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Interactive effects of genotype and N/S-supply on glucosinolates and glucosinolate breakdown products in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Summary

Chinese cabbage is rich in glucosinolates (GLS) and their breakdown products, mainly isothiocyanates (ITC), which are assumed to be human health-promoting compounds. Sulphur and nitrogen have been shown to influence concentrations and patterns of both. Little is known as to whether the effect of varying sulphur and nitrogen nutrition on glucosinolate and isothiocyanate content is influenced by the genotype. Therefore, two cultivars of *Brassica rapa* L. ssp. *pekinensis* were grown with increasing S (0.0, 0.3, and 0.6 g pot^{-1}) and N (1 and 2 g pot^{-1}) supply. Results show that total GLS concentration increased with higher N and S application, but ratios between individual GLS compounds remained unchanged. High N supply reduced the concentration of GLS, especially of the aliphatic ones, while the indole and aromatic GLS exhibited statistically insignificant responses to increasing N and S application. The profile of breakdown products was dominated by epithionitriles, followed by ITCs and nitriles. The ITCs were substantially reduced in response to increasing N and decreasing S supply. This was not observed for nitriles. Overall, GLS pattern were primarily influenced by the genetic background of the cultivar and less influenced by differential nutrition. Results show that selection of the cultivar is of utmost importance when glucosinolates and their breakdown products shall be increased by fertilization.

Keywords: *Brassica rapa* L. ssp. *pekinensis*, glucosinolates, isothiocyanates, breakdown products, nitrile, sulphur, nitrogen, genotype.

Abbreviations: 3But-ITC: 3-butenyl-ITC, 3But-CN: 4-pentenenitrile, CETB: 1-cyano-3,4-epithiobutane, 4Pent-ITC: 4-pentenyl-ITC, 4Pent-CN: 5-hexenenitrile, CETPent: 1-cyano-4,5-epithiopentane, OZT: 5-vinyl-1,3-oxazolidine-2-thione, 2OH3But-CN: 3-hydroxypentenenitrile, CHETB: 3-hydroxy-4,5-epithiopentylnitrile, 2PE-ITC: 2-phenylethyl ITC, 2PE-CN: 3-phenylpropanenitrile, IAN: indolacetonitrile, 4Met-IAN: 4-Methoxyindole-3-acetonitrile, DW: dry weight, ECN: Effective-carbon-number, FW: fresh weight, GC-FID: Gas-phase chromatograph-flame-ionisation-detector, GC-MS: Gas-phase chromatograph-mass spectrometry, GLS: glucosinolate. FM: Fresh matter, GLS: Glucosinolate, ITC: Isothiocyanate, OS: Chinese cabbage cultivar named Orient Surprise, Y: Chinese cabbage cultivar named Yuki.

Introduction

There is growing evidence that plant-derived bioactive compounds in the diet play an important role in promoting human health. About 10% of the world's vegetable production is generated from Brassicaceae, wherein *Brassica rapa* is a dominating species. Members of the *Brassicaceae* family include many important horticultural crops such as cabbage, kale, cauliflower, broccoli and kohlrabi that all contain a group of very potent phytochemicals, namely glucosinolates (GLS), and their breakdown products (Verkerk et al., 2009). They represent important aroma and flavour components. A high consumption of Brassica vegetables correlates negatively with the incidence of degenerative diseases in numerous epidemiological studies (Voorrips et al., 2000; Higdon et al., 2007; Wu et al., 2013). Particularly the indole GLS, glucoraphanin and the isothiocyanates (ITC), being breakdown products of GLS, are relevant for human health because of their anti-carcinogenic and antioxidant capacity (Mithen et al., 2001; Holst and Williamson, 2004; Schonhof et al., 2007).

GLS are located in vacuoles and the tonoplast separates them from contact with the degrading cytoplasmic enzyme myrosinase. However, upon maceration of the plant tissue, e.g. by herbivory attack, GLS get into contact with myrosinase (Fahey et al., 2001; Zimmermann et al., 2007). Myrosinase hydrolyses GLS into glucose, sulphate, and various other metabolites such as ITC, nitriles, thiocyanates, and epithionitriles (Fenwick, 1983; Hanschen et al., 2014). In the plant, this reaction is part of the chemical defence because ITCs act as repellent and have anti-microbial activity (Mithöfer and Boland, 2012). The formation of these different breakdown products depends on a multitude of factors like specifier proteins, pH, amino acid side chain and metal-ions (Rangkadilok et al., 2002).

Chinese cabbage is a relevant source of GLSs and ITCs because it is a popular vegetable in Asia (Kim et al., 2010; Zhu, 2006) and, moreover, dietary intake of Chinese cabbage leaves has also a tradition in parts of Europe (Gilbert and Khokar, 2008). In agricultural and horticultural production of GLS-rich foods, it is critical to control and optimize nitrogen (N) and sulphur (S) supply. This is because increasing availability of N raises the amount of GLSs initially, while excess of N negatively impacts the synthesis of GLSs (Omirou et al., 2009). This, however, also depends on the availability of S which is based on interactive effects between S and N (Zhao et al., 1994; Li et al., 2005; Kim et al., 2002; Rosen et al., 2005). In general, concentrations of GLSs in Chinese cabbage have been well described (Daxenbichler et al., 1979; Chen et al., 2008; Kübler et al., 2008; Kim et al., 2010). However, only few studies have investigated the interactive effects of S and N on individual GLS and ITCs, with a few exceptions: A correlation between GLS concentration and S supply, that was partly influenced by the plant tissue N concentration, has been reported (Schonhoff et al., 2007; Li et al., 2007; Gerendás et al., 2008a, Gerendás et al., 2008b; Chen et al., 2008; Omirou et al., 2009). Gerendás et al. (2008b) found that an increase of N fertilization at low S availability resulted in a decrease in total indole GLS. In this context, a high S supply result in increased ITC concentrations due to enhanced breakdown of GLSs. Despite this relevance, very little is known as to whether the effects of varying S and N supply on glucosinolate and isothiocyanate content are influenced by the plant genotype. With respect to genotypic effects, there is dearth of published data that thoroughly profile the interactive effects of S and N on various individual glucosinolates moieties including their enzymatic breakdown products. The extent of the genotypic preposition of pattern

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and concentration of GLSs and ITCs in dependency to variations in N and S supply is not well resolved so far. A genotypic effect can be hypothesized because it was reported that the glucosinolate concentration in *Brassica* depends on the species, the developmental stage and the environmental conditions (ROSA et al., 1997; VALLOJO et al., 2003; RADOVICH et al., 2005). The aim of this study has been to tackle this open question regarding the influence of the genotype. Here we present data that show how important the selection of a suitable genetic background is to enrich the *Brassica* vegetable Chinese cabbage in GLSs and their breakdown products.

Material and methods

Plant material and growth conditions

Experiments were conducted in Mitscherlich pots filled with 6.5 kg nutrient-poor sand (retrieved from Karkendamm, Schleswig-Holstein, Germany). Each pot received a basal fertilization of 0.5 g KCL, 0.9 g $Ca(H_2PO_4)$, 0.5 g $MgCl_2$, 200 mg Fe-EDTA, 30 mg $MnCl_2$, 20 mg $ZnCl_2$, 15 mg $CuCl_2$, 15 mg $CaCO_3$, 10 mg H_3BO_3 and 2 mg $(NH_4)_2Mo_7O_{24}$. Chinese cabbage seeds (*Brassica rapa* L. ssp. *pekinensis*, cv. Yuki and cv. Orient Surprise) were purchased from HILD Samen (Marbach, Germany). The presented study is based on the cvs. Yuki and Orient Surprise because a previous phenotyping study on the Chinese cabbage cvs. Bilko, China Express, Emiko, Granat, Hong Kong, Janin, Kasumi, Kilankin, Manoko, Orankin, Orient Surprise, Parkin, Richi, Sprinkin, Tabaluga, Treasure Island and Yuki revealed that the two cvs. Orient Surprise and Yuki contained the highest concentrations of both, total and indole GLS (HASLER and BÖHLENDORF, 2013). Chinese cabbage plants were cultivated for 10 weeks in a greenhouse with 14 h light at 20 °C and 10 h darkness at 16 °C and supplementary lighting. Light intensity was 200 µmol s^{-1} m^{-2}, on average (lamp, Professional Lighting, SON-K 400, Philips Deutschland GmbH, Hamburg, Germany; bulb, Philips SON-T Agro 400 watt, Philips Deutschland GmbH, Hamburg, Germany). Plants were treated with two levels of N (1.0 and 2.0 g pot^{-1}; given as KNO_3/NH_4NO_3) in combination with three levels of S (0, 0.3 and 0.6 g pot^{-1}; given as $CaSO_4$). A completely randomized design was established with one plant per pot and four biological replications per treatment. The fresh weight per pot was recorded directly after harvesting at the vegetative stage before head formation (BBCH 19 according to FELLER et al., 1995). Plant material was freeze-dried for dry matter determination and, after grinding, used for N, S, GLS and GLS breakdown product analysis.

GLS analyses

The HPLC method reported by KRUMMBEIN et al. (2005) was used for GLS determination. For this, 5 g freeze-dried and finely ground plant material was used whereas the myrosinase activity was heat inactivated by a 1-minute treatment at 75 °C. After extraction, 4 ml of a methanol/water mixture (70:3 v/v, T=75 °C) and 200 µl 5 mM 2-propenyl standard were added and the sample was incubated at 75 °C for 10 min and occasionally mixed. After adding 1 ml 0.4 M barium acetate, the sample was centrifuged at 4.000 rpm for 10 min. The supernatant was recovered and the sample mixed twice with 3 ml of the methanol/water mixture (70:3 v/v, T=75 °C) and centrifuged again. The combined extracts were diluted to 10 ml with the methanol/water mixture. Five ml of the extract was added to a 250 µl DEAE-Sephadex A-25 ion-exchange and rinsed with 10 ml distilled water. Next, 250 µl of purified aryl sulfatase solution was added. After 12 h, the desulpho compounds were eluted with 5 ml distilled water. After passing through a 0.45 m filter, 10 µl of the purified extract were used for GLS determination. The analysis was performed using an Agilent 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany) with a diode array detector (DAD) and a Spherisorb ODS2 column (5 µm, 250 × 4 mm; Bischoff, Leonberg, Germany). Samples were analyzed by using a gradient of 0% to 20% of acetonitrile in water (2-34 min), followed by 20% acetonitile in water (6 min) completing with 100% acetonitril (10 min). The analysis was carried out with a flow of 1.3 ml min^{-1} and detection at 229 nm. The individual GLSs were identified by comparisons of their retention time with that of individual GLSs in reference material. GLS concentration was calculated using 2-propenyl as internal standard.

GLS breakdown product analysis

Lyophilized and homogenized leaves (50 mg) are ground in 1 mL of water in 4 mL glass vials. After addition of 50 µl benzonitrile (100 ng/µl in methanol) as an internal standard, the samples were extracted with 2 × 2ml of dichloromethane. The combined organic phases were dried over Na_2SO_4, concentrated to about 500 µl in a nitrogen stream, and analyzed by GC with flame ionization detection (FID) using an Agilent 6890 series gas chromatograph with an HP5MS column (30 m × 0.25 mm × 0.25 µm), splitless injection at 200 °C (injection volume 1 µl) , and the following temperature program: 40 °C for 3 min, 10 °C/min to 250 °C, and 4-min final hold. Identity of the products was verified by GC-MS (LAMBRIX et al., 2001) in comparison with authentic standards and published MS spectra (SPENCER and DAXENBICHLER, 1980). Products were quantified using the molar FID-response-factors calculated by the ECN (effective-carbon-number) approach (SCANION and WILLIS, 1985): 3But-ITC (ECN 4.9, RF 1.29), 3But-CN (ECN 4.2, RF 1.5), CETB (ECN 4.3, RF 1.47), 4Pent-ITC (ECN 5.9, RF 1.07), 4Pent-CN (ECN 5.2, RF 1.21), CETPent (ECN 5.3, RF 1.19), OZT (ECN 4.4, RF 1.43), 2OH3But-CN (ECN 3.8, RF 1.66), CHETB (ECN 4.7, RF 1.34), 2PE-ITC (ECN 9.0, RF 0.70), 2PE-CN (ECN 8.3, RF 0.76), IAN (ECN 9.3, RF 0.68), 4Met-IAN (ECN 10.3, RF 0.61).

Plant N and S analysis

The N and S concentration were determined with elementary-analyser (Elementator Analysesystem GmbH, Hanau, Germany) as described by ZÖRB et al. (2013). For analysis, 5-10 mg of freeze dried plant material was used, combined with twice the amount of Wolfram-(IV)-oxide.

Statistical analysis

The experiments were set up in a complete randomized design. All data were assumed as approximately normally distributed with homogenous variances. Variables (FW, DW, GLS groups, break down product groups, %N, %S and N×S ratio) have been analyzed separately. First an ANOVA was applied, followed by multiple contrast tests for the factors varieties and N and S nutrition. All GLS breakdown products have been statistically analyzed simultaneously to incorporate there correlations. A multivariate ANOVA followed by a related multiple contrast test for multiple endpoints according to HASLER and HORTHORN (2011) were applied. Furthermore, a non-linear regression was done for the variables nitriles and total ITC concentration depending on the N×S ratio, following the results of GERENDÁS et al. (2008a) and ZIMMERMANN et al. (2007). All tests were performed at a significance level of $P < 0.05$. Calculations were carried out using the software R (R Development Core Team, 2010).

Results und discussion

Influence of N and S supply on biomass

Two cvs. of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) cultivated in soil were subjected to increasing concentration of N and

S in order to evaluate whether the effects of varying N and S supply on the GLS pattern and their human health-promoting breakdown products are affected by the genotype.

S-deprivation (0 g S per pot) resulted in the typical S-deficiency symptoms, namely chlorosis of younger leaves and decreased biomass (Fig. 1). When compared to the 0 S control, the addition of 0.3 g S per pot significantly increased whole shoot biomass whereas growth could not again be significantly increased by adding another 0.3 g S per pot (Fig. 2). This S effect on biomass formation was observed for both cvs. Yuki and Orient Surprise. The increase from 0.3 to 0.6 g S per pot was reflected by a concomitant increase in tissue S concentration (Tab. 1). Thus, Chinese cabbage contained excess leaf tissue S when 0.6 g S was given per pot and, consequently, lack of S can be ruled out as reason for the stop of biomass formation when S dose was increased from 0.3 to 0.6 g S. To the contrary, increase in the N supply from 1 to 2 g N per pot further increased the biomass in both varieties when plants were given ample S (0.6 g S per pot), indicating that 1 g N per pot was still inadequate. This is further supported by the appearance of N deficiency symptoms in these treatments (Fig. 1 A and C vs. B and D). After all, N is, besides phosphorus, a nutrient with very pronounced effects on growth (Ågren et al., 2012). Thus, highest fresh weight yield (432.3 g plant^{-1}) was observed under conditions when plants were supplied with 0.3 g S and 2 g N per pot (Fig. 1; Fig. 2). When no S was given, viz. when plants relied on the residual S from the soil, whole shoot biomass stagnated at 6.7 g plant^{-1}.

S supply has a larger impact on GLS accumulation than supply of N

The total GLS concentration ranged from 0.2-16.0 µmol g^{-1} DW when plants were grown with 1 g and 2 g of N, and 0.3 g and 0.6 g of S. Compared with other studies, the presented investigation revealed very low GLS concentration: Kim et al. (2010) reported a total GLS concentration in Chinese cabbage of 4.48-31.58 µmol g^{-1} DW. Since GLS concentration of *Brassica* varieties are strongly influenced by environmental conditions such as light, temperature, growing season length (Chartea et al., 2008; Ciska et al., 2000; Ludwig-Müller et al., 1996) or stage of harvest (De Maria et al., 2016) combined with the fact that our experiment was conducted in winter period in northern Germany (54° 19' 23.854" N 10° 7' 21.955" E) and plants were harvested at BBCH 19 according to Feller et al. (1995), we reason that such cues are attributable to the differences described here. With regard to the total GLS concentration, we further show that the effect of N nutrition was very confined (Fig. 3). Only for cv. Orient Surprise grown at the highest S level, there was a significant reduction by supply with excess N. In contrast to this, S fertilization had a major influence on GLS amounts. Here, the increase from 0.3 g to 0.6 g S per pot increased the total GLS concentration in the cv. Yuki, which was expected in view of frequent reports on the positive influence of higher S supply on GLS biosynthesis in *Brassica* (Gerendás et al., 2008a; Kim et al., 2002; Rosen et al., 2005; Zhao et al., 1994). Surprisingly, this effect was not observed for the cv. Orient Surprise when S supply was increased from 0.3 g to 0.6 g S. From these results, the question arises why the two cultivars reacted differently. Currently, this awaits clarification but – when compared to the cv. Orient Surprise – data in Fig. 6 suggest that the cv. Yuki might more efficiently remobilize S from glucosinolates by means of catabolic breakdown under conditions of S deficiency. This can be suggested because total concentration of isothiocyanates in Yuki was higher than in Orient Surprise when S was limited (Fig. 6). This higher amount of GLS breakdown products in the cv. Yuki might explain why the increase from 0.3 g to 0.6 g S per pot increased the total GLS concentration only in the cv. Yuki. Generally, further knowledge of genotypic specificities for the glucosinolate metabolism and its control under S-deficiency is required to achieve a better understanding of the role of these nutrient changes.

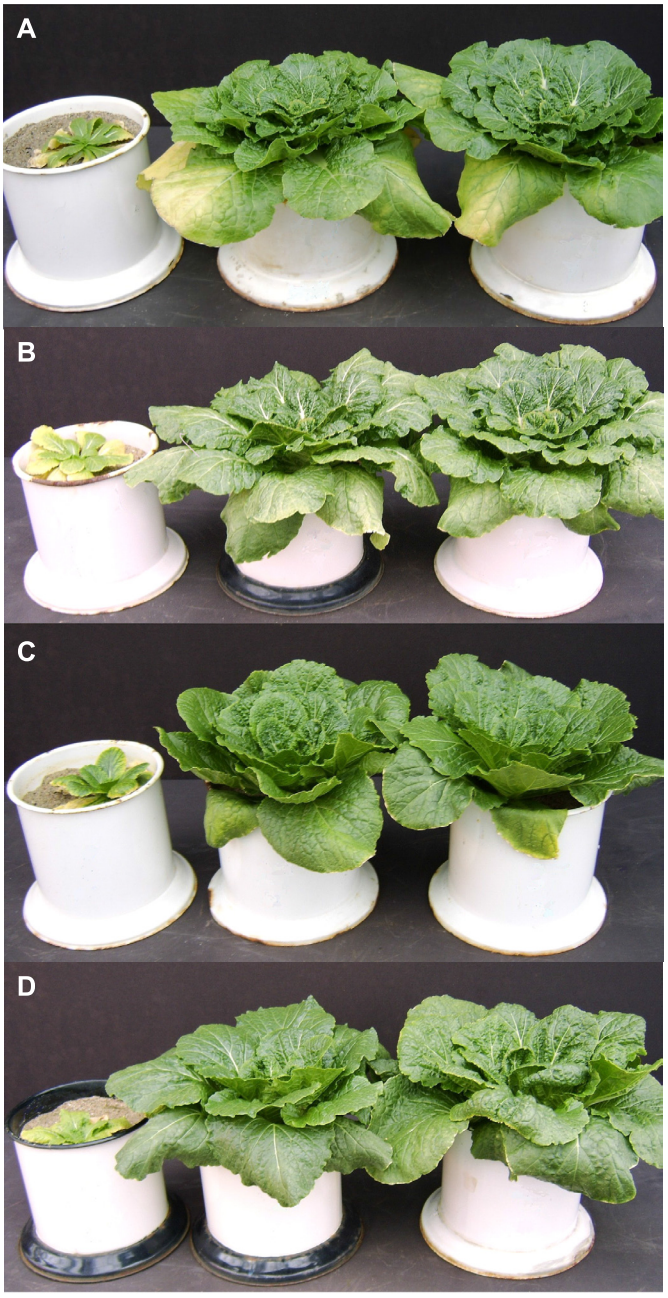

Fig. 1: Chinese cabbage cvs. Yuki and Orient Surprise in dependency to N (1.0 and 2.0 g pot^{-1}) and S (0, 0.3 and 0.6 g pot^{-1}) supply. In each sub-figure, increasing S supply is ranked in ascending order from 0.0 over 0.3 to 0.6 g S pot^{-1} from left to right. A, Yuki at 1 g nitrogen; B, Yuki at 2 g nitrogen; C, Orient Surprise at 1 g nitrogen; D, Orient Surprise at 2 g nitrogen.

It might be argued that N and S levels were chosen arbitrarily (in statistical terms resulting in model 'fixed'), limiting the eligibility of such interpretation. However, the fact that doubling the N supply – contrary to doubling the S supply – increased plant growth significantly indicates that increasing N supply from N1 to N2 was in the physiological range, promoting improved growth. This was not the case for S2 vs S1, still it had a more significant effect on the total GLS concentration. From the experiment we can also see that excess

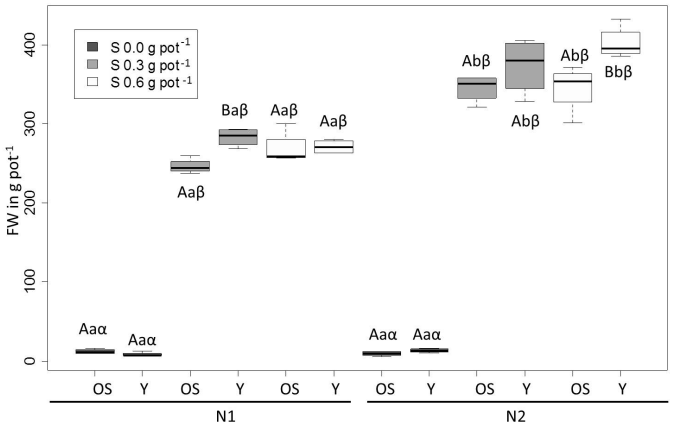

Fig. 2: Fresh weight (FW) of the cvs. orient Surprise (OS) and Yuki (Y) in dependency to the N (1.0 and 2.0 g pot^{-1}) and S (0, 0.3 and 0.6 g pot^{-1}) supply. N1, 1 g pot^{-1}; N2, 2 g pot^{-1}. Uppercase letters represent significant differences between the cvs., lower case letters represent significant differences between the N treatments, and Greek letters significant differences between the S treatments ($P < 0.05$).

Tab. 1: Percentage of N, S and N/S-ratio in shoots of Chinese cabbage.

Cultivar	g N per pot	g S per pot	%N	%S	N/S-ratio
Yuki	1	0.3	1.87	0.44	4.27
Orient Surprise	1	0.3	1.59	0.36	4.43
Yuki	2	0.3	3.44	0.53	6.56
Orient Surprise	2	0.3	3.02	0.47	6.45
Yuki	1	0.6	2.22	0.56	3.95
Orient Surprise	1	0.6	1.77	0.53	3.33
Yuki	2	0.6	2.76	0.7	3.93
Orient Surprise	2	0.6	2.63	0.64	4.12

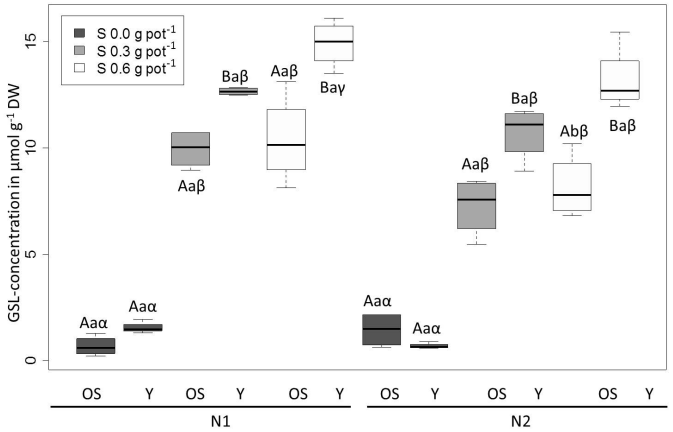

Fig. 3: Total GLS concentration in the cvs. orient Surprise (OS) and Yuki (Y) in dependency to the N (1.0 and 2.0 g pot^{-1}) and S (0, 0.3 and 0.6 g pot^{-1}) supply. N1, 1 g pot^{-1}; N2, 2 g pot^{-1}. Uppercase letters represent significant differences between the cvs., lower case letters represent significant differences between the N treatments, and Greek letters significant differences between the S treatments ($P < 0.05$).

N hampers this S-induced increase in total GLS in cv. Yuki. When plants treated with 0.6 g S were fertilized with 2 g N, total GLS content was lower compared to the situation when plants were fertilized with 0.6 g S and 1 g N. SCHONHOF et al. (2007) reported that a higher N supply strongly influences the GLS concentration, and KRUMBEIN et al. (2001) observed a high reduction of aliphatic GLS under high N-supply conditions. These results could be confirmed: A differential view on 3 sub-class GLS fractions, namely the groups indole GLS, aromatic GLS and aliphatic GLS, reveals that excess N (2 g in this case) reduced the concentrations of aliphatic GLS (Fig. 4). From all three fractions, the aliphatic GLSs dominate quantitatively over indole GLS and aromatic GLS. Thus, the decreased abundance of aliphatic GLS is responsible for the total GLS reduction upon supply with 2 g N. The increased abundance of indole GLS at 2 g N cannot counteract this reduction in total GLS because they accumulate only to approximately a fifth of the amount of the aliphatic GLS (Tab. 2;

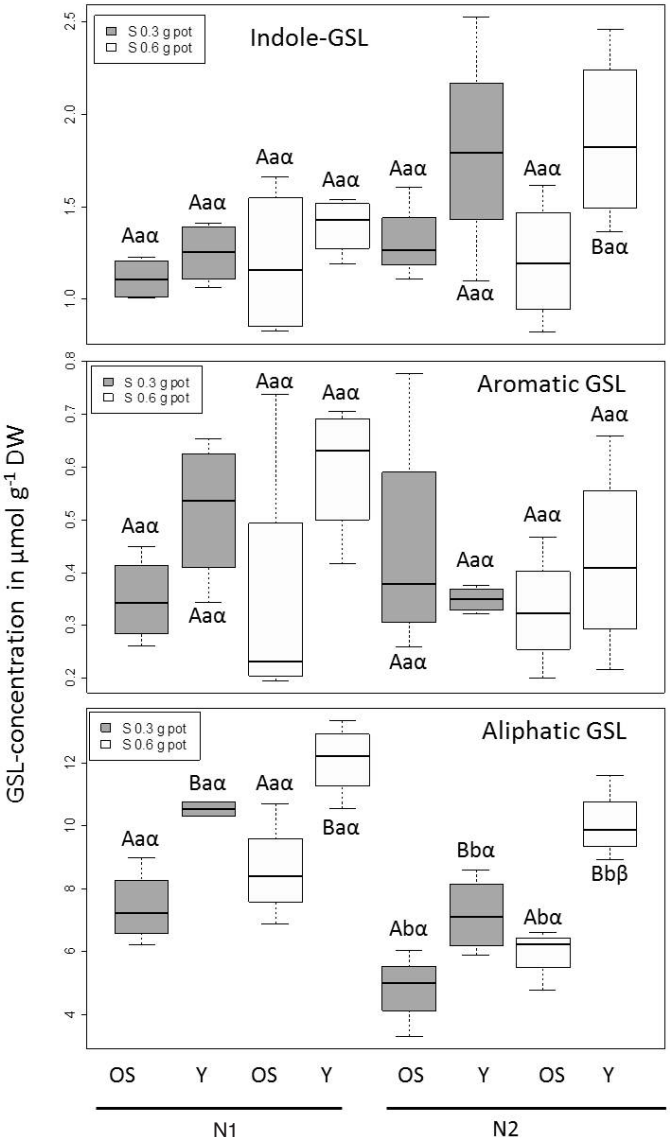

Fig. 4: Concentrations of indole GLSs, aromatic GLSs and aliphatic GLSs in the cvs. Orient Surprise (OS) and Yuki (Y) in dependency to the N (1.0 and 2.0 g pot^{-1}) and S (0, 0.3 and 0.6 g pot^{-1}) supply. N1, 1 g pot^{-1}; N2, 2 g pot^{-1}. Uppercase letters represent significant differences between the cvs., lower case letters represent significant differences between the N treatments, and Greek letters significant differences between the S treatments ($P < 0.05$).

Tab. 2: GLS concentration, indole GLS concentration, aliphatic GLS concentration, aromatic GLS concentration and the GLS ratio of different nutrient supply variation in Chinese cabbage cvs. Yuki (Y) and Orient Surprise (OS). Only significant differences were presented with uppercase letters represent significant differences between the cvs., lower case letters represent significant differences between the N treatments, and Greek letters significant differences between the S treatments ($P < 0.05$).

Cultivar	N-supply (mg/pot)	S-supply (mg/pot)	total GLS (µmol/g DM)	Indole ('in') GLS (µmol/g DM)	aliphatic ('al') GLS (µmol/g DM)	Aromatic ('ar') GLS (µmol/g DM)	ratio in:al:ar
Y	0.1	0.3	12.65	1.25	10.53^{Aa}	0.52	2.4:20.3:1
OS	0.1	0.3	9.93	1.11	7.41^{Ba}	0.35	3.2:21.2:1
Y	0.1	0.6	14.89	1.40	12.08^{Aa}	0.60	2.3:20.1:1
OS	0.1	0.6	10.38	1.20	8.59^{Ba}	0.35	3.4:24.5:1
Y	0.2	0.3	10.70	1.80	$7.17^{Ab\alpha}$	0.35	5.1:20.5:1
OS	0.2	0.3	7.26	1.31	4.84^{Bb}	0.45	2.9:10.8:1
Y	0.2	0.6	13.19	1.87^{A}	$10.06^{Ab\beta}$	0.43	4.3:23.4:1
OS	0.2	0.6	7.69	1.21^{B}	5.95^{Bb}	0.33	3.7:18.0:1

Fig. 4; Supplemental Tab. 2). This increase was only detected in cv. Yuki. Increased indole GLS concentration as result of increased N supply was also reported for *Brassica* crops by KIM et al. (2002) and ZHAO et al. (1994). The group of aliphatic GLSs also accounts for the genotypic differences with regard to total GLS accumulation at conditions of 0.6 g S and 1 g N (Fig. 3). This is reasoned because we detected a more pronounced S-induced increase in aliphatic GLSs for cv. Yuki compared to cv. Orient Surprise under 0.6 g S and 1 g N (Fig. 4).

Pattern of GLS degradation products resemble those of intact GLS under varying N and S supply

A total of eight different GLS were found in shoots of cvs. Orient Surprise and Yuki (not shown). Pattern of GLS composition and abundance were highly similar in both cvs., except for 3-butenyl GLS, which was found in higher concentrations in cv. Yuki, and for 4-(methylsulfinyl)butyl GLS, which was higher in cv. Orient Surprise.

From the eight GLS detected in both cvs., a total of 13 breakdown products from six GLS were found. The Chinese cabbage cvs. Orient Surprise and Yuki differed from one another not only with respect to their GLS profile, but also with respect to the spectrum of GLS breakdown products generated (Fig. 5). For instance, the intact GLS 3-butenyl was more abundant in cv. Yuki and so were the respective breakdown products 3-butenyl-ITC, 3-butenyl-CN and 1-cyano-3,4-epithiobutane (CETB). High cultivar-specific differences were also observed 5-vinyl-1,3-oxazolidine-2-thione (OZT), 3-hydroxypentenenitrile (2OH3But-CN) and 3-hydroxy-4,5-epithiopentylnitrile (CHETB) that all derived from 2(R)-2-hydroxy-3-butenyl GLS. Main degradation products formed were epithionitriles, followed by ITCs and then nitriles. With respect to N and S nutrition, only CETB increased significantly in response to increased S-supply from 0.3 to 0.6 g S per pot under conditions of 2 g N per pot (Fig. 5). Currently, to the best of our knowledge, the role of CETB is not very well resolved in situations when plants form GLS breakdown products. This lack of knowledge is based on a dearth of published data and the high complexity of the subtle differences in the individual GLS breakdown products. For this reason, it is not clear why CETB is the only breakdown product that is significantly affected by the differential nutritional regime. According to the nutrient supply, some products showed a tendency (not statistically confirmed) to increase abundance, such as CHETB, others a tendency to decrease abundance (4-pentenyl-CN) or no reaction, such as 3-hydroxypentenenitrile (2OH3But-CN) and indolacetonitrile (IAN). After grouping them into nitriles and ITCs, similar reactions could be detected: The ITCs were substantially reduced in response to increasing N and decreasing S supply (Fig. 6), which supports earlier data of GERENDÁS et al. (2008a). Our data show that the most abundant ITC in Chinese cabbage, the 4-pentenyl-ITC, was mainly reduced under these circumstances. Such an interrelationship could not be shown for nitriles (Fig. 7). They showed the opposite reaction.

Since ITCs can play a prominent role in cancer prevention (MITHEN et al., 2001) it would be desirable to stimulate the formation of these components. As shown in our study, a high N supply decreases ITCs, especially in cv. Orient Surprise. It could be argued that Chinese cabbage in Europe is grown commercially merely in autumn in the open, where light and other environmental conditions differ from greenhouse conditions. In this regard care was taken to bring, by providing supplementary lighting, at least the light supply in the range observed under field conditions, as earlier results indicated an influence of light supply on GLS pattern and total content in pakchoi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) (YANG et al., 2009). However, in view of the many factors that differ between greenhouse and field conditions, namely environmental conditions, and nutrient and water supply it is interesting to note that KANG et al. (2006) found that variations affiliated with the environmental conditions (greenhouse vs. open field) were comparatively minor. It is thus maintained that excessive N fertilization should be avoided while securing adequate S supply in order to allow a more favourable pattern of breakdown products to be formed, and that the ideal production procedures to follow under field conditions need to be fine tuned under the prevailing conditions.

Influence of the N×S ratio on the GLS breakdown products

GERENDÁS et al. (2008a) reported a strong exponential relationship between the N×S ratio and an increasing ITC concentration in kohlrabi. In our investigation only an N×S ratio of 1:3 to 1:7 could be detected (Fig. 6) which is lower than the recommended range of 1:11-1:12 in *Brassica* plants (LI et al., 2007; ROSEN et al., 2005). That could be caused by excess of N in our experiment. Former studies suggest, that not all GLS and there breakdown products response equally to the nutrient changes. GERENDÁS et al. (2008a) also reported that GLS whose side chains are not derivate from S-containing amino acids (indole and aromatic) can even show a positive response to increasing N supply and a less positive one to increasing S supply (LI et al., 2007; SCHONHOF et al., 2007; ZHAO et al., 1994). The weak relationship between the N×S ratio and the ITCs in our investigation do not recommend it as indicator for the enrichment of these

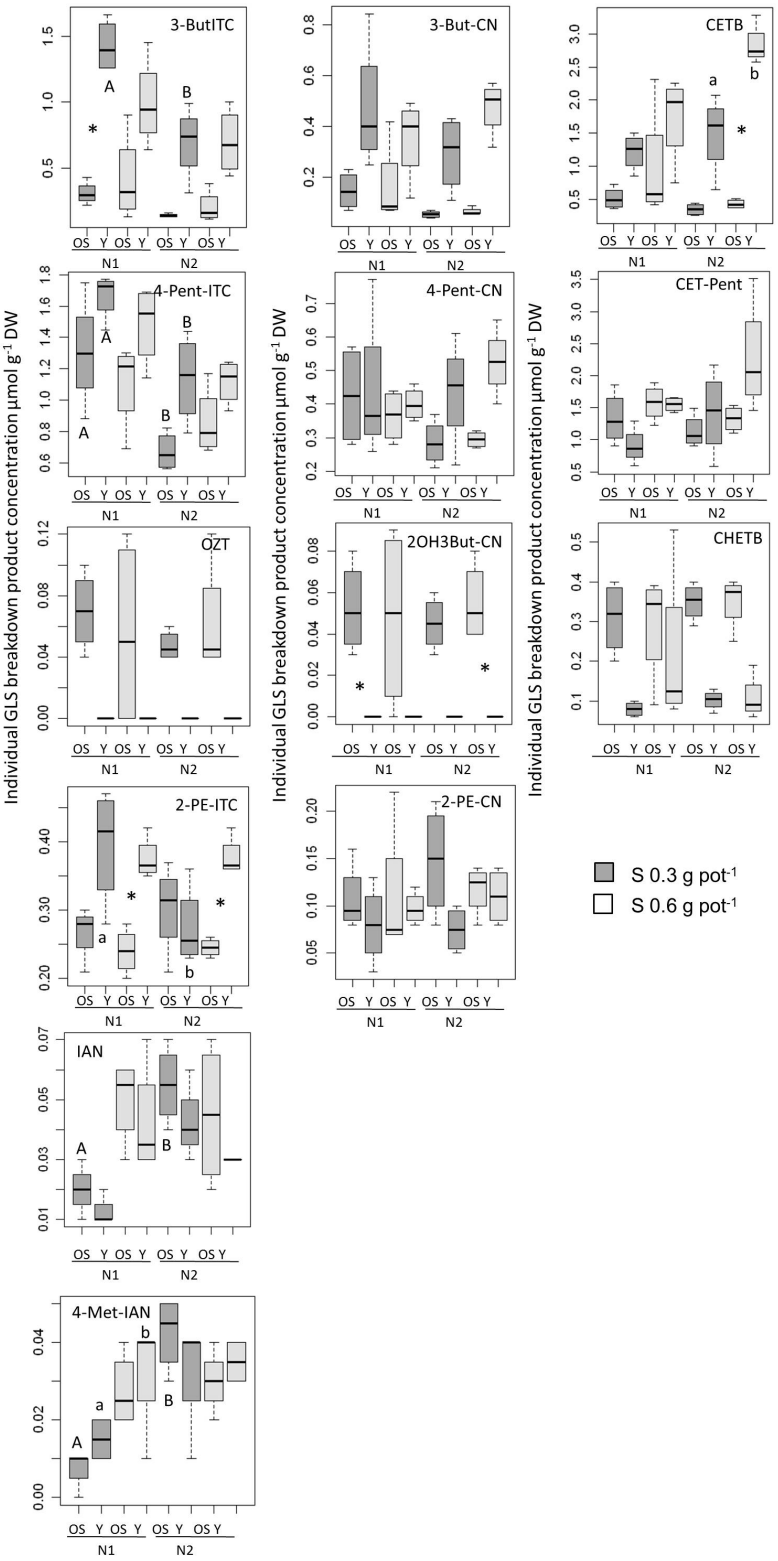

Fig. 5: Different GLS breakdown products extracted from the cvs. Orient Surprise (OS) and Yuki (Y) in dependency to the N (1.0 and 2.0 g pot^{-1}) and S (0.3 and 0.6 g pot^{-1}) supply. N1, 1 g pot^{-1}; N2, 2 g pot^{-1}. Abbreviations: 3But-ITC: 3-butenyl-ITC, 3But-CN: 4-pentenenitrile, CETB: 1-cyano-3,4-epithiobutane, 4Pent-ITC: 4-pentenyl-ITC, 4Pent-CN: 5-hexenenitrile, CETPent: 1-cyano-4,5-epithiopentane, OZT: 5-vinyl-1,3-oxazolidine-2-thione, 2OH3But-CN: 3-hydroxypentenenitrile, CHETB: 3-hydroxy-4,5-epithiopentylnitrile, 2PE-ITC: 2-phenylethyl ITC, 2PE-CN: 3-phenylpropanenitrile, IAN: indolacetonitrile, 4Met-IAN: 4-Methoxyindole-3-acetonitrile. Uppercase letters represent significant differences between the N treatments, lower case letters represent significant differences between S treatments and asterisks indicate significant differences between *B. rapa* cvs. Orient Surprise (OS) and Yuki (Y). The compounds are grouped according to intact GLS precursors. 3But-ITC, 3But-CN and CETB are derived from 3Butenyl GLS; 4Pent-ITC, 4Pent-CN and CETPent are derived from 4Pentenyl GLS; OZT, 2OH3But-CN and CHETB are derived from 2(*R*)-2OH3Butenyl GLS; 2PE-ITC and 2PE-CN are derived from 2Phenylethyl GLS; IAN is derived from 3Indolylmethyl GLS and 4-Met-IAN is derived from 4Methoxy-3-indolylmethyl GLS.

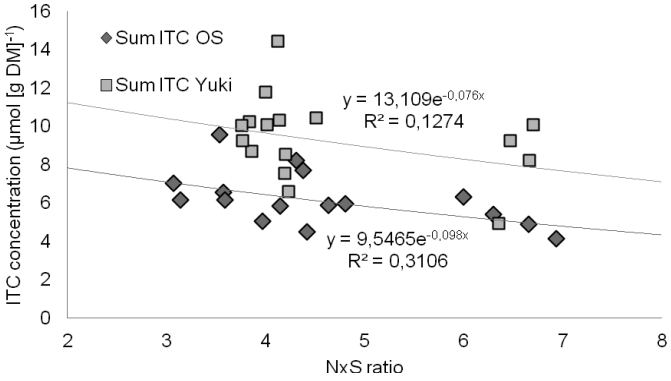

Fig. 6: Influence of different the cvs. Yuki (Y) and Orient Surprise (OS) and the N×S ratio on the ITC concentration in Chinese cabbage.

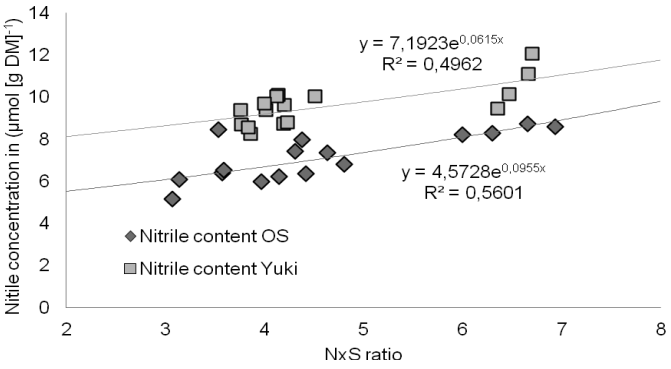

Fig. 7: Influence of different the cvs. Yuki (Y) and Orient Surprise (OS) and the N×S ratio on the nitrile concentration in Chinese cabbage.

components. Apart from that, our investigation showed to the best of our knowledge for the first time a strong positive relationship (r^2= 0.5 Y and r^2=0.56 OS; Fig. 7) between nitriles concentration and an increasing N×S ratio. That indicates that a higher N level conveyed the formation of nitriles in Chinese cabbage. Therefore high fertilization with N should be avoided and the adequate S supply must be ensured in order to optimize a high breakdown to ITCs.

Conclusions

The present study shed light on the interaction between plant nutrition and secondary metabolite composition. We found that the concentration and pattern of GLS and their breakdown products depend on the cv. and that the cvs. respond differentially to increased S and N supply. The GLS pattern was mainly influenced by the genetic background of the cv., but the pattern and concentration could be change by varied N and S application. The indole GLSs were slightly increased with higher N and S nutrition and the aliphatic GLSs were decreased. It is well known that the indole GLSs have health promoting effects, making us reasoning that an increasing concentration is desirable. It was shown that plants with higher N treatment accumulate rather nitriles than ITCs. Since ITCs can play a prominent role in cancer prevention, it is concluded that excessive fertilization with N should be avoided in order to ensure a more favourable pattern of GLS breakdown products.

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Supplemental Tab. 1: Content of individual glucosinolates in *B. rapa* cvs. Orient Surprise (OS) and Yuki (Y). Values are means of four biological replicates per treatment and concentrations are given in µmol g^{-1} dry matter. N1 and N2 represent both nitrogen levels of 1 g and 2 g, and S1 and S2 represent both sulfur levels of 0.3 g and 0.6 g.

	OS N1 S1	Y N1 S1	OS N1 S2	Y N1 S2	OS N2 S1	Y N2 S1	OS N2 S2	Y N2 S2
Aliphatic GLS								
3Butenyl (gluconapin)	1.41	4.88	1.99	5.01	0.85	2.94	0.94	4.75
4Pentenyl (glucobrassicanapin)	4.45	5.02	4.5	5.71	2.58	3.83	3.11	4.85
2(*R*)-2-Hydroxy-3-butenyl (progoitrin)	0.9	0.63	1.48	1.17	0.93	0.4	1.32	0.46
4(Methylsulfinyl)butyl (glucoraphanin)	0.65	0	0.62	0.2	0.47	0	0.58	0
Aromatic GLS								
2Phenylethyl (gluconasturtiin)	0.35	0.52	0.35	0.6	0.45	0.35	0.33	0.42
Indole GLS								
3Indolylmethyl (glucobrassicin)	0.39	0.33	0.41	0.38	0.45	0.43	0.39	0.52
4Methoxy-3-indolylmethyl (4methoxy-glucobrassicin)	0.56	0.71	0.63	0.79	0.71	0.98	0.68	0.94
1Methoxy-3-indolylmethyl (1methoxy-glucobrassicin)	0.15	0.2	0.16	0.22	0.15	0.39	0.13	0.41

Supplemental Tab. 2: Content of total , indole , aliphatic and aromatic GLS in *B. rapa* cvs. Orient Surprise (OS) and Yuki (Y) when no S was given. Values are means of four biological replicates per treatment and concentrations are given in µmol g^{-1} dry matter.

cultivar	N-supply (mg/pot)	S-supply (mg/pot)	total GSL (µmol g^{-1} DM)	indole GSL (µmol g^{-1} DM)	aliphatic GSL (µmol g^{-1} DW)	aromatic GSL (µmol g^{-1} DW)	ratio in:al:ar
Y	0.1	0.0	1.57	1.57	0.0	0.0	-
OS	0.1	0.0	0.67	0.67	0.0	0.0	-
Y	0.2	0.0	0.68	0.68	0.0	0.0	-
OS	0.2	0.0	1.44	1.44	0.0	0.0	-