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Isothiocyanate Concentration in Kohlrabi (*Brassica oleracea* L. Var. *gongylodes*) Plants As Influenced by Sulfur and Nitrogen Supply

JÓSKA GERENDÁS,[†] STEPHANIE BREUNING,[‡] THORSTEN STAHL,[§,||] VOLKER MERSCH-SUNDERMANN,[§,⊥] AND KARL H. MÜHLING*[,†,‡]

Institute for Plant Nutrition and Soil Science, Christian-Albrechts-University, D-24098 Kiel, Germany, and Institute for Plant Nutrition and Institute for Indoor and Environmental Toxicology, Justus-Liebig-University, D-35392 Giessen, Germany

Glucosinolates (GSss) represent bioactive compounds of *Brassica* vegetables whose health-promoting effects merely stem from their breakdown products, particularly the isothiocyanates (ITCs), released after hydrolysis of GSs by myrosinase. GSs are occasionally discussed as transient S reservoirs, but little is known concerning the interactive effect of S and N supply on ITC concentrations. Therefore, kohlrabi plants were grown in a pot experiment with varied S (0.00, 0.05, and 0.20 g pot^{-1}) and N (1, 2, and 4 g pot^{-1}) supplies. Plant growth exhibited a classical nutrient response curve with respect to both S and N. The ITC profile of kohlrabi tubers was dominated by methylthiobutyl ITC (11−1350 μmol (g DM)$^{-1}$), followed by sulforaphan (7−120 μmol (g DM)$^{-1}$), phenylethyl ITC (5−34 μmol (g DM)$^{-1}$), and allyl ITC (5−38 μmol (g DM)$^{-1}$), resulting from the hydrolysis of glucoerucin, glucoraphanin, gluconasturtiin, and sinigrin, respectively. The ITC profile was in agreement with reported data, and concentrations of all ITCs were substantially reduced in response to increasing N and decreasing S supply. A growth-induced dilution effect could be ruled out in most cases, and the results do not support the hypothesis that GS acts as transient reservoir with respect to S.

KEYWORDS: Fertilization; vegetable quality; sulforaphane; glucosinolates; myrosinase; β-glucosidase; N/S ratio; allyl isothiocyanate; methylthiobutyl isothiocyanate; phenylethyl isothiocyanate

INTRODUCTION

The beneficial health effects of frequent vegetable consumption are well-accepted. These are not merely related to primary nutrients but to secondary compounds, also called phytochemicals. Members of the Brassicaceae family, which include many important vegetable crops such as cabbage, kale, Brussels sprouts, cauliflower, broccoli, and kohlrabi, contain very potent phytochemicals, glucosinolates, and their breakdown products (*1*). Glucosinolates (GSs) bear a uniform structure, consisting of a thioglucose unit, a sulfonated oxime unit, and a variable side chain (**Figure 1**). In Brassicaceae, the side chain is derived from methionine, phenylalanine, or tyrosine and tryptophan in the case of aliphatic, aromatic, and indol GS, respectively (*1, 2*). Upon liberation of glucose by the action of myrosinase, a β-glucosidase, intramolecular rearrangements yield isothiocyanates (R−N═C═S), nitriles (R═C≡N), and epithionitriles depending on the GSs under consideration and the chemical environment (e.g., pH, Fe^{2+} concentration, and epithio-specific protein) (*2, 3*). Myrosinase and its substrates are well-compartmented in the intact plant, but rupture of the plant tissue by slicing, cutting, and chewing initiates an intense reaction. As these breakdown products deliver pungency and a strong smell, the plant biological significance of GSs merely has been discussed as a repellent (*3*) and in relation to disease defense (*2*). In animal and human nutrition, GSs and their breakdown products initially have been considered as antinutritive factors (reduced feed intake or antithyroid activity) (*1, 4, 5*), even though recent studies show that in human nutrition, critical intake rates hardly are reached (*2*). More importantly, recent investigations indicate that these breakdown products are responsible for several beneficial health effects (*1, 2*), of which the anticarcinogenic action is best documented (*6*), in addition to their well-known contribution to the typical smell and flavor of *Brassica* vegetables (*7, 8*). It is thus advisable to increase the concentration of these phytochemicals in the human diet by changing consumer behavior, plant breeding, and plant cultivation conditions, including fertilization.

* Corresponding author. E-mail: khmuehling@plantnutrition.uni-kiel.de.
[†] Christian-Albrechts-University.
[‡] Institute for Plant Nutrition, Justus-Liebig-University.
[§] Institute for Indoor and Environmental Toxicology, Justus-Liebig-University.
[||] Present address: Landesbetrieb Hessisches Landeslabor, Glasrusstrasse 6, D-65203 Wiesbaden, Germany.
[⊥] Present address: Institute of Environmental Medicine and Hospital Epidemiology, Freiburg University Hospital, Breisacher Strasse 115 B, 79106 Freiburg, Germany.

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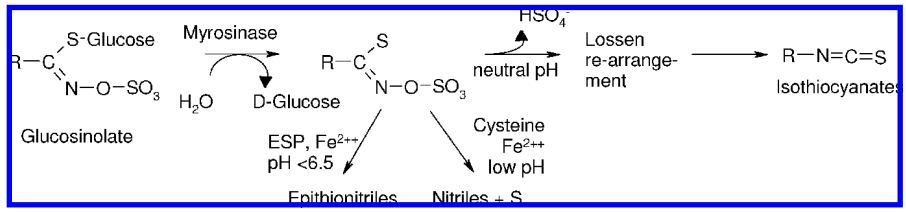

Figure 1. General structure of glucosinolates and scheme of their breakdown (ESP: epithiospecific protein).

As the side chains of GSs are derived from partly S-containing amino acids, the thiol bridge to glucose from cysteine, and the sulfate group from phospho-adenosin-phosphosulfate (*2*), a strong interaction of the N and S supply on contents of GSs can be envisaged (*9–11*). More so, as GSs have been discussed as a transient storage pool for S (*3, 12*), enhanced breakdown of GSs under conditions of S deficiency, resulting in the enhanced liberation of their breakdown products, also can be anticipated. Apparently, no studies have been reported on the interactive influence of N and S nutrition on the concentration of the pharmacologically most important breakdown products, isothiocyanates (ITCs), and the activity of myrosinase, the enzyme responsible for their liberation from GSs. In kohlrabi, an important *Brassica* vegetable worldwide, the most important ITCs include allyl ITC, methylthiobutyl ITC (MTB ITC), phenylethyl ITC, and sulforaphan, the latter being considered as one of the 40 most potent anticarcinogens (*13*). It is hypothesized that a reduced S supply will result in increased ITC concentrations due to enhanced breakdown of GSs, and this effect may be fostered by increasing the N supply, due to stimulated growth and protein synthesis, enhancing the competition for S-containing amino acids, their common substrate.

MATERIALS AND METHODS

Experimental Conditions. Experiments were conducted in Mitscherlich pots filled with 6 kg of nutrient-poor soil specified as follows: mass fractions of clay, silt, and sand were 0.207, 0.338, and 0.455, respectively, and total contents of C, N, and S were 2.37, 0.42, and 0.05 mg kg^{-1}, respectively. The soil contained 49.05 mg of available K kg^{-1} and 0.03 mg of available P kg^{-1} (*14*), and contents of DTPA-extractable trace nutrients (*15*) were 0.86 mg of Cu kg^{-1}, 13.58 mg of Mn kg^{-1}, 35.22 mg of Fe kg^{-1}, and 0.576 mg of Zn kg^{-1}. The soil pH ($CaCl_2$) was 5.8. Each pot received a basal dressing of 3.43 g of K, 1 g of Mg, and 5 g of P as KCl, $MgCO_3$, and $CaHPO_4$, respectively. Seeds of kohlrabi (*Brassica oleracea* L. var. *gongylodes* cv. 'Lanro') were sown directly into the soil on April 26, 2005. The experimental treatments consisted of a two-factorial, orthogonal design with three levels of N (1, 2, and 4 g pot^{-1}) and three levels of S (0, 0.05, and 0.2 g pot^{-1}) in six replications. N and S were administered as NH_4NO_4 and $CaSO_4 \cdot 2H_2O$, respectively. N application was split to avoid damage. The 1 g of N treatment received 0.25, 0.25, 0, and 0.5 g of N at seeding and on May 2, June 3, and June 13, 2005, respectively. Corresponding numbers for the 2 g of N and 4 g of N treatments were 1, 0.25, 0.5, and 0.5 and 2.5, 0.25, 0.5, and 0.5 g of N, respectively. Pots were kept in the open and arranged in a completely randomized design.

Harvesting and Determination of Growth Parameters. After about 8 weeks of growth, plants were harvested on June 23, 2005. Plants were separated into upper and lower leaves and the tuber, rinsed with distilled water, and weighed. Tubers were halved, one part being quickly chopped, frozen in liquid N_2, and kept at −80 °C. Later on, tuber pieces were powdered under liquid N_2 and stored at −20 °C until analysis. The other half of the tubers (after being chopped) and the leaf samples were dried to a constant weight at 80 °C and subsequently ground to pass through a 1.5 mm sieve.

Analytical Procedures. *Nutrient Analysis.* Concentrations of total N and S were determined using an automated elemental analyzer (MAX CNS, Elementar Analysensysteme GmbH, Hanau, Germany). For determination of anions (NO_3^- and SO_4^{2-}), hot water extracts were prepared by suspending 200 mg of ground plant material in 30 mL of distilled water in 50 mL volumetric flasks, which were kept in a boiling water bath with gentle agitation. The material was resuspended after 1.5 h, and after another 1.5 h, the flasks were cooled on ice. Having reached room temperature, the extracts were brought to volume and filtered (fluted filter 5951/2, Schleicher and Schüll, Dassel, Germany). Sample cleanup was accomplished using C_{18} cartridges (Bakerbond, Baker Inc., Gross-Gerau, Germany), before being analyzed by anion exchange chromatography (Metrohm Compact IC, Metrohm GmbH and Co., Filderstadt, Germany). Analytes were separated on a Metrosep A-Sup 4 column (250 mm × 4 mm), employing isocratic elution with 4 mM $NaHCO_3$ and 1 mM $NaCO_3$ at a flow rate of 1 mL min^{-1}, and were detected by conductivity after chemical suppression of background conductivity.

Ascorbic Acid. Ascorbic acid was analyzed following a protocol originally proposed for blood plasma (*16*), which was adapted for plant tissues (*17*). Exactly half a gram of frozen plant material was extracted with 4.5 mL of 150 mM NaK-phosphate buffer (prepared using appropriate amounts of KH_2PO_4 and Na_2HPO_4 to give a pH of 7.5). Aliquots of the extracts were mixed with 10% trichloroacetic acid in a 2:1 ratio to precipitate proteins and filtered. Ascorbic acid was determined by its reducing action on Fe^{3+} in the presence of 2,2′-bipyridyl, a specific chelator for Fe^{2+}. To include dehydroascorbic acid in the assay procedure, extracts were treated with dithiotreitol (DTT) and subsequently with *N*-ethylmaleimide to remove excess DTT.

ITC. For ITC extraction, three analytical replications of each plant sample were homogenized (Ultra Turax, Janke and Kunkel, IKA Labortechnik, Staufen, Germany) while being cooled with ice to minimize losses of ITC. Exactly 2.5 g of plant material was suspended twice with 5 mL of solvent (ethylacetate and cyclohexane 1:1) for 1 min, followed each time by centrifugation at 4500*g* and 4 °C. Supernatants were combined and filtered through 45 μm PTFE filter units. For ITC determination, a Varian CP-3800 equipped with a Varian FactorFour fused silica capillary column (VF-35 ms, 30 m × 0.25 mm (i.d.) and 0.25 μm film thickness) was used. The carrier gas (He) flow rate was 1 mL min^{-1}. A stepwise, linear temperature gradient was employed, starting at 30 °C, maintained for 0.5 min, then increasing at a rate of 5 °C min^{-1} to 90 °C, at which the temperature was held for 5 min. The temperature was further increased to 160 °C at a rate of 5 °C min^{-1} and at a rate of 30 °C min^{-1} up to 280 °C, which was maintained for 10 min. One microliter of the sample was injected, the injector operating in splitless mode. The injector and transfer line was set to 210 °C. On a Varian 1200 Quadrupole MS/MS instrument, mass spectra were obtained by electron ionization at 70 eV, and mass scans were recorded from 90 to 180 *m/z*. Compound quantification was accomplished by external standardization for each compound.

Myrosinase Activity. Myrosinase was extracted from powdered, frozen plant material by homogenizing 1.0 g of material with 3 mL of extraction buffer (100 mM K phosphate, pH 7.0, containing 5 mM DTT). After being vortexed for 1 min, samples were spun at 18000*g* at 4 °C. For protein precipitation, the clear supernatant was mixed with saturated $(NH_4)_2SO_4$ solution in a ratio of 1:2.6 and spun again at 18000*g* at 4 °C for 15 min. The protein pellet was resuspended in 500−1500 μL of reaction buffer (100 mM potassium phosphate, pH 7) and purified using NAP10 columns (Amersham Science). Three analytical replications and appropriate blanks containing no sinigrin were employed. The myrosinase activity was determined using a modification of a protocol that is based on the determination of the glucose released, using sinigrin as the substrate (*18*). Myrosinase activity was referenced to a glucose standard, and one unit of myrosinase activity is equivalent to 1 nmol of sinigrin hydrolyzed per minute.

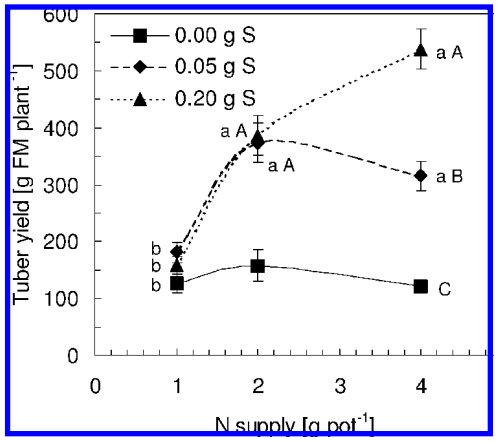

Figure 2. Tuber yield of kohlrabi plants as influenced by N and S supply. Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

Statistical Analysis. All statistical analyses were carried out using SAS (SAS Institute Inc., Cary, NC, release 8.02). Comparisons of means with respect to the influence of N and S supply were carried out using the GLM procedure considering a two-factorial, orthogonal, fully randomized design. Homogeneity of variance and normal distribution were evaluated by plotting studentized residues over predicted values (means) and by the Shapiro−Wilk test, respectively. Where appropriate, data were either square-root or log transformed to maintain the homogeneity of variance. To maintain an experimentwise α of $p < 0.05$, multiple *t* tests were adjusted according to Bonferoni−Holm (*19*). Statistical significance is indicated by lower- and uppercase letters for N and S levels, respectively. Nonlinear exponential functions (ITC concentration over N/S ratio) were fitted using SigmaPlot 2004 for Windows version 9.01 (Systat Software, Inc.).

RESULTS

Plant Growth. Varied nutrient supply induced a classical nutrient response, resulting in insignificant effects of incrementing provision of a nutrient as long as the other nutrient was in short supply, while the highest yield required adequate provision of both nutrients (**Figure 2**). When only one nutrient was in short supply, typical deficiency symptoms developed, namely, smaller, yellowish leaves and early development of chlorosis on older leaves in the case of N deficiency, while S deficiency was characterized by interveinal chlorosis particularly of younger leaves (not shown).

Nutrient Concentration. As expected, concentrations of N and S were significantly affected by the differentiated nutrient supply and also showed significant interactive effects, particularly in the tuber tissue (**Table 1**). With a few exceptions, the concentration of N was positively affected by an increased N and reduced S supply. Accordingly, the S concentrations were positively affected by high S and low N supply, although S effects were much less pronounced at the highest N level. As a consequence of this differential response, the N/S ratio, considered to be a suitable indicator for nutritional status, responded strongly in all plant parts investigated and in tubers showed a significant interaction of the treatments imposed.

The supply of N and S also significantly affected their major inorganic uptake and transport forms: nitrate and sulfate (**Table 2**). The nitrate concentration was elevated by increasing N and decreasing S supply in all plant parts investigated, and this effect was particularly strong in mature leaves, where nitrate is preferably translocated by the transpiration stream. With respect to the tissue investigated, the nitrate concentration was highest in mature leaves and lowest in tubers, indicating that kohlrabi plants do not accumulate nitrate preferably in their storage organ. The concentration of sulfate showed a less consistent response. A positive effect of enhanced S supply on the sulfate concentrations could only be observed in mature leaves at low N supply, while in several cases, the opposite was observed.

ITC Concentration. The ITC concentration was determined in the tuber tissue only, and data exhibited substantial variation. The lowest concentrations, in the range of 5−40 μmol (kg DM)$^{-1}$, were observed for allyl ITC and phenylethyl ITC, while the average sulforaphan concentration ranged between 6 and 120 μmol (g DM)$^{-1}$ (**Table 3**). The ITC profile was clearly dominated by MTB ITC, which reached more than 1300 μmol (kg DM)$^{-1}$. Analysis of variance revealed that the N effect was always significant, while the S effect was significant for MTB ITC and phenylethyl ITC only. A significant interaction of the two nutrients could be demonstrated with respect to sulforaphan. Generally, the ITC concentration was positively influenced by high S supply and low N supply, which was particularly pronounced in the case of MTB ITC. The concentration of sulforaphan also increased significantly at low N supply.

The sum of the concentrations of allyl ITC, MTB ITC, phenylethyl ITC, and sulforaphan is considered the total ITC concentration. Analysis of variance indicates that both N and S supply affected the total concentration of ITC significantly (**Figure 3A**). However, due to substantial variation of individual samples and the inconsistent influence of the treatments on individual ITC, most means did not differ significantly. Nonetheless, a clear trend could be observed, pointing to higher ITC concentrations with increasing S supply, provided that the provision of N was moderate. To avoid misinterpretations of the influence of nutrient supply on ITC stemming from concentration effects, the total ITC content per tuber was calculated (**Figure 3B**). As before, based on the analysis of variance, both N and S supply significantly affected the ITC content of kohlrabi tubers, and positive effects of increased S addition were particularly evident at low and intermediate N supply.

ITC is not translocated within the plant. Consequently, it seemed appropriate to consider graphical vector analysis (*20*) for ITC present in tubers, to evaluate as to whether the treatments affect ITC concentrations by changing the rate of synthesis or merely via dilution effects. This analysis allows interpreting changes of phytochemical content as excess and reduced synthesis, when consecutive treatments follow regression through the origin (straight lines in **Figure 4**), while changes perpendicular to these lines are interpreted as concentration and dilution effects. Horizontal vectors (constant concentrations, although total content change) indicate steady state conditions. Such analysis reveals that at a given N level, all vectors linking treatment means of increasing S supply are close to the straight line connecting the treatment mean with the origin (**Figure 4A**). This indicates that there is a truly enhanced release of ITC with increasing S supply, at all N levels tested. To the contrary, a reduced formation of ITC due to increasing N supply at a given S level was observed in most cases, although a strong dilution effect is apparent when the N supply increased from lowest to intermediate supply (**Figure 4B**), which is related to a strongly enhanced tuber yield (**Figure 2**).

The previous figures show that the supply of both N and S influences the ITC concentration. In an attempt to relate the ITC concentration to the N and S status, which is best described by the N/S ratio, the relationship between ITC concentration and this S status indicator was evaluated (**Figure 5**). All four ITCs exhibited a negative association with the N/S ratio, which

Table 1. N and S Concentration and N/S Ratio of Kohlrabi Plants As Influenced by N and S Supply[a]

nutrient supply (g pot^{-1})		young leaves			mature leaves			tuber		
N	S	N	S	N/S ratio	N	S	N/S ratio	N	S	N/S ratio
1	0.00	48.50 A	1.58 a C	30.94 c A	29.31 b A	1.21 B	24.20 A	30.42 c A	1.32 A	23.04 b A
1	0.05	38.81 ab AB	2.19 a B	17.71 c B	24.51 b AB	4.80 a A	8.01 b B	22.24 b B	2.68 a B	9.07 c B
1	0.20	30.59 B	4.39 a A	7.10 c C	21.32 ab B	3.38 A	6.56 b B	21.81 b B	5.84 a C	3.90 c C
2	0.00	44.74 A	1.10 b B	41.14 b A	32.00 b A	0.88	36.85 A	47.54 b A	0.89 B	53.60 a A
2	0.05	26.93 b B	1.07 b B	24.77 b B	19.44 b B	0.90 b	22.06 a AB	26.45 b B	1.34 b B	22.24 b B
2	0.20	24.49 B	2.18 b A	11.17 b C	15.33 b B	1.79	11.62 ab B	21.75 b B	2.83 b A	7.72 b C
4	0.00	57.45 A	0.91 b B	63.81 a A	61.58 a A	1.92	68.09	61.15 a A	1.13 B	55.52 a A
4	0.05	49.37 a AB	1.07 b B	46.64 a B	42.33 a B	1.80 b	34.83 a	46.73 a B	1.04 b B	45.13 a A
4	0.20	40.48 B	1.91 b A	21.21 a C	26.68 a C	1.23	21.64 a	34.20 a C	1.97 b A	17.47 a B
ANOVA	N	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	S	<0.0001	<0.0001	<0.0001	<0.0001	0.0200	<0.0001	<0.0001	<0.0001	<0.0001
	N × S	0.5545	0.0785	0.0722	<0.0001	0.0482	0.2292	<0.0001	0.0010	0.0006

[a] Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means due to the effect of N and S supply, respectively. S and N values are in g (kg DM)$^{-1}$.

Table 2. Nitrate and Sulfate Concentration of Kohlrabi Plants As Influenced by N and S Supply[a]

nutrient supply (g pot^{-1})		young leaves		mature leaves		tuber	
N	S	NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}
1	0.00	1.69 A	2.00	2.74 c A	2.15 b B	1.96 a A	1.58 A
1	0.05	0.25 B	1.38	0.86 b B	2.17 B	1.11 ab AB	1.45 A
1	0.20	0.04 B	1.31	0.08 C	3.97 a A	0.59 B	0.29 b B
2	0.00	3.98 A	2.00 A	6.42 b A	4.44 a A	0.85 b	1.22
2	0.05	0.53 B	0.77 B	0.45 b B	2.43 B	0.60 b	1.42
2	0.20	0.02 C	0.92 B	0.24 B	2.26 b B	0.46	1.54 a
4	0.00	6.73 A	1.26	23.10 a A	5.41 a A	2.17 a A	0.85
4	0.05	3.62 B	1.26	8.82 a B	3.44 B	1.48 a A	1.30
4	0.20	0.69 C	0.72	0.67 C	3.97 a AB	0.51 B	0.83 ab
ANOVA	N	<0.0001	0.0087	<0.0001	<0.0001	0.0002	0.1786
	S	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	0.0472
	N × S	0.0033	0.0531	<0.0001	<0.0001	0.0410	0.0191

[a] Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means due to the effect of N and S supply, respectively. All values are in g (kg DM)$^{-1}$.

Table 3. Concentration of ITC and Total Ascorbic Acid in Tubers of Kohlrabi Plants As Influenced by N and S Supply[a]

nutrient supply (g pot^{-1})		[μmol (kg DM)$^{-1}$]			[mg (g DM)$^{-1}$]	
N	S	allyl ITC	MTB ITC	phenylethyl ITC	sulforaphan	ascorbic acid
1	0.00	16.5	176.0	29.4 a	50.5 a	5.22
1	0.05	13.3	735.8 a	24.6 a	120.5 a	5.19
1	0.20	38.4 a	1348.9	34.1	94.4 a	6.67
2	0.00	8.9	11.0 B	5.4 b B	6.6 b B	5.94
2	0.05	10.3	87.5 ab AB	10.3 ab B	8.0 b AB	5.65
2	0.20	9.4 ab	657.0 A	31.1 A	42.0 ab A	4.73
4	0.00	4.9	28.0	6.6 b	6.9 b	6.59
4	0.05	6.0	16.7 b	7.8 b	7.1 b	4.86
4	0.20	6.5 b	112.9	19.3	15.3 b	4.25
ANOVA	N	0.0047	0.0002	<0001	<0.0001	0.8911
	S	0.4496	0.0021	<0.0001	0.0624	0.7253
	N × S	0.6697	0.1879	0.0692	0.0258	0.5452

[a] Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means due to the effect of N and S supply, respectively.

could be adequately described by an exponential decay in most cases. High ITC concentrations thus were expected in tubers of low N/S ratio, characterizing tissues relatively rich in S. This trend also was observed for the total ITC concentration (**Figure 6**). Within a given S supply level, the reduction of the N/S ratio was accompanied by a substantial increase in the ITC concentration, and this effect is particularly strong at intermediate and high S supplies.

In view of the potential function of GSs as a transient S storage compound, it seems appropriate to determine the potential contribution of GS breakdown to the S status of the tissue. On the grounds that the hydrolysis of GSs liberates equimolar amounts of ITC and sulfate (**Figure 1**), the potential contribution of glucosinolate breakdown to the metabolic S pool was evaluated. Apparently, the share of ITC-S increased with decreasing N supply, while the S supply had no significant effect (**Figure 7**). In any case, the share of ITC-S to the total S pool never exceeded 1.2%. In relation to the free sulfate pool, the contribution of ITC-S significantly responded to both N and S supply and reached ~40% at the lowest N and highest S supply levels, while in other treatments, its share on the free sulfate pool reached only a few percent.

Activity of Myrosinase. Myrosinase activity of individual samples showed substantial variation, and although no significant differences of individual means could be sustained, analysis

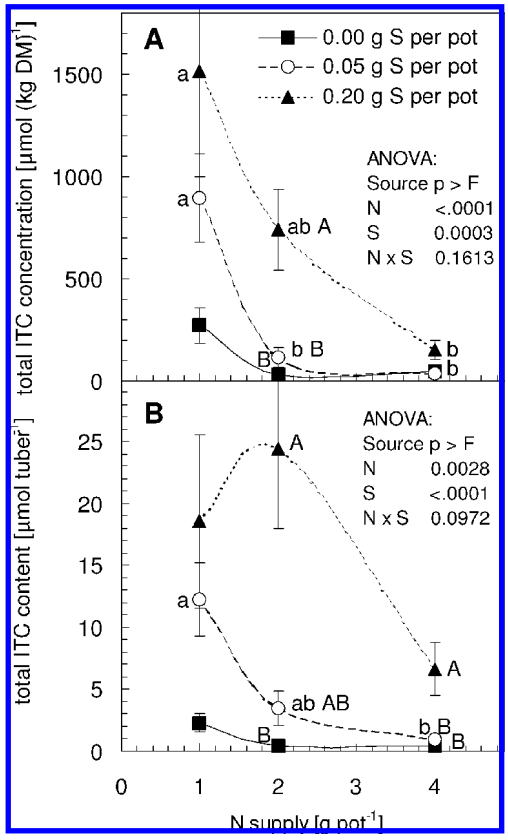

Figure 3. Concentration (**A**) and absolute content (**B**) of total ITC in tubers of kohlrabi plants as influenced by N and S supply. Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

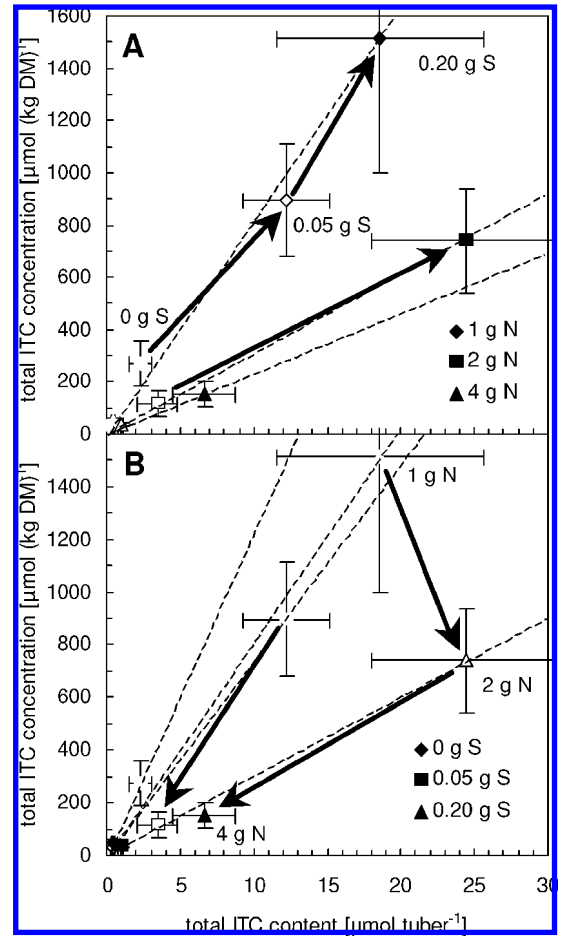

Figure 4. Graphical vector analysis of total ITC concentration in tubers of kohlrabi plants supplied with different S and N amounts (means ± SE). Analysis was carried out either with respect to the influence of S (**A**) or with respect to the influence of N (**B**). Solid, open, and gray symbols indicate the highest, intermediate, and lowest supply of S and N in panels **A** and **B**, respectively.

of variance clearly shows that the S supply had a significant effect on this enzyme activity (**Figure 8**).

DISCUSSION

Influence of N and S Supply on Nutritional Status and Growth. Kohlrabi plants responded strongly to the treatments imposed, exhibiting a typical interactive nutrient response (**Figure 2**). The growth response is thus reminiscent of the classical Law of the Optimum (i.e., a response that is stronger the closer the other nutrients are to their optima; *21, 22*).

To discuss the significance of the impact of N and S supply on ITC concentrations, it is necessary to assess the nutritional status of the plant material investigated. As expected, the nutrient supply strongly affects the nutrient contents of kohlrabi plants. An N concentration of 40−50 mg (g DM)$^{-1}$ might be considered to be adequate in just fully expanded (young) leaves of kohlrabi plants at the time of harvest, and an N/S ratio of 11−12 is considered to be adequate for most plant species (*23*), which translates into a critical S concentration of 3.5−4.3 mg (kg DM)$^{-1}$. In addition, a sulfate concentration of 12 mg (g DM)$^{-1}$ in the leaves is considered to be adequate for several *Brassica* vegetables (*23*). On the basis of these margins, the high N dose (4 g pot^{-1}) lead to adequate N status, while plants grown with 2 and 1 g of N pot^{-1} are considered to be of marginal and deficient N status, unless growth was hampered by inadequate S supply (**Table 1**). Overall, the S supply was less generous as indicated by the rather high N/S ratios and low sulfate concentrations (**Table 2**). Obviously, plants grown with the lowest S dose were strongly S-deficient, and even at intermediate S supply, their S status was inadequate unless growth was severely restricted by low N supply.

In the past, nitrate frequently was discussed in relation to health risks stemming from either the potential generation of *N*-nitroso compounds or met-hemoglobin anemia, but views have changed considerably in recent years (*24, 25*). Still, official limits for infant food (250 mg of nitrate (kg FM)$^{-1}$ (*26*)) and preserved spinach (2000 mg of nitrate (kg FM)$^{-1}$ (*27*)) are in effect. A recalculation of the data of **Table 2** reveals that the nitrate concentration did not exceed 200 mg (g FM)$^{-1}$ in any treatment. Nonetheless, a significant increase in the tuber nitrate concentration in response to inadequate S supply is evident (**Table 2**), which stems from the lack of S-containing amino acids and further metabolites required for protein synthesis and overall growth, resulting in an accumulation of N-rich precursors including nitrate (*28*).

Does the ITC Profile Correspond to the GSs Profile of Kohlrabi? Four ITCs could be identified, and the ITC profile was dominated by MTB ITC, followed by sulforaphan, phenylethyl ITC, and allyl ITC (**Table 3**), resulting from the hydrolysis of glucoerucin, glucoraphanin, gluconasturtiin, and sinigrin, respectively (*2, 3*). The GSs contents were not analyzed in these kohlrabi tubers. Apparently, the GSs profile of kohlrabi is dominated by glucoraphanin, glucoerucin, glucoiberin, and glucobrassicin, followed by progoitrin, glucoraphenin, glucoibervirin, and 4-OH-glucobrassicin (*29*). Neoglucobrassicin, sinigrin, gluconasturtiin, and 4-methoxy-glucobrassicin were detected in trace amounts, as were the breakdown products

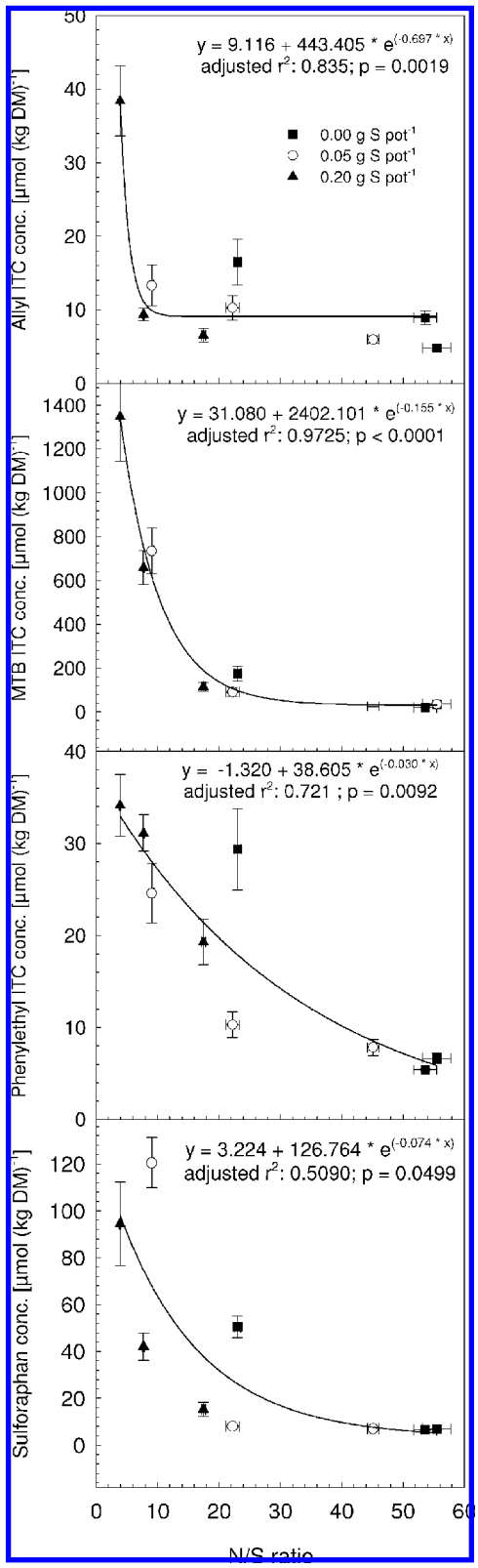

Figure 5. Relationship between concentration of individual ITC and N/S ratio in tubers of kohlrabi plants (means ± SE).

phenylethyl ITC and allyl ITC derived from gluconasturtiin and sinigrin, respectively, in the present study. Another study also identified glucoraphanin (6.4 μmol (g FM^{-1})) as the dominant GSs in kohlrabi, followed by glucoiberin (4 μmol (g FM)$^{-1}$)) and glucoerucin (0.7 μmol (g FM)$^{-1}$)) (*30*). In kohlrabi seeds, the major GSss found in decreasing order are glucoraphanin, glucoiberin, 4-hydroxyglucobrassicin, progoitrin, and glucoeru-

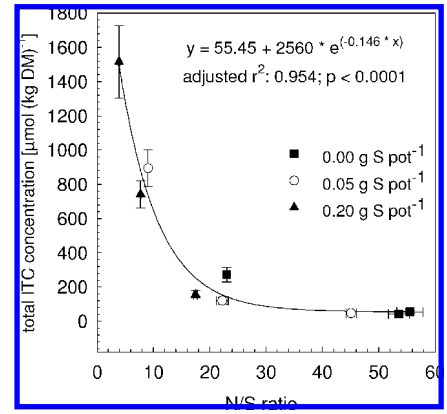

Figure 6. Relationship between total ITC concentration and N/S ratio in tubers of kohlrabi plants (means ± SE).

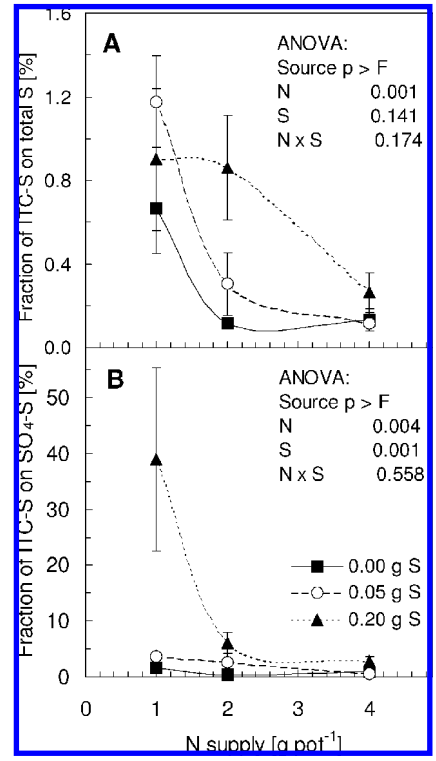

Figure 7. Fraction of ITC-S on total S (**A**) and SO_4^{2-}-S (**B**) concentration in tubers of kohlrabi plants as influenced by N and S supply. Lower- and uppercase letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

cin (*31*). Traces of gluconapin, gluconasturtiin, sinigrin, and glucoibervirin also were detected. Among the volatile oil constituents of kohlrabi seeds obtained by steam-distillation, 13 ITC could be identified, and the profile was dominated by MTB ITC, followed by 3-butenyl ITC, allyl ITC, 3-methylthiopropyl ITC, and 2-phenylethyl ITC (*32*). The large share of sulforaphan of the ITC profile is thus in full accordance with the GSs profile being dominated by glucoraphanin, its immediate precursor (**Table 3**). However, the major ITC observed was MTB ITC, although the corresponding GSs glucoerucin is found in much lower quantities than glucoraphanin, suggesting preferred metabolic conversion and thus accumulation of the breakdown product MTB ITC. In accordance, GSss account for ~57% of the aroma volatiles of green kohlrabi, with MTB ITC, 3-methylthiopropyl ITC (derived from glucoibervirin), and 2-phenethyl ITC contribut-

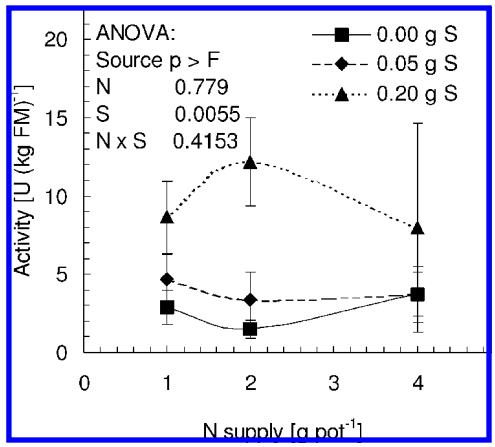

Figure 8. Activity of myrosinase in tubers of kohlrabi plants as influenced by N and S supply (means ± SE).

ing 35, 16, and 4%, respectively (*33*). Sulforaphan was not detected presumably due to its low vapor pressure. In agreement, another study also identified ITCs as the dominant fraction of flavor compounds of kohlrabi, accounting for 70−85% of all flavor volatiles, depending on nutritional conditions (*34*). The profile was dominated by 3-methylthiopropyl ITC (derived from glucoibervirin) and MTB ITC, while allyl ITC was detected in much smaller quantities in addition to several unidentified ITC compounds.

Overall, the profile of ITC detected is in agreement with GSs reported to be present in kohlrabi. In addition, it should be kept in mind that GSs may yield other breakdown products such as nitriles, thiocyanates, and epithionitriles (*2, 3, 32, 33*), which were not considered in the present study (**Figure 1**).

Interactive Effect of N and S Supply on Total ITC Concentration. While the impact of N and S supply on the concentration of GSs has been studied in some detail (*9–11*), nothing is known concerning the interactive effect of these nutrients with respect to ITCs. It has been hypothesized that GSs, in addition to other functions, may act as a transient reservoir for S, hydrolyzed to liberate sulfate and ITCs in the course of S starvation (*3, 12*). However, the results presented here do not suggest that GSs are broken down at enhanced rates in S-deprived plants. In fact, concentrations were increased when plants were adequately supplied with S (**Table 3** and **Figure 5**), and as the S status is best described by the N/S ratio, a unique functional relationship between this parameter and the total ITC concentration could be established (**Figure 6**). This relationship was ascertained despite a slightly increased myrosinase activity in S-deficient plants (**Figure 8**). However, enzyme and substrate of the glucosinolate−myrosinase system are strictly compartmentalized (*2*), even though views differ on their exact location. While compartmentation at the tissue level—myrosinase being concentrated in specialized myrosin cells—has been widely accepted (*1, 3, 35*), compartmentation at the cellular level—GSs and myrosinase being confined to vacuole and cytosol, respectively—also has been suggested (*2, 3, 36*). Obviously, even in the intact plant, compartmentation of enzyme and substrate is more critical for ITC liberation than the myrosinase activity itself, which holds true for the sulfur bomb concept without a doubt (*3*). No information on the interactive influence of N and S supply on GSs compartmentation is available. Furthermore, it has been suggested that myrosinase activity is regulated by ascorbic acid (*12, 37*), the concentration of which is known to respond negatively to excessive N supply (*38*). However, the total amount of ascorbic acid, determined as the sum of ascorbate and dehydroascorbate, was not affected by either N or S supply (**Table 3**). Actually, in a meta analysis of published data on the impact of increasing N supply on vitamin C contents of vegetables, the frequently quoted reduction of ascorbic acid content only was confirmed in about half the studies (*39*).

In any case, calculating the total ITC amount per tuber to account for dilution effects and graphical vector analysis clearly identifies an enhanced generation of ITCs upon increasing the S supply as the genuine mechanism leading to enhanced ITC concentrations in plants of high S status (**Figures 3** and **4**). A dilution effect only was involved when the severe N deficiency (low N and high S supply) was relieved by increasing the provision of N (**Figure 4**).

Even when the response of the total ITC concentration does not support the hypothesis that it is released in response to S deficiency by enhanced GSs breakdown, it is interesting to see as to what contribution to the metabolisable S pool is expected. On the grounds that GSs breakdown liberates equimolar concentrations of sulfate and side-chain residues (including ITC), it becomes evident that sulfate release makes a significant contribution to the sulfate pool only under conditions of high S and low N supply (**Figure 7**), when the plant's metabolism is in the least demand for additional sulfate.

While the response of GSs contents to S supply is generally a positive one, the response to N is less uniformly described, as positive (*40*), neutral (*41*), and negative relationships (*28, 42*) have been reported. Reduced GSs concentrations are frequently observed when N fertilization induces a strong growth response in combination to limited S supply, leading to substantial dilution effects (*10, 43*). This is tentatively supported by vector analysis, indicating that a substantial dilution effect occurred when the severe S deficiency was relieved (**Figure 4**). In any case, increasing the N supply led to lower ITC concentrations (**Figure 3**), which is supported by early data on the impact of variable N and K supply on flavor compounds of kohlrabi (*34*). In that investigation, the ITC concentration also substantially was reduced at elevated N supply, while the response to K underwent an optimum.

Interactive Effect of N and S Supply on ITC Profile. From studies considering the impact of varied N and S supply on the GSs profile, it is known that individual GSs do not respond uniformly. Frequently, GSs whose side chains are not derived from S-containing amino acids, namely, indolyl and aromatic GSs derived from tryptophan and aromatic amino acids, respectively, show a less negative—or even positive—response to increasing N supply (*9, 11, 43*) and a less positive one to increasing S supply (*7, 9, 11, 36*). No results are documented with respect to the ITC profile, but in this investigation, all four ITC responded in a roughly similar fashion to the N/S ratio (**Figure 5**). However, a closer looks reveals that at ample S provision, increasing N supply affected the concentration of phenylethyl ITC only slightly, while the concentrations of all other ITCs were substantially reduced (**Table 3**). Similarly, at low N supply, provision of S did not affect phenylethyl ITC, derived from gluconasturtiin. It seems that a less sensitive response of GSs that derive their side chain from tyrosine—as in the case of gluconasturtiin—also is seen in the response of the ITC profile.

In conclusion, this study demonstrated for the first time that N and S supplies exert a strong interactive impact not only on plant growth and mineral composition but also on the concentration of ITC, whose profile was in accordance with the supposed GSs spectrum of this species. However, the results do not support the hypothesis that liberation of ITC is enhanced in response to S limitation, as concentrations were substantially

reduced in response to low S and high N supply. The results also indicate that moderate N and adequate S supply are suitable measures to improve not only the nutritional quality of the harvested product but also its flavor characteristics.

ABBREVIATIONS USED

GSs, glucosinolate; ITC, isothiocyanate; MTB, methylthiobutyl; DTPA, diethylene-triaminepentaacetic acid; DTT, dithiothreitol; FM, fresh matter; DM, dry matter.

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