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Effect of NaCl treatments on glucosinolate metabolism in broccoli sprouts*

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Abstract: To understand the regulation mechanism of NaCl on glucosinolate metabolism in broccoli sprouts, the germination rate, fresh weight, contents of glucosinolates and sulforaphane, as well as myrosinase activity of broccoli sprouts germinated under 0, 20, 40, 60, 80, and 100 mmol/L of NaCl were investigated in our experiment. The results showed that glucoerucin, glucobrassicin, and 4-hydroxy glucobrassicin in 7-d-old broccoli sprouts were significantly enhanced and the activity of myrosinase was inhibited by 100 mmol/L of NaCl. However, the total glucosinolate content in 7-d-old broccoli sprouts was markedly decreased although the fresh weight was significantly increased after treatment with NaCl at relatively low concentrations (20, 40, and 60 mmol/L). NaCl treatment at the concentration of 60 mmol/L for 5 d maintained higher biomass and comparatively higher content of glucosinolates in sprouts of broccoli with decreased myrosinase activity. A relatively high level of NaCl treatment (100 mmol/L) significantly increased the content of sulforaphane in 7-d-old broccoli sprouts compared with the control. These results indicate that broccoli sprouts grown under a suitable concentration of NaCl could be desirable for human nutrition.

Key words: Glucosinolates, *Brassica oleracea*, Sulforaphane, Myrosinase, NaCl
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1 Introduction

Glucosinolates are a class of amino-acid-derived secondary metabolites, especially found in *Arabidopsis thaliana* and *Brassica* crops (Chen and Andreasson, 2001; Fahey *et al*., 2001). Under optimal metabolic conditions, vacuole-existed glucosinolates are generally spatially separated from their hydrolysis enzymes, known as myrosinases (thioglucoside glucohydrolase, EC 3.2.1.147), which are located in specific proteins of plant cells (van Eylen *et al*., 2006; Kissen *et al*., 2009). The myrosinases are distributed throughout the organs of the plant, including leaves, roots, flowers, fruit, and seeds (Textor and Gershenzon, 2009).

Glucosinolate-myrosinase is a unique substrate-enzyme system, in which myrosinases are responsible for glucosinolate turnover (van Eylen *et al*., 2006). Glucosinolates themselves are biologically inactive and must be enzymatically hydrolyzed to produce bioactive compounds. When exposed to wounding or insect attack, the intact cells are disrupted, causing myrosinase to come into contact with glucosinolates and hydrolyze them to glucose and unstable intermediates. These unstable intermediates spontaneously decompose into hydrogen sulfate and isothiocyanates, nitriles, thiocyanates, or epithionitriles (van Eylen *et al*., 2006). One of these decomposition products,

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sulforaphane, an isothiocyanate metabolite produced from glucoraphanin (4-methylsulphinylbutyl glucosinolate) by myrosinase, can protect rodents from chemically induced cancer and inhibit the development of cancer in transgenic mice. Sulforaphane is also found to prevent the growth of human cancer cells as well as cell cycle arrest and reactive oxygen species-dependent apoptosis (Sharma *et al*., 2010). An appropriate consumption of cruciferous vegetables has been shown to be beneficial to human health (Okulicz, 2010).

The sprouts of broccoli (*Brassica oleracea* var. *italica*) are rich sources of glucosinolates and their hydrolysis products. For example, glucoraphanin, the main glucosinolate in broccoli sprouts, is present in sprouts in concentrations of 15 times of that in mature plants, while the content of sulforaphane, one of the degradation products of glucoraphanin, is 10 times higher than that of mature plants (Fahey *et al*., 1997; Nakagawa *et al*., 2006). Akhlaghi and Bandy (2010) reported that eating broccoli sprouts for a relatively short time could notably reduce damage such as oxidative stress and cell death caused by ischemia-reperfusion on the heart (Akhlaghi and Bandy, 2010).

The glucosinolate-myrosinase system of plants is affected by various environmental and ontogenic factors. Temperature (van Eylen *et al*., 2008), growth conditions such as nitrogen and sulfur assimilation (Pérez-Balibrea *et al*., 2010), selenium application (Nyberg, 1991), water deficiency (Schreiner *et al*., 2009), and phenological stage (Velasco *et al*., 2007) can all make an impact on the content of glucosinolates in plants. In addition, cooking methods may also affect the content of glucosinolates in *Brassica rapa* (Francisco *et al*., 2010). The influence of salt stress on the primary metabolism such as photosynthesis and metabolism of antioxidants in mustard (*Brassica juncea* L.) has been well documented (Khan *et al.*, 2009). Moreover, salt stress was also found to influence secondary metabolism in plants. It had also been demonstrated that the content of glucosinolates in radish (*Raphanus sativus* L.) sprouts (Yuan *et al*., 2010), canola (*Brassica napus* L.) (Qasim *et al*., 2003), and broccoli (*Brassica oleracea* L.) florets (López-Berenguer *et al*., 2008) can be changed by the application of NaCl.

However, limited information is available about the influence of NaCl treatments on the metabolism of glucosinolates in broccoli sprouts. The aim of the present study was to evaluate the glucosinolate metabolism in broccoli sprouts under NaCl treatment by determining the glucosinolate composition and their contents. The activity of its hydrolysis enzyme, myrosinase, and the content of the main hydrolysis product, sulforaphane, in broccoli sprouts were also analyzed in the present study.

2 Materials and methods

2.1 Plant materials and cultivation conditions

Seeds of broccoli (*Brassica oleracea* var. *italica* cv. Youxiu) were ordered from the Sakata Seed Corporation (Japan). The seeds were cultivated and harvested as previously described (Yuan *et al*., 2009) except that the treatment sprouts were grown on 0.5% agar (5 g/L) with 20, 40, 60, 80, or 100 mmol/L of NaCl added separately in culture flasks. For each treatment, three replicates were taken for analysis.

2.2 Glucosinolate assay

Glucosinolates were extracted and analyzed as previously described with minor modifications (Yuan *et al*., 2009). A total of 500 mg sprouts were boiled in 3 ml water for two times respectively, and 1 ml of the combined aqueous extract was applied to a DEAE-Sephadex A-25 (35 mg) column (GE Healthcare, Piscataway, NJ, USA). The desulfoglucosinolates were obtained according to the procedure of Yuan *et al*. (2009). Then the extraction was analyzed by high performance liquid chromatography (HPLC). The glucosinolate concentration was expressed as μmol/g fresh weight of broccoli sprouts.

2.3 Myrosinase activity determination

Myrosinase activity was determined using spectrophotometry as described previously by Yuan *et al.* (2009). A total of 500 mg broccoli sprouts were ground with 1.8 ml of 50 mmol/L 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.0) to homogenate at 0 °C, and then incubated at 25 °C for 5 min. After centrifuging at 10000 r/min and 4 °C for 10 min, the supernatants were collected for measurements. The assay followed the procedure of Yuan *et al.* (2009). The myrosinase activity was expressed as nmol glucose formed per minute and mg total protein.

2.4 Sulforaphane measurement

Sulforaphane content was determined as described previously by Guo *et al.* (2011). A total of 500 mg broccoli sprouts were homogenized with 2 ml of 50 mmol/L MES buffer solution (pH 6.0) at room temperature. After incubating for 5 min, the mixture was centrifuged at 8000 r/min for 5 min, and then 400 μl supernatant was analyzed using a Shimadzu 2014 series gas chromatograph according to the procedure of Guo *et al.* (2011). The sulforaphane concentration was expressed as mg/g fresh weight of broccoli sprouts.

2.5 Statistical analyses

Statistical analysis was performed using the SPSS package program version 11.5 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance (ANOVA), followed by Turkey's Honestly Significant Difference (HSD) multiple comparison test. The values are reported as means with their standard error for all results. Differences were considered significant at $P<0.05$.

3 Results

3.1 Effect of NaCl on the germination rate and fresh weight in broccoli sprouts

The seed germination rate was not notably influenced by NaCl treatments at the concentrations of 20, 40, and 60 mmol/L, while it was inhibited at the concentrations of 80 and 100 mmol/L as compared to the control (Fig. 1a).

In short, the fresh weights of broccoli sprouts were markedly enhanced during the experimental period (Fig. 1b). The biomass of 3-d-old broccoli sprouts was significantly enhanced after treatments with 20, 40, and 60 mmol/L of NaCl, while it declined after application of 80 and 100 mmol/L of NaCl as compared to the control. The fresh weights of 5-d-old and 7-d-old broccoli sprouts were increased by applications of 20, 40, 60, and 80 mmol/L of NaCl. However, the fresh weights under treatment with 100 mmol/L of NaCl did not differ significantly from those of the control.

3.2 Effect of NaCl on the content of glucosinolates in broccoli sprouts

The current experiment found seven kinds of glucosinolates in "Youxiu" broccoli sprouts (Table 1; Fig. 2). These included three aliphatic glucosinolates including glucoraphanin, glucoerucin, and glucoalyssin

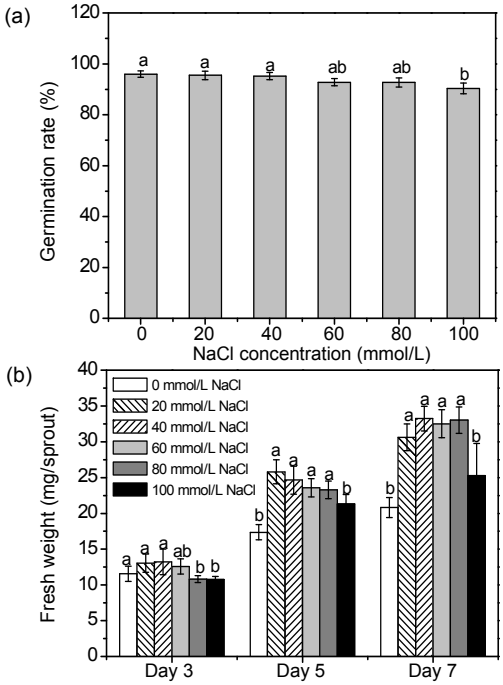

Fig. 1 Germination rate (a) and fresh weight (b) of broccoli sprouts under different saline treatments (0, 20, 40, 60, 80, and 100 mmol/L of NaCl)

Each data point is the mean of three replicates per treatment and time point (mean±standard error). Values not sharing a common letter are significantly different at $P<0.05$

Table 1 Glucosinolate profiles in broccoli sprouts

Retention time (min)	Abbreviation	Trivial name	Chemical name	Relative response factor
7.787	GRA	Glucoraphanin	4-Methylsulfinylbutyl-	0.9
12.410	GL	Glucoalyssin	5-Methylthiopentyl-	0.9
15.669	4-OHIM	4-Hydroxy glucobrassicin	4-Hydroxy-indol-3-ylmethyl-	0.3
19.213	GER	Glucoerucin	4-Methylthiobutyl-	0.9
20.951	IM	Glucobrassicin	Indol-3-ylmethyl-	0.3
22.928	4-IM	4-Methoxy glucobrassicin	4-Methoxy-indol-3-ylmethyl-	0.3
27.996	1-IM	Neoglucobrassicin	1-Methoxyindol-3-ylmethyl-	0.2

as well as four indole ones including 4-hydroxy glucobrassicin, glucobrassicin, 4-methoxy glucobrassicin, and neoglucobrassicin which were detected by HPLC. Among these glucosinolates, glucoraphanin was the most abundant one in "Youxiu" sprouts, accounting for 67% of total glucosinolate content. The most predominant indole glucosinolate was found to be 4-methoxy glucobrassicin (Table 2).

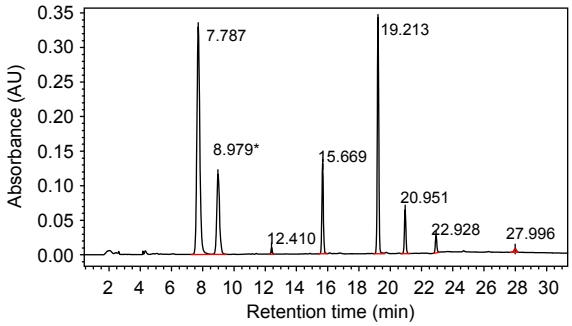

Fig. 2 Glucosinolate profiles in broccoli sprouts
* Standard: sinigrin (8.979 min)

Generally, total glucosinolates in 3-d-old broccoli sprouts treated with different concentrations of NaCl maintained at the same level as that in the control. However, the content of glucosinolates in 7-d-old broccoli sprouts was significantly decreased by 20, 40, 60, and 80 mmol/L of NaCl treatment and maintained the same level in broccoli sprouts treated by 100 mmol/L of NaCl as that in the control.

Individual glucosinolate responded differently to the NaCl treatment. Glucoraphanin was decreased significantly in 7-d-old sprouts treated by different concentrations of NaCl. At the same time, glucoerucin and three kinds of indole glucosinolates, neoglucobrassicin, 4-hydroxy glucobrassicin, and glucobrassicin in 7-d-old sprouts were increased by relatively high concentration NaCl (100 mmol/L) treatment as compared to the control. On the other hand, low and medium concentrations of NaCl (20 and 40 mmol/L) treatments reduced the contents of glucobrassicin, 4-methoxy glucobrassicin, and

Table 2 Total and individual glucosinolate contents in broccoli sprouts under different NaCl treatments at different growth stages

GLS	Time	Glucosinolate content (μmol/g fresh weight)					
		0 mmol/L NaCl	20 mmol/L NaCl	40 mmol/L NaCl	60 mmol/L NaCl	80 mmol/L NaCl	100 mmol/L NaCl
GRA	Day 3	11.99±5.21a	9.57±0.59a	10.76±3.46a	10.50±3.43a	9.10±2.00a	8.91±4.13a
	Day 5	6.94±3.31a	5.12±0.40ab	5.89±0.95ab	6.20±0.95a	3.61±1.35b	2.43±0.71c
	Day 7	5.29±2.36a	2.58±1.04b	2.33±0.05b	2.36±0.05b	1.84±0.99b	2.70±0.83b
GL	Day 3	0.12±0.03b	0.11±0.02b	0.12±0.03b	0.11±0.02b	0.19±0.03a	0.18±0.04a
	Day 5	0.07±0.02a	0.06±0.00ab	0.06±0.02ab	0.07±0.02a	0.06±0.00ab	0.05±0.02b
	Day 7	0.05±0.02a	0.03±0.00a	0.03±0.02a	0.03±0.02a	0.04±0.02a	0.06±0.02a
GER	Day 3	5.42±0.95b	4.86±1.07b	5.65±2.15b	5.43±1.61b	12.30±3.71a	12.29±5.06a
	Day 5	4.34±1.33a	2.72±0.61a	3.09±0.81a	3.08±0.29a	5.30±2.94a	3.18±1.09a
	Day 7	2.09±1.30bc	1.39±0.36bc	1.16±0.43c	1.82±1.20bc	3.41±1.37b	5.76±2.41a
4-OHIM	Day 3	0.85±0.52a	0.55±0.24a	0.72±0.21a	0.81±0.28a	1.02±0.26a	0.94±0.33a
	Day 5	0.30±0.31a	0.16±0.12a	0.13±0.05a	0.23±0.05a	0.22±0.02a	0.13±0.07a
	Day 7	0.10±0.07b	0.02±0.02c	0.02±0.02c	0.03±0.00c	0.07±0.02bc	0.20±0.02a
IM	Day 3	0.41±0.05b	0.31±0.12b	0.34±0.05b	0.45±0.21b	0.94±0.31a	0.81±0.05a
	Day 5	0.16±0.14ab	0.10±0.03b	0.08±0.02b	0.15±0.07ab	0.22±0.07a	0.14±0.05ab
	Day 7	0.08±0.03b	0.02±0.00c	0.03±0.02c	0.04±0.03c	0.10±0.03b	0.29±0.02a
4-IM	Day 3	0.15±0.05a	0.20±0.09a	0.15±0.05a	0.15±0.07a	0.21±0.05a	0.23±0.10a
	Day 5	0.32±0.10a	0.20±0.12ab	0.28±0.10ab	0.23±0.10ab	0.27±0.03ab	0.16±0.09b
	Day 7	0.35±0.09a	0.21±0.02b	0.19±0.05b	0.22±0.09ab	0.20±0.10b	0.27±0.16ab
1-IM	Day 3	0.07±0.02a	0.04±0.00a	0.05±0.03a	0.06±0.03a	0.10±0.03a	0.09±0.00a
	Day 5	0.07±0.03a	0.03±0.02a	0.04±0.02a	0.04±0.00a	0.06±0.05a	0.03±0.02a
	Day 7	0.04±0.02c	0.02±0.00c	0.01±0.02c	0.02±0.02c	0.11±0.05b	0.17±0.02a
Total GLS	Day 3	18.95±6.77a	15.58±0.74a	17.73±5.91a	17.46±5.44a	23.79±6.20a	23.38±9.62a
	Day 5	12.10±2.82a	8.32±0.81bc	9.48±1.66ab	9.92±0.97ab	9.66±2.56ab	6.05±1.97c
	Day 7	7.89±3.78ab	4.20±1.30b	3.71±0.38c	4.45±1.73b	5.72±2.49ab	9.35±3.36a

Each value is the mean of three replicates per treatment and time point (mean±standard error). Values not sharing a common letter are significantly different at $P<0.05$. GLS: glucosinolate; GRA: glucoraphanin; GER: glucoerucin; GL: glucoalyssin; IM: glucobrassicin; 4-OHIM: 4-hydroxy glucobrassicin; 1-IM: neoglucobrassicin; 4-IM: 4-methoxy glucobrassicin

4-hydroxy glucobrassicin while they exerted no significant influence on the accumulations of neoglucobrassicin, glucoalyssin, and glucoerucin.

3.3 Effect of NaCl on the activity of myrosinase in broccoli sprouts

As shown in Fig. 3, the activity of myrosinase was increased with the growth of broccoli sprouts, which was consistent with the developmental decrease of the glucosinolates in sprouts. Treatment with 80 and 100 mmol/L of NaCl increased the activity of myrosinase in 5-d-old broccoli sprouts as compared to the control. However, the activity of myrosinase in 7-d-old sprouts was significantly inhibited after all the treatments with NaCl compared to the control.

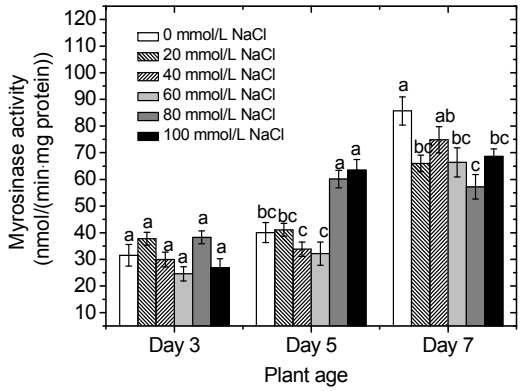

Fig. 3 Myrosinase activity of broccoli sprouts under different saline treatments (0, 20, 40, 60, 80, and 100 mmol/L NaCl)

Each value is the mean of three replicates per treatment and time point (mean±standard error). Values not sharing a common letter are significantly different at $P<0.05$

3.4 Effect of NaCl on the content of sulforaphane in broccoli sprouts

Sulforaphane, with glucoraphanin as substrate, was the main product of glucosinolate degradation catalyzed by myrosinase. The sulforaphane content in broccoli sprouts treated by various concentrations of NaCl during different developmental stages is shown in Fig. 4 and decreased with the growth of broccoli sprouts. The application of 100 mmol/L of NaCl significantly increased the content of sulforaphane in 3-d-old and 7-d-old broccoli sprouts over that of the control. The content of sulforaphane in 7-d-old broccoli sprouts treated by 100 mmol/L of NaCl was 2.1 times of that in the control sprouts.

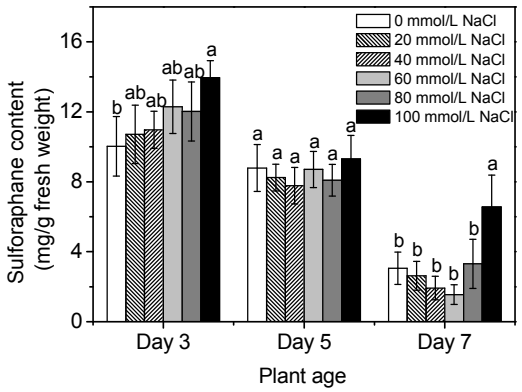

Fig. 4 Sulforaphane content of broccoli sprouts under different saline treatments (0, 20, 40, 60, 80, and 100 mmol/L NaCl)

Each value is the mean of three replicates per treatment and time point (mean±standard error). Values not sharing a common letter are significantly different at $P<0.05$

4 Discussion

The germination rate, fresh weight, contents of glucosinolates and sulforaphane, as well as myrosinase activity of broccoli sprouts under 0 (control), 20, 40, 60, 80, and 100 mmol/L of NaCl were investigated in the current study. Seed germination was inhibited by the application of 80 and 100 mmol/L of NaCl, which was similar to the results in other species of vegetable such as radish and beetroot (Zapata *et al*., 2004; Yuan *et al*., 2010). The fresh weights of 5-d-old and 7-d-old "Youxiu" sprouts treated with 20, 40, 60, and 80 mmol/L of NaCl were significantly heavier than that of the control. This was in accordance with the findings that 10 and 50 mmol/L of NaCl treatments increased the fresh weight of radish sprouts (Yuan *et al*., 2010). This may be due to the nutrients that Na^+ and $C1^-$ ions provide and the reserves of cotyledons which are sufficient to permit the sprouts to adjust to the osmotic stress, maintaining turgor and allowing the growth of sprouts (Scialabba and Melati, 1990; Yuan *et al*., 2009). The differences between the 3-d-old sprouts and 5- and 7-d-old sprouts treated with 80 and 100 mmol/L of NaCl indicated that the older the sprouts are, the more able to readily adapt to their environment.

In the research by Aires *et al*. (2006), the glucosinolates in broccoli include 3-methyl-sulfinylpropyl glucosinolate and 2-phenyl-ethyl glucosinolate, which were not found in the present study. However, two

indole glucosinolates, 4-methoxy glucobrassicin (4-IM) and neoglucobrassicin (1-IM), were detected in our research. These discrepancies might be due to the different varieties and varied developmental stages used in our study. Moreover, glucoraphanin was still the most substantial glucosinolate in "Youxiu" sprouts.

The glucosinolates in sprouts were found to decrease with sprout growth (Fahey *et al*., 1997; Pereira *et al*., 2002). This trend was also observed in the current survey. The contents of total as well as individual glucosinolates were significantly decreased with the growth of sprouts and the 3-d-old sprouts had higher contents of total as well as individual glucosinolates in our study.

It had been observed that NaCl exerted a positive influence on the content of glucosinolates in canola (*Brassica napus* L.) (Qasim *et al*., 2003) and radish (*Raphanus sativus* L.) sprouts (Yuan *et al*., 2010). In the present study, the content of glucosinolates in 7-d-old broccoli sprouts under treatment of 100 mmol/L of NaCl stayed at the same level as that in the control. The different effects of NaCl on canola, radish, and broccoli sprouts could be due to the different glucosinolate profiles of these species. In the broccoli sprouts, glucoraphanin is the dominant glucosinolate while 2-hydroxy-3-butenyl glucosinolate and glucoraphasatin are dominant in *Brassica napus* and radish sprouts, respectively. The glucoraphanin content in 5- and 7-d-old broccoli sprouts was dropped significantly after treatment with NaCl. In contrast, the glucoerucin content in 7-d-old broccoli sprouts was raised by NaCl treatment. The rapid growth of sprouts benefiting from the nutrition of Na^+ and $C1^-$ ions after treatment with NaCl of 40 mmol/L may be the reason for the decline of the glucosinolate contents in sprouts with the plant growth.

Myrosinase was reported to be vulnerable to environmental variations such as phototropic stimulation (Yamada *et al*., 2003) and methyl jasmonate treatment (Kim *et al*., 2006). In our study, the myrosinase activity was increased in 5-d-old sprouts and inhibited in 7-d-old sprouts by 80 and 100 mmol/L of NaCl compared to the control, which was consistent with the change of glucosinolates in broccoli sprouts.

Sulforaphane is affected by various factors, such as pressure (van Eylen *et al*., 2008) and cooking methods (Jones *et al*., 2010). The changing trend of sulforaphane content was similar with the content of glucosinolates, especially glucoraphanin in broccoli sprouts during developmental stages in the present study. The highest content of sulforaphane, the glucoraphanin degradation product, was found in 3-d-old broccoli sprouts and it decreased with the growth of broccoli sprouts. The reduction of sulforaphane content with the growth of sprouts could be due to the osmotic adjustment of glucosinolate degradation by myrosinase in the present study.

Sulforaphane has been implicated in the cancer-protective effects of *Brassica* vegetables (Singh *et al*., 2009; Sharma *et al*., 2010). The accumulation of sulforaphane in 7-d-old sprouts treated with 100 mmol/L NaCl was 2.1 times of that in the control sprouts in the present study, suggesting that sprouts treated with high concentration of NaCl may be desirable from a health perspective. On the other hand, the myrosinase activity of sprouts treated by 100 mmol/L of NaCl was lower than that in the control. Therefore, this could be due to another mechanism for the sulforaphane formation from glucoraphanin.

5 Conclusions

In conclusion, NaCl treatment has a concentration-dependent effect on glucosinolate-myrosinase system in broccoli sprouts. NaCl treatments at relatively low concentrations enhanced the growth of the broccoli sprouts. NaCl treatment at the concentration of 60 mmol/L for 5 d maintained higher biomass and comparatively higher content of glucosinolates in broccoli sprouts with decreased myrosinase activity. The application of high concentration, especially 100 mmol/L of NaCl, for 3 d can endow broccoli sprouts with abundant glucosinolates and sulforaphane. The 100 mmol/L NaCl treatment significantly increased the sulforaphane content in 7-d-old broccoli sprouts as compared to the control. These results indicate that broccoli sprouts grown under a suitable concentration of NaCl could be desirable for human nutrition.

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