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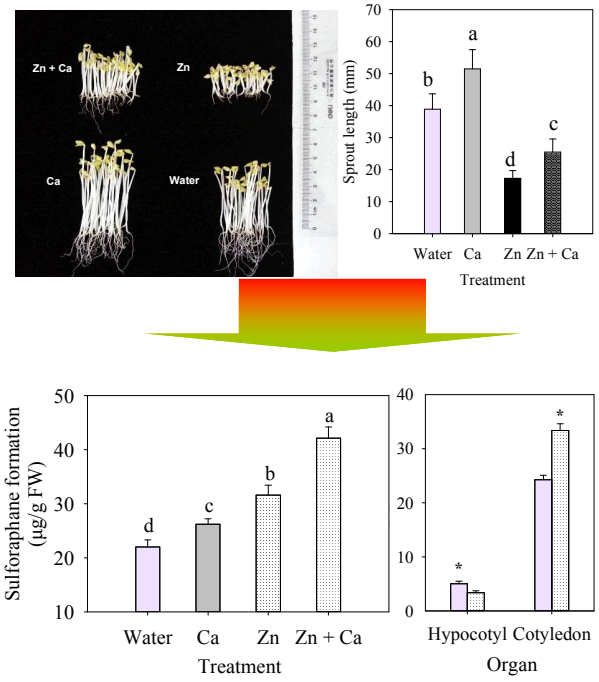
Graphical Abstract:



Calcium mitigates the stress caused by $ZnSO_4$ as sulphur fertilizer and enhances sulforaphane formation of broccoli sprouts

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Abstract

In order to improve the growth condition of broccoli sprouts under $ZnSO_4$ application, exogenous $CaCl_2$ was added in the cultural medium. Then the growth profiles, key bioactive substances (glucosinolates, sulforaphane, ascorbic acid and phenolic compounds), antioxidant capacity, myrosinase activity and related genes expression of broccoli sprouts were evaluated. Results showed that the stressful condition caused by $ZnSO_4$ was effectively mitigated by $CaCl_2$. Consequently, ascorbic acid and total phenolics content as well as antioxidant capacity of broccoli sprouts decreased compared with that of sole $ZnSO_4$ treatment. However, sulforaphane formation increased since the higher glucoraphanin content, myrosinase activity and the related genes expression induced after $CaCl_2$ treatment. Glucoraphanin content and sulforaphane formation of the water-treated sprouts decreased steadily during germination. However, sulforaphane formation of sprouts treated by $ZnSO_4$ plus $CaCl_2$ increased after germinating for 2 days. These results suggested that $CaCl_2$ could mitigate the stress caused by $ZnSO_4$ and enhance the sulforaphane formation of broccoli sprouts.

Keywords: Broccoli sprouts, calcium, $ZnSO_4$ stress, glucoraphanin, sulforaphane, myrosinase

1 Introduction

Compared with mature counterparts, broccoli sprouts are more globally consumed as a promising food since they are rich in health-promoting compounds such as folic acid, phenolics, ascorbic acid and glucosinolates.[1, 2] Regular consumption of broccoli sprouts can reduce the risk of developing chronic diseases and different types of cancer.[3] These beneficial effects are mainly due to the presence of glucosinolates, especially glucoraphanin that accounts for over 50% of total glucosinolate in most broccoli cultivars.[4, 5] Upon a disruption of plant tissues, glucoraphanin will be hydrolyzed by myrosinase into sulforaphane. Sulforaphane is proved to be a natural inducer of the phase II detoxication enzyme to detoxify cancer-causing chemicals.[3] In plants, sulforaphane formation depends on the content of glucoraphanin biosynthesized by flavin-containing monooxygenase (FMO). Except for being hydrolyzed by myrosinase, glucoraphanin also serves as a precursor for biosynthesis of alkenyl-glucosinolates by *AOP2*. [6] In addition, sulforaphane formation also depends on myrosinase and epithiospecifier protein (ESP) activity. High ESP activity is beneficial for sulforaphane nitrile formation. Hence, it is necessary to investigate these enzymes gene expression and sulforaphane formation of broccoli sprouts.

Glucosinolates as one kind of sulphur-containing compound may be considered as a source of sulphur for sprouts growth under low-sulphur conditions.[7] Simultaneously, the biosynthesis of glucosinolates also needs sulphur.[7] Hence, sulphur has been applied to broccoli sprouts growth in order to accumulate glucosinolates.[8, 9] However, the exceeded application of sulphur would decrease broccoli sprouts yield, significantly.[8] In our previous study, we compared the 2 mmol/L of $ZnSO_4$, K_2SO_4 and methionine (Met) on growth and glucosinolates contents of broccoli sprouts. The results showed that only $ZnSO_4$ increased glucosinolate content but inhibited broccoli sprouts

growth, significantly (Fig. S1, Supplementary data). Hence, how to mitigate the stress caused by $ZnSO_4$ and increase the yield of the sprouts is very important.

Calcium is known to be involved in signal transduction in plant through binding with Ca^{2+}-sensors such as calmodulin to induce appropriate physiological cellular responses.[10] Besides, Ca^{2+} plays an important role in maintaining the functional and structural integrity of cell membranes.[11] Epstein [12] clearly demonstrated that the addition of supplemental Ca^{2+} in the growth medium could enhance salt tolerance of plant. Therefore, it is hypothesized that whether Ca^{2+} could mitigate the stress caused by $ZnSO_4$ during broccoli sprouts growth and further improve the content of bioactive substances.

In the present study, we attempted to add Ca^{2+} in the growth medium of broccoli sprouts paralleled with $ZnSO_4$ application. Subsequently, the growth profiles, key bioactive substances (glucosinolates, sulforaphane, ascorbic acid and phenolic compounds), antioxidant capacity, myrosinase activity and related genes expression of broccoli sprouts were evaluated. The changing patterns of glucoraphanin content, sulforaphane formation and myrosinase activity of germinating broccoli seeds and sprouts were also investigated.

2 Materials and methods

2.1 Materials and reagents

Seeds of broccoli (*B. oleracea* L. var. *italica*) were purchased from Nanjing Jinshengda Seed Co. Ltd. (Jiangsu, China). Standard samples of sulforaphane and glucotropaeolin were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade and purchased from Shanghai Institute of Biochemistry (Shanghai, China).

2.2 Seed germination

Dry seeds were immersed in 1.5% sodium hypochlorite for 15 min, then drained and washed with distilled water until they reached neutral pH. They were then placed in distilled water and soaked at 30 °C for 4 h. Soaked seeds were germinated on a filter paper in petri dishes (15 cm in diameter) filled with sterilized quartz sand and grew in the incubators at 30 °C in darkness for 4 days. Sprout samples were rapidly and gently collected from the surface of the filter paper. Then the fresh weight (FW) of the broccoli sprouts was weighed, after which they were immediately frozen in liquid nitrogen and kept in polyethylene bags at -70 °C for further experiments.

2.3 $ZnSO_4$ and $CaCl_2$ treatment

Seeds in each petri dish were supplied with 60 mL distilled water for the first day of germination and then every 12 h with 20 mL of the following testing solutions respectively: (i) distilled water as the control (Water); (ii) 4 mmol/L $ZnSO_4$ (Zn); (iii) 11 mmol/L $CaCl_2$ (Ca); (iv) 4 mmol/L $ZnSO_4$ plus additional mixture of 11 mmol/L $CaCl_2$ (Zn+Ca). The $ZnSO_4$ concentration of 4 mmol/L was pre-selected with the highest sulforaphane formation while inhibited sprouts growth, significantly (Fig. S2, Supplementary data). As for the $CaCl_2$ concentration, we did a series of concentration including 0, 3, 5, 7, 9, 11, 13 and 15, respectively. Then the 11 mmol/L was chosen since it could get the highest sulforaphane formation of sprouts and also increase the sprouts length under stress of 4 mmol/L $ZnSO_4$ (Fig. S3, Supplementary data).

2.4 Sprout length measurement

Twenty sprouts were taken as one sample group. Their length was measured using a vernier caliper (Shanghai Precision Instruments Co., Ltd., Shanghai, China), directly.

2.5 Malondialdehyde (MDA) level determination

MDA was determined using the method of 2-thiobarbituric acid reaction. Fresh sprouts (0.5 g)

were homogenized with 5 mL of 5% trichloroacetic acid. The mixture was centrifuged at 10,000 rpm for 17 min (4 °C). The supernatant was used to determine MDA content as described by Yang *et al*.[13] MDA content was expressed as nmol/g FW.

2.6 Ascorbic acid content determination

Ascorbic acid content was determined according to Volden *et al*.[14] Fresh broccoli sprouts (0.3 g) were extracted with 5 mL of 1.0% (w/v) oxalic acid. After centrifuged at 10,000 rpm for 15 min, the supernatant was filtered through a 0.45 μm Millipore before injected into HPLC (Agilent 1200, USA) with Agilent 1200 tunable absorbance detector and a reversed-phase C18 column (4.6×250 mm, 5 μm, ZORBAX. Eclips) and detected at 254 nm. Ascorbic acid was calculated by external standard curve and expressed as mg per 100 g FW.

2.7 Total phenolics content determination

Total phenolics of the sprouts (0.2 g) was extracted with 5.0 mL of 50% methanol. The mixture was centrifuged at 10,000 rpm for 15 min. One mL of the supernatant was mixed with 1 mL of 0.2 mmol/L Folin-Ciocalteu reagent and 2.0 mL of 2% Na_2CO_3. The mixture reacted at room temperature in the darkness for 2 h. Its absorbance was read at 765 nm. Gallic acid was used as the standard. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g FW.

2.8 Antioxidant capacity test

The sample extract was prepared the same as that of total phenolics. The antioxidant capacity was evaluated using the T-AOC Kit (Nanjing Jiancheng Biotech Inc., Jiangsu, China). One unit of antioxidant capacity was defined as an increase in absorbance of 0.01 at 520 nm per min per gram at 37 °C of the reaction system.

2.9 Glucosinolates analysis

Glucosinolates were extracted and analyzed as previously reported by Guo *et al*.[15] Briefly, samples (500 mg) were extracted with 2 mL of 75% methanol at 80 ℃ for 15 min in a temperature-controlled water bath, with vortexing at regular intervals. The supernatant was collected after centrifugation (5 min, 10,000 rpm), and the residues were extracted once again by using 2 mL of 75% methanol, centrifuged. One milliliter of the combined supernatant was applied to a 1 mL DEAE Sephadex A-25 column (acetic acid activated) and rinsed with 2 mL of 0.02 mol/L sodium acetate. After addition of 200 μL of arylsulfatase solution and incubation for 16 h at 35 ℃, the desulphoglucoraphanin was eluted with 4 mL of Milli-Q water and filtered through a 0.45 μm membrane filter. Separation and detection were performed on an Agilent 1200 HPLC system (AgilentTechnologies Co. Ltd., Palo Alto, CA, USA) equipped with a G1314B UV detector and a G1311A quat pump, using a Eclipse XDB-C18 column (5 μmparticle size, 4.6 × 150 mm; AgilentTechnologies Co. Ltd.) at 226 nm. Determination was conducted at a flow-rate of 1.0 mL/min in a linear gradient starting with 0% acetonitrile for 1 min, reaching 20% acetonitrile at 21 min, 0% acetonitrile at 26 min. Sinigrin (2-propenyl glucosinolate) (Sigma, St. Louis, MO, USA) was added to each sample as an internal standard before the first extraction. The total glucosinolate and glucoraphanin content was calculated and expressed as μmol/g fresh weight of broccoli sprouts.

2.10 Sulforaphane formation determination

Sulforaphane formation was measured by the method of Gu et al.[16] with some modifications. Sprouts (0.2 g) were homogenized with 4.0 mL of distilled water. The mixture was hydrolyzed at 37 ℃ for 3 h in a shaking water bath. After hydrolyzing, it was extracted three times with 10 mL of ethyl acetate. The ethyl acetate fraction was dried at 37 ℃ under vacuum on a rotary evaporator. After dissolving in 2.0 mL of 10% acetonitrile, filtered through a 0.45 μm membrane filter before

injected into HPLC. Agilent 1200 HPLC system (AgilentTechnologies Co. Ltd., Palo Alto, CA, USA) equipped with a Eclipse XDB-C18 column (5 μmparticle size, 4.6 × 150 mm; Agilent Technologies Co. Ltd.) was used.

2.11 Myrosinase activity determination

Myrosinase activity determination was conducted as described previously by Guo et al.[15] The protein content of the supernatant was determined according to Bradford (1976) using bovine serum albumin as the standard. One myrosinase activity unit corresponded to 1.0 nmol glucose formed per minute. The specific activity was expressed as units per milligram of protein.

2.12 Gene expression assay (QRT-PCR, Quantitative Real-Time PCR)

Total RNA from broccoli sprouts was isolated using a E.Z.N.A.™ Plant RNA Kit (OMEGA, R6827-01). Approximately 2 μg total RNA were used as a template for the first strand cDNA synthesis, which was performed with a RT-PCR Kit (TaKaRa: DR027S). First-strand cDNA was synthesized as described previously.[17] The PCR amplification was performed using TaKaRa *Ex-TaqTM* polymerase for target genes and *Actin*. For QRT-PCR analysis, the sequence-specific primers used were shown in Table 1. Triplicate quantitative assays were performed on each cDNA with the SYBR®*Premix Ex Taq*™ (TAKARA: RR420A) with the ABI 7500 sequence detection system according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions used were as follows: 1 cycle of 95 ℃ for 30 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s.

Table 1 The primers used in the present study

Gene	Primer name	Primer sequences (5'→3')
$FMO_{GS\text{-}OX}$	Sense	TCGTCTTGGTGTCGTCGGTC
	Ant-sense	ATCCTCATAAGCCTCCGCAAT
AOP2	Sense	GAGTAACGGAAAGAAAGAAGACAAGG

	Ant-sense	ATAAGCGTGAAGAGTAGAACGAGGT
MYR	Sense	AAGGTCATCAGGGAGAAGGGTG
	Ant-sense	TGTTTGGCAGGGTTCTTAGTGG
ESP	Sense	ACATTTGGGACCAGGGACG
	Ant-sense	TTTCCATACACGGTGGCAGTC
Actin	Sense	CTGTTCCAATCTACGAGGGTTTCT
	Ant-sense	GCTCGGCTGTGGTGGTGAA

2.13 Statistical analysis

Experimental data were expressed as the mean ± standard deviation (SD) with three replications (n = 3) and subjected to statistical analysis with SPSS 18.0 (SPSS Inc., Chicago, IL). Means were performed by Duncan's multiple-range tests. Differences at $p < 0.05$ were considered to be significant.

3 Results and analysis

3.1 Growth profiles of broccoli sprouts

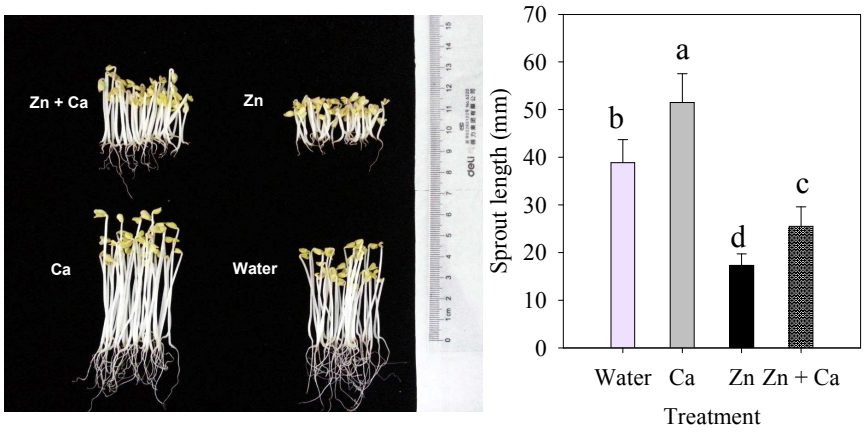


Fig. 1. The growth profiles of broccoli sprouts grown under different treatments. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters are significantly different at $p < 0.05$. After 4-day germination, the sprouts were collected and washed, and measured the sprouts length using a vernier calipers, directly.

The growth profiles of 4 day-old broccoli sprouts in photo and sprout length were presented in Fig. 1. $ZnSO_4$ application significantly ($p < 0.05$) inhibited sprouts growth with the shortest length of 17.3 mm, whereas that of the water-treated was 38.9 mm. After spraying supplementary Ca^{2+}, the $ZnSO_4$-induced stress was significantly mitigated, where the sprouts elongation was accelerated. Interestingly, the application of sole Ca^{2+} also increased the sprout length by 32.4% compared with the control.

3.2 Fresh weight and MDA content of broccoli sprouts

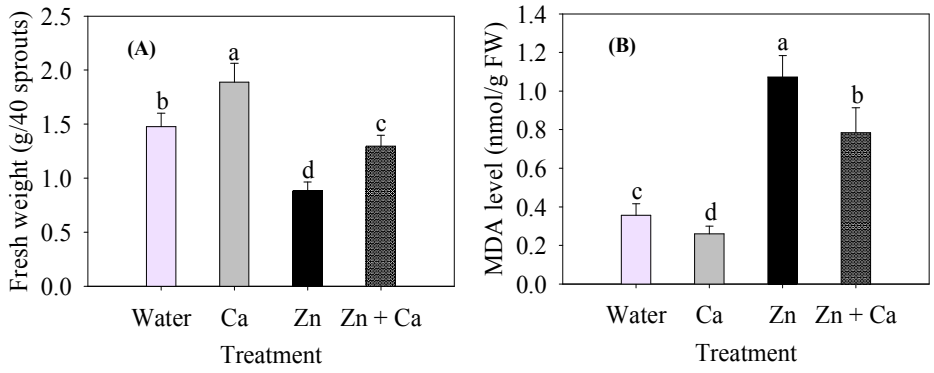


Fig. 2. The fresh weight (A) and MDA content (B) of broccoli sprouts grown under different treatments. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters are significantly different at $p < 0.05$. After 4-day germination, the sprouts were collected, washed and sipped up. The fresh weight and MDA level were measured immediately.

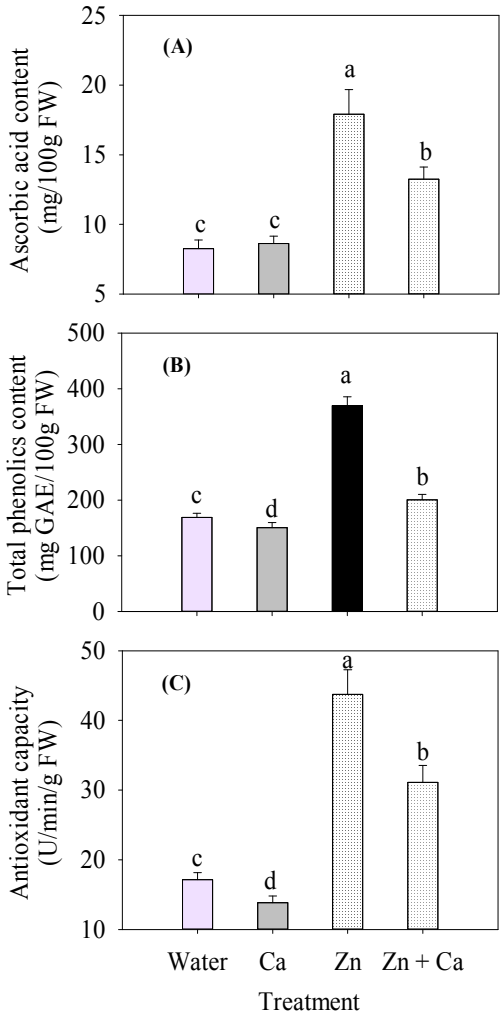
The fresh weight of sprouts grown under $ZnSO_4$ was the least, indicating the growth of sprouts was markedly inhibited ($p < 0.05$) (Fig. 2A). Spraying $CaCl_2$ alone favored sprout elongation, whose fresh weight increased by 27.9% compared with the control. $CaCl_2$ addition alleviated the $ZnSO_4$ stress, generating an increment of 47.0% in fresh weight in comparison with the

191 $ZnSO_4$-treated sprouts.

192 MDA content exhibited an opposite trend of fresh weight (Fig. 2B). $ZnSO_4$ application led to
193 the highest accumulation of MDA in sprouts compared with the control. After adding $CaCl_2$ based
194 on $ZnSO_4$ treatment, the sprouts' MDA content decreased by 26.9%. Water (Control) and $CaCl_2$
195 alone did not cause stress condition, where the MDA content of sprouts kept at a very low level.

196 **3.3 Ascorbic acid, total phenolics content and antioxidant capacity of broccoli sprouts**

197



198

199 **Fig. 3.** Ascorbic acid content (A), total phenolics content (B) and antioxidant capacity (C) of
200 broccoli sprouts grown under different treatments. Values are the means of triplicate analyses. Error

bars show the standard deviation. Those with different lower case letters are significantly different at $p < 0.05$. After 4-day germination, the sprouts were collected, washed and sipped up. The ascorbic acid content, total phenolics content and antioxidant capacity of fresh samples were measured immediately.

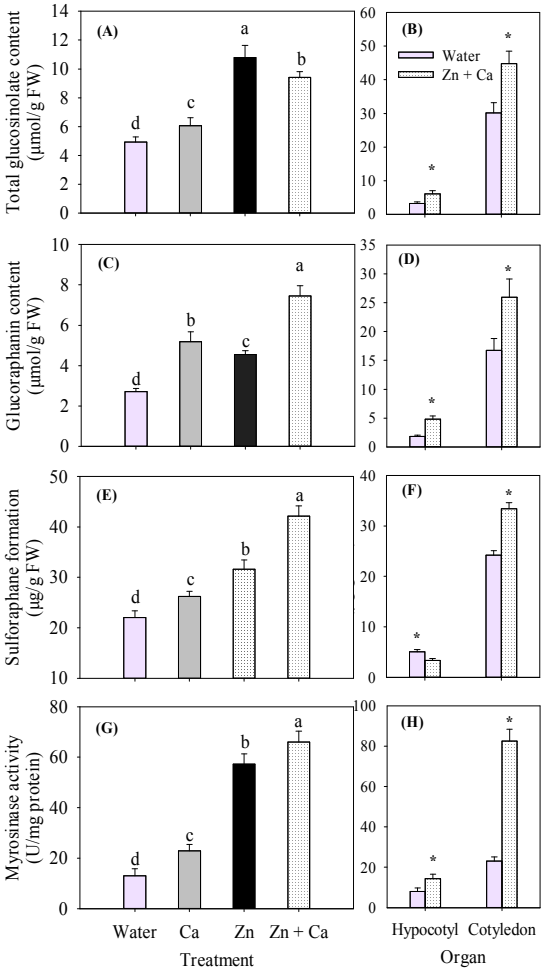
The sprouts treated with $ZnSO_4$ had the highest contents of ascorbic acid and total phenolics, followed by that of $ZnSO_4$ plus $CaCl_2$, water and $CaCl_2$ alone (Fig. 3A and 3B). $CaCl_2$ treatment alone had no significant effect ($p > 0.05$) on ascorbic acid content while decreased that of total phenolics as compared with the control. The contents of ascorbic acid and total phenolics of broccoli sprouts grown under $ZnSO_4$ were 3.17- and 3. 91-fold of the control, respectively. Whereas, additional $CaCl_2$ treatment decreased their contents by 36.47% and 44.23% compared with $ZnSO_4$ treatment, respectively.

As shown in Fig. 3C, the antioxidant capacity of $ZnSO_4$ treated sprouts was the highest, which was 2.55-fold of the control. Adding $CaCl_2$ on the basis of $ZnSO_4$ treatment decreased the antioxidant capacity. Compared with the control, spraying $CaCl_2$ alone also decreased the sprouts antioxidant capacity by 19.1%.

3.4 Glucosinolate content, sulforaphane formation and myrosinase activity of broccoli sprouts

Compared with the control (Water), $CaCl_2$, $ZnSO_4$ and $ZnSO_4$ plus $CaCl_2$ treatments significantly increased total glucosinolate content in broccoli sprouts (Fig. 4A). $ZnSO_4$ led the highest content of total glucosinolate, which was 2.13-fold of the control. However, $ZnSO_4$ plus $CaCl_2$ treatment decreased content of total glucosinolate compared with $ZnSO_4$ treatment. In both hypocotyl and cotyledon, total glucosinolate content in $ZnSO_4$ plus $CaCl_2$ treated sprouts increased

223 significantly by 1.01- and 0.43- fold, respectively, compared with the control (Fig. 4B). Similar to
224 total glucosinolate content, $CaCl_2$, $ZnSO_4$ and $ZnSO_4$ plus $CaCl_2$ treatments all increased
225 glucoraphanin content, but the treatment of $ZnSO_4$ plus $CaCl_2$ best favored the accumulation of
226 glucoraphanin (Fig. 4C). In both hypocotyl and cotyledon, glucoraphanin content in $ZnSO_4$ plus
227 $CaCl_2$ treated sprouts increased significantly by 1.59- and 0.55- fold, respectively, compared with
228 the control (Fig. 4D).



229

230 **Fig. 4.** Total glucosinolate (A, B) and glucoraphanin (C, D) content, sulforaphane formation (E, F)
231 and myrosinase activity (G, H) of broccoli sprouts under different treatments. Values are the means
232 of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters

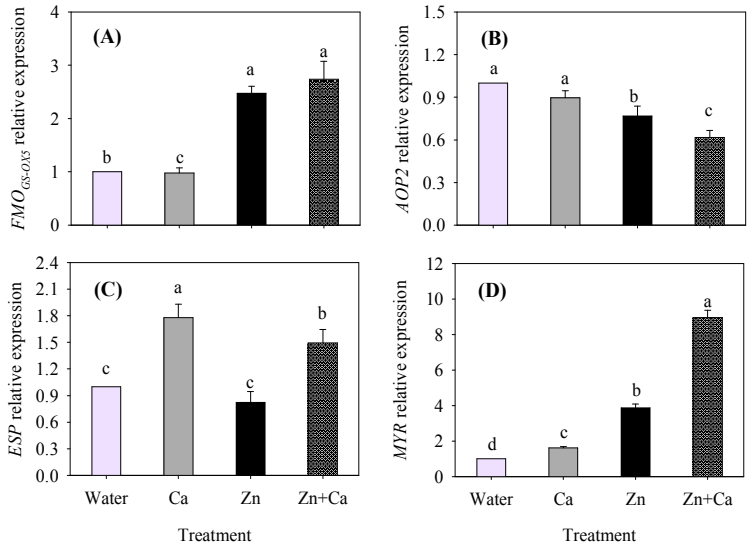
are significantly different at $p < 0.05$. * Represents significant differences at $p < 0.05$. After 4-day germination, the sprouts were collected, washed and sipped up. Then frozen in liquid nitrogen. Total glucosinolate and glucoraphanin content, sulforaphane formation and myrosinase activity were measured within 2 days.

Compared with the control, treatments of $CaCl_2$ alone, $ZnSO_4$ and $ZnSO_4$ plus $CaCl_2$ were all beneficial for the formation of sulforaphane (Fig. 4E). They increased sulforaphane formation by 18.9%, 43.5% and 91.2%, respectively. However, $ZnSO_4$ plus $CaCl_2$ decreased sulforaphane formation in hypocotyl by 33.62% and increased that in cotyledon significantly compared with the control (Fig. 4F).

Myrosinase activity of broccoli sprouts was enhanced by 3.40-fold under $ZnSO_4$ treatment in comparison with the control (Fig. 4G). Supplementary $CaCl_2$ further stimulated myrosinase activity by 15.3% compared with $ZnSO_4$ treatment. Spraying $CaCl_2$ alone increased the activity by 75.9% compared with the control. Similar to glucoraphanin content, myrosinase activity increased under $ZnSO_4$ plus $CaCl_2$ treatment significantly and by 0.78 and 2.57- fold in hypocotyl and cotyledon respectively compared with the control (Fig. 4H).

3.5 Genes expression related to sulforaphane formation

$ZnSO_4$ and $ZnSO_4$ plus $CaCl_2$ treatments induced $FMO_{GS\text{-}OX}$ expressions which were 2.48- and 2.67-fold of the control, respectively (Fig. 5A). However, they decreased *AOP2* expression (Fig. 5B), significantly. $CaCl_2$ addition significantly enhanced the expression of *ESP* (Fig. 5C). $ZnSO_4$ and $ZnSO_4$ plus $CaCl_2$ treatments all induced *MYR* expression which was 1.97-, 4.12- and 9.45-fold of the control, respectively (Fig. 5D).



255

256 **Fig. 5.** The relative expression of FMO_{GS-OX} (A), *AOP2* (B), *ESP* (C) and *MYR* (D) of broccoli
257 sprouts under different treatments. Each datum is the mean and standard deviation of three
258 replicates per treatment. Values not sharing the same letter are significantly different at $p < 0.05$.
259 After 4-day germination, the sprouts were collected, washed and sipped up. Total RNA was
260 extracted immediately using a kit.

261

262 **3.6 Changes of glucoraphanin content, sulforaphane formation and myrosinase activity of**
263 **broccoli sprouts during germination**

264 Germination brought a significant ($p < 0.05$) decrease in glucoraphanin content both in control
265 and the $ZnSO_4$ plus $CaCl_2$ treatment sprouts (Fig. 6A). Glucoraphanin content of sprouts grown
266 under water and $ZnSO_4$ plus $CaCl_2$ decreased by 96.5% and 90.4% within 4 days of germination,
267 respectively. However, after germinating for 1 day, glucoraphanin content of $ZnSO_4$ plus $CaCl_2$
268 treated sprouts was significantly ($p < 0.05$) higher than that of the control at the same time point.

269 Similar to glucoraphanin content, sulforaphane formation of the control sprouts decreased
270 steadily with time elongation (Fig. 6B). Whereas, $ZnSO_4$ plus $CaCl_2$ treatment led to an gradual

increase in sulforaphane formation after 2 days of germination. No significant ($p > 0.05$) difference was observed between the control and $ZnSO_4$ plus $CaCl_2$ treatment at the first 2 days of germination. However, sulforaphane formation of 3 day- and 4 day-old sprouts were significantly ($p < 0.05$) higher than that of the control.

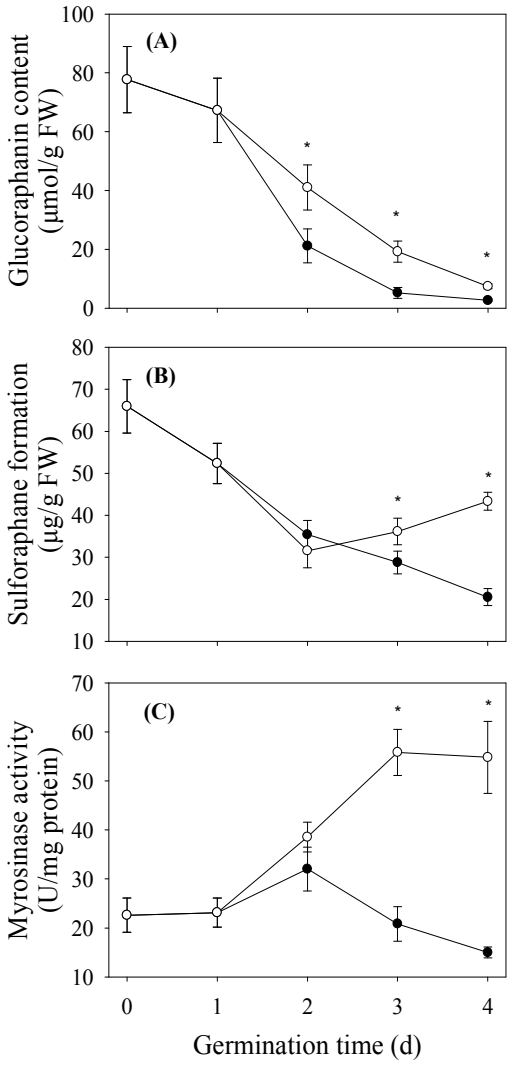


Fig. 6. Glucoraphanin content (A), sulforaphane formation (B) and myrosinase activity (C) of broccoli seeds and sprouts during germination period under control and $ZnSO_4$ plus $CaCl_2$ treatments. Values are the means of triplicate analyses. Error bars show the standard deviation. * Represents significant differences at $p < 0.05$. During germination, sprouts were sampling every day

and frozen in liquid nitrogen. Glucoraphanin content, sulforaphane formation and myrosinase activity were measured within 2 days.

Myrosinase activity of sprouts sprayed with water increased to 2nd day of germination and decreased afterwards (Fig. 6C). In contrast, the activity increased continuously to 3rd day of germination and kept stable when sprayed with $ZnSO_4$ plus $CaCl_2$. No significant ($p > 0.05$) change occurred at the first day of germination.

4 Discussion

The addition of $CaCl_2$ to seed germination medium under salinity stress have been reported to enhance germination rate and seedling growth in wimmera ryegrass,[18] cotton,[19] tomato and cucumber.[20] However, the concentration of $CaCl_2$ should be clearly defined since lower or higher concentration would have significant different effects, which depends upon the salinity stress source and plant species or cultivars. In wimmera ryegrass, the 5.0 mmol/L Ca^{2+} showed the best effect on NaCl stress, while 10.0 mmol/L Ca^{2+} was optimal on $MgCl_2$ stress.[18] Besides, the author found that increasing Ca^{2+} concentration to 10.0 mmol/L under NaCl stress decreased the radicle length compared with that of 5.0 mmol/L. In this study, it was also detected that a concentration higher than 11 mmol/L would decrease broccoli sprout length (Fig. S2, Supplementary data). Apart from directly adding Ca^{2+} to sprout growth medium, several studies chose to pre-treat seeds through soaking in Ca^{2+} solutions.[21, 22] They both found the salinity tolerance of tested seeds was greatly enhanced. These results suggested that the mitigation effect of Ca^{2+} might also be achieved via soaking seeds prior to germination.

The salinity environment is usually created by NaCl treatment during sprouts growth.[23, 24] In

broccoli sprouts cultivation, in order to enhance glucoraphanin biosynthesis, sulphur-containing chemicals are commonly supplied.[7] However, since the sprouts are very salt sensitive,[25] it is difficult to find the optimal sulphur concentration without influencing sprouts growth. It has been reported that unsuitable concentrations of sodium thiosulphate[8] and K_2SO_4[25] inhibited sprouts growth and thus decreased the positive effects of sulphur fertilization. Here, the stress was caused by spraying $ZnSO_4$, which was originally expected to be a sulphur fertilizer. For one probable reason, the stress of $ZnSO_4$ was due to the excessive level of Zn^{2+} because Zn is a micronutrient for sprout growth. The excessive Zn^{2+} was not utilized promptly and thus created an osmotic stress condition where the water absorption system was interrupted. Another one might be that $ZnSO_4$ presents a similar negative effect as NaCl does. Hence, towards different broccoli cultivars and growth medium, the optimal sulphur type and concentration should be pre-selected depending on sprouts sulphur uptake abilities and partitioning of sulphur into glucoraphanin.[26]

Ascorbic acid content and the activities of its regenerating enzymes in plants are highly related to environmental stresses such as light, temperature, salt, and drought.[27] Nishikawa *et al.*[28] investigated the ascorbic acid metabolism in postharvest broccoli florets, and found that ascorbate-regenerating system could be activated to protect plant organs from environmental stresses. In this study, the ascorbic acid content in broccoli sprouts was accelerated by $ZnSO_4$ application, which could also be attributed to the activation of the regenerating enzymes under $ZnSO_4$ stress.[27] Phenolic compounds are secondary metabolites produced when plants are facing environmental stresses.[29] In the present study, $ZnSO_4$ application significantly promoted the biosynthesis of phenolic compounds as a result of the possible osmotic stress (Fig. 3B). However, when the stress situation was likely mitigated by exogenous $CaCl_2$, the total phenolics content of

broccoli sprouts was decreased as compared with that of $ZnSO_4$.

Ascorbic acid and phenolic compounds both have high antioxidant activities in plants,[30, 31] hence, the antioxidant capacity of plant tissues is closely associated with their contents.[32] Significant positive correlation between ascorbic acid, phenolic compounds and antioxidant capacity was observed in radish[33] and broccoli sprouts.[5] In the present study, the antioxidant capacity of broccoli sprouts of the tested four treatments had a same changing trend with the contents of ascorbic acid and phenolic compounds (Fig. 3). The highest antioxidant capacity was observed in sprouts having the highest ascorbic acid and phenolic compounds contents. Exogenous Ca^{2+} decreased the antioxidant capacity since the contents of ascorbic acid and phenolic compounds were decreased.[5] Barillari *et al.*[34] reported that 4-methylthio-3-butenyl glucosinolate isolated from *Raphanus sativus* sprouts was related to its total antioxidant capacity. Guo *et al.* [5] also suggested that glucosinolates in broccoli sprouts had some antioxidant capacity. However, in this study, no obvious relations were observed between total antioxidant and total glucosinolates or glucoraphanin content ($p > 0.05$). The discrepancy could be attributed to different cultivars of broccoli in which the content of antioxidant components is different.

Falk *et al.*[35] highlighted that sulphur fertilization could lead to an increase in glucosinolate content ranging from 0.25- to more than 50-fold, which relies on sulphur types and treating styles. Here, as a sulphur-containing chemical, $ZnSO_4$ increased total glucosinolate content, but $ZnSO_4$ plus $CaCl_2$ treatment decreased it content (Fig. 4A). Interestingly, the content of glucoraphanin was further enhanced by $CaCl_2$ addition (Fig. 4C). Applying $CaCl_2$ alone to sprouts increased glucoraphanin content compared with that of the control. Besides, it was noted that glucoraphanin content of sprouts under sole $CaCl_2$ treatment was higher than that of sole $ZnSO_4$ treatment. In

addition, compared with $ZnSO_4$ treatment, $ZnSO_4$ plus $CaCl_2$ treatment increased genes expression related to glucoraphanin biosynthesis (Fig. 5A), but decreased *AOP2* expression which is related to glucoraphanin degradation (Fig. 5B). These indicated that $CaCl_2$ could induce glucoraphanin biosynthesis in broccoli sprouts.

In both $ZnSO_4$ plus $CaCl_2$ treated and control sprouts, glucoraphanin content decreased steadily with germination time (Fig. 6A), which agreed with previous studies.[2, 36] The decrease in glucoraphanin content might result from 'dilution effects' of water absorption during growth.[37] Another possibility could be that glucoraphanin was degraded as sulphur supply to support sprouts tissue expansion and formation of other sulphur-containing compounds.[7] Under $ZnSO_4$ plus $CaCl_2$ treatment, the decrease of glucoraphanin was delayed to some extent, which was probably a result of sulphur supply. Pérez-Balibrea *et al.*[9] had previously reported that glucoraphanin content of broccoli sprouts treated with K_2SO_4 kept stable when grew from 6 to 12 days.

Sulforaphane is hydrolyzed from glucoraphanin by myrosinase, whose formation largely depends on both glucoraphanin content and myrosinase activity.[7] In this study, sulforaphane formation of broccoli sprouts treated with $ZnSO_4$ plus $CaCl_2$ was the highest (Fig. 4D). This might be due to a higher glucoraphanin content (Fig. 4B), myrosinase activity (Fig. 4E) and its higher expression (Fig. 5D). Although the glucoraphanin content of sprouts under sole $CaCl_2$ treatment was higher than that of sole $ZnSO_4$, its sulforaphane formation was less because of a lower myrosinase activity (Fig. 4E) and its lower expression (Fig. 5D) as well as the higher expression of *ESP* (Fig. 5C). Liang *et al.*[38] reported that Zn^{2+} could enhance myrosinase activity and thus increase sulforaphane formation. The finding in the present study was similar to theirs. It was clearly noticed that glucoraphanin content of broccoli sprouts under $ZnSO_4$ plus $CaCl_2$ and the control treatments

368 both decreased steadily during germination (Fig. 6A). However, the sulforaphane formation of
369 sprouts treated with $ZnSO_4$ plus $CaCl_2$ did not decrease steadily as expected (Fig. 6B). In contrast,
370 its content increased after germinating for 2 days. This could be due to the fact that myrosinase
371 activity increased to a high level after 1 day of germination. These results suggested that the
372 glucoraphanin-myrosinase system is complicated and needs further investigation.

373 **5 Conclusion**

374 $CaCl_2$ application effectively mitigated stressful condition caused by $ZnSO_4$. Consequently, the
375 antioxidant compounds such as ascorbic acid and total phenolics content decreased. However,
376 sulforaphane formation increased since the higher glucoraphanin content, myrosinase activity and
377 the related genes expression induced after $CaCl_2$ treatment.

378 **Conflicts of interest**

379 The authors declare no conflict of interest.

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