N and S fertility levels did not influence the concentrations of either individual or total glucosinolate in broccoli (*B. oleracea* L. var. Italica) or broccoli sprouts (*B. oleracea* L. var. Italica cv. Marathon) (*25, 26*).

While increasing the number of servings consumed can be one way to increase dietary phytonutrients, enhancement efforts to increase phytonutrient concentrations per serving could make a significant impact on health. Therefore, our research objectives were to determine how several levels of N and S fertility interact to affect watercress plant tissue biomass production, tissue C/N ratios, concentrations of plant pigments, and glucosinolate concentrations. Higher concentrations of glucosinolate and carotenoid compounds would be expected to increase watercress dietary impacts.

MATERIALS AND METHODS

Plant Culture for Watercress. Seeds of watercress (Johnny's Selected Seed, Winslow, ME) were sown into 2.5 × 2.5 cm growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a model E15 growth chamber (Conviron, Winnipeg, Manitoba) at 20 °C day/night under a 16 h photoperiod on 9 June 2006. The growing cubes were submerged in water and fertilized with a half-strength Hoagland's nutrient solution upon seedling emergence (*30*). At 16 days after planting (DAP), the plantlets were transferred to 11-L containers (Rubbermaid Inc., Wooster, OH) filled with 9 L of a nutrient solution (*30*). Each container held six individual plants placed into 2-cm round holes set at 11 × 9 cm spacing on the lid. A completely randomized design was replicated in each of four growth chambers. Treatments consisted of a three by three factorial arrangement, with three S and three N fertility concentrations. Sulfur treatments consisted of 8, 16, and 32 mg/L S, which were delivered by the addition of 62, 123, and 246 mg/L $MgSO_4 \cdot 7H_2O$, respectively. Magnesium (Mg) was balanced by the addition of 339, 288, and 186 mg/L $MgCl_2 \cdot 6H_2O$ to the 8, 16, and 32 mg/L S treatments, respectively. The N treatments consisted of 6, 56, and 106 mg/L N. The 6 mg/L N treatment was achieved by adding 24 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 14 mg/L KNO_3, and 13 mg/L $NH_4H_2PO_4$, the 56 mg/L N treatment was achieved by addition of 210 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 124 mg/L KNO_3, and 115 mg/L $NH_4H_2PO_4$, and the 106 mg/L N treatment was achieved by addition of 378 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 239 mg/L KNO_3, and 230 mg/L $NH_4H_2PO_4$. Potassium (K) and phosphorus (P) were balanced by addition of 387, 245, and 82 mg/L KH_2PO_4 to the 8, 16, and 32 mg/L S treatments, respectively. Calcium (Ca) was balanced by addition of 279, 177, and 59 mg/L $CaCl_2 \cdot 2H_2O$ to the 8, 16, and 32 mg/L S treatments, respectively. All solutions contained 5 mg/L Fe chelate (derived from sodium ferric ethylenediamine di-(*o*-hydroxyphenylacetate) from Sprint 138 (Becker Underwood, Inc., Ames, IA), 1.43 mg/L H_3BO_3, 0.90 mg/L $MnCl_2$ $4H_2O$, 0.04 mg/L $CuSO_4$ $7H_2O$, 0.11 mg/L $ZnSO_4$ $7H_2O$, and 0.02 mg/L Na_2MoO_4 $2H_2O$. Photosynthetically active radiation (PAR) was measured using a model QSO-ELEC light meter (Apogee Instruments; Logan, UT) at four locations on top of each container (without plants present) at the four corner plant holes and were averaged for each of nine containers. Irradiance inside each chamber was measured at the beginning and confirmed at the end of each replication and averaged 500 ± 100 μmol/m^2/s. Solutions were aerated via a model 25E133W222 air blower (Spencer; Winsor, CT) connected to air stones. Deionized water was added daily to maintain initial solution volumes in each container. Plants were harvested 28 DAP. At harvest, shoot and root tissues were collected from each containers (6 plants each), and fresh mass (FW) recorded. Shoot tissues were lyophilized for 48 h and stored at −80 °C prior to extractions and analysis.

Tissue %N, %C, and C/N Ratio Determination for Watercress. A 0.01-g sample of freeze-dried watercress shoot tissue was placed in a tin crucible and analyzed for total %N and %C using a FlashEA 1112 series NC Soil Analyzer (Thermo, Waltham, MA). Data generated was used to calculate the C/N ratio for each experimental sample.

Carotenoid Determination for Watercress. *Tissue Extraction.* Plant pigments were extracted from freeze-dried tissues according to Kopsell et al. (*31*) and analyzed according to Emenhiser et al. (*32*). A 0.10 g subsample was rehydrated with 0.8 mL of ultra pure H_2O at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl-β-8′-*apo*-carotenoate (Sigma Chemical Co., St. Louis, MO) was added to determine extraction efficiency. The addition of 2.5 mL of tetrahydrofuran (THF) stabilized with 25 mg/L of 2,6-di-*tert*-butyl-4-methoxyphenol (BHT) was performed after sample hydration. The sample was then homogenized in a Potter-Elvehjem tissue grinding tube (Kontes, Vineland, NJ) using ∼25 insertions with a pestle attached to a drill press set at 540 rpm. During homogenation, the tube was immersed in ice to dissipate heat. The tube was then placed into a clinical centrifuge for 3 min at 500g_n. The supernatant was removed, and the sample pellet was resuspended in 2 mL of THF and homogenized again with the same extraction technique. The procedure was repeated for a total of four extractions to obtain a colorless supernatant. The combined supernatants were reduced to 1.5 mL under a stream of nitrogen gas and were brought up to a final volume of 5 mL with methanol (MeOH). A 2 mL aliquot was filtered through a 0.2-μm Econofilter PTFE 25/20 polytetrafluoroethylene filter (Agilent Technologies, Wilmington, DE) using a 5-mL syringe prior to high-performance liquid chromatography (HPLC) analysis.

Carotenoid Liquid Chromatography Analysis. An Agilent 1100 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA) was used for pigment separation. Chromatographic separations were achieved using a 250 × 4.6 mm i.d., 5 μm analytical scale polymeric C_{30} reverse-phase column (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar carotenoid compounds. The column was equipped with a 10 × 4.0 mm i.d. guard cartridge and holder (ProntoSIL) and was maintained at 30 °C using a thermostated column compartment. All separations were achieved isocratically using a binary mobile phase of 11% methyl *tert*-butyl ethanol (MTBE), 88.9% MeOH, and 0.1% triethylamine (TEA) (v/v). The flow rate was 1.0 mL/min, with a run time of 53 min, followed by a 10 min equilibration prior to the next injection. Eluted compounds from a 20 μL injection loop were detected at 453 (carotenoids and internal standard), 652 [chlorophyll *a* (Chl *a*)], and 665 [chlorophyll *b* (Chl *b*)] nm, and the data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Internal standard % recovery ranged from 62 to 100%, with a mean for all samples at 80%. Peak assignment for individual pigments was performed by comparison of retention times and line spectra obtained from photodiode array detection using external standards of antheraxanthin, neoxanthin, 5,6-epoxylutein, lutein, vio-

laxanthin, and zeaxanthin (Carotenature, Lupsingen, Switzerland) and β-carotene, Chl *a*, Chl *b* (Sigma Chemical Co.). The concentration of the external pigment standards were determined spectrophotometrically using methods described by Davies and Köst (*33*). Slurried Spinach 2385 standard reference material (National Institute of Science and Technology, Gaithersburg, MD) was used for method validation.

Glucosinolate Determination for Watercress. *Tissue Extraction.* Glucosinalbin (4-hydroxybenzyl), gluconapin (3-butenyl), 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl), glucobrassicin (3-indolylmethyl), 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), and gluconasturtiin (2-phenethyl) were extracted from freeze-dried watercress leaf tissue. For glucosinolate analysis, 0.2 g of freeze-dried tissue sample was combined with 1 mL of benzyl glucosinolate solution (1 mM) as an internal standard, 2.0 mL of MeOH, and 0.1 mL of barium−lead acetate (0.6 M) in a 16 × 100-mm culture tube, and the mixture was shaken at 60 rpm for 1 h. Each tube was then centrifuged at 2000 g_n for 10 min. A 0.5 mL aliquot of supernatant was then added to a 1-mL column containing 0.3 mL DEAE Sephadex A-25 (Sigma-Aldrich, St. Louis, MO). The sample was desulfated by the procedure of Raney and McGregor (*34*).

Glucosinolate Liquid Chromatography Analysis. Extracted desulfoglucosinolates were separated with an HPLC unit with a photodiode array detector (1100 series, Agilent Technologies) using a reverse-phase 250 × 4.6 mm i.d., 5-μm Luna C_{18} column (Phenomenex, Inc., Torrance, CA, USA) at a wavelength of 230 nm. The column temperature was set at 40 °C, with a flow rate of 1 mL/min. The gradient elution parameters were 100% water for 1 min, followed by a 15 min linear gradient to 75% water/25% acetonitrile. Solvent levels were then held constant for 5 min, and returned to 100% water for the final final 5 min. Desulfoglucosinolates were identified by comparison with retention times of authentic standards or previously reported results (*35, 36*).

Statistical Analysis. Data was subjected to analysis of variance (ANOVA), regression, and contrast procedures to test the significance of main effects and possible interactions using SAS statistical software (JMP, version 6.0.0, SAS Institute, Cary, NC). Interactions among fertility treatments for dependent variables were not observed; therefore, interpretations and discussions center on the main effects. Orthogonal polynomials were used to study changes associated with increases in either N or S fertility concentrations by partitioning the sum of squares into components associated with linear or quadratic terms.

RESULTS

Watercress Tissue Biomass. Watercress shoot tissue biomass was influenced by N ($F = 631.8$; $P \leq 0.001$) but was unaffected by changes in S concentrations or by the interaction of N × S. Average watercress shoot biomass ranged from 4.5 to 38.6 g/plant. Watercress root tissue biomass was influenced by N ($F = 34.6$; $P \leq 0.001$) but was unaffected by changes in S concentrations or by the interaction of N × S. Average watercress root biomass ranged from 3.1 to 5.6 g/plant. Shoot FM increased linearly [shoot FM = 4.53 + 0.34 (trt)] in response to increasing N concentrations in nutrient solutions. Root FM increased linearly [root FM = 3.52 + 0.02 (trt)] in response to increasing N concentrations in nutrient solutions (**Table 1**).

Watercress %N, %C, and C/N Ratio. Shoot tissue %N was influenced by N treatment concentrations ($F = 114.1$; $P \leq 0.001$) but was unaffected by S concentrations or through N × S interactions. Average shoot tissue %N ranged from 1.1 to 4.8% for the N treatments of 6 and 106 mg N/L, respectively. Watercress shoot tissue %N increased linearly [tissue %N = 0.97 + 0.04 (trt)] in response to increasing N treatment concentrations (**Table 2**). The main effect of N treatment concentrations influenced watercress biomass production; therefore, we examined shoot tissue %C and C/N ratios among the main effect treatments. Shoot tissue %C was not influenced by the experimental treatments. The C/N ratio in watercress shoot tissues was influenced by N treatments ($F = 888.4$; $P \leq 0.001$) but was unaffected by S concentrations or through N × S interactions. The C/N ratio of watercress tissues decreased in a linear trend [C/N ratio = 34.51−0.28 (trt)] in response to increasing N concentrations in nutrient solutions (**Table 2**).

Table 1. Mean Biomass[a] for Nitrogen and Sulfur Fertility Treatments for Watercress Grown in Nutrient Solutions

treatment (mg/L)	tissue biomass (g)	
	shoot fresh mass	root fresh mass
	Nitrogen	
6	4.55 ± 0.36	3.07 ± 0.27
56	27.64 ± 2.03	6.14 ± 1.20
106	38.60 ± 3.41	5.57 ± 0.96
linear contrast	$P \leq 0.001$	$P \leq 0.001$
	Sulfur	
8	23.76 ± 15.50	4.97 ± 1.62
16	24.30 ± 15.39	5.03 ± 1.50
32	22.75 ± 14.49	4.76 ± 1.83
linear contrast	ns[b]	ns[b]

[a] Values are for six plants per replication ± standard deviation. [b] ns = not significant.

Table 2. Mean Leaf Tissue Nitrogen (N), Carbon (C), and the C/N Ratio[a] on a Dry Mass basis for Nitrogen (N) and Sulfur (S) Fertility Treatments for Watercress Grown in Nutrient Solutions

treatment (mg/L)	tissue %N	tissue %C	C/N ratio
	Nitrogen		
6	1.07 ± 0.05	38.59 ± 1.43	36.38 ± 1.78
56	3.26 ± 0.49	39.22 ± 1.71	12.25 ± 1.62
106	4.75 ± 0.79	39.91 ± 0.77	7.97 ± 2.70
linear contrast	$P \leq 0.001$	ns[b]	$P \leq 0.001$
	Sulfur		
8	2.92 ± 1.61	39.05 ± 1.67	19.49 ± 13.06
16	3.29 ± 1.75	39.47 ± 0.74	17.65 ± 12.78
32	2.91 ± 1.54	39.63 ± 0.78	17.90 ± 13.22
linear contrast	ns[b]	ns[b]	ns[b]

[a] Values are for six plants per replication ± standard deviation. [b] ns = nonsignificant.

Watercress Carotenoid and Chlorophyll Compounds. The watercress shoot tissue carotenoid concentrations of β-carotene ($F = 14.5$; $P \leq 0.001$), lutein ($F = 87.6$; $P \leq 0.001$), 5,6-epoxylutein ($F = 16.9$; $P \leq 0.001$), neoxanthin ($F = 44.1$; $P \leq 0.001$), and zeaxanthin ($F = 31.5$; $P \leq 0.001$) all responded to changes in N treatment concentrations but were unaffected by S concentrations or through N × S interactions. Watercress tissue β-carotene ranged from 0.3 to 0.9 mg/100 g FM, lutein ranged from 2.4 to 9.5 mg/100 g FM, 5,6-epoxylutein ranged from 0.2 to 0.4 mg/100 g FM, neoxanthin ranged from 0.4 to 2.4 mg/100 g FM, and zeaxanthin ranged from 0.2 to 0.6 mg/100 g FM. Watercress tissue carotenoid pigments increased linearly [β-carotene = 0.29 + 0.01 (trt); lutein = 2.42 + 0.07 (trt); 5,6-epoxylutein = 0.24 + 0.01 (trt); neoxanthin = 0.44 + 0.02 (trt); zeaxanthin = 0.25 + 0.01 (trt)] in response to increasing N concentrations in nutrient solutions (**Table 3**). None of the carotenoids in the watercress tissue responded to S concentrations or the interaction of N × S. Shoot tissue concentrations of antheraxanthin and violaxanthin were not influenced by the experimental treatments (data not shown). Watercress tissue chlorophyll *a* concentrations ($F = 123.5$; $P \leq 0.001$) responded to changes in N treatments concentrations but not to changes in S treatments or by the interaction of N × S. Chlorophyll *a* concentrations in the watercress shoot tissues ranged from 10.6 to 70.5 mg/100 g FM. Tissue chlorophyll *b* concentrations were influenced by N treatments ($F = 127.5$; P

Table 3. Mean Carotenoid Pigment Concentrations[a] Expressed on a Fresh Mass Basis for Nitrogen and Sulfur Fertility Treatments for Watercress Grown in Nutrient Solutions

	pigment concentration (mg/100 g)				
treatment (mg/L)	β-carotene	lutein	5,6-epoxy lutein	neoxanthin	zeaxanthin
			Nitrogen		
6	0.33 ± 0.05	2.43 ± 0.40	0.23 ± 0.03	0.42 ± 0.15	0.23 ± 0.07
56	0.63 ± 0.28	7.38 ± 0.93	0.39 ± 0.08	1.80 ± 0.42	0.53 ± 0.09
106	0.93 ± 0.27	9.52 ± 1.41	0.42 ± 0.09	2.35 ± 0.49	0.60 ± 0.11
linear contrast	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$
			Sulfur		
8	0.53 ± 0.37	6.18 ± 3.01	0.34 ± 0.10	1.54 ± 0.88	0.41 ± 0.18
16	0.59 ± 0.24	6.26 ± 3.09	0.36 ± 0.12	1.55 ± 0.99	0.48 ± 0.20
32	0.71 ± 0.36	6.86 ± 3.59	0.35 ± 0.11	1.46 ± 0.92	0.48 ± 0.18
linear contrast	ns[b]	ns[b]	ns[b]	ns[b]	ns[b]

[a] Values are for six plants per replication ± standard deviation. [b] ns = nonsignificant.

Table 4. Mean Chlorophyll Pigment Concentrations[a] Expressed on a Fresh Mass Basis for Nitrogen and Sulfur Fertility Treatments for Watercress Grown in Nutrient Solutions

	pigment concentration (mg/100 g)			
treatment (mg/L)	chlorophyll *a*	chlorophyll *b*	total chlorophyll	chlorophyll *a*/*b* ratio
		Nitrogen		
6	10.64 ± 3.12	5.75 ± 1.29	16.39 ± 4.39	1.82 ± 0.21
56	50.03 ± 10.45	21.47 ± 4.02	71.49 ± 14.23	2.33 ± 0.18
106	70.45 ± 11.97	28.32 ± 4.37	98.77 ± 15.88	2.50 ± 0.23
linear contrast	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$
		Sulfur		
8	43.54 ± 27.68	18.28 ± 10.18	61.82 ± 37.71	2.23 ± 0.41
16	40.22 ± 24.58	17.44 ± 9.58	57.66 ± 34.09	2.15 ± 0.37
32	47.36 ± 29.67	19.81 ± 11.42	67.18 ± 41.02	2.26 ± 0.29
linear contrast	ns[b]	ns[b]	ns[b]	ns[b]

[a] Values are for six plants per replication ± standard deviation. [b] ns = nonsignificant.

Table 5. Mean Group Glucosinolate Concentrations[a] Expressed on a Dry Mass Basis for Nitrogen and Sulfur Fertility Treatments for Watercress Grown in Nutrient Solutions

treatment	glucosinolate concentration (μmol/g)			
(mg/L)	aliphatic	aromatic	indole	total
		Nitrogen		
6	0.08 ± 0.09	7.33 ± 2.76	0.78 ± 0.51	8.19 ± 2.85
56	0.09 ± 0.05	11.98 ± 3.38	0.50 ± 0.20	12.56 ± 3.58
106	0.04 ± 0.05	9.07 ± 5.91	0.33 ± 0.23	9.44 ± 6.12
linear contrast	ns[b]	ns[b]	$P = 0.003$	ns[b]
quadratic contrast	ns[b]	$P = 0.01$	ns[b]	$P = 0.05$
		Sulfur		
8	0.03 ± 0.04	7.52 ± 3.48	0.41 ± 0.27	7.96 ± 3.61
16	0.09 ± 0.09	9.30 ± 4.87	0.65 ± 0.57	10.03 ± 4.98
32	0.09 ± 0.04	11.55 ± 4.61	0.55 ± 0.16	12.19 ± 4.67
linear contrast	$P = 0.04$	$P = 0.03$	ns[b]	$P = 0.03$
quadratic contrast	ns[b]	ns[b]	ns[b]	ns[b]

[a] Values are for six plants per replication ± standard deviation. [b] ns = Nonsignificant.

≤ 0.001), but not S or the interaction of N × S. Chlorophyll *b* concentrations in the watercress shoot tissues ranged from 5.8 to 28.3 mg/100 g FM. Total chlorophyll concentrations in the watercress tissues were influenced by N treatments ($F = 130.5$; $P \leq 0.001$), but not S or the interaction of N × S. Concentrations of watercress total chlorophyll ranged from 16.4 to 98.8 mg/100 g FM. The chlorophyll *a*/*b* ratio in the watercress tissues was influenced by N treatments ($F = 8.03$; $P \leq 0.001$), but not S or the interaction of N × S. Watercress tissue chlorophyll pigments increased linearly [chlorophyll $a = 10.21 + 0.59$ (trt); chlorophyll $b = 5.87 + 0.23$ (trt); total chlorophyll $= 16.08 + 0.83$ (trt); *a*/*b* ratio $= 1.84 + 0.01$ (trt)] in response to increasing N concentrations in nutrient solutions (**Table 4**).

Watercress Glucosinolate Compounds. Watercress tissue aromatic glucosinolates responded to changes in N ($F = 4.08$; $P = 0.03$) and S ($F = 3.01$; $P = 0.06$) treatment concentrations, but not to N × S interactions. Indole glucosinolates responded to changes in N ($F = 5.22$; $P = 0.01$) treatment concentrations, but not to S or N × S interactions. Total glucosinolate concentrations responded to changes in N ($F = 3.46$; $P = 0.04$) and S ($F = 3.05$; $P = 0.06$) treatment concentrations, but not to N × S interactions. Concentrations of indole glucosinolates increased linearly [indole glucosinolates $= 0.78 + 0.01$ (trt)] in response to increases in N treatment concentrations in nutrient solutions, while concentrations of aromatic and total glucosinolates increased, then decreased [aromatic glucosinolates $= 11.09 + 0.02$ (trt) $- 0.001$ (trt^2); total glucosinolates $= 11.86 + 0.01$ (trt) $- 0.001$ (trt^2)] in response to increases in N treatment concentrations in nutrient solutions (**Table 5**). Watercress tissue concentrations of aliphatic glucosinolates were influenced by S ($F = 4.33$; $P = 0.02$), but not N or the interaction of N × S. Aliphatic glucosinolates increased linearly [aliphatic glucosinolates $= 0.03 + 0.01$ (trt)] in response to increasing S concentrations in nutrient solutions (**Table 5**). Watercress gluconasturtiin concentrations were responsive to N ($F = 3.86$; $P = 0.03$) and S ($F = 2.25$, $P = 0.05$) treatment concentrations, but not to N × S interactions. 4-methoxyglucobrassicin responded to N ($F = 57.61$; $P \leq 0.001$) treatments, but did not respond to S and N × S treatments. Glucobrassicin concentrations were responsive to N ($F = 3.44$; $P = 0.05$) and S ($F = 2.08$, $P = 0.07$) treatment concentrations, but not to N × S interactions. Concentrations of 4-methoxyglucobrassicin glucosinolates increased linearly [4-methoxyglucobrassicin $= 0.20 + 0.002$ (trt)] in response to increases in N treatment concentrations in nutrient solutions, while concentrations of gluconasturiin [gluconasturiin $= 10.02 + 0.02$ (trt) $- 0.001$ (trt^2)] and glucobrassicin [glucobrassicin $= 0.49 + 0.001$ (trt) $- 0.001$ (trt^2)] glucosinolates increased, then decreased in response to increases in N treatment concentrations in nutrient solutions. Glucobrassicin [glucobrassicin $= 0.23 + 0.007$ (trt)] and gluconasturiin [gluconasturiin $= 5.40 + 0.16$ (trt)] increased linearly in response to increasing S treatment concentrations (**Table 6**). Gluconapin concentrations responded to S ($F = 4.33$;

Table 6. Mean Individual Glucosinolate Concentrations[a] Expressed on a Dry Mass Basis for Nitrogen and Sulfur Fertility Treatments for Watercress Grown in Nutrient Solutions

	glucosinolate concentration (μmol/g)					
treatment (mg/L)	glucosinalbin	gluconapin	4-hydroxyl-glucobrassicin	glucobrassicin	4-methoxy-glucobrassicin	gluconasturiin
			Nitrogen			
6	0.82 ± 0.44	0.08 ± 0.09	0.19 ± 0.47	0.37 ± 0.11	0.22 ± 0.05	6.51 ± 2.54
56	1.11 ± 0.24	0.09 ± 0.05	nd[b]	0.44 ± 0.18	0.05 ± 0.03	10.87 ± 3.23
106	1.05 ± 0.20	0.04 ± 0.05	0.02 ± 0.08	0.27 ± 0.21	0.04 ± 0.05	8.02 ± 5.90
linear contrast	ns[c]	ns[c]	ns[c]	ns[c]	$P \leq 0.001$	ns[c]
quadratic contrast	ns[c]	ns[c]	ns[c]	$P = 0.05$	ns[c]	$P = 0.02$
			Sulfur			
8	1.05 ± 0.26	0.03 ± 0.04	0.04 ± 0.13	0.27 ± 0.17	0.10 ± 0.09	6.47 ± 3.38
16	0.91 ± 0.49	0.09 ± 0.09	0.37 ± 0.21	0.37 ± 0.21	0.11 ± 0.10	8.39 ± 4.60
32	1.02 ± 0.16	0.09 ± 0.04	0.44 ± 0.13	0.44 ± 0.13	0.10 ± 0.10	10.53 ± 4.55
linear contrast	ns[c]	$P = 0.04$	ns[c]	$P = 0.03$	ns[c]	$P = 0.02$
quadratic contrast	ns[c]	ns[c]	ns[c]	ns[c]	ns[c]	ns[c]

[a] Values are for six plants per replication ± standard deviation. [b] nd = nondetectable. [c] ns = nonsignificant.

$P \leq 0.02$) treatments; however, N treatments and N × S interactions had no effect. Gluconapin glucosinolates increased linearly [gluconapin = 0.03 + 0.02 (trt)] in response to increasing S concentrations in nutrient solutions (**Table 6**). Individual glucosinolates concentrations of glucosinalbin and 4-hydroxyglucobrassicin were not influenced by experimental treatments (**Table 6**).

DISCUSSION

Increases in N concentrations in nutrient solutions increased shoot and root tissue biomass production in watercress. Biomass increases resulting from increases in N concentrations could be expected because biomass production in the herbal crop parsley (*Petroselinum crispum* Nym.) and in several kale cultivars increased under similar nutrient solution N treatment concentrations (*37, 38*). Watercress biomass did not respond to increases in S fertility. Similarly, kale biomass accumulations were unaffected by increases in nutrient solution S concentrations (*24*).

Since watercress tissue biomass responded to increases in N concentrations, we investigated possible treatment influences on tissue %N, %C, and the C/N ratio. Tissue %N in the watercress increased significantly with increasing solution culture N concentrations. Similar results occurred with parsley (*37*). Watercress %C in shoot tissues was unaffected by N treatment concentrations. Therefore, C/N ratios in watercress shoot tissues significantly decreased with increasing N treatment concentrations. These findings are in agreement with those reported for leave tissue of young oaks (*Quercus petraea* L. and *Q. robur* L.) grown under increasing N concentrations (*39*).

The influences of N on plant growth parameters are well understood; however, knowledge of the influence of N fertility on protein, vitamin, and phytonutrient contents in crop plants is limited and often contradictory. In the current study, increasing N treatment concentrations significantly increased watercress tissue β-carotene, lutein, 5,6-epoxylutein, neoxanthin, and zeaxanthin. In a past review, Mozafar (*40*) concluded that increasing rates of N fertilizers resulted in the decrease of vitamins and phytonutrients, while often increasing tissue NO_3-N levels. Several early studies demonstrate increases in carotene carotenoid content under increasing N fertilization for members of the Brassicaceae and Chenopodiaceae (*41–43*). More recently, linear increases in lutein/zeaxanthin and β-carotene were observed in parsley grown under increasing N concentrations in nutrient solution culture (*37*). Tissue carotenoid concentrations generally increased with increasing N concentration rates for carrot (*Daucus carota* L.) (*44, 45*). However, increasing N fertility rates were reported to decrease carotene content in carrots (*46*) or to have no effect on the accumulation of carotenoid pigments in kale (*38*). The response in carotenoid phytonutrients to N fertility appears to be species specific.

Nitrogen is essential for chlorophyll pigment production, and deficiencies of this macronutrient manifest as chlorosis in leaf tissues. In the current study, increasing N fertility significantly increased watercress tissue chlorophyll pigments and chlorophyll *a*/*b* ratios. Similarities in behavior of carotenoids and chlorophylls are reported for different crop species. Chlorophyll pigments were found to correlate highly with carotenoid levels in the leaves of tobacco (*Nicotiana tabacum* L.) (*47*), Swiss chard (*Beta vulgaris* L.) (*48*), several *B. oleracea* cultigens (*31*), and lettuce (*Lactuca sativa* L.) genotypes (*49*). Sulfur fertility did not influence chlorophyll concentrations for watercress (**Table 4**); on the other hand, a comparison between 0 and 1000 kg S/ha revealed significant increases in total chlorophyll in the leaves of two Italian ecotypes of *B. rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort (*50*). However, changes in chlorophyll production may be expected when deficient (0 kg/ha) and luxuriant (1000 kg/ha) S treatments are compared. Genomic analysis has revealed signaling functions among chlorophyll biosynthetic pathway intermediate compounds which regulate transcriptional production of light-harvesting chlorophyll-binding proteins (carotene and xanthophyll carotenoids) (*51*). Further, the biosynthesis of chlorophyll molecules appears to be linked to the occurrence and production of light-harvesting complex polypeptides (*52*). Together, genomic and analytical data demonstrate the close connections between chlorophyll and carotenoid biosynthetic pathways.

Glucosinolates are S-rich, anionic natural products that upon hydrolysis by an endogenous thioglucosidase, called myrosinase, produce isothiocyanates, thiocyanates, and nitriles. The identity of the side chains of glucosinolates, which include aliphatic, aromatic, or heteroaromatic groups, determine the nature of the isothiocyanate, thiocyanate, or nitrile formed (*53*). Glucosinolate concentrations and their hydrolysis products can change in response to S fertility levels in plants that synthesize these compounds (*24–27*). Previously, decreasing N/S ratios (increasing S proportions) in nutrient solutions increased 2-phenethyl isothiocyanate concentrations in watercress after endogenous gluconasturiin hydrolysis (*29*). In broccoli and broccoli sprouts, increases in N and S fertility levels did not influence the concentrations of either individual or total glucosinolate (*25, 26*). The concentration of glucosinolate compounds significantly

increased in kale as nutrient solution S treatment concentrations increased from 4 to 64 mg/L (*24*). In the current study, S treatments increased aliphatic, aromatic, and total glucosinolates (**Table 5**). Increases in S treatment concentrations also increased the individual glucosinolates of gluconapin, glucobrassicin, and gluconasturiin (**Table 6**). Increases in N treatment concentrations significantly influenced aromatic, indole, and total glucosinolate concentrations, as well as influencing the concentrations of glucobrassicin, 4-methoxyglucobrassicin, and gluconaturiin in the watercress plants (**Tables 5** and **6**).

In the current study, we examined the influence of N and S fertility on growth parameters, pigment accumulation, and glucosinolate production in the perennial herb watercress. The results from this experiment indicate that the main effect of N fertility influenced biomass production, C/N ratios, carotenoid and chlorophyll pigment accumulations, aromatic glucosinolates, indole glucosinolates, total glucosinolates, and several individual glucosinolate compounds. Increases in S fertility increased aliphatic glucosinolates, aromatic glucosinolates, and total glucosinolates. Sulfur fertility also influenced watercress gluconapin, glucobrassicin, 4-methoxyglucobrassicin, and gluconasturiin concentrations. Previous studies have concluded that a proper balance between N and S will maximize glucosinolate concentrations in *Brassica* species (*25, 26*). Increases in carotenoid and glucosinolate concentrations through fertility management would be expected to influence the nutritional value of watercress in human diets.

ABBREVIATIONS USED

ANOVA, analysis of variance; BHT, 2,6-di-*tert*-butyl-4-methoxyphenol; DAP, days after planting; DM, dry mass; ETOH, ethanol; FM, fresh mass; HPLC, high-performance liquid chromatography; MeOH, methanol; TEA, triethylamine; THF, tetrahydrofuran.

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