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ARTICLE

Impact of Nitrogen and Sulfur Fertilization on the Composition of Glucosinolates in Relation to Sulfur Assimilation in Different Plant Organs of Broccoli

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Broccoli (*Brassica oleracea* var. *italica*) is one of the most important winter season vegetables and a rich source of chemoprotective molecules, including glucosinolates (GSL). The aim of this study was to investigate the impact of nitrogen (N) and sulfur (S) fertilization on GSL concentration and composition in different parts of broccoli plants. A greenhouse experiment was performed, with four different treatments of sulfur (10, 30, 70, and 150 kg/ha) and three treatments of nitrogen (50, 250, and 600 kg/ha). GSL concentrations and plant growth responded to the N supply, but this was not observed above the 250 kg N/ha dose. On the contrary, plant growth did not respond to the S supply, whereas GSL concentrations showed a sharp response to the whole range of S applications (from 10 to 150 kg/ha). Glucosinolate composition was altered differentially in the examined plant parts. Aliphatic GSL were more abundant in the florets and leaves, whereas indolyl GSLs were dominant in roots, in which aromatic GSL were also observed. High nitrogen fertilization had a higher impact on indolyl compared to aliphatic GSLs concentration. More importantly, a high concentration of aliphatic GSL, >2.4 μmol/g dry weight (dw), and high S assimilation into aliphatic GSL were consistently observed in the florets compared to other broccoli parts, indicating adaptable processes for nitrogen and sulfur regarding synthesis and transport of aliphatic GSL for these organs.

KEYWORDS: broccoli; glucosinolates; sulfur; nitrogen fertilization; HPLC

INTRODUCTION

In Mediterranean countries, broccoli (*Brassica oleracea* var. *italica*) is one of the most important vegetables because it is a major winter crop and a rich source of health promoting substances. It contains significant amounts of ascorbic acid, β-carotene, vitamin E, various flavonoids, fibers, and minerals such as magnesium (Mg) and calcium (Ca) (*1*). In addition, like other *Brassica* species, broccoli contains significant quantities of glucosinolates (GSL), compounds rich in nitrogen (N) and sulfur (S), consisting of a thioglucose unit, a sulfonated oxime unit, and a variable side chain (*2*). Glucosinolates are secondary metabolites that can be divided into three major groups, namely, aliphatic, aromatic, and indolyl GSLs, depending on the amino acid that they derive from, which may be methionine, phenylalanine, and tryptophan, respectively (*2*). Glucosinolates are located in the cytoplasm and vacuoles and as intact molecules they are inactive and have no known function. In all GSL-containing plants, a thioglucosidase (E.C. 3.2.3.1), also known as myrosinase, is present and compartmentalized in special cells named myrosin cells (*3*). Myrosinase hydrolyses GSL into glucose, sulfate, and various other metabolites such as isothiocyanates, nitriles, thiocyanates, and epithionitriles after cell disruption. These compounds, characterized by high in vitro bioactivity, have attracted the interest of the scientific community due to their potential anticancer activity and ability to control or suppress soil plant pathogens (*4*−*8*).

Glucosinolate content and profile in *Brassica* species are influenced both from plant factors, such as species and developmental stage, as well as from environmental conditions (*9*−*11*). The latter include nutrient availability, especially N and S supply (*12*−*17*). As anticipated, S has a strong influence on GSL content in *Brassica* species and an increase in S supply resulted in all cases in a significant increase of GSL content (*13*, *15*, *16*).

On the other hand, data on the effect of N supply to the GSL content in plants appear rather contradictory. For example, increase of N supply resulted in an increase of indolyl GSL content in watercress (*Nasturtium officinale* R. Br.), fresh turnip

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(*Brassica rapa* ssp. *rapifera* L.) roots, turnip rape (*Brassica rapa*), and packhoi (*Brassica rapa* var. *chinensis* L.) (*12*, *15*−*17*), whereas Li et al. (*16*) observed a decrease of aliphatic GSL content in fresh turnip roots, by increasing nitrogen supply dose. Kopsell et al. (*15*) found that aliphatic GSL remained constant by increasing N supply. Moreover, a number of reports have shown that there is a significant interaction between N and S availability. For example, Schonhof et al. (*13*) reported that an increase of N fertilization at low S availability resulted in a decrease in total indolyl GSL. Nevertheless, information is limited on GSL distribution in different plant parts of broccoli and how this is influenced by N and S supply. Optimization of S and N for content of chemoprotective GSL in edible plant parts (florets) could influence significantly GSL concentration in plant parts of broccoli other than florets and consequently influence the physiological role that these molecules have in the various plant tissues. The aim of the present study was to examine the influence of N and S supply on GSL distribution in different plant parts of broccoli.

MATERIALS AND METHODS

Plant Material and Experimental Design. Broccoli cultivar Marathon plants were transplanted in large pots (12 L) in a cooled greenhouse 700 m^2. Each pot contained 10 kg of air-dried sand collected from a river basin area near Astromeritis village, central Cyprus, containing no detectable available forms of N or S, and traces of total N, with pH 7.9 and 14% $CaCO_3$.

Each pot contained 1 plant and received the following macronutrients: $Mg(NO_3)_2$ 1.27 g, $CaH_4(PO_4)_2$ 2.88 g. Micronutrients were supplied through an irrigation system by mixing 100 mL of the following nutrient solution H_3BO_3 2.86 g/L, $MnSO_4 \cdot H_2O$ 3.4 g/L, $CuSO_4 \cdot 5H_2O$ 0.1 g/L, $ZnSO_4 \cdot 5H_2O$ 2.2 g/L, and $(Na)_2Mo_4O_4 \cdot 2H_2O$ 1 g/L in 1000 L of water. Iron was added separately by dissolving 25.56 g of Fe-EDDHA in the same water tank. Three different levels of nitrogen were established, supplied in the form of NH_4NO_3 at 0.38 g/pot, 3.54 g/pot, and 9.08 g/pot. This, together with the quantity of nitrogen added through $Mg(NO_3)_2$ corresponded to 50, 250, and 600 kg N/ha, respectively. Sulfur was applied at four different rates 10, 30, 70, and 150 kg/ha in the form of potassium sulfate and each pot received 0.22, 0.81, 1.96, and 4.92 g, respectively. Potassium was balanced by the addition of the corresponding quantity of potassium chloride. Each pot was irrigated according to common practices and any pot leachate was collected and back-transferred to the plants to avoid nutrient losses.

A completely randomized design assembled in a factorial arrangement was established; each replicate consisted of 10 pots (10 plants) and repeated four times in each treatment.

Sampling and Plant Analysis. After harvesting the mature florets, broccoli plants were removed and separated into shoots (leaves and stems) and roots. The youngest fully expanded leaves (YFEL) were used for GSL, N, and S determination, whereas the petioles were detached and separately collected for sulfate analysis. Four replicate subsamples (florets, YFEL, roots, and petioles) from each treatment were bulked (100 g) and frozen immediately at −60 °C and freeze-dried. Freeze-dried material was ground and stored at −20 °C until analysis. From the remaining plant tissues (florets, shoots, and roots), samples were weighed just after sampling and were used for dry matter determination after drying to achieve constant weight at 80 °C. Finally, oven-dried florets, shoots, and root samples were bulked, ground, and stored at −20 °C for total nitrogen and sulfur determination.

Total N, S, and Sulfate Determination. Tin capsules containing 100 mg of freeze-dried or oven-dried plant material plus 300 mg WO_3 were transferred to a CNS analyzer (Elementar, Germany), and a combustion procedure was carried out for the quantitation of N and S content. The system was calibrated daily before analysis using as reference standard sulfanilamide (Sigma-Aldrich, Germany). The tissue accumulation of the N and S in broccoli plant parts was calculated as the product of N and S concentration in florets, shoots and roots by their corresponding dry weight.

Sulfates were measured by extracting 30 mg of lyophilized petioles in 30 mL of deionized water at 90 °C for 2 h, after which the extract was filtered through filter paper (Whatman No. 42). Sulfate concentrations in the extracts were determined by ion chromatography (Dionex) using an AS14A-5 μm separation column fitted with an AG14A-5 μm guard column (Dionex, Sunnyvale, CA). The eluent solution consisted of 0.08 mM Na_2CO_3, 0.01 mM $NaHCO_3$, and the regenerant of 0.072 N H_2SO_4.

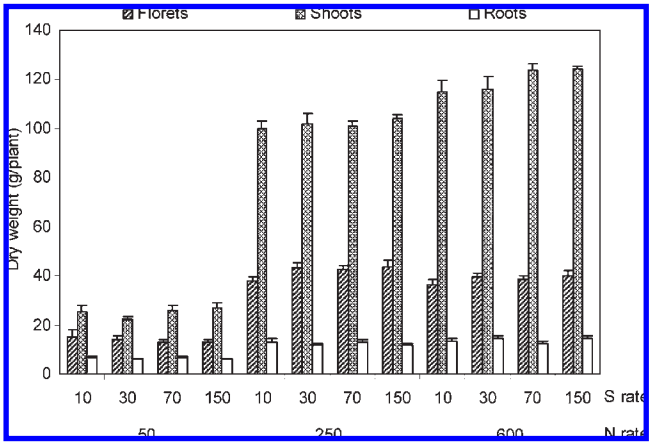

Figure 1. Dry weight (g/plant) production in various broccoli plant organs. Spreads represent standard errors of means ($n = 10$) for each treatment.

Glucosinolates Analysis. Glucosinolates were determined according to the E.U. official method (ISO 9167-1) with some modifications. Briefly, 300 mg of freeze-dried material was transferred into a 50 mL polypropylene conical tube with 5 mL of 70 °C EtOH 70% and 150 μL sinigrin (6.65 mM) as an internal standard and homogenized with a U-Turrax at 14500 rpm (T18, IKA Germany) for 3 min. Samples were centrifuged for 10 min at 11500 rpm, at 4 °C, the supernatants were transferred in a 10 mL volumetric flask and the procedure was repeated once more. Finally, the supernatants were combined and the extract volume was adjusted to 10 mL. Afterwards, sample (1 mL) was loaded onto a DEAE−Sephadex A25 column (Amersham, Biosciences) preconditioned with 0.025 M sodium acetate buffer, pH 5.6. Then the column was washed three times by applying 1 mL of the sodium acetate buffer and the elutate was discarded. Finally, purified sulfatase *Helix pomatia* type I (Sigma, Germany) was loaded onto the resin and was left overnight at room temperature. The desulfo-GLs (DS-GSL) were then collected by applying 3 mL of distilled water to the column. Separation of DS-GLs was performed on a Schimadzu HPLC system (Prominence) coupled with Photo Diode Array detector (SPD-M20A), equipped with an Inertsil ODS3 column (250 × 4.6 mm, 5 μm) and a gradient elution consisting of acetonitrile and water according to the following program: 0 to 1 min, isocratically 1%, 30% ACN over 30 min, and linear gradient returning to 1% ACN over 5 min. Chromatograms were analyzed using LC solution software.

Statistical Analysis. Analysis of variance (ANOVA) was used to examine the main effects of nitrogen and sulfur treatments and their interaction on individual and total GSL. Mean values were compared with Tukey−Kramer HSD test using StatSoft, Inc. (2004), *STATISTICA* (data analysis software system), version 7 (www.statsoft.com).

RESULTS AND DISCUSSION

Dry Matter Production. Nitrogen fertilization up to 250 kg/ha, resulted in significant increases in dry matter production of all broccoli plant parts (**Figure 1**). However, treatments of 600 kg N/ha had no further effect on dry biomass of florets and roots and a marginal effect on shoots. These results are in line with the general response of plants to nitrogen (*18*, *19*) and specific previous reports indicating that dry matter production is strongly affected by nitrogen fertilization (*13*, *20*−*22*). Increase of nitrogen fertilization also resulted in an increase in broccoli yields and fresh biomass (data not presented). To the contrary, increased sulfur fertilization did not have a significant effect on the dry matter production of the broccoli tissues examined (**Figure 1**) despite the deficiency symptoms (chlorotic leaves) that were observed at low sulfur fertilization. The results are in accordance

Table 1. Influence of Nitrogen and Sulfur Supply (kg/ha) on N and S (mmol/g dry weight), Individual Glucosinolates, Total, Total Aliphatic, and Total Indolyl Glucosinolates (μmol/g dry weight) in Broccoli Florets

N supply	S supply	N	S	GIB	GRA	4-OH GBS	GBS	4-MeO GBS	neo GBS	total aliphatic	total indolyl	total GSL
50	10	1718.14[a]A[b]a[c]	60.16 Aa	0.22 Aa	2.92 Ba	nd[d]	0.25 Aa	0.23 Aa	0.27 Aa	3.15 Ba	0.75 Aa	3.89 Aa
	30	1796.25 Aa	70.31 Aa	0.29 Aa	3.48 Ab	nd	0.26 Aa	0.32 Aa	0.35 Aa	3.77 Bb	0.93 Aa	4.70 Ab
	70	1803.21 Aa	114.30 Ab	0.28 Aa	3.95 Ac	0.22 Aa	0.48 Ab	0.30 Aa	0.40 Aab	4.23 Ac	1.39 Ab	5.62 Ac
	150	1766.75 Aa	118.59 Ab	0.34 Aa	4.30 Ad	0.20 Aa	0.62 Ab	0.34 Aa	0.54 Ab	4.64 Ad	1.70 Ac	6.34 Ad
250	10	2362.50 Ba	93.75 Ba	0.14 Aa	2.29 Aa	0.14 Aa	0.63 Ba	0.70 Ba	0.46 Ba	2.42 Aa	1.92 Ba	4.34 Ba
	30	2280.80 Ba	109.68 Ba	0.20 Aab	3.36 Ab	0.16 Aa	0.79 Bb	0.64 Ba	0.65 Bb	3.56 ABb	2.24 Ba	5.79 Bb
	70	2342.32 Ba	233.25 Bb	0.30 Abc	4.49 Bc	0.24 Aa	1.27 Bc	0.73 Ba	1.25 Bc	4.79 Bc	3.49 Bb	8.28 Bc
	150	2170.71 Ba	251.16 Bb	0.34 Ac	6.82 Cd	0.28 Aa	1.30 Bc	0.80 Ba	1.35 Bc	7.16 Cd	3.72 Bb	10.88 Bd
600	10	2402.14 Ca	96.48 Ba	0.13 Aa	2.32 Aa	0.19 Aa	0.65 Ba	0.64 Ba	0.52 Ba	2.45 Aa	1.99 Ba	4.44 Ba
	30	2393.57 Ba	108.28 Ba	0.20 Aa	3.25 Ab	0.21 Aa	0.84 Bb	0.69 Ba	0.69 Ba	3.45 Bb	2.43 Bb	5.88 Bb
	70	2498.57 Ca	220.94 ABb	0.38 Ab	4.51 Bc	0.29 Aa	1.44 Bc	0.81 Ba	1.22 Bb	4.89 Bc	3.75 Cc	8.64 Bc
	150	2440.71 Ba	288.75 Bb	0.35 Ab	5.78 Bd	0.27 Aa	1.63 Cd	1.09 Cb	1.29 Bb	6.13 Bd	4.28 Cd	10.41 Bd

[a] Mean value of four bulk sub samples from each treatment. [b] Lack of capital letters in common indicate differences ($p < 0.05$) between treatments at the same sulfur supply. [c] Lack of small letters in common indicate differences ($p < 0.05$) between treatments at the same nitrogen supply (Tuckey's HSD test). [d] nd = not detected. GIB = glucoiberin; GRA = glucoraphanin; 4-OH-GBS = 4-hydroxy-glucobrassisin; GBS = glucobrassisin; 4-MeO-GBS = 4-methoxy-glucobrassisin; GST = gluconasturtiin; neo-GBS = neo-glucobrassisin.

Table 2. Influence of Nitrogen and Sulfur Supply (kg/ha) on N and S Concentration (mmol/g dw), Individual Glucosinolates, Total, Total Aliphatic, and Total Indolyl Glucosinolates (μmol/g dry weight) in Broccoli Leaves

N supply	S supply	N	S	GIB	GRA	GBS	Neo-GBS	total aliphatic	total indolyl	total GSL
50	10	648.21[a] A[b]a[c]	31.25 Aa	0.10 Aa	0.36 Ba	0.10 Aa	0.11 Aa	0.46 Aa	0.20 Aa	0.66 Aa
	30	792.86 Aa	56.25 Aa	0.34 Aa	0.77 Ab	0.16 Aab	0.25 Ab	1.10 Ab	0.41 Ab	1.51 Ab
	70	730.36 Aa	103.13Ab	0.21 Aa	0.77 Ab	0.26 Abc	0.27 Ab	0.97 Ab	0.53 Ab	1.50 Ab
	150	658.93 Aa	151.56 Ac	0.29 Aa	0.80 Ab	0.29 Ac	0.29 Ab	1.08 Ab	0.57 Ab	1.65 Ab
250	10	864.29 Ba	62.50 ABa	0.14 Aa	0.52 Ca	0.45 Ba	0.22 Ba	0.66 Ba	0.66 Ba	1.32 Ba
	30	914.29 Aa	118.75 Bab	0.18 Aa	1.73 Bb	0.48 Ba	0.27 Aa	1.91 Bb	0.74 Ba	2.65 Bb
	70	871.43 Aa	162.50 Bbc	0.19 Aa	2.99 Bc	0.50 Ba	0.30 Aa	3.18 Bc	0.80 Bab	3.98 Bc
	150	985.71 Ba	207.73Ac	0.26 Aa	4.62 Bd	0.68 Bb	0.36 Aa	4.88 Bd	1.05 Bb	5.93 Bd
600	10	1264.29Ca	94.53 Ba	0.11 Aa	0.24 Aa	0.65 Ca	0.20 Ba	0.35 Aa	0.85 Ca	1.20 Ba
	30	1250.00 Ba	110.16 Bab	0.32 Ab	1.68 Bb	0.82 Ca	0.21 Aa	2.00 Bb	1.03 Ba	3.03 Bb
	70	1414.29 Ba	153.13 ABbc	0.42 Bbc	2.74 Bb	1.32 Cb	0.30 Aa	3.15 Bb	1.62 Cb	4.78 Cc
	150	1371.43 Ca	192.97 Ac	0.52 Bc	4.07 Bc	1.55 Cb	0.31 Aa	4.59 Bc	1.86 Cb	6.44 Bd

[a] Mean value of four bulk sub samples from each treatment. [b] Lack of capital letters in common indicate differences ($p < 0.05$) between treatments at the same sulfur supply. [c] Lack of small letters in common indicate differences ($p < 0.05$) between treatments at the same nitrogen supply (Tuckey's HSD test). GIB = glucoiberin; GRA = glucoraphanin; GBS = glucobrassisin; neo-GBS = neo-glucobrassisin.

with other reports, showing that no impact on dry matter production at vegetative and green head stage was observed by the increase of S to three broccoli varieties through gypsum applications (*10*) and that the dry matter of eight plant species remained constant when sulfur deficiency was not severe (*23*).

N and S Accumulation in Plant Tissues. Both N and S concentrations were significantly influenced by fertilization in all examined organs (**Tables 1**−**3**). No interaction between N and S fertilization was observed regarding N concentration in the examined organs, the effects of N being independent of the S level. Higher nitrogen was found in the florets, followed by leaves and roots. When N fertilization increased from 50 to 250 kg/ha, a significant increase of about 30% in the nitrogen concentration of broccoli florets was observed. However, a further increase in N fertilization had a marginal, if any, impact (**Table 1**). A similar pattern was observed in broccoli roots (but the response from 50 to 250 kg N/ha was even more dramatic), whereas in leaves nitrogen content increased even when N fertilization exceeded 250 kg/ha (**Tables 2** and **3**). Response to N fertilization has been recently reported for broccoli florets; at higher nitrogen levels the harvest index increased sharply along with nitrogen content increase (*24*). Accordingly, we observed that the florets-to-leaves N ratio was lowered from 2.52 for the 50 and 250 kg N/ha dose to 2.27 for the 600 kg N/ha dose; apparently, excessive N fertilization reduced the need for N translocation from leaves to florets and N accumulated in the leaves. This explains the response to the excessive 600 kg N/ha application in leaves only.

Sulfur concentration increased along with S fertilization in broccoli florets, leaves, and roots; this effect was observed at all N fertilization levels and showed its sharpest increase from 30 to 70 kg S/ha (**Tables 1**−**3**). However, in contrast to effects on N concentration, a significant interaction was observed between N and S fertilization regarding the S concentration in the examined organs: S concentration was enhanced by N fertilization an effect observed mostly at the low S fertilization levels in leaves and roots and at all S fertilization levels for florets. This result is in line with the well supported dependency of sulfur uptake and assimilation on nitrogen availability (*25*).

The response of S concentration to N supply is generally observed. For example, foliar sulfur increased significantly in oilseed rape (*Brassica napus*) when sulfur fertilization was high and nitrogen fertilization dose was also increasing (*26*). Similar observations were noticed in sunflower (*Helianthus annuus*) vegetative tissues where the S concentration increased during high sulfur and nitrogen fertilization doses (*27*). Different results were obtained by Schonhof et al. (*13*) in broccoli florets, who found that at adequate nitrogen doses the total concentration of S decreased even when sulfur supply was sufficient. They proposed that under S sufficiency conditions, metabolites such as cysteine or phytohormones, that is, cytokinins, may down-regulate sulfate assimilation. Alternatively, and as suggested by Falk et al. (*28*), the decline of the S content they observed in the florets at high N supply was partly due to a dilution effect derived from the respective 3-fold increase in the floret mass. The cross-talk

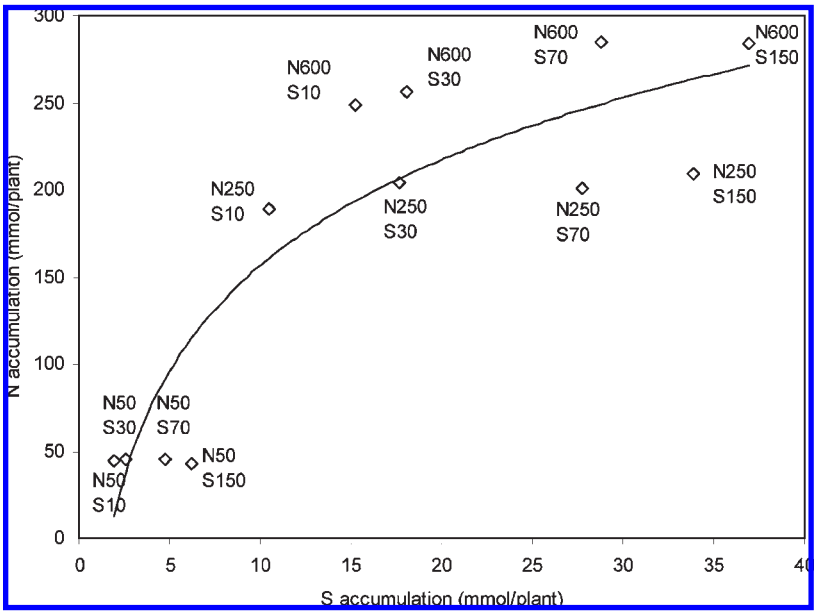

Figure 2. Correlation curve between N and S accumulation values at maturity in broccoli plants. Each point represents a mean value of four replications.

Table 3. Influence of Nitrogen and Sulfur Supply (kg/ha) on N and S Concentration (mmol/g dw), Individual Glucosinolates, Total, Total Aliphatic, Total Indolyl, and Aromatic (GST) Glucosinolates (μmol/g dry weight) in Broccoli Roots

N supply	S supply	N	S	GRA	GST	GBS	4-MeO GBS	Neo GBS	total aliphatic	total indolyl	total GSL
50	10	263.57[a]A[b] a[c]	35.00 Aa	0.15 Aa	1.00 Aa	0.14 Aa	0.37 Aa	0.16 Aa	0.15 Aa	0.66 Aa	1.80 Aa
	30	352.14 Aa	49.69 Aab	0.22 Aa	0.98 Aa	0.15 Aa	0.50 Aab	0.24 Aab	0.22 Aa	0.89 Ab	2.09 Aa
	70	322.32 Aa	74.92 Abc	0.17 Aa	1.09 Aa	0.30 Ab	0.66 Ab	0.30 Ab	0.17 Aa	1.25 Ac	2.51 Ab
	150	330.36 Aa	84.77 Ac	0.26 Aa	1.12 Ba	0.35 Ab	0.65 Ab	0.44 Ac	0.26 Aa	1.44 Ac	2.81 Ab
250	10	939.29 Ba	52.34 ABa	0.18 Aa	0.89 Aa	0.16 Aa	0.43 Aa	0.27 ABa	0.18 Aa	0.86 ABa	1.93 ABa
	30	1003.57 Ba	71.09 Aab	0.49 Bb	1.00 Aa	0.31 Bb	0.64 Aa	0.44 Bb	0.49 Bb	1.39 Bb	2.88 Bb
	70	1008.93 Ba	110.94 ABbc	0.67 Bc	1.39 Bb	0.58 Bc	1.10 Bb	0.49 Bb	0.67 Bc	2.17 Bc	4.23 Bc
	150	1075.00 Ba	115.63 Ac	0.74 Bc	1.35 Bb	0.65 Bc	1.17 Bb	0.56 ABb	0.74 Bc	2.37 Bc	4.45 Bd
600	10	1125.00 Ca	59.38 Ba	0.15 Aa	1.02 Aa	0.17 Aa	0.44 Aa	0.31 Ba	0.15 Aa	0.91 Ba	2.08 Ba
	30	1146.43 Ba	66.41 Aa	0.51 Bb	0.90 Aa	0.40 Bb	0.54 Aa	0.48 Ba	0.51 Bb	1.43 Bb	2.83 Bb
	70	1071.43 Ba	106.33 Bb	0.75 Bc	1.41 Bb	0.56 Bc	1.16 Bb	0.46 ABa	0.75 Bc	2.18 Bc	4.34 Bc
	150	1108.93 Ba	96.88 Ab	0.73 Bc	1.43 Bb	0.67 Bc	1.33 Cb	0.70 Bb	0.73 Bc	2.71 Cd	4.87 Cd

[a] Mean value of four bulk sub samples from each treatment. [b] Lack of capital letters in common indicate differences ($p < 0.05$) between treatments at the same sulfur supply. [c] Lack of small letters in common indicate differences ($p < 0.05$) between treatments at the same nitrogen supply (Tuckey's HSD test). GRA = glucoraphanin; GBS = glucobrassisin; 4-MeO-GBS = 4-methoxy-glucobrassisin; GST = gluconasturtiin; neo-GBS= neo-glucobrassisin.

between sulfur and nitrogen (and carbon) metabolism is not as yet clarified in the various plant species. Thus, it is imperative to perform both elaborate and targeted assessments of the different parameters that govern the complex regulation of sulfate accumulation and assimilation (*29*). The results obtained in the present study clearly show that, in broccoli grown under the fertilization scheme followed, S accumulation responds to N supply at an increasing pace, and that N accumulation tends to reach a plateau, whereas S accumulation continues to increase leading to higher S-to-N ratios (**Figure 2**). This is in accordance with studies on the mechanisms that govern the coordination of nitrogen and sulfur assimilation and in line with the proposed precursor of cysteine, *O*-acetyl-serine as the signal of plant N status toward S assimilation (*25*).

To elaborate on the translocation of sulfur, we further examined the sulfate-S concentrations in the petioles of the youngest fully expanded leaves. At all levels of nitrogen (50, 250, and 600 kg/ha), sulfate-S increased dramatically at the two high S fertilizations (**Figure 3**). However, an interaction was observed; gradual decrease of sulfate-S under higher nitrogen fertilization occurred, probably attributed to the increased assimilation of sulfur at higher nitrogen levels and the decreased reallocation

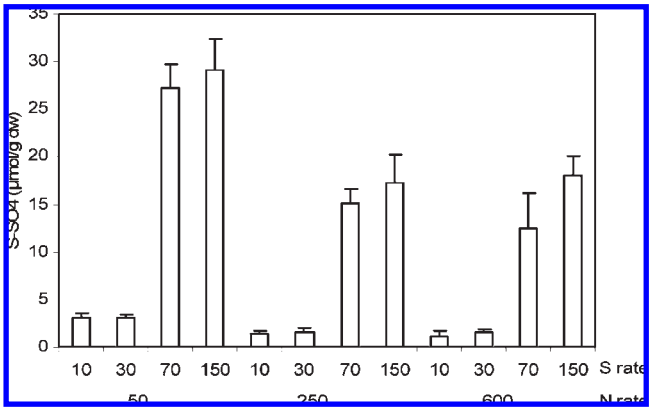

Figure 3. Sulfate−sulfur concentration (μmol/g dry weight) in broccoli petioles of the youngest fully expanded leaves. Spreads indicate standard errors of means ($n = 5$) for each treatment.

of sulfate into the phloem. Under high N supply, the plant growth is accelerated and more sulfate-S is incorporated into plant proteins and other sulfated molecules. Recent results showed that sulfate transporters are induced under high nitrogen supply

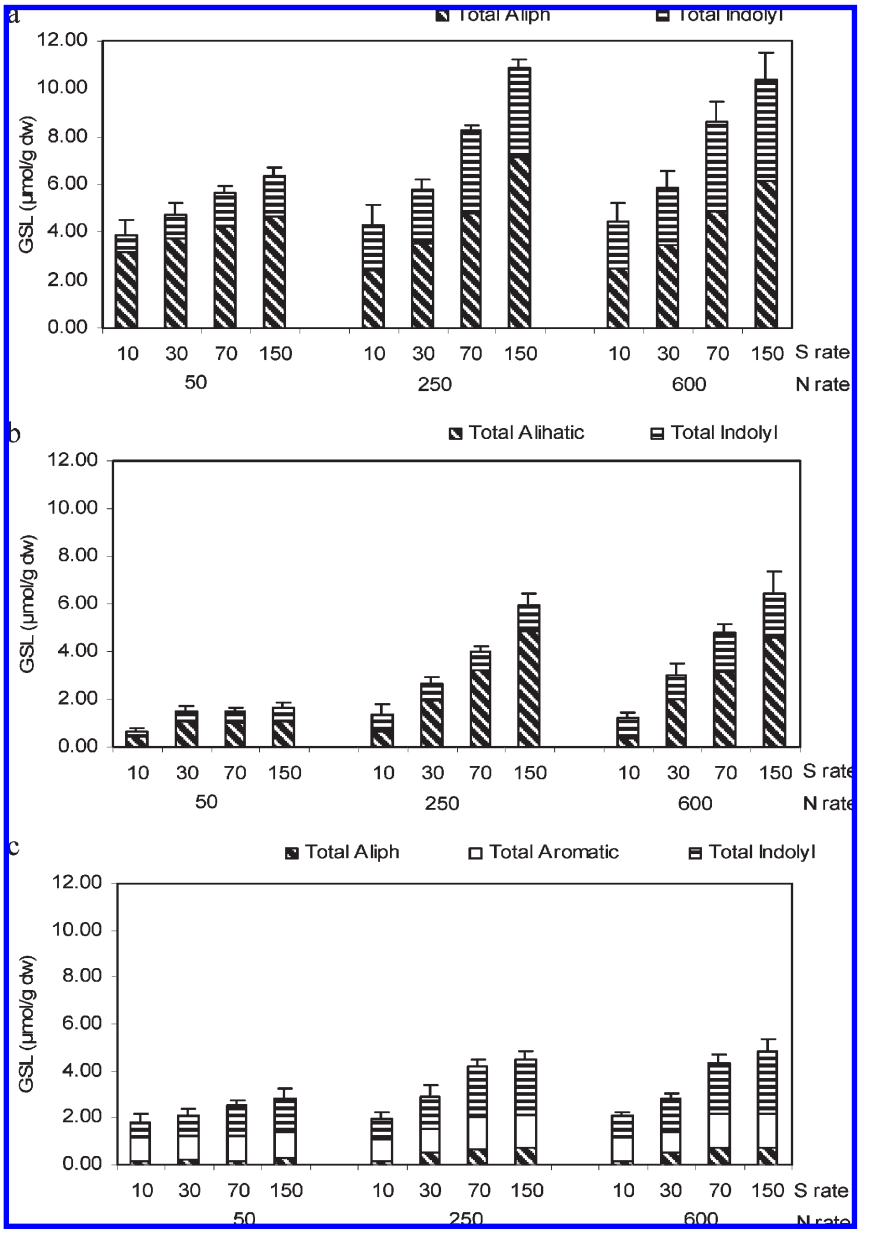

Figure 4. Total aliphatic, aromatic, and indolyl glucosinolate concentrations (μmol/g dry weight) in broccoli florets (a), leaves (b), and roots (c) under different nitrogen and sulfur supply doses. Spreads represent standard errors of means (n = 4) for each treatment.

conditions and may, thereby, increase sulfate assimilation (*30*, *31*). Consequently, the sulfate-S content in transportation plant parts (petioles) is reduced. The above are in accordance with reports suggesting that sulfate assimilation is negatively regulated by nitrogen starvation in *Lemna minor* and in tobacco (*Nicotiana tabacum*) culture cells (*32*, *33*). Furthermore, a sulfur cycling study in tobacco plants showed that less sulfate was reallocated into the phloem and the reduction of sulfur was increased when nitrogen and sulfur were sufficiently supplied (*34*).

Glucosinolate Content and Composition in Relation to Nitrogen and Sulfur. Individual glucosinolates, namely, the aliphatic glucoraphanin (GRA) and glucoiberin (GIB), the aromatic gluconasturtiin (GST) and the indolyl GSL, 4-OH-glucobrassisin (4-OH-GBS), glucobrassisin (GBS), 4-MeO-glucobrassisin (4-MeO-GBS), and neo-glucobrassicin (neo-GBS), were quantitatively determined in florets, leaves, and roots of broccoli plants grown under the fertilization scheme described (**Tables 1**−**3**). Individual GSL were detected differentially in the various plant tissues, that is, the aromatic GSL, gluconasturtiin was found exclusively in roots and 4-OH-GBS only in florets. In general, we detected all previously described GSL (*1*, *9*) in broccoli tissues; however, a systematic monitoring of the GSL profiles in the different broccoli tissues and organs was not performed.

The highest total GSL concentration (the sum of the individual GSL) was observed in the florets (3.9−10.9 μmol/g dw) and dropped to roughly half in the leaves and roots (**Figure 4**). The higher GSL concentration in the florets of broccoli is in line with their function in plant defense (*35*−*37*) because it is anticipated that the plant needs to increase the protection of its reproductive organs. This cannot rule out the possibility that the accumulation of GSL in florets may have a different, as yet elusive, role for the physiology of the plant. The response of total GSL concentration to N fertilization was similar to the response of the plant biomass and of the S concentration in the plant organs. Glucosinolate concentration was low in the 50 kg N/ha treatments in all plant organs, but it did not respond to N applications above 250 kg/ha (**Figure 4**). Reduced concentration of GSL at low N fertilization levels was also observed by Schonhof et al. (*13*) in broccoli florets

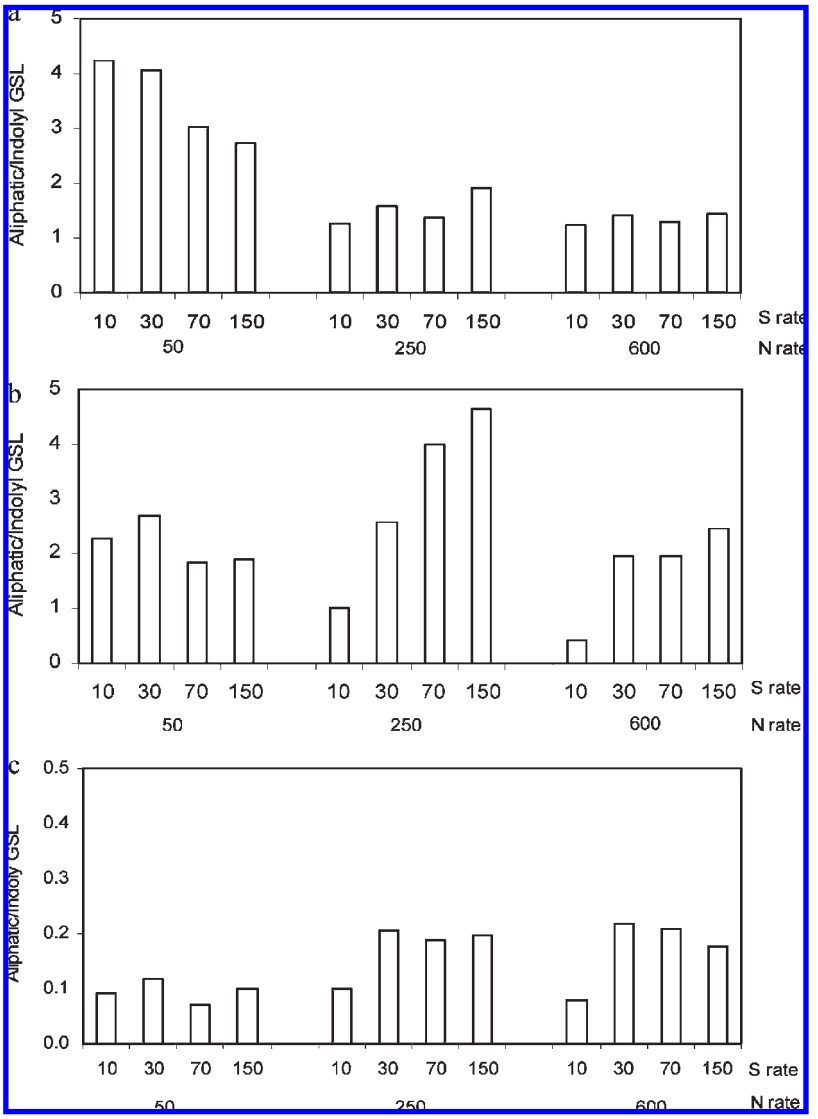

Figure 5. Aliphatic-to-indolyl glucosinolate ratio in broccoli florets (a), leaves (b), and roots (c) under different nitrogen and sulfur supply doses (kg/ha). Notice the 1 order of magnitude smaller range in c, compared to a and b.

and is in line with the crucial role of N in the formation of GSL precursor amino acids, in chlorophyll formation, and in S uptake.

Total GSL concentrations, however, clearly responded to increasing sulfur applications within the whole wide range of S applications (from 10 to 150 kg/ha) in all broccoli organs (**Figure 4**). This indicates that sulfur is a main determinant of the concentration of total GSL in the plant organs. Apart from the direct role of S in GSL biosynthesis (*28*), degradation of GSLs by myrosinase-like proteins under S limited conditions (*38*−*40*) may be involved in the demonstration of this strong effect. The response to S was linear in the florets and leaves and characterized by a steep dose−response when N availability was not a limiting factor (250 and 600 kg N /ha). However, under limited N availability (50 kg N/ ha), a reduced dose−response was observed in the florets (apparently due to N limitation, restricting both aliphatic and indolyl GSL biosynthesis). Furthermore, under limited N availability low GSL concentrations and a complete lack of response above 30 kg S/ha were observed in the leaves, consistent with GSL translocation to the florets.

As the ratio of nitrogen to sulfur increases, the GSL concentration is often reduced, probably due to vegetative growth, which outpaces the biosynthesis of these molecules, particularly in the case of S-demanding aliphatic GSLs, resulting in a dilution of GSL content (*13*, *41*, *42*). However, this was shown in this study not to be generally applicable, especially under adequate S supply.

Effects of nitrogen and sulfur supply on the concentration and on the composition of glucosinolates have been reported for a number of plant species, such as turnip (*16*, *41*), cabbage (*Brassica oleracea*) (*42*), rape seed (*43*), watercress (*15*) broccoli sprouts (*44*), and also broccoli (*13*). Although clear effects were described in all these studies, it is obvious that there exist species-specific responses to the N and S supply and that environmental parameters may influence these responses, as for example the growth period (*45*) and plant tolerance to salts (*44*).

Aliphatic GSL were the major fraction of total GSL in the broccoli florets and leaves, corresponding to 55−81% and 29−80% of total GSL respectively and the most abundant individual GSL was glucoraphanin (GRA), which is transformed via the myrosinase enzyme to sulforaphane, a well-established anticancer and antimicrobial isothiocyanate compound (*46*, *47*). In broccoli roots, however, aliphatic GSL were only 6−17% of total GSL; aromatic and indolyl GSL made the major GSL fraction, and the most abundant individual GSL was gluconasturtiin (GST; **Tables 1**−**3**).

In all organs, increasing sulfur resulted in a significant increase of both aliphatic and indolyl GSL at each nitrogen level

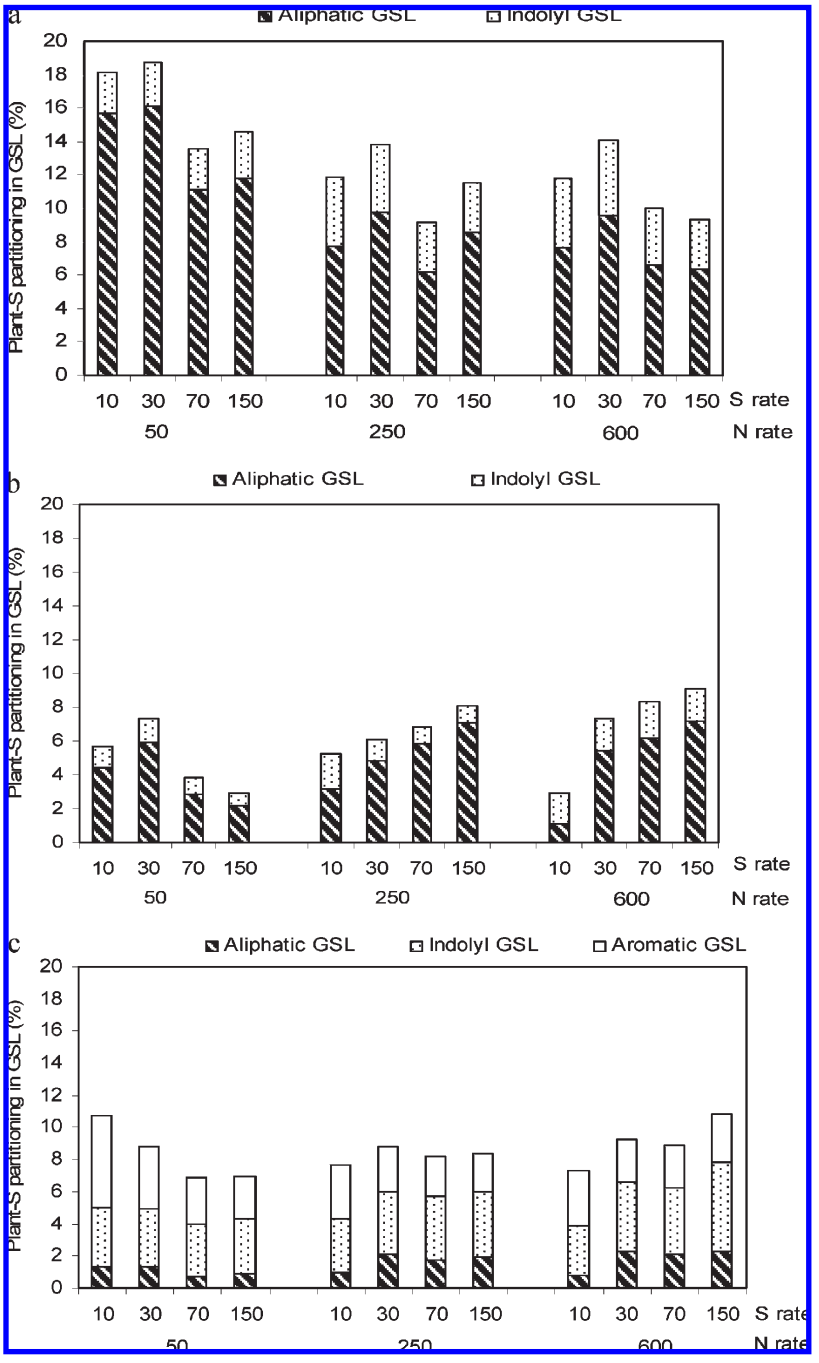

Figure 6. Percentage (%) of applied S that is assimilated into aliphatic, indolyl, and aromatic glucosinolates in broccoli florets (a), leaves (b), and roots (c) under different nitrogen and sulfur supply doses (kg/ha).

(**Figure 4**). Across N levels, however, S-limited conditions (10 kg/ha) led to a decrease in aliphatic GSL in the florets and leaves as N supply increased (**Figure 4**a,b). This lead to lowered aliphatic-to-indolyl GSL ratios in the respective S-limited treatments (**Figure 5**a,b). The opposite was detected for indolyl GSL, the concentration of which, under S-limited conditions, exhibited a multifold increase when N supply increased (**Figure 4**a and **4**b). The rise of indolyl GSL in sulfur-starved plants under increasing N supply may be expected, because in *Arabidopsis*, S deficiency was linked to a higher tryptophan content, the precursor amino acid for indolyl GSL (*39*). Moreover, more S is needed to synthesize aliphatic GSL, than indolyl GSL because three atoms of sulfur instead of two are needed for the biosynthesis of aliphatic and indolyl GSL, respectively. This also explains the higher incorporation of plant tissue sulfur in aliphatic GSL compared to indolyl GSL (**Figure 6**) in respect to their total amounts (**Figure 4**). It could be suggested that the plant is managing the limited nutrient in the most effective way. This is further corroborated by our findings that the percentage of total sulfur allocated to indolyl GSL was doubled at low sulfur and adequate nitrogen supply (compared to low sulfur and low nitrogen supply). Under these conditions, the percentage of total sulfur allocated in aliphatic GSL decreased (**Figure 6**a,b).

Indolyl GSL concentrations were generally high in the roots (**Figure 4**c) and the ratio of aliphatic-to-indolyl GSL very low (**Figure 5**c). Indolyl GSLs and IAA are both secondary plant metabolites derived from tryptophan and their metabolic formation pathways are cross-linked via the formation of indole-3-acetaldoxime (IAOx) that may be converted to both compounds (*48*, *49*). The proposed breakdown mechanism of indolyl

GSL in the roots under limited S availability, to form indole-3-acetic acid (IAA) and help stimulate S uptake (*28*, *50*), is in line with the sharp response of indolyl GSL concentrations in the roots to S supply (**Figure 4**c), but specific investigation is needed to elucidate the complex metabolic processes leading to the final root GSL composition, in which a relatively constant concentration of aromatic GSLs was also observed (**Figure 4**c).

Contrary to indolyl GSLs, aliphatic GSL concentrations were small in the roots (**Figure 4**c) and were further decreased under limited N or S supply leading to the smallest aliphatic-to-indolyl GSL ratios, around 0.2 (**Figure 5**c). This same trend for low aliphatic GSL concentrations under limited N or S supply was also observed in the leaves (**Figure 4**b). However, in the florets, their concentration did not fall below 2.4 μmol/g dw and the aliphatic-to-indolyl GSL ratios did not fall below 1.2, indicating possible translocation processes from leaves and roots to keep minimum aliphatic GSL concentrations in the florets even at low S application doses. GSLs are hydrophilic molecules and the transport of GSLs or desulfo-GSLs has been well documented (*36*, *51*).

Lack of N suppressed indolyl GSL in the florets (**Figure 4**a) leading to higher aliphatic-to-indolyl GSL ratios (**Figure 5**a). Under these conditions, the percentage of total sulfur incorporated into indolyl GSL decreased, whereas the percentage of total sulfur incorporated into aliphatic GSL increased (**Figure 6**a). The results indicate that nitrogen is an important factor that determines the composition of the total GSL in broccoli florets. Moreover, our data suggest that in broccoli florets there exists a need for the biosynthesis (or transport) of aliphatic GSL, which is satisfied even at sulfur or nitrogen starvation conditions, supporting the importance of these molecules for the plant.

The sulfate-S concentration measured in the petioles was greatly increased at high sulfur doses (**Figure 3**). Apparently, especially under N-limited conditions, the surplus of sulfur was not assimilated in GSL in the leaves. In accordance, the percentage of total sulfur incorporated in GSL was drastically reduced under high S (70 and 150 kg/ha) but limited N supply (50 kg/ha; **Figure 6**b). Under long-term sulfur deficiency conditions such as examined in our study, sulfate transporters may be activated to remobilize sulfate through the vasculature (*52*). This is also in line with the physiological role of mature leaves as donor tissue to satisfy the increased demand of young tissues for protein and other organic compound syntheses, although the regulatory aspects of the remobilization of sulfur from mature leaves is not well-known (*53*). Interestingly, at high nitrogen fertilization doses (600 kg/ha) and at low sulfur supply (10 kg/ha) the percent of total sulfur in broccoli leaves assimilated into GSL was also reduced significantly (**Figure 6**b), perhaps reflecting the extremely low concentration of aliphatic glucosinolates and their possible breakdown and translocation to the florets. Glucosinolates could play the role of sulfur storage molecules (*54*, *55*). In support of this, sulfur starvation in *Arabidopsis* promoted the production of a thioglucosidase (*39*, *40*), which potentially could be involved in the decomposition of GSL through a mechanism that also involves phytohormones and particularly auxin (*50*). The importance of glucosinolates as an alternative sulfur source when plants are under limited sulfur supply has also been implicated in an integrative transcriptome and metabolome study, where the link between primary metabolism (and consequently partitioning), sulfur, and nitrogen nutrient status and glucosinolate metabolism has been revealed (*54*, *56*).

In conclusion, the biomass of all broccoli tissues depended on nitrogen and had reached a plateau at a N fertilization dose standing for 250 kg/ha, whereas S applications as low as 10 kg/ha did not show any detrimental biomass effects. Glucosinolate concentrations responded to the whole range of S applications (from 10 to 150 kg/ha). This response was steep and linear when N was not a limiting factor for plant growth. Nitrogen applications above 250 kg/ha did not result in increased GSL concentrations. Aliphatic GSLs dominated in the leaves and florets in contrast to roots, where indolyl GSLs were dominant. High N application resulted in a drastic increase of indolyl GSL concentrations in the leaves and florets, whereas a concomitant poor S supply induced a reduction in aliphatic GSLs. It appears that the broccoli metabolic and transport processes were adapted to keep a threshold concentration of 2.4 μmol/g dw of aliphatic GSL in the florets even at sulfur or nitrogen starvation conditions, supporting the importance of these molecules for broccoli reproductive organs.

ABBREVIATIONS USED

ANOVA, analysis of variance; GSLs, glucosinolates; GIB, glucoiberin; GRA, glucoraphanin; 4-OH-GBS, 4-hydroxy-glucobrassisin; GBS, glucobrassisin; 4-MeO-GBS, 4-methoxy-glucobrassisin; GST, gluconasturtiin; neo-GBS, neo-Glucobrassisin; N, nitrogen; S, sulfur; DW, dry weight.

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