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Sulfur but not nitrogen supply increases the ITC/Nitrile ratio in Pak Choi (*Brassica rapa* subsp. chinensis (L.) Hanelt)

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Summary

Glucosinolates (GLS) are a serendipitous class of secondary metabolites found in pak choi, a Chinese cabbage (*Brassica rapa* subsp. *chinensis* (L.) Hanelt). GLS are hydrolyzed by the enzyme myrosinase to obtain isothiocyanates (ITC), nitriles, and epithionitriles. GLS hydrolysis products (GLS-HP) are responsible for the typical flavor and odour of pak choi. Little is known about the influence of S and N interactions on pak choi GLS and their hydrolysis products (GLS-HP), especially nitriles.

We investigated the effect of S and N concentrations on pak choi GLS, isothiocyanates, and nitriles content under varying nitrogen (0.75 and 1.5 g N pot^{-1}) and sulfur (0, 0.06, and 0.3 g S pot^{-1}) supply. Increasing the S supply but not N resulted in a reciprocal increase of the total GLS. The GLS concentration decreased under S deficiency. S supply delivered an optimized GLS pattern, and substantially enhanced the synthesis of aliphatic GLS and ITC in particular. In contrast, N-rich nutrition favored the synthesis of indolic GLS and nitriles, the latter are known to have less health beneficial potential and even showed harmful effects. The study indicates, for the first time, that the ITC/nitrile ratio increases under S supply.

GLS and their GLS degradation products in pak choi showed a strong response to sulfur supply. Moreover, the ITC/nitrile ratio might be used as a physiological trait to compare nutritional quality and health benefits of brassica species.

Keywords: Pak choi, glucosinolates, isothiocyanates, nitriles, sulfur nutrition, ITC/nitrile ratio

Introduction

Brassica rapa is a vegetable crop species with significant economic value. It is produced globally as an oil and vegetable crop (Sun, 2015). Importantly, Asia possesses a main diversification area of vegetable *B. rapa* crops (Cartea and Francisco, 2011). Brassica vegetables such as broccoli, cauliflower and cabbage are known for their important medicinal benefit and nutritional value, which have been mainly attributed to the presence of sulfur-containing secondary metabolites known as glucosinolates (Abdalla and Mühling, 2019). These important metabolites are enzymatically degraded to deliver isothiocyanates, nitriles, and epithionitriles. Isothiocyanates might be responsible for the chemopreventive effect of cruciferous vegetables (Thornalley, 2002; Hanschen and Schreiner, 2017), in addition to their antimicrobial (Saladino et al., 2016), and anti-inflammatory potential (Fahey et al., 1997; Vo et al., 2013; Mitsiogianniet et al., 2018). For example, cabbage or pak choi are known to contain alkenyl glucosinolates, which can be enzymatically degraded to obtain epithionitriles and, to a lesser degree, isothiocyanates (Hanschen et al., 2019).

More than 200 glucosinolates have been identified in nature (Koprivova and Kopriva, 2016). Their structures depend on the precursor amino acid and they are subsequently classified into three groups: aliphatic (alanine, leucine, isoleucine, methionine or valine), indolic (tryptophan) and phenylalkyl (phenylalanine or tyrosine) glucosinolates, as listed in Fig. 1 (Lambrix et al., 2001; Hanschen et al., 2014; Kopriva and Gigolashvili, 2016).

Different glucosinolates induce the defense mechanism of plants, so that they can cope with various abiotic and biotic stresses (Abdalla and Mühling, 2019). Moreover, they are known as defensive compounds in plants, which might induce their resistance to disease (Bloem et al., 2015). Importantly, they have demonstrated promising neuroprotective properties (Venditti and Bianco, 2018), anti-inflammatory effects (Vo et al., 2013), and antimicrobial activities (Saladino et al., 2016). It is well-established that ITCs have been involved in the inhibition of carcinogenesis as indicated previously (Zhang and Talalay, 1994; Traka and Mithen, 2009; Palliyaguru et al., 2018; Mitsiogianniet et al., 2018; Blažević et al., 2020), in addition to its antimicrobial, and anti-inflammatory effects (Hanschen et al., 2014; Veeranki et al., 2015). In contrast to the above-mentioned beneficial effects of glucosinolates, it has been reported that some glucosinolates such as progoitrin and indolylic GLS converted to goitrin and thiocyanate, respectively, might cause minimal adverse risks on thyroid hormone production (Felker et al., 2016). Additionally, ruminants are found to be more tolerant to glucosinolate-containing plants intake in addition to adults, which are more tolerant in comparison to young animals (Mandiki et al., 2002). However, it has been reported that long-term feeding on glucosinolate-containing diets caused hypothyroidism and goiter in addition to elevated plasma levels of thiocyanates (Tripathi et al., 2001).

A leafy Chinese cabbage, pak choi *Brassica rapa* subsp. *chinensis* (L.) Hanelt, Family: Brassicaceae) is known as the most important and popular vegetable in human nutrition in East, Northeast and Southeast Asia (Zhu et al., 2013). Interestingly, China is responsible for 30-40% of its production volume (Zhu et al., 2013). The consumption of pak choi in Europe and North America has increased owing to its relatively mild flavor (Kim et al., 2017). Moreover, Kaliabis-Rippel reported that it showed great marketability and acceptance among consumers especially in Germany (Kallabis-Rippel, 2000). Furthermore, pak choi is a rich source of secondary metabolites, especially glucosinolates, which have been reported to be present at concentrations of 19-63 µmol/g DW (Wiesner et al., 2014). Several glucosinolates such as gluconapin, glucobrassicanapin, progoitrin, glucobrassicin and neoglucobrassicin have been detected in pak choi (Wiesner et al., 2014; Zang et al., 2015). Glucosinolates are well known for their significant role in plant defense (Abdalla and Mühling, 2019). Various factors including insect damage, physical wounding, and abiotic stress (such as drought, UV light, and salt stress), can induce glucosinolate biosynthesis. The concentration of glucosinolates and their hydrolysis products in *Brassica* vegetables can be regulated by sulfur and nitrogen fertilization (Li et al., 2007; Gerendás et al., 2008a; 2008b; 2009; Geilfus et al., 2016). For instance, Gerendás et al. (2008b) indicated that an increase of N supply caused a decrease in indolic glucosinolates in cress (*Lepidium sativum* L.) plants. Additionally, Gerendás et al. (2008a) investigated the accumulation of isothiocyanate in Kohlrabi

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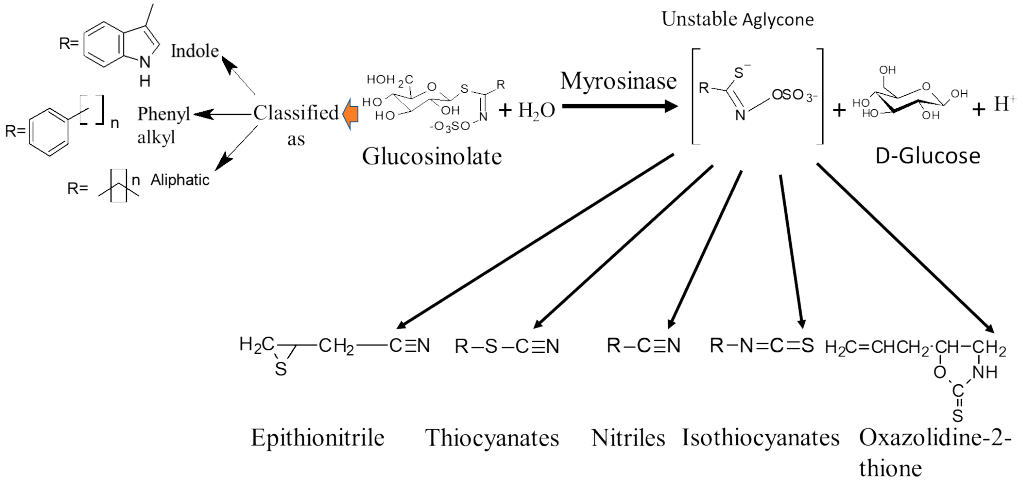

Fig. 1: The enzymatic hydrolysis products of glucosinolates modified from (Lambrix et al., 2001; Hanschen et al., 2014). The main glucosinolate side chain classifications (R) Hanschen et al., 2014; Kopriva and Gigolashvili, 2016).

(*Brassica oleracea* L. Var. gongylodes) plants in response to S and N fertilization. The authors found that the concentration of all ITC including methylthiobutyl ITC, sulforaphane, phenylethyl ITC, and allyl ITC were substantially decreased with increasing N and decreasing S supply.

Although pak choi has recently attracted significant interest, few studies have investigated the concentration of its glucosinolates and their hydrolysis products in response to nitrogen/sulfur nutrition to determine the optimum growth conditions and to enhance the nutritional quality and health benefits of pak choi.

In this report we investigated the effect of increasing S supply on pak choi GLS and GLS-HP concentration under contrasting N supply. We followed the hypotheses: 1. S nutrition is necessary to a certain extent to increase the ITC concentration. 2. A higher N supply decreases the ITC/nitrile ratio. Furthermore, the study set out to determine the S level that is required to increase the ITC/nitrile ratio under varied N supply. The ITC/nitrile ratio might be used as a physiological trait to evaluate the nutritional value and medicinal significance of pak choi.

Tab. 1: The compositions of the nutrient solution (g pot^{-1})

Nutrients	g pot^{-1}
Macronutrients	
$Ca(H_2PO_4)_2$	0.3
$CaCO_3$	1.5
KNO_3	0.6
$Mg(NO_3)_2$	0.3
NH_4NO_3	0.75 and 1.5
$CaSO_4$	0.06 and 0.3
Micronutrients	
$FeCl_2$	0.05
$MnCl_2$	0.03
$CuCl_2$	0.01
$ZnCl_2$	0.015
H_3BO_3	0.01
$(NH_4)_6Mo_7O_{24}{\cdot}H_2O$	0.002

Materials and methods

Plant material and growth conditions: The samples of *Brassica rapa* L. subsp. *chinensis* cv. Joi Choi obtained from Kiepenkerl (Germany) were pre-cultivated in nutrient-poor sand (retrieved from Oldenburg, Germany). After ten days, seedlings were transferred into Mitscherlich pots filled with 7.2 kg soil (2:1:1 Quartz sand 1.2-1.4 mm:quartz sand 0.4-0.8 mm:Ostenfeld). The soil Ostenfeld contained 170 mg P_2O_5 kg^{-1}, 120 mg K_2O kg^{-1}, 4.9 mg S kg^{-1}, 0.13% N_{total}, and 23 g organic S kg^{-1}. The compositions of the nutrient solution added to each pot (g pot^{-1}) are listed in Tab. 1.

Plants were cultivated during wintertime in a greenhouse with 14 h light at 20 °C and 10 h darkness at 16 °C in addition to 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). Pack choi plants were treated with two N supply (N1=0.75 and N2=1.5 g pot^{-1}) given as NH_4NO_3 in combination with three S concentrations (S0=0, S1=0.06, and S2=0.3 g pot^{-1}) given as $CaSO_4$. N and S treatments were applied 15 days from germination. Four biological replications per treatment were established to deliver a total of 24 pots. Plant shoots were harvested after 66 days and the fresh matter (FM) was directly recorded at the vegetative stage. Subsequently, plant material was immediately placed in a freeze-dryer at -53 °C for 72h (Gamma1-20, Christ, Osterode am Harz, Germany) to determine the dry matter (DM). Plant material was then ground to a fine powder for further analysis of N, S, GLS, and GLS breakdown products.

Determination of sulfur and nitrogen content: To determine the amount of sulfur and nitrogen in the plants, 5 ± 0.1 mg of freeze-dried plant material was used and combined with 5-10 mg Wolfram-VI-oxide for optimal combustion. The concentrations of S and N were determined using an elemental analyzer, Flash EA1112 (Thermo Fisher Scientific, Milano, Italy) as described by Zörb et al. (2013).

Determination of sulfate and nitrate content: Ion chromatography (Dionex ICS 2500, Dionex Softron, Germering, Germany) was used to determine the sulfate and nitrate concentrations in the freeze-dried plant materials. For this, 30 mg of plant material and 1.5 mL deionized water were incubated at 75 °C for 5 min. Samples were cooled to room temperature, centrifuged (5 min, 13 000 rpm) (Eppendorf Centrifuge 5415R, Hamburg, Germany), diluted 1:10 with deionized water, and four to five drops of chloroform were

added. After centrifugation (13 000 rpm, 3 min) the upper phase was added to C-18 cartridges and measured by ion chromatography.

Glucosinolate (GLS) analysis: The concentration of glucosinolates was determined by using high-performance liquid chromatography (HPLC) (Agilent 1100 HPLC, Agilent Technologies, Waldbronn) as described by KRUMBEIN et al. (2005). For this analysis, 0.5 g of the finely crushed plant material was incubated at 75 °C for 1 min to deactivate the myrosinase. Subsequently, a mixture of 4 mL methanol/water (70:30 v/v) and 200 µL of sinigrin standard were added and the mixture was incubated at 75 °C for 10 min. Protein precipitation was performed by adding 1 mL of 0.4 M barium acetate, then the samples were centrifuged at 4 000 rpm at 10 °C for 10 min. The supernatant was recovered and extracted twice with 3 mL methanol/water mixture (70:30 v/v) and centrifuged. All combined supernatants were diluted to 10 mL with the methanol/water mixture. Five milliliters of the prepared sample was added to a 250 µl DEAE Sephadex A-25 ion-exchange column and rinsed with 10 mL deionized water. Then, 250 µL of purified arylsulfatase solution was applied to the column and incubated overnight. Subsequently, desulpho compounds were eluted using 5 mL distilled water. After passing through a 0.45 m filter, 10 µl of the purified extract was used for determination of the glucosinolates. The GLS analysis was carried out 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). The gradient started from 0% to 20% of acetonitrile/water for 2-34 min, followed by 20% acetonitile/water for 6 min and finally, 100% acetonitrile was run isocratically for a further 10 min. The flow rate was 1.3 ml min^{-1} and the detection operated at 229 nm. The individual glucosinolates were characterized by comparisons of their retention time peaks with that of individual glucosinolates in reference material. The quantification of the GLS was performed using sinigrin as internal standard.

Glucosinolate breakdown products (GLS-HP) analysis: Freeze-dried and finely crushed leaves (0.5 g) were added to 1 mL deionized water and 50 µL internal standard benzyl nitrile (100 ng/µL in methanol). After 30 min of incubation, 2 × 2 mL of dichloromethane were added. The sample was centrifuged at 3.000 rpm for 25 min at room temperature. The organic phase was collected in 5 mL test tubes and the extraction was repeated. The organic phase was then mixed with Na_2SO_4 to remove residual water, centrifuged, and concentrated under a nitrogen stream. For the identification of GLS-HP, the analysis was performed by gas chromatography (GC) with a flame ionization detector (FID), using an Agilent 6890 series gas chromatograph, with a HP5MS column (30 m × 0.25 mm × 25 µm), and hydrogen was used as a carrier gas. Splitless injection was performed at 200 °C (injection volume was 1 µl), and the oven program started with 40 °C for 3 min and increased again to 250 °C at 10 °C min^{-1}. Characterization of the products was confirmed by GC-MS by comparison with authentic standards and published MS spectra (SPENCER and DAXENBICHLER, 1980). The concentration of GLS-HP was calculated in µmol g^{-1} DM. GLS-HP were quantified using the molar FID-response factors calculated by using the ECN (Effective-Carbon-Number) method (SCANLON and WILLIS, 1985): 2PE-ITC (ECN 9.0, RF 0.70), 3But-ITC (ECN 4.9, RF 1.29), 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), 2OH3But-CN (ECN 3.8, RF 1.66), IACN (ECN 9.3, RF 0.68).

Statistical analysis: Statistical analysis was performed using the statistical software package R (R Development Core Team, 2013). For this, a generalized linear least squares-model was defined for all parameters (CARROLL and RUPPERT, 1988). All measured variables were assumed to be normally distributed with homogenous variances, depending on the influence of factor S and N supply. For all parameters, a significance level of 0.05 was chosen. The significances were marked by capital letters for S effect and small letters for N effect within the figures. Heteroscedastic variables (N, S, N/S ratio, NO_3, SO_4, fresh matter (FM), dry matter (DM), GLS, aliphatic, phenylalkyl and indolic GLS), and homoscedastic variables (total concentration, GLS-HPs, ITCs, nitriles and epithionitriles (Epi), and ITC/nitrile ratio) were analyzed separately. Based on this information, a multivariate ANOVA (HARTUNG et al., 2005) was applied, followed by a related multiple contrast test for multiple endpoints according to BRETZ et al. (2010). Therefore, a pseudo one way model was provided (SCHAARSCHMIDT and VAAS, 2009).

Results and discussion

Influence of N and S supply on growth and nutritional status

Pak choi Chinese cabbage (*Brassica rapa* subsp. *chinensis* (L.) cv. Joi Choi was cultivated in soil and exposed to N and S supply to evaluate the effects of varying N and S supply on the nutritional parameters (FM, DM, N, S, N/S ratio, nitrate, and sulfate) of the investigated plant. The concentration of N, S N/S ratio, nitrate, and sulfate in addition to the fresh and dry matter content in response to nitrogen and sulfur supply are listed in Tab. 2.

The nutritional status of the pak choi was affected in response to N and S supply. S deprivation (0 g S per pot^{-1}) caused the typical S-deficiency symptoms including chlorosis of younger leaves and decreased the biomass, as shown in Fig. 2 and Tab. 2. Additionally, increasing the S supply (0.06 and 0.3 g S per pot) significantly increased the whole shoot biomass of pak choi. The highest fresh matter (FM) (589.8 g $plant^{-1}$) and dry matter (DM) (48.0 g $plant^{-1}$) were observed under conditions when the plants were subjected to higher N (1.5 g N per pot) and S (0.3 g S per pot) supply. Furthermore, increasing the S supply to 0.06 and 0.3 g S per pot resulted in a concomitant increase in S concentration in the plant tissue (Tab. 2).

The N/S ratio increased if the amount of N fertilization increased from moderate to sufficient N levels (to 0.75 and 1.5 g N per pot). The nitrate concentration increased with higher N supply (1.5 g N pot^{-1}) and was not affected by a lower S level. A higher S supply resulted in a decrease in the nitrate concentration owing to a stimulated nitrate assimilation. An increase in the sulfate concentration under the conditions of higher S supply was observed, which has also been reported in a previous study (GERENDÀS et al., 2008a).

S fertilization enhanced GLS accumulation in pak choi

The total concentration of GLS was 0.44 µmol/g DM under the conditions of N and S deficiency, whereas the concentration was elevated to 2.45 and 7.05 µmol/g DM owing to increased S supply of 0.06 and 0.3 g S pot^{-1}, respectively (Fig. 3). Moreover, below a N supply of 1.5 N per pot the total concentration of GLS was 0.29 and 1.46 µmol/g if the S supply was 0.06 g S pot^{-1} but this increased to 5.99 µmol/g if a higher supply of S was used (0.3 g S pot^{-1}).

The total concentration of aliphatic GLS under the conditions of lower N supply and S deficiency was close to zero (0.36 µmol g^{-1} DM) which increased to 2.03 and 6.23 µmol g^{-1} DM with increased S supply to 0.06 and 0.3 respectively. Under a higher N supply the aliphatic GLS concentrations were 0.23, 1.15 and 5.05 µmol g^{-1} DM. A similar trend was observed for phenylalkyl GLS, but at lower concentrations (Fig. 3). The concentrations of indolic GLS were 0.07, 0.22 and 0.40 µmol g^{-1} DM under low N supply and increased with higher N supply to 0.01, 0.20 and 0.60 µmol g^{-1} DM. The total concentration of GLSs, in addition to the concentration of aliphatic, phenylalkyl, and indolic GLS, is shown in Fig. 3.

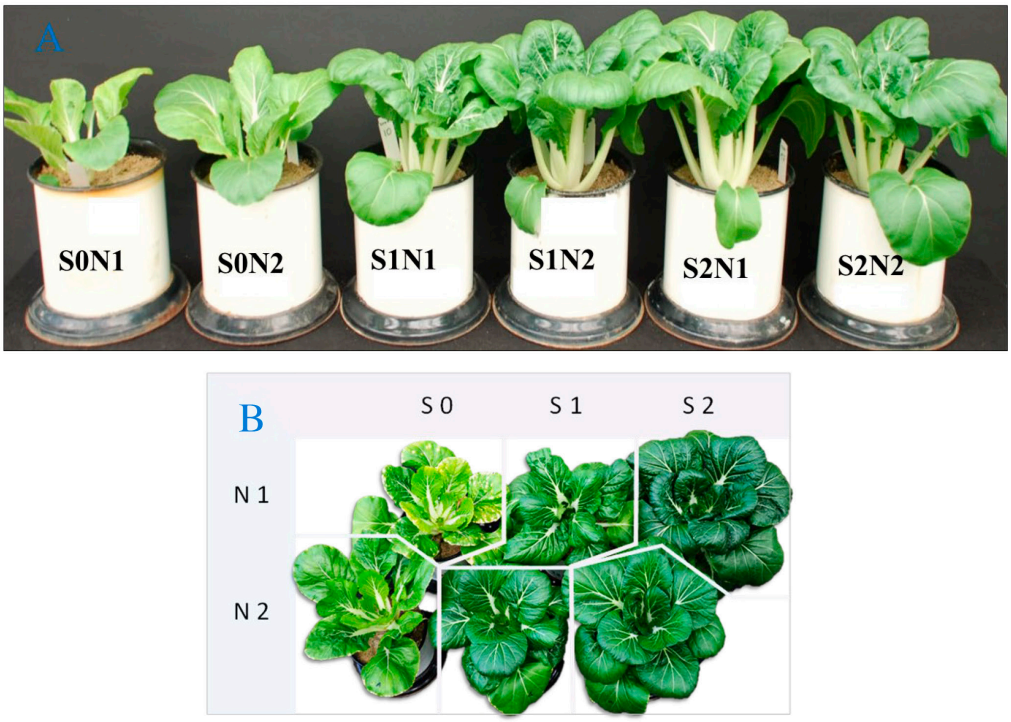

Fig. 2: Development of the biomass by means of varied nitrogen (N1 = 0.75 g N, N2 = 1.5 g N) and sulfur (S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S) fertilization in Pak Choi (A) side view (B) Top view. S deprivation (S0N1 and S0N2) caused the typical S-deficiency symptoms, and decreased biomass. Increasing the S supply (0.06 and 0.3 g S pot^{-1}) significantly enhanced the whole shoot biomass

Tab. 2: The concentration of N, S, N/S ratio, nitrate, and sulfate, fresh matter and dry matter in response to varied nitrogen and sulfur supply.

N	S	FM	DM	N	S	N/S ratio	nitrate	sulfate
N1	S0	323.2 Aa	32.9 Aa	2.51 Aa	0.09 Aa	27.02 Aa	6.36 Aa	0.15 Aa
N1	S1	437.8 Ba	35.5 Aa	2.35 Aa	0.24 Ba	9.39 Ba	7.02 Aa	1.14 Ba
N1	S2	448.1 Ba	37.7 Aa	2.27 Aa	0.48 Ca	4.49 Ca	3.97 Aa	3.95 Ca
N2	S0	325.0 Aa	35.1 Aa	3.61 Ab	0.08 Aa	46.89 Ab	43.71 Ab	0.09 Aa
N2	S1	511.5 Ba	37.2 Ba	3.05 Bb	0.25 Ba	12.92 Bb	52.58 Ab	1.35 Ba
N2	S2	589.8 Bb	48.0 Cb	2.79 Bb	0.48Ca	6.47 Ca	22.18 Bb	3.84 Ca
ANOVA	N	0.0120	0.0002	0.0001	0.1834	0.0001	0.0001	0.0001
	S	0.0001	0.0001	0.0053	0.0001	0.0001	0.0001	0.6041
	N × S	0.0561	0.0072	0.1430	0.5001	0.0079	0.0004	0.5789

Lower- and uppercase letters indicate significant difference ($p<0.05$) between means owing to the effect of N and S supply, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N

In total, eleven different GLS compounds were determined quantitatively. These GLS were 3-methylsulfinylpropyl GLS (glucoiberin; GI), (2*R*)-2-hydroxybut-3-enyl GSL (progoitrin; PRO), (R_S)-4-(methylsulfinyl)butyl GSL (glucoraphanin; GRA), but-3-enyl GSL (gluconapin; GNP), 4-Hydroxyindol-3-ylmethyl GSL (4-hydroxyglucobrassicin; 4OH), pent-4-enyl GSL (glucobrassicanapin; GBC), indol-3-ylmethyl GSL (glucobrassicin; GB), 2-phenylethyl GSL (gluconasturtiin; GNI), (R_S, 3*E*)-4-(methylsulfinyl)but-3-enyl GSL (glucoraphanin; GRE), 4-methoxyindol-3-ylmethyl GSL (4-methoxyglucobrassicin; 4M) and 1-methoxyindol-3-ylmethyl GSL, (neoglucobrassicin; NGB). GLS compounds were classified as six aliphatic (GI, PRO, GRA, GNP, GBC, GRE), one phenylalkyl (GNI) and four indolic (4OH, GB, 4M, NGB) GLS.

All detected aliphatic GLS (GI, PRO, GRA, GNP, GBC, GRE) were derived from the S-containing amino acid methionine. Therefore, increased S supply had a significant effect on the accumulation of these compounds (Fig. 4, F, H, I).

In particular, partly reduced concentrations of the alkenyl derivatives were observed with higher N supply (Fig. 4, B, C, D, H). The only phenylalkyl GLS compound found in the pak choi plant was GNI (Fig. 4, H) (Chen et al., 2006). This compound was clearly induced by S supply, which has also been reported by Vallejo et al., (2003). Glucosinolates are derived from amino acids and constitute a core structure that has a β-d-glucopyranose residue connected to a (*Z*)-*N*-hydroximino sulfate ester through a sulfur atom in addition to a variable side chain. As reviewed by the group of De Kok, it can be confirmed that the glucosinolate content can be affected by the sulfur nutritional status (Aghajanzadeh et al., 2014). This was in agreement with the observed results, as S deficient plants had a low GLS concentration. Consequently, a high S supply enhanced the GLS concentration to 2.45 and 7.05 µmol g^{-1} DM (Fig. 3). During GLS synthesis, cysteine and gluthatione (GSH) are needed as S donors (Geu-Flores et al., 2011). Under conditions of S deficiency, these substances are insufficient and the GLS concentration is reduced;

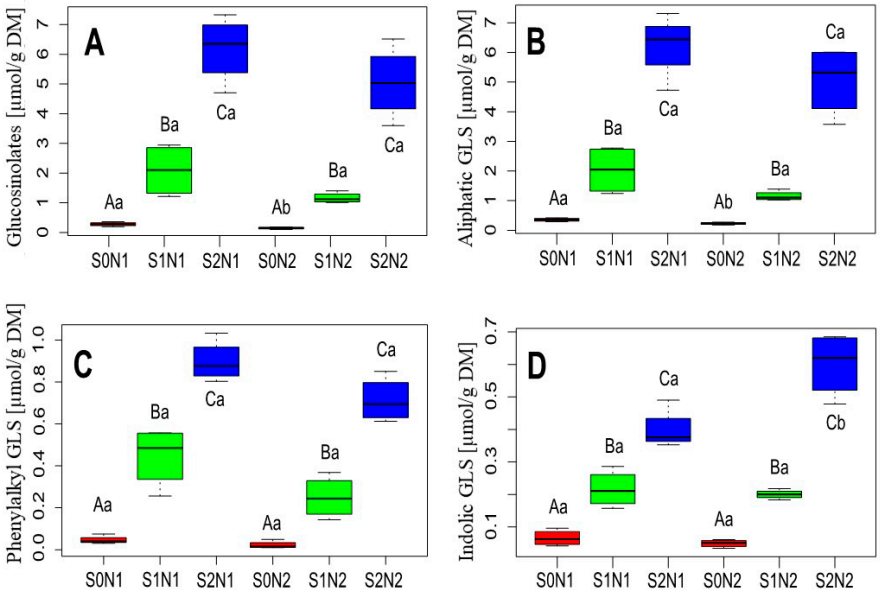

Fig. 3: The total concentration of glucosinolates (GLS) detected in pak choi (A) and the concentrations after classification into aliphatic (B), phenylalkyl (C), and indolic glucosinolates (D). Lower- and uppercase letters indicate significant difference ($p<0.05$) between means owing to the effect of N and S supply respectively, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N.

if the S supply is increased, the GLS concentration is elevated (Schnug and Haneklaus, 1993; Halkier and Gershenzon, 2006). On the other hand, under conditions of S deficiency, remobilization of S from GLS can occur owing to catabolic breakdown, resulting in high GLS hydrolysis product concentration and reduced GLS concentration (Du and Halkier, 1998; Gerendàs et al., 2008b). This hypothesis could not be confirmed because it was clearly shown that the ITC concentration increases with S supply (Gerendàs et al., 2008b; Gerendás et al., 2009). Under higher N supply, the GLS concentration increased in the presence of higher S supply (Fig. 3). Although several studies have reported that N increases the GLS concentration (Zhao et al., 1994; Omirou et al., 2009), other researchers have reported that N supply has a reducing effect (Li et al., 2007). In this study, N had no effect on the GLS concentration (Fig. 3).

The contradictory effect of N on the GLS concentration might be explained by the variable GLS patterns. GLS can be classified into aliphatic, indolic and phenylalkyl GLS, which can be affected differently by N and S supply.

In this study, the concentration of aliphatic GLS increased with enhanced S supply, especially under high S and lower N fertilization (Fig. 3), whereas S deficiency and higher N supply decreased its concentration. Chen et al. (2006) reported that aliphatic GLS responds sensibly on a varied N and S supply, rather than indolic and phenylalkyl GLS. This can be attributed to the chemical structure of the aliphatic GLS, which are classified as alkenyl, alkyl, and thioalkyl of which the latter incorporates an additional S atom (Zhao et al., 1994). Therefore, this can explain how an individual aliphatic GLS can be affected by fertilization.

In contrast, a higher N supply and 60 mg S pot^{-1} led to a reduced concentration of GNI (Fig. 3 H), as also found previously (Kim et al., 2002; Vallejo et al., 2003). Our results showed that in addition to aliphatic or phenylalkyl GLS, indolic GLS were also synthesized under conditions of high S supply (Fig. 4, E, G, J, K). Under conditions of 0.06 g S and 0.75 g N pot^{-1}, aliphatic GLS were detected at a concentration of 2.03 µmol g^{-1} DM, whereas only 0.22 µmol g^{-1} DM indolic GLS were found at the same fertilization conditions. This result suggested that the aliphatic GLS dominate quantitatively over indolic and phenylalkyl GLS, which was in agreement with the findings of Geilfus et al. (2016). Therefore, the positive effect of the N and S supply on aliphatic GLS accumulation might affect the total GLS concentration.

Influence of N and S supply on isothiocyanates, nitriles and epithionitrile concentration

The formation of glucosinolate hydrolysis products decreased under conditions of lower N and S supply. Higher levels from 0.67 to 1.22 µmol g^{-1} FM (Fig. 5, A) were detected in response to higher S supply (0.3 g S pot^{-1}). On the other hand, higher N supply (1.5 g N pot^{-1}) led to a concentration of 0.00, 0.41 and 1.14 µmol g^{-1} FM. The concentration of released ITC under lower N supply (0.75 g N pot^{-1}) was 0.00, 0.04 and 0.12 µmol g^{-1} FM and enhanced with higher N supply to 0.00, 0.01 and 0.07 µmol g^{-1} FM (Fig. 5, B). The concentrations of nitriles and epithionitriles are both shown in Fig. 3.

In fresh plant material, eight individual glucosinolate hydrolysis products were determined quantitatively, alkenyl aliphatic ITCs (3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate), aliphatic nitrile 3-hydroxypentenenitrile (2OH3But-CN), and 5-hexenenitrile (4-pent-CN), phenylalkyl ITC (2-phenylethyl-isothiocyanate), indolic nitrile (indole-3-acetonitrile), and aliphatic epithionitrile (1-cyano-3,4-epithiobutane a product of 3butenyl GLS, and 1-cyano-4,5-epithiopentane or 5,6-epithiohexanenitrile (CETPent) a product of 4-pentenyl GLS) are listed in Fig. 6, A-H. Under S deficient conditions, no GLS-HP were detected owing to the lack of S resource (Fig. 5, A), as also reported earlier by (Wrigley et al. (1980). Consequently, when the S supply was increased the GLS-HP concentration increased (Fig 4, A).

On the other hand, N did not have an effect on the GLS-HP concentration. Nevertheless, the concentration of the main groups of GLS-HP including ITC, nitriles and epithionitriles can be affected by S fertilization.

With respect to N and S nutrition, the main degradation products formed were epithionitriles, as the epithionitrile concentration was 10-fold higher as the ITC concentration.

The 1-cyano-3,4-epithiobutane (CETB) concentration gradually increased with increasing S fertilization (0.28 and 0.46 µmol/g FM) (Fig. 6, F). Increased N fertilization resulted in higher concentrations

of 1-cyano-3,4-epithiobutane (0.44 µmol/g FM) with respect to the S slope with only the highest S fertilization level. Moreover, a significant increase in the concentration of 1-cyano-4,5-epithiopentane (CETPent) to 0.54 µmol/g FM was observed only at the highest N and S concentration (Fig. 6, G).

These results indicated that S nutrition induced the accumulation of not only GLS, but also the corresponding GLS-HP. Previous reports studied the control, recovery strategies and stability of glucosinolates and their hydrolysis products (ANGELINO et al., 2015; HANSCHEN et al., 2015; GALANAKIS, 2020). For instance, HANSCHEN et al. (2015) stated that analyzing glucosinolates content of plant materials before and after tissue homogenization and formation of the GLS-HP showed that hydrolysis of glucosinolates to GLS-HP was more than 99%, additionally, the recovery of corresponding GLS-HP varied between 67 and 99%. Glucosinolates can be found either in free or bounded forms within biological sources (ANGELINO et al., 2015). Therefor, measurement systems can play a crucial role in the determination of GLS-HP such as both tissue-bound ITC and free ITC (ANGELINO et al., 2015).

The ITC concentration was substantially reduced in response to increasing N and decreasing S supply, and subsequently enhanced with S supply. For example, there was no significant increase in the concentration of 2PE-ITC (0.010 µmol/g FM) under low S supply (0.06 g S pot^{-1}) and both lower and higher N supply (0.75 and 1.5 g N pot^{-1}). Only an increase in the S level had a significant effect on the concentration of 2PE-ITC; at higher N and lower S levels, the concentration was 0.003 µmol/g FM, which increased to 0.012 µmol/g FM only at the higher S level. The same trend was observed for 3But-ITC, which was detected at a concentration of 0.013 µmol/g at a lower S level, which increased to 0.038 µmol/g FM at the highest

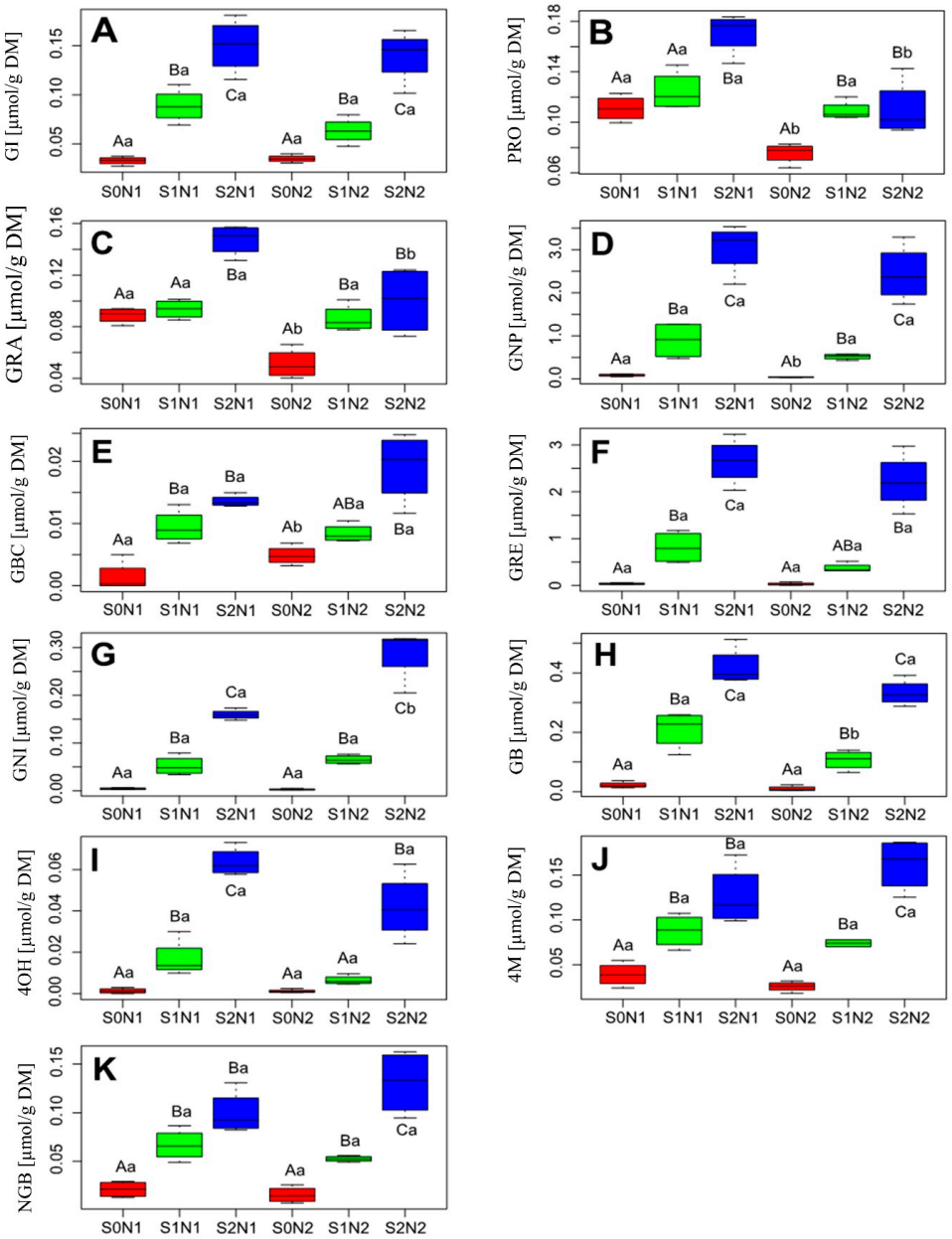

Fig. 4: Effect of S and N supply on the glucosinolate pattern: glucoiberin; GI (A), progoitrin; PRO (B), glucoraphanin; GRA (C), gluconapin; GNP (D), glucobrassicanapin; GBC (E), glucoraphanin; GRE (F), gluconasturtiin, GNI (G), glucobrassicin, GB (H), 4-hydroxyglucobrassicin; 4OH (I), 4-methoxyglucobrassicin, 4M (J), neoglucobrassicin, NGB (K). Lower- and uppercase letters indicate significant diff,erence ($p<0.05$) between means owing to effect of N and S supply respectively, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N

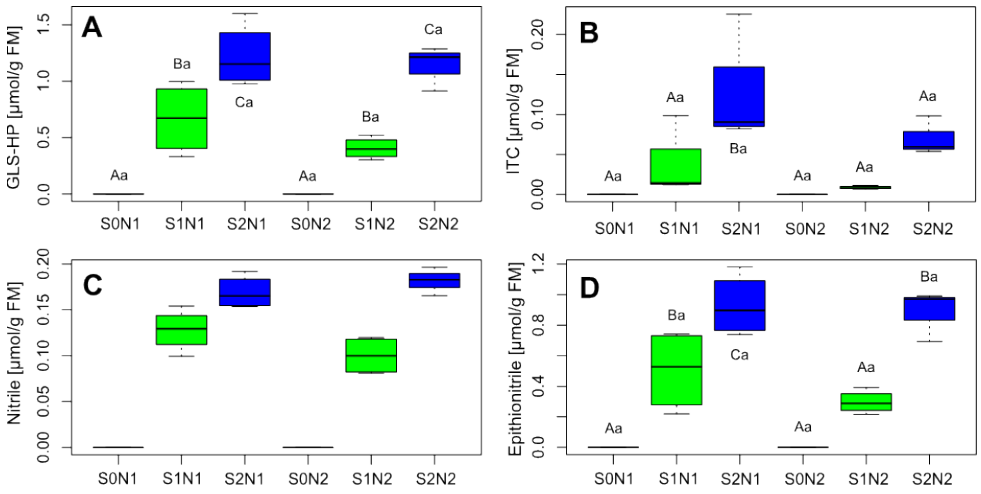

Fig. 5: Total concentration of glucosinolate hydrolysis products (A) as well as isothiocyanates (B), nitriles (C), and epithionitriles (D) in pak choi fertilized with a varied N and S supply. Lower- and uppercase letters indicate significant difference ($p<0.05$) between means owing to the effect of N and S supply respectively, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N.

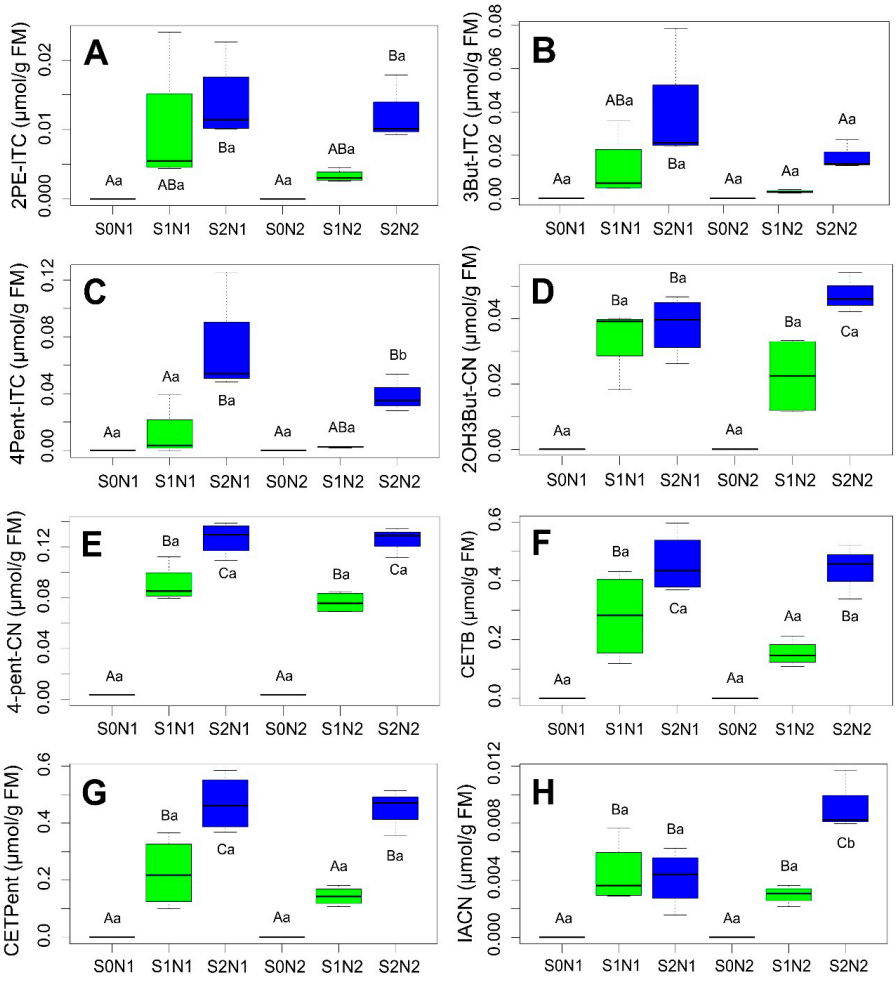

Fig. 6: Individual glucosinolate hydrolysis products under different N and S fertilization conditions in pak choi plants: 2-phenylethyl-isothiocyanate (2PE-ITC) (A), 3-butenyl-isothiocyanate (3But-ITC) (B), 4-pentenyl-isothiocyanate (4Pent-ITC) (C), 3-hydroxypentenenitrile (2OH3But-CN) (D), 5-hexenenitrile (4-pent-CN) (E), 1-cyano-3,4-epithiobutane (CETB) (F), 5,6-epithiohexanenitrile (CETPent) (G), indolacetonitrile (IACN) (H). Lower- and uppercase letters indicate a significant difference ($p<0.05$) between means owing to the effect of N and S supply respectively, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N.

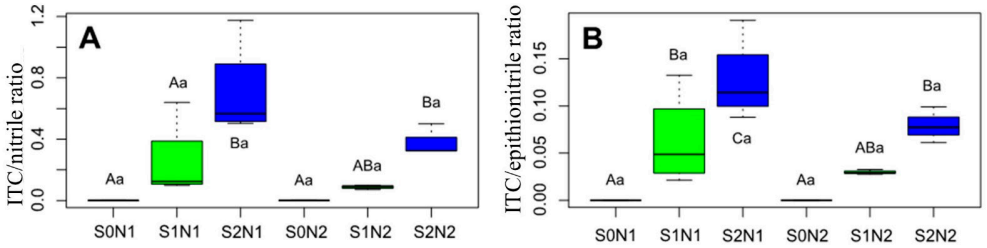

Fig. 7: ITC/nitrile ratio (A) and ITC/epithionitrile ratio (B) in pak choi plants fertilized with varied N and S supply. Lower- and uppercase letters indicate significant di ference ($p<0.05$) between means owing to the effect of N and S supply respectively, S0 = 0 g S, S1 = 0.06 g S, S2 = 0.3 g S, N1 = 0.75 g N, N2 = 1.5 g N.

S level (Fig. 6, B). These findings suggested that there was an obvious S effect but not N effect (Fig. 6, A). These results support the findings of Gerendàs et al. (2008b).

Regarding the total nitrile concentration, this study showed that N supply did not affect the nitrile concentration as strongly as S supply (Fig. 3, C). Considering individual nitriles, indolacetonitrile (IACN) exhibited a significant increase with higher N supply (Fig. 6, H). However, the other nitriles, 3-hydroxypentenenitrile (2OH3But-CN) and 5-hexenenitrile (4-pent-CN) (Fig. 6, D, E), which are known as nitriles found previously in pak choi (Klopsch et al., 2018; Chen et al., 2019), were less affected by a higher N supply, as also reported by Geilfus et al. (2016).

Moderate S under low N supply stimulates ITC synthesis and improves the ITC/nitrile

Because nitriles and epithionitriles have been reported to have fewer health benefits than ITCs and even showed harmful effects in animal nutrition, it is of significant interest to produce food rich in ITCs (Hanschen et al., 2017; Hanschen and Schreiner, 2017).

ITC/nitrile ratio quantifies the changes in the amount of ITC in relation to nitrile, when exposed to S and N supply. The current study demonstrated that under a lower N supply the ITC/nitrile ratio was 0 and increased to 0.25 and 0.70 in response to a higher S supply. At a higher N supply, the ITC/nitrile ratio was 0.0, 0.09, and 0.38 (Fig. 7). Therefore, a higher N supply decreased the ITCs, which was also reported by Geilfus et al. (2016). Additionally, at a higher N supply the ITC/epithionitrile ratio was 0.063 and increased to 0.127 in response to higher S supply (Fig. 7).

Because ITCs can play a pivotal role in cancer prevention as chemopreventive agents, it would be desirable to induce the accumulation of these metabolites. In this study, an increase in the ITC/nitrile ratio was observed with increased S supply, leading to the highest ratio with 0.3 g S pot^{-1} (Fig. 7). A higher N supply reduced the ratio to approximately 50% (S2N1 = 0.7, S2N2 = 0.38). This is the first report to highlight the high ITC/nitrile ratio, which might support the anti-carcinogenic effects of *Brassica* vegetables. For future investigation, we recommend further determination of the chemopreventive potential to indicate that ITC/nitrile ratio can be used as a physiological trait for chemopreventive capacity in pak choi.

Conclusions

GLS and their corresponding GLS-HP are clearly affected by increased S and N supply in pak choi. Owing to the well-known chemopreventive effects of isothiocyanates, the evaluation of the concentration of ITC in comparison with nitriles could be a useful approach to gain an understanding of the potential improvement in the health promoting effects in pak choi plants. To the best of our knowledge, this is the first study that has shown a strong positive relationship between S/N nutrition and ITC/nitrile ratio; low N and high S nutrition enhanced the concentration of ITC in Chinese cabbage pak choi and increased the ITC/nitrile ratio. Furthermore, fertilization with a high N supply should be avoided, and a S supply is required to optimize high breakdown to ITCs. Therefore, the ITC/nitrile ratio might be used as a reference for the medicinal potential in pak choi and other *Brassica* vegetable species. Therefore, our study recommends further investigation of the anti-carcinogenic effect of ITC in pak choi plants grown under varied S and N supply.

Conflict of interest

No potential conflict of interest was reported by the authors.

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