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Effect of NH_4^+/NO_3^- ratios on the growth and bolting stem glucosinolate content of Chinese kale (*Brassica alboglabra* L.H. Bailey)

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Abstract

Glucosinolates (GSs) are a group of plant secondary metabolites mainly found in *Brassica* that contain abundant nitrogen (N) and have anticarcinogenic effects on human health. GS levels are affected by nutrient availability, including the NH_4^+/NO_3^- ratio of the soil. Our objective was to examine the influence of NH_4^+/NO_3^- ratio on the growth and bolting stem GS content of Chinese kale (*Brassica alboglabra* L.H. Bailey). Seedlings were grown in five culture solutions with the same nitrogen level (15 mmol L^{-1}), but different ratios of NH_4^+/NO_3^- (100/0, 75/25, 50/50, 25/75 and 0/100). As the NH_4^+/NO_3^- ratio increased from 0/100 to 75/25, there was no significant difference in growth characteristics ($P > 0.05$), but in the NH_4^+ only treatment the height and aboveground fresh weight decreased significantly ($P < 0.05$), by 9.02 cm and 2.55 g $plant^{-1}$, respectively, because the leaves withered and no bolting stem emerged because of ammonia toxicity. The highest values for total GS and total aliphatic GS content in bolting stems were observed in the 50/50 treatment ($P < 0.05$), while total indolyl GS was highest in the 75/25 treatment, which indicated that different glucosinolate groups responded to different NH_4^+/NO_3^- ratios. The bolting stem N/S ratio did not change among the 75/25, 50/50, 25/75 and 0/100 treatments ($P > 0.05$) indicating that there was no direct correlation between the glucosinolate content and N/S ratio change. Our results suggest that a NH_4^+/NO_3^- ratio of 50/50 in fertilizer application can increase the GS content in bolting stems, but the underlying mechanisms need to be studied further.

Keywords: NH_4^+/NO_3^- ratio; Chinese kale (*Brassica alboglabra* L.H. Bailey); Glucosinolates; Growth; Bolting stem; Nitrogen/sulfur ratio (N/S).
Abbreviations: GS - glucosinolate; N - nitrogen; S - sulfur; HPLC - high-performance liquid chromatography; MS - mass spectrometry; LC/MSD - liquid chromatography/mass spectrometric detector; ICP-MS - inductively coupled plasma atomic emission spectrometer; LSD - least significant difference.

Introduction

The epidemiological data show that a diet rich in cruciferous vegetables such as broccoli, Brussels sprouts and cauliflower can lessen the risk of a number of cancers (Higdon et al., 2007; Verkerk et al., 2009). Recently, some of the cancer chemoprotective activities of these vegetables have become widely recognized to be due to their high content of glucosinolates (GSs) (Traka and Mithen, 2009; Zhang and Tang, 2007). GSs are a group of plant secondary metabolites mainly found in the *Brassica* family (Hennig et al., 2012; Verkerk et al., 2009). Studies have shown that GSs are derived from amino acids and can be divided into three groups—aliphatic, aromatic and indolyl—according to the amino acid precursor from which they originate (Sønderby et al., 2010). The GS levels and chemical forms in plants can be strongly affected by both genetic and environmental factors, such as plant variety, climate, nutritional supply, and so on (Verkerk et al., 2009; Wang et al., 2012; Jia et al., 2009). Among the environmental factors, the application of nitrogen (N) and sulfur (S) fertilizers have been shown to significantly affect GS synthesis and levels (Rosa et al., 1997; Chen et al., 2006a; Grant et al., 2011). There have been investigations into the interaction between N and S levels in many crops, including the *Brassica* vegetables (Verkerk et al., 2009), but there has been no consistent conclusion on which is more important for GS synthesis, N or S (Chen et al., 2006a; Falk et al., 2007). However, increasing numbers of results suggest that the proper balance between N and S levels can maximize the GS content (Kim et al., 2002; Falk et al., 2007). Besides the overall N and S levels, N forms also affect plant GS accumulation. Plants can use both ammonium (NH_4^+) and nitrate (NO_3^-) as a nitrogen source (Ciampitti and Vyn, 2012) but research has indicated that the effect of these two forms on plant growth and chemical content is dependent not only on the plant species, but also on the NH_4^+/NO_3^- ratio and concentrations (Guo et al., 2002). However, the effects of NH_4^+ /NO_3^- ratios on glucosinolate levels are not clear. In rapeseed (*B. napus* L.), no glucosinolate changes were observed when N was supplied as NO_3^- only, NH_4^+ only, or both NH_4^+ and NO_3^- (Josefsson, 1970). However, in salad rocket (*Eruca sativa* Mill.), the NH_4^+/NO_3^- ratio significantly affected the GS content, with the highest content occurring at a NH_4^+/NO_3^- ratio of 50/50 and the lowest value in the NH_4^+ only treatment (Kim et al., 2006). However, little is known about the effects of NH_4^+/NO_3^- ratios on *Brassica* vegetables. Chinese kale (*Brassica alboglabra* L.H. Bailey) belongs to a group of *Brassica* vegetables produced in China. Recent studies have shown that the bolting stems of Chinese kale, the main edible part, are the best source of GSs, and are particularly rich in glucoraphanin (La et al., 2009, Si et al., 2009; Sun et al., 2011), the precursor of sulforaphane, which

Table 1. Effect of NH_4^+/NO_3^- ratios on the growth of Chinese kale.

NH_4^+/NO_3^- ratios	Height (cm)	Fresh weight (g plant^{-1})	
		Aerial parts	Bolting stem
100/0	9.02±1.25b†	2.55±0.81b	No-bolting
75/25	35.42±10.59a	69.27±8.03a	26.85±1.23a
50/50	36.15±4.26a	76.66±17.52a	29.29±3.85a
25/75	41.22±5.54a	72.47±5.24a	31.35±3.58a
0/100	40.35±11.45a	75.90±12.70a	27.85±1.98a
F	18.17***‡	71.79***	0.85ns

†Data followed by the same lowercase letter in each column are not significantly different at $P < 0.05$. Values are mean ± standard deviation ($n = 6$).
‡ Significance levels: ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

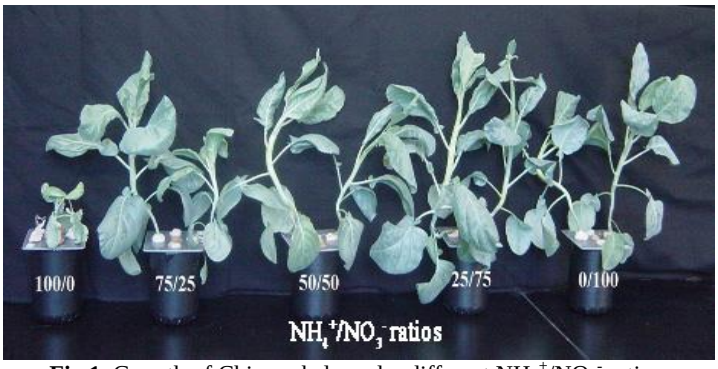

Fig 1. Growth of Chinese kale under different NH_4^+/NO_3^- ratios.

is one of the most potent naturally occurring inducers of phase II enzymes in anticancer metabolic processes (Grubb and Abel, 2006; Traka and Mithen, 2009). Chinese kale has spread quickly in China and Japan in recent decades because of its high levels of GSs and potential health benefits as a cancer preventive agent (La et al., 2009, Si et al., 2009; Sun et al., 2011). The objective of this research was to evaluate the effect of different NH_4^+/NO_3^- ratios on the growth and GS content of bolting stems of Chinese kale.

Results

Chinese kale growth

Changes in the NH_4^+/NO_3^- ratio significantly affected plant height and the fresh weight of the aboveground parts (Table 1). Plant height and aboveground fresh weight were significantly reduced in the NH_4^+ only treatment compared with the other four NH_4^+/NO_3^- ratios ($P < 0.05$), but there was no significant difference among the NH_4^+/NO_3^- ratios 75/25, 50/50, 25/75 and 0/100 ($P > 0.05$). When NH_4^+ was supplied as the sole nitrogen source in the nutrient solution, the Chinese kale did not grow normally; the leaves were withered and showed chlorotic and necrotic phenomena, and no bolting stems emerged (Fig. 1). Additionally, there was no significant difference in bolting stem fresh weight among the 75/25, 50/50, 25/75 and 0/100 treatments.

Glucosinolate content of Chinese kale bolting stems

A typical HPLC chromatogram of desulfo-GSs in bolting stems of Chinese kale is shown in Fig. 2. Chemically, the desulfo-GSs share a common glucosyl structure besides the diversity of the side-chain R. Identification was performed according to characteristic fragments in the ESI data. $[M+K]^+$, $[M+Na]^+$, $[M+H]^+$ and [M-glucosyl+H]$^+$ quasi-molecular ions were available in the positive ESI spectrum for the identification of desulfo-GSs, while $[M+Cl]^-$ and [M-glucosyl-H]$^-$ were the characteristic quasimolecular ions in the negative ESI spectrum (Macfarlane-Smith and Griffiths, 1988). Eleven individual GSs were identified in the bolting stems of Chinese kale from all treatments except the NH_4^+ only treatment, because no bolting stems emerged (Table 2). These included seven aliphatic glucosinolates (glucoiberin, progoitrin, sinigrin, glucoraphanin, glucoalyssin, gluconapin and glucoerucin) and four indolyl glucosinolates (4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin). The total GS content in bolting stems of Chinese kale ranged from 6.37–9.74 μmol g^{-1} DW (Fig. 3). The aliphatic GSs were predominant and accounted for 95.39% of the total GS content on average, while indolyl GSs made up a small proportion of the total GS content. Gluconapin was the major GS, accounting for 70.8% of total GS content on average, while glucoraphanin accounted for 8.9%, sinigrin 6.5% and glucoiberin 6.2%. The other glucosinolates accounted for less than 5% of the total GS content. The NH_4^+/NO_3^- ratio significantly affected the total GS content, total aliphatic GS content, total indolyl GS content and all individual GS contents except for glucoalyssin, gluconapin, and glucobrassicin (Fig. 3, Tables 3 and 4). The total GS and total aliphatic GS concentrations were greatest in the NH_4^+/NO_3^- 50/50 treatment, intermediate in the 75/25 treatment and lowest in the other two treatments. The total indolyl GS concentration increased significantly from the NO_3^- only to the 75/25 NH_4^+/NO_3^- treatment. For the individual GSs, the response to the NH_4^+/NO_3^- ratios differed. The highest values for progoitrin, glucoraphanin, glucoerucin were obtained in the 50/50 treatment, for glucoiberin and 4-methoxyglucobrassicin at 75/25, and for sinigrin at NH_4^+/NO_3^- ratios of 75/25 and 50/50. The 4-methoxyglucobrassicin content decreased as NH4+ increased, while the other indolyl GSs did not change consistently.

Table 2. Glucosinolates identified in bolting stems of Chinese kale by LC/MSD.

Trivial names	Structure of R groups	Desulfated molecular weight	Response factor†
Aliphatic			
Glucoiberin	CH_3-SO-$(CH_2)_3$-	343	1.07
Progoitrin	CH_2=CHCH(OH)CH_2-	309	1.09
Glucoraphanin	CH_3-SO-$(CH_2)_4$-	357	1.07
Sinigrin	CH_2=CH-CH_2-	279	1.00
Glucoalyssin	CH_3-SO-$(CH_2)_5$-	371	1.07
Gluconapin	CH_2=CH-$(CH_2)_2$-	293	1.11
Glucoerucin	CH_3-S-$(CH_2)_4$-	341	1.00‡
Indolyl			
4-Hydroxyglucobrassicin	Indole-4-OH-3-CH_2-	384	0.28
Glucobrassicin	Indole-3-CH_2-	368	0.29
4-Methoxyglucobrassicin	Indole-4-OCH_3-3-CH_2-	398	0.25
Neoglucobrassicin	Indole(OCH_3)-3-CH_2-	398	0.20

† The response factors relative to the standard sinigrin were experimentally determined with HPLC by the International Organization for Standardization (ISO 9167-1, 1992) in 1992 for individual GS content in rapeseed.
‡ Not yet determined by the ISO.

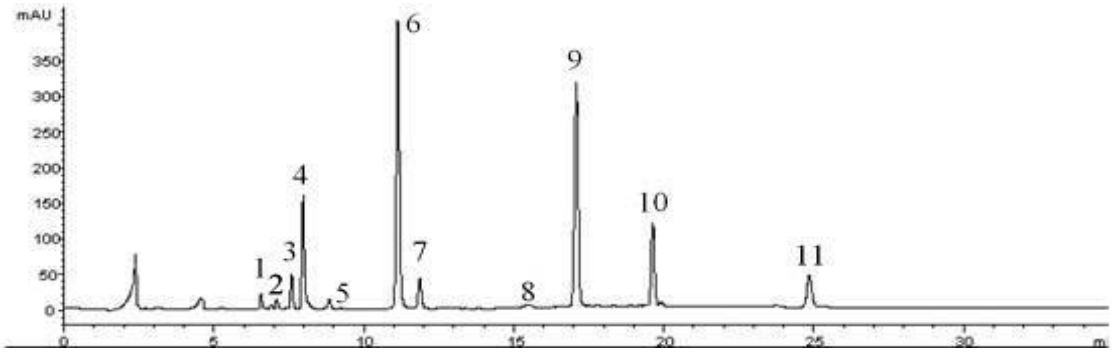

Fig 2. Typical HPLC elution profile of desulfated glucosinolates in bolting stems of Chinese kale. 1.Glucoiberin; 2. Progoitrin; 3.Glucoraphanin; 4. Sinigrin; 5. Glucoalyssin; 6. Gluconapin; 7. 4-Hydroxyglucobrassicin; 8. Glucoerucin; 9. Glucobrassicin;10. 4-Methoxyglucobrassicin; 11. Neoglucobrassicin.

Nitrogen (N) and sulfur (S) contents of Chinese kale bolting stems

The effect of NH_4^+/NO_3^- ratio on the bolting stem total N and S contents was investigated (Table 5). The NH_4^+/NO_3^- ratios affected the total N significantly ($P < 0.001$), but not the total S content and N/S ratio ($P > 0.05$). The bolting stem N content was greatest at a NH_4^+/NO_3^- ratio of 75/25, intermediate in the 50/50 treatment and lowest in the other treatments.

Discussion

Plants can usually absorb both nitrate and ammonia as nitrogen sources (Bruck and Guo, 2006; Ciampitti and Vyn, 2012). However, research has indicated that for most plants, the highest yield is obtained by supplying both nitrogen forms (Ali et al., 2001; Guo et al., 2002). In this study, growth (height and fresh weight of aboveground parts) was inhibited when NH_4^+ was supplied as the sole nitrogen source in the nutrient solution. In the NH_4^+/NO_3^- 100/0 treatment, the leaves withered and no bolting stem emerged. This result is similar to a report on the effects of NH_4^+/NO_3^- ratio on the growth of salad rocket (Kim et al., 2006), which indicated that salad rocket grows slowly and withers when supplied with only NH_4^+ in the nutrient solution. This may be related to the plant's inability to resist or adapt itself to NH_4^+ toxicity (Kim et al., 2006). Once plants absorb NH_4^+ it must be immediately biosynthesized during plant growth to avoid accumulation to toxic levels (Gill and Reisenauer, 1993). Otherwise, the high NH_4^+ concentration leads to serious physiological and morphological disorders resulting in chlorosis, restricted growth and even death (Barker et al., 1966; Santamaria et al., 1998). Glucosinolates are a uniform class of thioglucosides. They are derived from amino acids and are grouped into aliphatic, indolyl and aromatic GSs according to their amino acid structure (Sønderby et al., 2010). In the present experiment, seven aliphatic and four indolyl GSs were detected in the bolting stems of Chinese kale, which is in agreement with previous studies on the same material (La et al., 2009; Sun et al., 2011). The presence of glucoraphanin in bolting stems of Chinese kale should be studied more extensively, because this aliphatic GS is the precursor of sulforaphane, which is considered to be one of the most potent inducers of phase II proteins (Grubb and Abel, 2006; Traka and Mithen, 2009). Isothiocyanates derived from the hydrolysis of sinigrin and glucoiberin have been found to have anticarcinogenic and antimutagenic effects (Barillari et al., 2005; Cartea and Velasco, 2008). Moreover, it is known that isothiocyanates derived from sinigrin can cause a reduction in cholesterol levels in mice (Chen et al., 2006b). Another beneficial effect attributed to sinigrin is suppression of the growth of nematodes, fungi, and other soil microorganisms (Farnham et al., 2004), although this GS also contributes, as do glucoiberin and gluconasturtiin, to the presence of some specialist pests (Rosa et al., 1997;

Table 3. Effect of NH_4^+/NO_3^- ratios on the individual and total aliphatic glucosinolate concentrations in bolting stems of Chinese kale.

NH_4^+:NO_3^- ratio	Aliphatic GSs (μmol g^{-1} DW)							
	GIB[§]	PRO	GRA	SIN	GAL	GNP	GRU	Total
100/0	No-bolting	No-bolting	No-bolting	No-bolting	No-bolting	No-bolting	No-bolting	No-bolting
75/25	1.02±0.11 a[†]	0.45±0.01 b	0.47±0.05 bc	0.85±0.09 a	0.03±0.01 ab	5.13±0.43a	0.05±0.03b	8.00±0.56 b
50/50	0.57±0.05 b	0.68±0.07 a	1.48±0.34 a	0.83±0.07 a	0.03±0.01 a	5.01±0.47a	0.41±0.09a	9.00±0.30 a
25/75	0.14±0.03 c	0.26±0.07 c	0.69±0.07 b	0.29±0.23 b	0.05±0.02 a	5.31±0.34a	0.02±0.01b	6.76±0.51 c
0/100	0.27±0.06 c	0.13±0.00 d	0.23±0.01 c	0.14±0.02 b	0.05±0.01 a	5.65±0.46a	0.02±0.01b	6.48±0.42 c
F	96.64***[‡]	67.25***	28.29***	24.77***	3.39ns	1.26ns	47.16***	19.46***

§ GIB, glucoiberin; PRO, progoitrin; GRA, glucoraphanin; SIN, sinigrin; GAL, glucoalyssin; GNP, gluconapin; GRU, glucoerucin.
[†] Data followed by the same lowercase letter in each row are not significantly different at $P < 0.05$. Values are mean ± standard deviation ($n = 3$).
[‡] Significance levels: ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

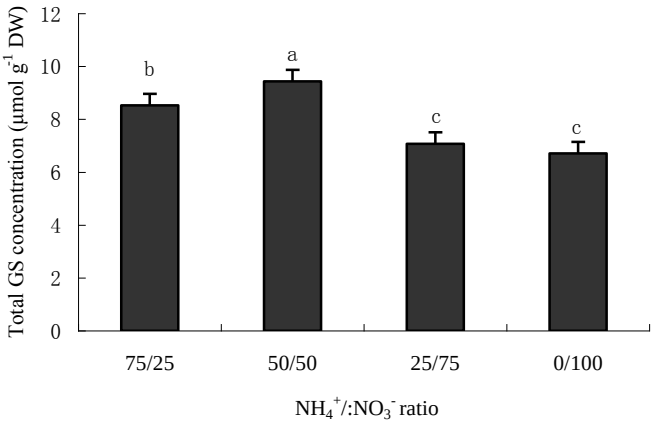

Fig 3. Effect of different NH_4^+/NO_3^- ratios on total GS concentration in bolting stems of Chinese kale. Columns with the same letter(s) indicate no significant difference at the $P < 0.05$ level. The lines represent the standard error. No bolting stems were harvested in the NH_4^+/NO_3^- 100/0 treatment.

Balasinska et al., 2005). The total GS and total aliphatic GS concentrations in bolting stems were greatest in the NH_4^+/NO_3^- 50/50 treatment, while the highest value for total indolyl GS concentration was achieved at 75/25. There has been little information about the effect of different forms of nitrogen on GS content. Kim et al. (2006) first reported the effect of different ratios of ammonium and nitrate on the GS concentrations in salad rocket. In salad rocket, the highest GS concentration was achieved in a NH_4^+/NO_3^- 50/50 treatment.

N and S are two essential constituents of amino acids and the main factors that affect GS synthesis (Rosen et al., 2005; Schonhof et al., 2007; Falk et al., 2007). Therefore, it is reasonable to assume that there would be a relationship between these two elements and GS content in plants. There have been some reports on the relationship between N/S ratio in plant tissues and the total GS content (Kim et al., 2002; Rosen et al., 2005; Aries et al., 2006). In watercress (*Nasturtium officinale* R. Br.), 2-phenethyl isothiocyanate concentrations increased with a decrease in N/S ratio (increased S) in the nutrient solution (Kopsell et al., 2007). In broccoli (*B. oleracea* L. var. *italica*), there was a correlation between N/S ratio and glucosinolate content; the contents of the methylsulfinylalkyl glucosinolates glucoraphanin and glucoiberin in broccoli inflorescences increased as the N/S ratio decreased (Schonhof et al., 2007). In turnip rape (*B. rapa* L.), there was no distinct correlation between the ratio of leaf N/S and GS content (Kim et al., 2002). However, in cabbage (*B. oleracea* L. var. *capitata*), tissue N was negatively correlated and tissue S and S/N ratio were positively correlated with total glucosinolate concentration, although all correlations were generally weak ($r^2 < 0.5$) (Rosen et al., 2005). In this study, the N content differed significantly while the S content and N/S did not, suggesting that the GS content change in bolting stems was not because of changes in S or N/S ratio. However, the further study is needed on the mechanisms of the GS response to different NH_4^+/NO_3^- ratios.

Materials and methods

Plant preparation

Experiments were carried out in a computer-controlled greenhouse at the Huajiachi campus of Zhejiang University, Hangzhou, China. Nutrient solutions were prepared according to NH_4^+/NO_3^- ratios of 100/0, 75/25, 50/50, 25/75 and 0/100 at the 15 mmol L^{-1} N level recommended for use in Chinese kale cultivation in China (100/0, 75/25, 50/50, 25/75 and 0/100 will be used to refer to these treatments later) (Chen et al., 2005). The compositions of the nutrient solutions for the different treatments are listed in Table 6. Micronutrients were supplied in all treatments as: $MnCl_2 \cdot H_2O$ 1.81 g L^{-1}, H_3BO_3 2.86 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.22 g L^{-1}, H_2MoO_4 0.072 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.08 g L^{-1} and

Table 4. Effect of NH_4^+/NO_3^- ratios on individual indolyl GS concentrations in bolting stems of Chinese kale.

	Indolyl GSs (μmol g^{-1} DW)				
NH_4^+/NO_3^-	4HGB[§]	GBS	4MGB	NGBS	Total
100/0	No-bolting	No-bolting	No-bolting	No-bolting	No-bolting
75/25	0.00±0.00c	0.06±0.02a	0.28±0.02a	0.18±0.04a	0.52±0.05a
50/50	0.02±0.00 a	0.04±0.00a	0.24±0.01b	0.14±0.02ab	0.43±0.01b
25/75	0.01± 0.00b	0.06±0.01a	0.12±0.01c	0.13±0.02b	0.31±0.04c
0/100	0.02± 0.00a	0.04±0.01a	0.07±0.01d	0.10±0.02b	0.23±0.04d
F	109.92***[‡]	3.42ns	171.60***	5.37*	37.61***

§ 4HGB, 4-hydroxyglucobrassicin; GBS, glucobrassicin; 4MGB, 4-methoxyglucobrassicin; NGBS, neoglucobrassicin.
[†] Data followed by the same letter(s) indicate no significant difference at the $P < 0.05$ level.
Values are means ± standard deviation.
[‡] Significance levels: ns, not significant; *P<0.05; ** P<0.01; *** P<0.001.

Table 5. Effect of NH_4^+/NO_3^- ratios on the nitrogen (N) content, sulfur (S) content and N/S ratio in bolting stems of Chinese kale.

NH_4^+/NO_3^-	N content %	S content %	N/S ratio
100/0	No-bolting	No-bolting	No-bolting
75/25	4.27±0.13a[†]	0.90±0.05a	4.77±0.27a
50/50	3.88±0.20b	0.90±0.14a	4.40±0.95a
25/75	3.31±0.14c	0.89±0.15a	3.73±0.23a
0/100	3.50±0.21c	0.86±0.19a	4.44±0.14a
F	17.78***[‡]	0.23ns	2.14ns

[†] Data followed by the same letter(s) indicate no significant difference at the $P < 0.05$ level. Values are means ± standard deviation.
[‡] Significance levels: ns, not significant; ***$P < 0.001$.

Table 6. Compositions (mmol L^{-1}) of nutrient solutions at the same N levels (15 mmol L^{-1}) with different NH_4^+/NO_3^- ratios.

Nutrient source	NH_4^+/NO_3^- ratios				
	100/0	75/25	50/50	25/75	0/100
KCl	4	4	4	2.5	0
KNO_3	0	0	0	1.5	4
$NaNO_3$	0	0	0	0	5
$MgCl_2$	2	2	0	0	0
$MgSO_4$	0	0	2	2	2
$CaCl_2$	3	3	0	0	0
$Ca(NO_3)_2$	0	0	3	3	3
NH_4Cl	11	3.5	2	0	0
NH_4NO_3	0	3.75	1.5	3.75	0
K_2HPO_4	1	1	1	1	1
$(NH_4)_2SO_4$	2	2	2	0	0
Micronutrients*	+	+	+	+	+

*Micronutrients were the same for all treatments and were applied according to Hoagland-type solutions (Hoagland and Arnon, 1950).

ethylenediamine tetraacetic acid monosodium ferric salt (EDTA-NaFe) 13.21 g L^{-1} (Hoagland and Arnon, 1950). Each two seedlings of Chinese kale (*B. alboglabra*) with three fully expanded leaves were transplanted into a 2 L black plastic container full of nutrient solution. There were six containers (twelve seedlings) for each treatment and the containers were randomly placed in the greenhouse. The growth conditions in the greenhouse were as follows: relative humidity of 65%, day/night temperatures of 23/18 °C and photosynthetically active radiation of 500 μmol m^{-2} s^{-1} for 16 h per day. The culture solutions were continually aerated and adjusted to pH 6.0 using diluted NaOH or HCl every day and renewed every three days. Forty days after transplantation, the height and the bolting stem diameter of the plants were measured using a ruler, and one plant from every container was randomly sampled for each treatment. The aboveground parts of the samples were weighed and the bolting stems were separated from the aboveground parts and frozen immediately in liquid nitrogen. After being lyophilized and ground into powder, the samples were stored in a desiccator at -20 °C prior to analysis.

Extract preparation for glucosinolate analysis

The desulfo-GSs were prepared according to the procedure described by Kiddle et al. (2001) with some modifications. Triplicate samples (0.1 g) of freeze-dried bolting stem powder were each weighed into 5-mL tubes, and crude GSs were extracted with 1.5 mL 70% methanol at 70 °C for 10 min in a water bath. The mixture was centrifuged at 5000 × *g* for 10 min at 4 °C and the supernatant was decanted into another tube. The extraction was repeated twice on the pellet using the same procedure. The three supernatants were combined and made up to a final volume of 5 mL. Next, 2 mL of each GS extract was added to a mini-column filled with DEAE Sephadex A-25 (100 mg dry matter) (Amersham Biosciences, Sweden) and 100 μL sulfatase (28.4 units) (Sigma-Aldrich Co., USA). After a desulfation reaction at room temperature overnight (16 h), the desulfated GSs (desulfo-GSs) were eluted with 0.5 × 4 mL deionized water (Millipore, USA) and stored at -20 °C prior to high-performance liquid chromatography (HPLC) analysis. 2-Propenyl GS (sinigrin) (S1647, Sigma-Aldrich) was used as an external standard for quantitative GS analysis.

High-performance liquid chromatography

For each sample, 20 μL desulfated GS extract was analyzed by HPLC (Beckman, USA) using a Hypersil ODS2 column (250 mm × 4.6 mm, 5 μm; Elite, China). The detection

wavelength was set at 227 nm and the temperature of the column was set at 35 °C. The mobile phase was a mixture of deionized water (A) and acetonitrile (B), and run at a flow rate of 1 mL min^{-1}. The elution program consisted of a linear gradient from 0 to 20% (B) in 18 min and constant 20% (B) for a further 16 min (Macfarlane-Smith and Griffiths, 1988).

Mass spectrometry analysis

The separated compounds were identified according to the mass spectrometry (MS) data obtained with an LC/MSD system (Agilent 1100 LC/MSD, Agilent Co., USA). The conditions used for the electrospray source were: ionspray mode, positive and negative; capillary voltage, 4 kV; nebulizer pressure, 60 psi; fragment voltage, 100 V; curtain gas, nitrogen; drying gas flow, 13 L min^{-1}, desolvation gas temperature, 350 °C.

Bolting stem nitrogen (N) and sulfur (S) content analysis

Bolting stem N content was determined according to Pruden et al. (1985), using the Kjeldahl procedure with salicylic acid, sodium thiosulfate and zinc as catalysts. Bolting stem S content was determined by an inductively coupled plasma atomic emission spectrometer (ICP-MS; Agilent, 7500a, USA) after digestion with HNO_3 and $HClO_4$.

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) to determine the statistical significance of the treatment with SPSS for Windows, version 9.0 (SPSS INC., Chicago, IL, USA) using $P < 0.05$. Differences between means were analyzed using Fisher's protected least significant difference (LSD) procedure.

Conclusion

The NH_4^+/NO_3^- ratio affected the growth and glucosinolate content in bolting stems of Chinese kale (*Brassica alboglabra* L.H. Bailey) significantly ($P < 0.05$). As the NH_4^+/NO_3^- ratio increased from 0/100 to 75/25, there was no significant difference in growth characteristics ($P > 0.05$), whereas in the NH_4^+ only treatment the height and aboveground parts fresh weight decreased significantly ($P < 0.05$) as the leaves withered and no bolting stem emerged due to ammonia toxicity. The highest values for total GS and total aliphatic GS content in bolting stems of Chinese kale were observed in the NH_4^+/NO_3^- 50/50 treatment, while total indolyl GS was highest in the 75/25 treatment. The bolting stem N/S ratio did not change among the 75/25, 50/50, 25/75 and 0/100 treatments, which indicated that there was no direct correlation between glucosinolate content and N/S ratio change. Our results indicate that 50/50 NH_4^+/NO_3^- fertilizer application does not increase the growth of Chinese kale, but can increase the GS content in bolting stems.

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