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Interactive Effects of Sulfur and Nitrogen Supply on the Concentration of Sinigrin and Allyl Isothiocyanate in Indian Mustard (*Brassica juncea* L.)

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Food derived from *Brassica* species is rich in glucosinolates. Hydrolysis of these compounds by myrosinase yields isothiocyanates and other breakdown products, which due to their pungency represent the primary purpose of Indian mustard cultivation. Strong interactive effects of S (0.0, 0.2, and 0.6 g pot^{-1}) and N (1, 2, and 4 g pot^{-1}) supply on growth, seed yield, and the concentrations of glucosinolates and isothiocyanates in seeds were observed in growth experiments, reflecting the involvement of S-containing amino acids in both protein and glucosinolate synthesis. At intermediate S supply, a strong N-induced S limitation was apparent, resulting in high concentrations of sinigrin (12 μmol g^{-1} of DM) and allyl isothiocyanate (213 μmol kg^{-1} of DM) at low N supply only. Myrosinase activity in seeds increased under low N and low S supply, but the results do not suggest that sinigrin functions as a transient reservoir for S.

KEYWORDS: Allyl glucosinolate; allyl isothiocyanate; glucosinolates; myrosinase; nitrogen; sinigrin; sulfur

INTRODUCTION

Mustard seeds are used globally as a pungent condiment because of the spicy taste associated with the isothiocyanates that are formed by the breakdown of glucosinolates. The structure of the latter consists of a thioglucose unit, a sulfonated oxime unit, and a variable side chain (**Figure 1**). In Indian mustard, the glucosinolate profile is dominated by sinigrin (allyl glucosinolate, 2-propenyl glucosinolate) and gluconapin (but-3-enyl glucosinolate) (*1*), and hydrolysis by myrosinase yields allyl isothiocyanate (**1** in **Figure 1**) and 3-butenyl isothiocyanate, respectively (*2*). Myrosinase and its substrates, the glucosinolates, are compartmentalized in the intact tissue, but rupture of the plant tissue by grinding or chewing initiates an intense reaction (*3*). In plants, glucosinolates are considered to be important as repellents (*4*) and in relation to disease defense (*3*). However, they also appear to be significant from the perspective of human nutrition, as recent investigations indicate that isothiocyanates are responsible for several beneficial health effects (*3*, *5*), of which the anticarcinogenic action is best documented (*6*). This suggests that it would be desirable to increase the concentration of isothiocyanates in the human diet by changing consumer behavior, plant breeding, and cultivation conditions including fertilization.

A strong interaction between the N and S supply on the contents of glucosinolates can be envisaged (*7*, *8*), as their side chains are derived from S-containing or S-free amino acids, the thiol bridge to glucose from cysteine, and the sulfonate group from phospho-adenosin-phosphosulfate (*3*). It is hypothesized that high S supply combined with adequate provision of N will increase the concentration of sinigrin in mustard seeds. Moreover, as glucosinolates have been discussed as a transient storage pool for S (*4*, *9*, *10*), it is further hypothesized that a reduced S supply will result in enhanced breakdown of glucosinolates and the concomitant enhanced release of isothiocyanate, presumably resulting from less efficient compartmentalization of glucosinolates from myrosinase. Sulfate released by myrosinase activity (**Figure 1**) is easily consumed by metabolic processes, but further conversion of low molecular weight isothiocyanates might be restricted because of their inefficient compartmentalization. Whereas energy-coupled uptake by protoplasts (*11*) and site-directed translocation of intact glucosinolates have been documented (*12*), this seems not to be the case for isothiocyanates. The effect of S limitation might be particularly strong in plants grown with marginal S, but ample N, supply. Under these conditions plants face S deficiency toward the end of seed filling due to

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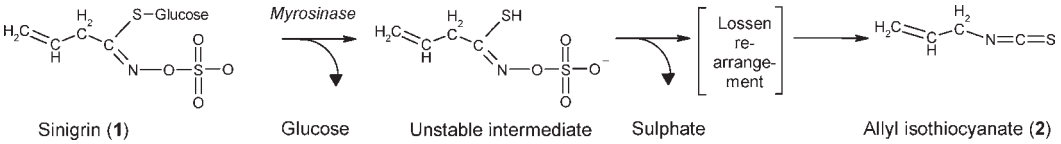

Figure 1. Structure of sinigrin (allyl glucosinolate, **1**) and the scheme for its decomposition to allyl isothiocyanate (**2**) after hydrolysis by myrosinase.

stimulated growth and protein synthesis, enhancing the competition for S-containing amino acids, their common substrate. Indian mustard (*Brassica juncea* L.), also referred to as brown or sarepta mustard, was chosen as a suitable model to test these hypotheses and to obtain information on the impact of the N/S fertilization on the concentrations of glucosinolates and isothiocyanates.

MATERIAL AND METHODS

Experimental Conditions. Experiments were conducted in Mitscherlich pots filled with 6 kg of nutrient-poor soil specified as follows: 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} [according to Schüller (*13*)], and contents of DTPA-extractable trace nutrients (*14*) 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}, respectively. 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 Indian mustard (*B. juncea* L. cv. line 'SF01', provided by Develey Senf & Feinkost GmbH, Unterhaching, Germany) were sown on May 15, 2006. 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, 0.2, and 0.6 g pot^{-1}) using six replications. N was administered as NH_4NO_3 and S as $CaSO_4 \cdot H_2O$ (basal dressing) and $MgSO_4 \cdot 7H_2O$. Applications of N and S were split to avoid salt stress, the second dressing applied 4 weeks after sowing. Four plants per pot were cultivated in the open and arranged in a completely randomized design.

Harvesting and Determination of Growth Parameters. Senescent leaves were continuously secured, and after 12 weeks of growth, plants were separated into stems, leaves, and intact siliques. The number of siliques per pot was recorded, and after they had been dried at 40 °C to constant weight, seeds were kept in a desiccator at room temperature. All other plant parts were dried at 60 °C to constant weight. For further analysis leaves were ground to pass a 1.5 mm sieve, and aliquots of seeds, after grinding in liquid N_2, were kept at −80 °C.

Analytical Procedures. *Nutrient Contents*. Total N and S were determined using an automated elemental analyzer (MAX CNS, Elementar Analysensysteme GmbH, Hanau, Germany). For determination of anions (NO_3^-, 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 595 1/2, Schleicher and Schüll, Dassel, Germany). Sample cleanup was accomplished using C_{18} cartridges (Bakerbond, Baker Inc., Gross-Gerau, Germany), before analysis by anion exchange chromatography (Metrohm Compact IC, Metrohm GmbH & Co, Filderstadt, Germany). Analytes were separated on a Metrosep A-Sup 4 column (250 × 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.

Glucosinolates. Sinigrin was analyzed as intact glucosinolates after methanol extraction (methanol 70% aqueous, 70 °C), evaporated to dryness and redissolved in pure water (18 Mohm) (*15*). Analyses were performed using an Agilent 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany) with a diode array detector (DAD). For detection of intact sinigrin a Varian Polaris (Varian Deutschland GmbH, 64289 Darmstadt, Germany) C18-Ether column (250 × 4.6 mm i.d.; particle size = 3 μm) was used with gradient elution of pure water (eluent A) and acetonitrile (eluent B), both acidified by 0.1% trifluoroacetic acid. Initially eluent A was used at 100% for 20 min, reduced to 5% within 42 min, kept there for 5 min, and then raised back to 100% to re-equilibrate for 12 min. The flow rate was set to 0.8 mL min^{-1} and the column temperature maintained at 25 °C. Detection wavelength was set to 229 nm. Commercially available sinigrin (sinigrin potassium salt, purity > 97%, PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) served as external standard.

Isothiocyanates. Two hundred and fifty milligrams of ground seed material, kept at −80 °C, was vortexed twice for 1 min with 1 mL of solvent (ethyl acetate/cyclohexane 1:1 by volume, both of analytical grade from Fluka Chemie AG, Buchs, Switzerland) followed each time by centrifugation for 10 min at 4500*g* and 4 °C. Supernatants were combined, filtered through 45 μm PTFE filter units, and stored at −80 °C. Preliminary tests indicated that a third extraction is not required for quantitative analysis (*16*). Each sample was extracted and analyzed in triplicate. For the chemical analysis a Varian gas chromatograph CP-3800 (Varian Deutschland GmbH, Darmstadt, Germany) equipped with a CombiPAL autosampler and a FactorFour fused silica capillary column (VF-35 ms, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Varian Deutschland GmbH) was used. The column temperature was set to 30 °C and held for 0.5 min. Then the temperature was increased to 90 °C at a rate of 5 °C min^{-1} and held for 5 min. Afterward, the temperature was increased to 160 °C at a rate of 5 °C min^{-1} before reaching a final temperature of 280 °C with an increase rate of 30 °C min^{-1}. This temperature was held for 10 min. The flow rate of the helium carrier gas was 1 mL min^{-1} using the splitless mode and injection volume of 1 μL. The injector temperature was set to 210 °C. A Varian 1200 quadrupole MS/MS acted as the detector. The transfer line was set to 210 °C. Mass spectra were obtained by electron ionization at 70 eV, and mass scan was from 90 to 180. Quantification was carried out on the basis of external calibration using commercially available allyl isothiocyanate (purity = 95%, Sigma-Aldrich Chemie Gmbh, Munich, Germany). The entire analytical procedure for determining isothiocyanates has been carefully evaluated and optimized as described in detail elsewhere (*16*)

Myrosinase Activity. Five hundred milligrams of ground seed material was extracted with 3.6 mL of extraction buffer [100 mM K phosphate, pH 7.0, containing 5 mM DL-dithiothreitol (purity = 99%, Sigma-Aldrich Chemie Gmbh, Munich, Germany)]. After four vortexes every 15 s, samples were kept on ice for another 5 min and spun for 15 min at 18000*g* at 4 °C. The myrosinase activity was determined by measuring the release of glucose (*17*), employing a modification of the protocol described elsewhere (*18*), and using commercial sinigrin (sinigrin monohydrat, purity > 99%, Sigma-Aldrich Chemie Gmbh) as substrate. The protocol also included a sampled blank to account for endogenous glucose. One unit of myrosinase activity is equivalent to 1 nmol of sinigrin hydrolyzed min^{-1}.

Statistical Analysis. All statistical analysis was carried out using SAS (SAS Institute Inc., Cary, NC, release 8.02, 2001). 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 was evaluated by plotting studentized residues over predicted values (means) and by the Shapiro−Wilk test, respectively. When appropriate, data were either square root or log transformed to maintain homogeneity of variance. To maintain an experimentwise type I error of $p < 0.05$, multiple t tests were adjusted according to Bonferoni−Holm (*18*). Statistical significance is indicated by lower and upper case letters for the N and S levels, respectively.

RESULTS

Plant Growth and Yield. Varying the N and S supply induced a classical nutrient response, resulting in insignificant effects of incrementing nutrient supply as long as the other nutrient was growth limiting, whereas highest seed yield required adequate provision of both nutrients (**Figure 2**). As the supply level of one nutrient depended heavily on the provision of the other, the interaction of the two nutrients was significant for all growth and yield parameters tested. Note that at the lowest S supply an improved N supply actually caused a yield decrease. Increasing N supply resulted in more leaf material being produced (**Table 1**) and induced more branches, but plant height was reduced (data not shown). Whereas the consequences of increasing S supply for leaf DM (**Table 1**) and branching (not shown) were similar to those for N, plant height was reduced by insufficient S supply. Interactive effects of N and S supply were particularly strong with respect to the weight and number of siliques per pot. Whereas S supply hardly affected these parameters under N-deficient conditions, increasing provision of S at ample N supply substantially increased both traits.

Nutrient Concentration. Supply of N and S interactively affected the N concentration in vegetative tissue of mustard plants (**Table 2**). Whereas low N supply led to low N concentrations throughout, at higher N levels N accumulated due to a concentrating effect when growth (**Table 1**) was retarded by inadequate S availability. Compared to N, the concentration of S was much less affected, and only the impact of the S supply level was significant. Consequently, the N/S ratio responded strongly to the provision of these elements, with values being higher the lower the S and the higher the N supply, whereas their interaction was not significant. The supply of N and S also significantly affected the concentration of nitrate, the major inorganic uptake and transport form of N. Except for N-limited conditions, the nitrate concentration was elevated by increasing N and decreasing S supply, and the latter response was particularly strong when N was generously provided.

Concentrations of Sinigrin and Allyl Isothiocyanate. The concentration of sinigrin also responded strongly to the nutritional regime (**Figure 3**). Maximal concentrations reached 16 μmol g^{-1} (equivalent to 5.75 mg g^{-1}), whereas in seeds from plants grown without supplementary S, but intermediate to high levels of N, sinigrin was barely detectable. In the remediation of strong sulfur deficiency (i.e., increasing S supply to intermediate level), the seed sinigrin concentration was more responsive to additional S the less N was provided, whereas at the highest S level sinigrin concentrations did not differ significantly between different N treatments. As the seed yield was also significantly affected (**Figure 2**), accumulation of the highest amounts of sinigrin required intermediate to high N supply at the highest S level chosen, whereas—in agreement with respect to the sinigrin concentration—the sinigrin amount produced in plants grown with low N supply reached a plateau much earlier in response to increasing S supply (**Figure 3**), due to the very limited response of the seed yield in these treatments (**Figure 2**).

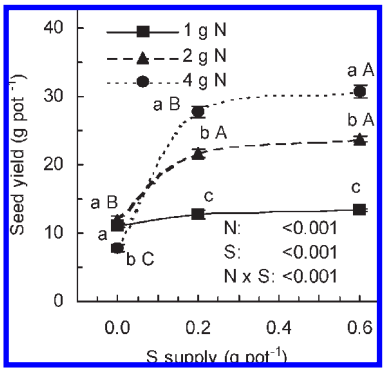

Figure 2. Effect of N and S supply on the seed yield of Indian mustard plants. Lower and upper case letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

The concentration of allyl isothiocyanate, the major breakdown product of sinigrin, reveals a rather different response to the nutritional regimes imposed (**Figure 4A**). Concentration of allyl isothiocyanate in seeds produced with the highest N supply always remained low and barely exceeded 10 μmol kg^{-1} (approximately 1 mg kg^{-1}). However, mustard seeds produced with intermediate N supply contained more allyl isothiocyanate the lower the S supply, accumulating > 100 μmol kg^{-1} (approximately 10 mg kg^{-1}) at the lowest S supply. In contrast, when plants received insufficient N and intermediate to high S levels, their seeds were characterized by high allyl isothiocyanate concentrations of 213 and 172 μmol kg^{-1}, respectively, whereas when grown without additional S, allyl isothiocyanate was virtually absent from the seeds of these plants. As the interactive effects of N and S supply on allyl isothiocyanate concentration were much larger than on seed yield (**Figure 2**), the response of the allyl isothiocyanate content did not differ markedly from the response of its concentration (**Figure 4B**).

To evaluate whether the nutritional regime affects the concentrations of sinigrin and allyl isothiocyanate by changing the rates of synthesis and decomposition, or merely via dilution effects graphical vector analysis (GVA) was employed (*20*). Such an analysis requires that the phytochemical under consideration is not retranslocated from the seeds to any significant extent, which seems reasonable to assume for seed sinigrin and the isothiocyanates. This analysis allows interpreting changes of phytochemical concentrations as excess and reduced synthesis, when consecutive treatment means follow a straight line linking the treatment means with the origin (*20*), whereas vertical changes are interpreted as concentration and dilution effects. Horizontal vectors (constant concentrations although total contents change) would refer to steady state conditions. This analysis reveals that at a given N level all vectors linking treatment means of increasing S supply exhibit a constant trend (**Figure 5A**), indicating a truly enhanced synthesis of sinigrin with increasing S supply, at all N levels tested. With respect to the effect of increasing N supply at a constant S level (**Figure 5B**) it becomes apparent that at ample S supply the sinigrin accumulation seems to be in a steady state as its concentration is rather stable. In contrast, at intermediate S supply increasing

Table 1. Effect of N and S Supply on the Growth of Indian Mustard Plants[a]

nutrient supply (g pot^{-1})						
N	S	stem DM (g pot^{-1})	leaf DM (g pot^{-1})	silique DM (g pot^{-1})	straw DM (g pot^{-1})	silique no. (pot^{-1})
1	0.0	29.1 ± 0.3 b B	9.6 ± 0.2 b B	25.0 ± 0.6	63.8 ± 1.0 B	585 ± 16 b
1	0.2	35.0 ± 1.0 b A	11.3 ± 0.3 c A	26.2 ± 0.3 c	72.4 ± 1.1 c A	623 ± 19 c
1	0.6	34.6 ± 0.4 b A	11.5 ± 0.4 c A	27.3 ± 0.2 c	73.4 ± 0.7 c A	662 ± 9 c
2	0.0	29.8 ± 1.4 ab B	10.8 ± 0.5 ab B	26.5 ± 1.1 B	67.1 ± 2.6 B	800 ± 32 a B
2	0.2	51.8 ± 1.4 a A	20.2 ± 0.4 b A	45.8 ± 1.0 b A	117.8 ± 1.6 b A	1177 ± 22 b A
2	0.6	50.2 ± 0.9 a A	21.1 ± 0.5 b A	47.0 ± 0.9 b A	118.3 ± 1.7 b A	1156 ± 375 b A
4	0.0	34.1 ± 1.8 a B	12.8 ± 0.7 a B	23.9 ± 1.2 B	70.8 ± 3.3 C	744 ± 48 a C
4	0.2	50.4 ± 1.4 a A	24.2 ± 1.5 a A	53.7 ± 1.2 a A	128.2 ± 1.8 a B	1352 ± 25 a B
4	0.6	51.6 ± 1.1 a A	27.6 ± 1.1 a A	57.0 ± 1.3 a A	136.1 ± 1.3 a A	1618 ± 47 a A
ANOVA	N	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	S	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	N × S	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

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

Table 2. Effect of N and S Supply on the Concentrations N and S, Their Ratio, and the Concentration of Nitrate in Leaves of Indian Mustard[a]

nutrient supply (g pot^{-1})					
N	S	N (mg g^{-1}of DM)	S (mg g^{-1} of DM)	N/S ratio	NO_3-N (mg kg^{-1} of DM)
1	0.0	11.06 ± 0.32 c	2.15 ± 0.92 B	8.00 ± 1.49 A	7 ± 2 c
1	0.2	10.28 ± 0.12 c	2.72 ± 0.15 AB	3.85 ± 0.25 AB	4 ± 2 b
1	0.6	10.28 ± 0.23 b	5.07 ± 0.15 a A	2.03 ± 0.06 b B	6 ± 3 c
2	0.0	21.58 ± 1.69 b A	2.52 ± 0.89	12.27 ± 2.77 A	1057 ± 187 b A
2	0.2	12.49 ± 0.35 b B	2.48 ± 1.02	7.27 ± 1.10 AB	42 ± 14 b B
2	0.6	12.03 ± 0.31 b B	4.89 ± 1.04 ab	2.88 ± 0.43 b B	244 ± 148 b B
4	0.0	28.71 ± 1.02 a A	2.27 ± 0.82	18.18 ± 3.47	1984 ± 51 a A
4	0.2	20.63 ± 0.71 a B	2.70 ± 0.75	9.63 ± 1.54	708 ± 158 a B
4	0.6	19.21 ± 0.51 a B	1.95 ± 0.10 b	10.06 ± 0.78 a	818 ± 78 a B
ANOVA	N	<0.0001	0.1476	<0.0001	<0.0001
	S	<0.0001	0.0056	<0.0001	<0.0001
	N × S	<0.0001	0.0747	0.1124	<0.0001

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

provision of N initially reduces the sinigrin concentration, whereas its content remains rather constant, indicating a dilution effect. Further increasing the N supply results in an impaired synthesis of sinigrin, as both its concentration and content are reduced.

The results presented so far show that the supply of both N and S influences the concentration of both sinigrin and its derivative in the seeds. In an attempt to relate these concentrations to the nutritional status, which is best described by the N/S ratio of the leaves, the relationship between these parameters and the N/S ratio was evaluated (**Figure 6**). Overall, a negative relationship between the sinigrin concentration and the N/S ratio of the leaves is apparent, and this trend is very strong for increasing S supply at low and intermediate N level (**Figure 6A**). In contrast, at ample provision of S, the sinigrin concentration did not respond to the S supply and, hence, the N/S ratio. The relationship between the N/S ratio and the concentration of allyl isothiocyanate is less consistent (**Figure 6B**). For the lowest and highest N supply the allyl isothiocyanate concentration in the seeds appears to be inversely related to the N/S ratio of the leaves, but at intermediate N supply the opposite relationship is observed.

Myrosinase Activity. Myrosinase is responsible for the hydrolysis of the glucose moiety and thus for the breakdown of sinigrin to allyl isothiocyanate. This enzyme activity exhibited substantial interactions with the treatments imposed (**Figure 7**). A significant increase in myrosinase activity was observed as the S supply was increased at the lowest N level, whereas this trend was insignificant at higher levels of N supply. Hence, the highest myrosinase activity was observed in seeds of plants grown with 1 g of N per pot without supplementary S.

DISCUSSION

Influence of N and S Supply on Growth and Nutritional Status. Growth and yield of Indian mustard plants responded strongly to the treatments imposed, exhibiting a classical interactive nutrient response (**Figure 2**; **Table 1**). Increasing provision of one element was more effective the closer the other element was to its optimum, as expected from the classical "Law of the Optimum" (*21*, *22*). Indeed, increasing provision of N without supplementary S supply even retarded yield formation. This phenomenon most likely stems from stimulated vegetative growth, and hence sequestration of S, by generous N supply, delaying remobilization of S during the generative phase (*23*). This limits endogenous S availability during seed formation and ultimately reduces seed yield (**Figure 2**). Under conditions of ample S supply, increasing provision of N increased seed yield dramatically (**Figure 2**), as well as all vegetative plant biomass, particularly the leaf DM and silique number (**Table 1**).

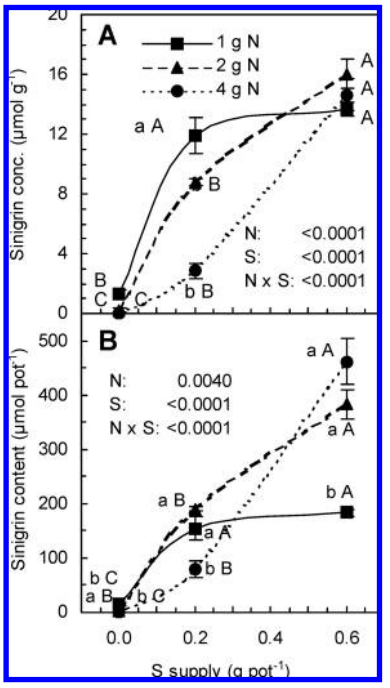

Figure 3. Effect of N and S supply on the concentration (**A**) and absolute content (**B**) of sinigrin in seeds of Indian mustard. Lower and upper case letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

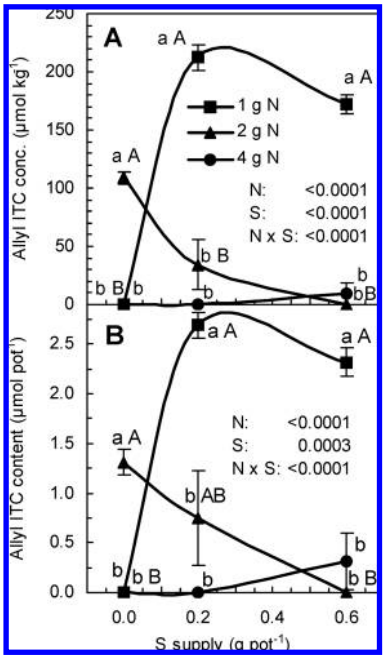

Figure 4. Effect of N and S supply on the concentration (**A**) and absolute content (**B**) of allyl isothiocyanate in seeds of Indian mustard. Lower and upper case letters are used to indicate significant differences ($p < 0.05$) between means (±SE) due to the effect of N and S supply, respectively.

To evaluate the impact of N and S supply on sinigrin and allyl isothiocyanate accumulation the nutritional status with respect to N and S needs to be considered. Critical values for Indian mustard are not tabulated. In leaves of young rapeseed plants and of cabbage plants at peak harvest, N concentrations of 33−64 and 20−26 mg of N (g DM)$^{-1}$, respectively, are considered to be adequate (*24*), whereas 4.5−6.5 mg of S (g DM)$^{-1}$ is considered to be adequate in leaves of rapeseed plants at early shooting (*25*) and 3.5 and

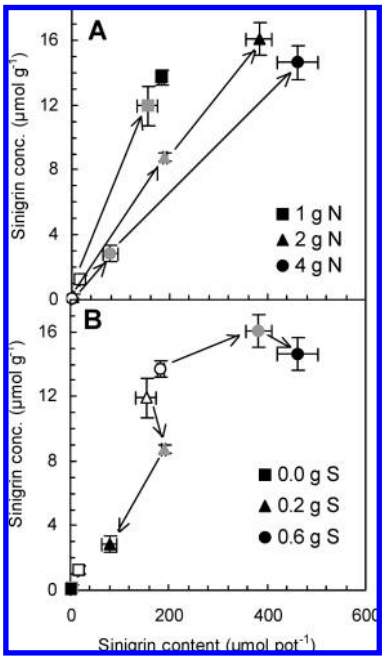

Figure 5. Graphical vector analysis, carried out for a fixed supply of either N (**A**) or S (**B**), of sinigrin concentration over sinigrin content accumulated in seeds of Indian mustard supplied with different S and N amounts (means ± SE). Open, gray, and solid symbols indicate the lowest, intermediate, and highest supply of S (**A**) or N (**B**), respectively.

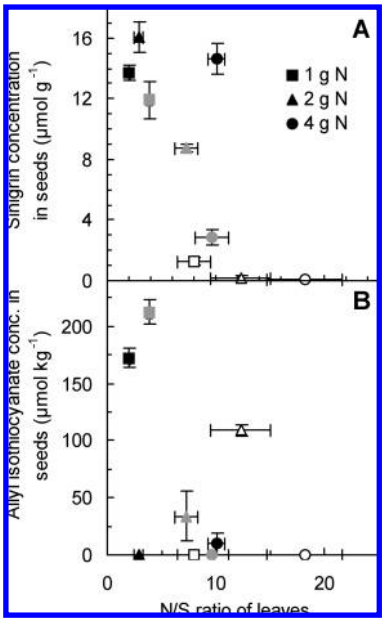

Figure 6. Relationship between the concentration of sinigrin (**A**) and allyl isothiocyanate (**B**) and the N/S ratio in Indian mustard seeds (means ± SE). Open, gray, and solid symbols indicate the lowest, intermediate, and highest supply of S.

2.6 mg of S (g DM)$^{-1}$ are considered to be critical during early and late flowering (*24*). In view of these numbers the N and S status was inadequate, unless N and S were generously supplied or unless concentration effects occurred due to serious growth restrictions when one of the two nutrients was in short supply (**Table 2**). However, as leaves were collected when senescing, the critical values used to assess the nutritional status of vigorously growing young plants do not apply, as typically a significant dilution effect is to be considered (*24*, *25*). In any case, as the S concentration does

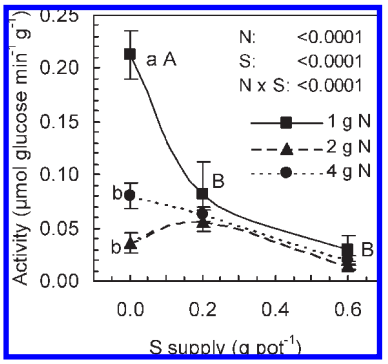

Figure 7. Effect of N and S supply on the activity of myrosinase in seeds of Indian mustard. Lower and upper case letters are used to indicate significant differences ($p < 0.05$) between means due to the effect of N and S supply, respectively (means ± SE).

not fall below a value of about 2 mg of S (g DM)$^{-1}$, this seems to represent the physiological bottom line for senescing mustard leaves under our experimental conditions.

The N/S ratio of leaves is frequently used to characterize the nutritional status of crops (*24*), and as well as having a physiological basis in the common presence of these nutrients in proteins, this parameter also has the merit of being less dependent on the plant organ selected and the developmental stage sampled (*25*). For rapeseed, during early and late flowering, adequate N/S ratios of 12 and 10.8, respectively, have been suggested (*24*). The N/S ratio indeed responded strongly to the treatments imposed, and it was higher the more N and the less S were supplied (**Table 2**). On the basis of the margins mentioned, the S status of plants grown with 1 g of N pot^{-1} is considered to be sufficient, as growth was primarily retarded by N deficiency. Plants that were grown with intermediate N represent a valuable treatment, as their S status was marginal when grown without S supplementation, and this restriction was continuously lifted as the S supply was increased. In contrast, when N was generously administered (4 g of N pot^{-1}), plants suffered severe S deficiency when grown without S supplementation, which was improved to marginal level as the S supply increased.

The nutrient elements N and S represent key elements used in amino acid formation and hence protein synthesis. Protein synthesis is impaired if the S supply is insufficient, leading to an accumulation of free amino acids and nitrate, the protein's immediate and ultimate precursors, respectively (*26*). Nitrate also accumulated in the leaves of the plants used here with increasing N supply, and this response was particularly strong when S was in short supply (**Figure 2**; **Table 2**). A similar response, namely, an accumulation of nitrate in S-deficient plants, is frequently observed in leafy vegetables and vegetative tissues (*18*, *27*).

Profile and Concentration Range of Glucosinolates and Isothiocyanates. Surprisingly little information is available concerning the interactive effects of N and S supply on the glucosinolate concentration of Indian mustard, and this applies even more so with respect to the isothiocyanate concentration in the seed. The glucosinolate profile of the Indian mustard variety used was dominated by sinigrin, indicating that it belongs to the sinigrin chemotaxonomic class (*1*), although the universal applicability of this classification has been questioned later (*29*). Gluconapin, the other glucosinolate frequently present in Indian mustard genotypes, was present only below the limits of quantitation, which agrees with a recent survey including *Sinapis alba* (*2*).

The actual sinigrin concentration in the seeds of plants adequately supplied with S reached 17 μmol (g^{-1} of seed) (**Figure 3**). For field-grown Indian mustard grown in Australia, Wisconsin, and Poland, up to 120, 126, and 165 μmol (g^{-1} of seed), respectively, were reported (*29*−*31*), considering a mean oil content of 32% (*30*). Canadian *B. juncea* mustard was reported to contain up to 99 μmol (g^{-1} of seed) (*32*). It is unclear whether the rather low glucosinolate concentration reported here stems from environmental effects or genotypic differences. Indeed, substantial genotypic variations of seed glucosinolate concentrations of a given *Brassica* species have been reported (*29*, *33*), in addition to a strong dependency on the S supply as discussed in the following.

Impact of the N and S Nutritional Status on Sinigrin Accumulation. As all glucosinolates contain at least two S groups, namely, the thiol bridge and the sulfate ester group (**Figure 1**), a strong influence of the S supply on the glucosinolate concentration can be envisaged (**Figure 3**) (*34*). Indeed, it was shown that without supplementary S the seed glucosinolate concentration might decrease to < 0.6 μmol (g^{-1} of seed) (*32*), and it is speculated that the S status was not sufficient to allow for a genotype-specific glucosinolate formation. As the side chain of sinigrin is derived from methionine, a S-containing amino acid, and the thiol group from cysteine, a strong influence of the N supply and an interactive effect of both nutrients, as observed here, is to be expected (**Figure 3**). Apparently, such a strong interaction has not been reported for Indian mustard seeds before, although it has been demonstrated to some extent for low-glucosinolate oilseed rape (*35*). For vegetative tissues such as kohlrabi tubers (*18*), turnip roots (*36*), and broccoli inflorescences (*7*) strong interactive effects of N and S supply have also been reported. In the absence of supplementary S, varied N supply did not affect the sinigrin concentration due to absolute S deficit. At the highest S supply tested, an increased N supply did not reduce the glucosinolate concentration in the seeds as frequently reported for other species (*27*, *37*), presumably because the ample S supply circumvented a dilution effect induced by largely increased seed yield (**Figure 2**). This resulted in significantly increased sinigrin accumulation (**Figure 3B**) and hence demand for S. In a pot experiment with rapeseed it was demonstrated that S deficiency strongly reduced the glucosinolate concentration in the seed (*38*), whereas the concentrations of methionine and cysteine were marginally and strongly reduced, respectively (*39*). In contrast, at intermediate S supply, which has been classified as marginal (see above), increasing provision of N led to a significant reduction of the sinigrin concentration and content, pointing to a strong dilution effect due to significantly enhanced yield (**Figure 2**). Apparently, S in vegetative parts was not mobilized sufficiently to meet the S requirement for seed production, and this can be explained by the different responses to N and S deficiency. Whereas a shortage of N induces a breakdown of proteins and earlier senescence of older leaves, this response is not initiated to a sufficient extent under S-deficient conditions, resulting in younger plant parts being deprived of S (*23*).

GVA indicates that any increase of the S supply resulted in an increase of both concentration and content of sinigrin for all N supplies tested, indicative for a truly enhanced synthesis (**Figure 5**). In contrast and in agreement with the interpretations given above, increasing N supply in a high S environment resulted in steady state conditions as indicated by the fairly stable sinigrin concentration (**Figure 5B**).

Increasing N supply at marginal S status induced a growth-induced dilution effect initially, followed by a genuine diminution of sinigrin synthesis as the N supply was further increased. The N/S ratio in vegetative plant parts has frequently been used to characterize the S status (*25*), and an inverse relationship between this variable and the glucosinolate concentration has been observed in both vegetative (*18*, *28*) and generative tissues (*40*). At first glance the relationship observed here is not very close (**Figure 6A**). However, as discussed before, at high and low S supply the sinigrin concentration does not respond to large extent to the N/S ratio, as the S status is excessive and severely limiting, respectively. In contrast, the sinigrin concentration is closely related to the N/S ratio at intermediate S supply.

Impact of the N and S Nutritional Status on the Concentration of Allyl Isothiocyanate. It has been argued that glucosinolates represent a transient storage pool for S that is broken down under conditions of limited S availability (*4*, *9*, *10*). This would result not only in reduced glucosinolate concentrations (**Figure 3**) but also in the liberation of isothiocyanates and their accumulation (**Figure 1**). Although the presence of isothiocyanates has been reported in a wide range of *Brassica* seeds (*33*, *41*), apparently this investigation reports the interactive effects of N and S supply on seed isothiocyanate concentrations for the first time. The isothiocyanate concentration shows a distinct response to the N and S supply, and this response also applies to the absolute contents accumulated in the seeds (**Figure 4**). Whereas the isothiocyanate concentration did not respond to the S supply when N was generously provided, it responded positively to S when N was in short supply, in line with the response of the sinigrin concentration (**Figure 3**). In contrast, at intermediate N supply increasing the S status led to a significant reduction of the isothiocyanate concentration, exhibiting the opposite response from sinigrin. Should this support the concept of glucosinolates acting as a transient reservoir for S, initiating less glucosinolate breakdown and hence less isothiocyanate accumulation as the S deficiency is relieved (*10*)? Evaluating the response of the myrosinase, the key enzyme responsible for glucosinolate breakdown, reveals that its activity was not significantly affected by the N status, provided that S was not in short supply (**Figure 7**). Moreover, in the absence of S supplementation, N deficiency stimulated myrosinase activity, but this did not lead to a liberation of allyl isothiocyanate (**Figure 4**), presumably because its substrate was in short supply (**Figure 3**). Under N-deficient conditions an improved S status led to a diminishing myrosinase activity, opposing the observations in *S. alba* (*10*) and contradicting the concept of glucosinolates acting as transient S reservoir. However, it should be noted that enzyme and substrate of the glucosinolate−myrosinase−system are strictly compartmentalized (*3*), even though views differ on their exact location. This compartmentalization of enzyme and substrate is likely to be more critical for isothiocyanate liberation than the myrosinase activity itself, especially because the concentration of the substrate, sinigrin, is 100 times higher than the concentration of its product allyl isothiocyanate. No information on the interactive influence of N and S supply on glucosinolate compartmentalization is available.

Analyzing the response of the isothiocyanate concentration to increasing N supply at intermediate S provision, leading to N-induced S deficiency, clearly shows that isothiocyanate liberation is lower the higher the N status and does not suggest that glucosinolates are broken down in response to N-induced S starvation. The relationship between the N/S ratio and the allyl isothiocyanate concentrations supports the unique response of plants receiving intermediate N doses to increasing S supply and hence N/S ratios (**Figure 6B**). It is possible that this effect stems from the relief of the physiological S deprivation of generative organs due to effective sequestration of S and N in vegetative parts. This S sequestration is diminishing as the S status is improved, not only because more S is available but also because the greatly increased sink capacity (**Figure 2**) is likely to further stimulate protein breakdown in mature leaves, leading to a further boost of S available for the formation of seeds and sinigrin therein (**Figure 3**).

In conclusion, this study demonstrated for the first time that N and S supply exert a strong interactive impact not only on yield formation and glucosinolate accumulation but also on the concentration of allyl isothiocyanate in Indian mustard. However, linking the concentrations of substrate (sinigrin) and product (allyl isothiocyanate) to the myrosinase activity was not possible, presumably due to the compartmentalization at cellular level governing accessibility of the substrate. The results do not support the hypothesis that liberation of isothiocyanates is enhanced in response to S limitation. However, it was demonstrated that a high N supply not only increases seed yield, but—contrary to views frequently encountered—did not reduce the sinigrin concentration provided that S was adequately supplied. This further stresses the significance of adequate provision of S to improve not only the profitability of mustard cultivation for its seed but also its flavor characteristics and nutritional value.

ABBREVIATIONS USED

DTPA, diethylenetriaminepentaacetic acid; DM, dry matter; FM, fresh matter; GVA, graphical vector analysis.

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LITERATURE CITED

(1) Gland, A.; Robbelen, G.; Thies, W. Variation of alkenyl glucosinolates in seeds of *Brassica* species. *Z. Pflanzenzuecht*. **1981**, *87*, 96–110.

(2) Song, L.; Iori, R.; Thornalley, P. J. Purification of major glucosinolates from *Brassicaceae* seeds and preparation of isothiocyanate and amine metabolites. *J. Sci. Food Agric*. **2006**, *86*, 1271–1280.

(3) Fahey, J. W.; Zalcmann, A. T.; Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochem*. **2001**, *56*, 5–51.

(4) Bones, A. M.; Rossiter, J. T. The myrosinase−glucosinolate system, its organisation and biochemistry. *Physiol. Plant*. **1996**, *97*, 194–208.

(5) Mithen, R. F.; Dekker, M.; Verkerk, R.; Rabot, S.; Johnson, I. T. The nutritional significance, biosynthesis and biovailability of glucosinolates in human foods. *J. Sci. Agric*. **2000**, *80*, 967–984.

(6) Higdon, J. *Isothiocyanates*; Linus Pauling Institute, Oregon State University: Corvallis, OR, 2005.

(7) Schonhof, I.; Blankenburg, D.; Müller, S.; Krumbein, A. Sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. *J. Plant Nutr. Soil Sci*. **2007**, *170*, 65–72.

(8) Zimmermann, N.; Krumbein, A.; Zhu, Z.; Gerendás, J. Influence of N and S supply on contents of glucosinolates and their

precursor amino acids in Bai Cai (*Brassica campestris* L. ssp. *chinensis*). *Plant Nutrition for Food Security, Human Health and Environmental Protection*; Li, C. J., et al. , Eds.; Tsinghua University Press: Beijing, China, 2005; pp 390−391.

(9) Schnug, E.; Haneklaus, S.; Borchers, A.; Polle, A. Relations between sulphur supply and glutathione and ascorbate concentrations in *Brassica napus*. *Z. Pflanzenernaehr. Bodenk.* **1995**, *158*, 67–69.

(10) Bones, A. M.; Visvalingam, S.; Thangstad, O. P. Sulphate can induce differential expression of thioglucoside glucohydrolases (myrosinases). *Planta* **1994**, *193*, 558–566.

(11) Chen, S.; Halkier, B. A. Characterization of glucosinolate uptake by leaf protoplasts of *Brassica napus*. *J. Biol. Chem.* **2000**, *275*, 22955–22960.

(12) Brudenell, A. J. P.; Griffiths, H.; Rossiter, J. T.; Baker, D. A. The phloem mobility of glucosinolates. *J. Exp. Bot.* **1999**, *50*, 745–756.

(13) Schüller, H. Die CAL-Methode, eine neue Methode zur Bestimmung des pflanzenverfügbaren Phosphates in Böden. *Z. Pflanzenernaehr. Bodenk.* **1969**, *123*, 48–63.

(14) Lindsay, W. L.; Norvell, W. A. Development of a DTPA soil test for zinc, iron, manganese and copper. *Soil Sci. Soc. Am. J.* **1978**, *42*, 422–428.

(15) Bennett, R. N.; Mellon, F. A.; Kroon, P. A. Screening crucifer seeds as sources of specific intact glucosinolates using ion-pair high-performance liquid chromatography negative ion electrospray mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 428–438.

(16) Zeuner, C. R. Isothiocyante aus Brassicaceae—quantitative und qualitative Aspekte. Master's thesis, Institute for Nutritional Sciences, Justus Liebig University, Giessen, Germany, 2005.

(17) Bones, A.; Slupphaug, G. Purification, characterization and partial amino acid sequencing of β-thioglucosidase from *Brassica napus* L. *J. Plant Physiol.* **1989**, *134*, 722–729.

(18) Gerendás, J.; Breuning, S.; Stahl, T.; Mersch-Sundermann, V.; Mühling, K. H. Isothiocyanate concentration in kohlrabi (*Brassica oleracea* L. Var. *gongylodes*) plants as influenced by sulfur and nitrogen supply. *J. Agric. Food Chem.* **2008**, *56*, 8334–8342.

(19) Horn, M.; Vollandt, R. *Multiple Tests und Auswahlverfahren*; Gustav Fischer Verlag: Stuttgart, Germany, 1995.

(20) Koricheva, J. Interpreting phenotypic variation in plant allelochemistry: problems with the use of concentrations. *Oecologia* **1999**, *119*, 467–473.

(21) Wollny, E. Das Grundgesetz der Pflanzenproduktion. *Illust. Monatshefte Gesamt-Interessen Gartenbaues* 1887, *6*, 232−237, 257−260, 290−293.

(22) Liebscher, G. Untersuchungen über die Bestimmung des Düngerbedürfnisses der Ackerböden und Kulturpflanzen. *J. Landwirtsch.* **1895**, *43*, 49–216.

(23) Marschner, H. *Mineral Nutrition of Higher Plants*, 2nd ed.; Academic Press: Amsterdam, The Netherlands, 1995.

(24) Reuter, D. J.; Robinson, J. B. *Plant Analysis—An Interpretation Manual*, 2nd ed.; Inkata Press: Sydney, Australia, 1988.

(25) Bergmann, W. *Ernährungsstörungen bei Kulturpflanzen*, 3rd ed.; Gustav Fischer Verlag: Jena, Germany, 1993.

(26) Eagle, D. R.; Eaton, F. M. Sulphur nutrition of cotton. *Plant Physiol.* **1951**, *26*, 639–654.

(27) Schnug, E. Sulphur nutrition and quality of vegetables. *Sulphur Agric.* **1990**, *14*, 875–877.

(28) Gerendás, J.; Sailer, M.; Fendrich, M.-L.; Stahl, T.; Mersch-Sundermann, V.; Mühling, K. H. Influence of sulfur and nitrogen supply on growth, nutrient status and concentration of benzyl-isothiocyanate in cress (*Lepidium sativum* L.). *J. Sci. Food Agric.* **2008**, *88*, 2576–2580.

(29) Palmer, M. V.; Sang, J. P.; Oram, R. N.; Tran, D. A.; Salisbury, P. A. Variation in seed glucosinolate concentrations of Indian mustard (*Brassica juncea* (L.) Czern. + Coss.). *Aust. J. Exp. Agric.* **1988**, *28*, 779–782.

(30) Nowak-Polakowska, H.; Czaplicki, S.; Tánska, M.; Jankowski, K. Chemical composition of white and sarepta mustard seeds as affected by differentiated conditions of nitrogen top-dressing at sowing-preceding fertilization with sulphur and magnesium. *Pol. J. Nat. Sc.* **2005**, *18*, 25–37.

(31) Carlson, D. G.; Daxenbichler, M. E.; VanEtten, C. H.; Kwolek, W. F.; Williams, P. H. Glucosinolates in crucifer vegetables: broccoli, Brussels sprouts, cauliflower, collards, kale, mustard greens, and kohlrabi. *J. Am. Soc. Hortic. Sci.* **1987**, *112*, 173–178.

(32) Malhi, S. S.; Gan, Y.; Raney, J. P. Yield, seed quality and sulfur uptake of *Brassica* oilseed crops in response to sulfur fertilization. *Agron. J.* **2007**, *99*, 570–577.

(33) West, L. G.; Meyer, K. A.; Balch, B. A.; Rossi, F. J.; Schultz, M. R.; Haas, G. W. Glucoraphanin and 4-hydroxyglucobrassicin contents in seeds of 59 cultivars of broccoli, raab, kohlrabi, radish, cauliflower, brussels sprouts, kale, and cabbage. *J. Agric. Food Chem.* **2004**, *52*, 916–926.

(34) Marquard, R.; Kühn, H.; Linser, H. Der Einfluss der Schwefelernährung auf die Senfölbildung. *Z. Pflanzenernaehr. Bodenk.* **1968**, *121*, 221–230.

(35) Zhao, F. J.; Evans, E. J.; Bilsborrow, P. E.; Syers, J. K. Influence of sulfur and nitrogen on seed yield and quality of low glucosinolate oilseed rape (*Brassica napus* L.). *J. Sci. Food Agric.* **1993**, *63*, 29–37.

(36) Li, S.; Schonhof, I.; Krumbein, A.; Li, L.; Stützel, H.; Schreiner, M. Glucosinolate concentration in turnip (*Brassica rapa* ssp. *rapifera* L.) roots as affected by nitrogen and sulfur supply. *J. Agric. Food Chem.* **2007**, *55*, 8452–8457.

(37) Josefsson, E. Method for quatitative determination of p-hydroxybenzyl isothiocyanate in digests of seed meal of *Sinapis alba* L. *J. Sci. Food Agric.* **1968**, *19*, 192–194.

(38) Josefsson, E.; Appelqvist, L. A. Glucosinolates in seed of rape and turnip rape as affected by variety and environment. *J. Sci. Food Agric.* **1968**, *19*, 564–570.

(39) Josefsson, E. Glucosinolate content and amino acid composition of rapeseed (*Brassica napus*) meal as affected by sulphur and nitrogen nutrition. *J. Sci. Food Agric.* **1970**, *21* 98–103.

(40) Fismes, J.; Vong, P. C.; Guckert, A.; Frossard, E. Influence of sulfur on apparent N-use efficiency, yield and quality of oilseed rape (*Brassica napus* L.) grown on a calcareous soil. *Eur. J. Agron.* **2000**, *12*, 127–141.

(41) Afsharypuor, S.; Suleimany, M. Volatile oil constituents of *Brassica oleracea* var. *gongylodes* seeds. *J. Essent. Oil Res.* **2002**, *14*, 18–19.

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