Emirates Journal of Food and Agriculture. 2017. 29(2): 123-130
doi: 10.9755/ejfa.2016-06-758
http://www.ejfa.me/

REGULAR ARTICLE

NaCl-$CaCl_2$ treatment enhancing nutritional and functional quality of mung bean sprouts

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ABSTRACT

In this study, the effects of NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on the growth profiles, nutritional quality, phytic acid degradation-lower inositol phosphate formation system of germinated mung bean were investigated. Mung bean seeds were germinated at 30°C in darkness for 4 days and sprayed solutions containing distilled water, 1.6 mM NaCl, 6 mM $CaCl_2$, and 1.6 mM NaCl plus 6 mM $CaCl_2$ every 1 h. Samples were collected every 2 days in liquid nitrogen for experiments and each experiment was repeated three times. The results showed that the NaCl-$CaCl_2$ treatment improved nutritional quality significantly for the higher content of ascorbic acid, soluble sugar and free amino acid content in mung bean sprouts. Meanwhile, NaCl-$CaCl_2$ treatment was also the most effective way to improve the degradation of phytic acid by enhancing phytase activity and the related gene expressions. This led to a higher level of the lower inositol phosphate content in mung bean sprouts. These results suggest that NaCl-$CaCl_2$ treatment had a better influence on improving nutritional and functional quality of mung bean sprouts than NaCl and $CaCl_2$.

Keywords: Mung bean sprouts; NaCl-$CaCl_2$ treatment; Nutritional and functional quality; Phytic acid

INTRODUCTION

As an important kind of legume, mung bean has been cultivated for more than 2000 years in China. Mung bean is an especially important source of dietary proteins and also rich in vitamins and minerals including calcium, phosphorus and iron. It plays an important role in cholesterol metabolism and blood cholesterol levels control (Vayupharp, 2013). However, the application of mung bean was limited by anti-nutritional factors like phytic acid which is also named as hexphosphates and has adverse effects for human nutrition. This is because phytic acid cannot be easily digested by human bodies, and it can interact with minerals and protein molecule which is positively charged due to its strong chelating capability (Jin et al., 2015). Germination is one of the effective and simple ways to overcome the problem, during germination, the increase of phytase activity and decline of phytic acid content were found. Phytic acid will be disintegrated into inositol phosphate and free inorganic phosphorus. As phytic acid degradation products, inositol phosphate has many positive functions such as antioxidant and anticancer capacity but has no function of anti-nutrition (Maffucci et al., 2005; Muzquiz et al., 1997). There are many studies which focused on the ways of reducing phytic acid content during food processing. Many factors, such as temperature and germination treatment, have a crucial influence on appearance and metabolite profiles during mung bean germination (Jom, 2012). It is reported that phytase activity increased under NaCl stress in lettuce seedling (Nasri et al., 2011) and Ca^{2+} could activate the β-spiral phytase of lily pollen (Barrientos et al., 1994). However, there are few available studies on the effects of inorganic salt on phytic acid degradation of mung bean sprouts.

Nutrients change a lot during germination, for example, macromolecule carbohydrate and protein are converted into small molecules including soluble sugars and amino acids, respectively. Previous studies also suggested that inorganic salt treatments could increase the nutrients content. Keutgen and Pawelzik (Keutgen et al., 2008) found that NaCl enhanced the content of ascorbic acid and free amino acids in strawberries. The growth-promoting ability of $CaCl_2$ was found in soybeans (Baohong, 1997), so it

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Received: 04 July 2016; **Revised:** 12 January 2017; **Accepted:** 21 January 2017; **Published Online:** 02 February 2017

may accelerate anabolic metabolism and catabolism of the materials. Though numbers of investigations conducted on the effects of salts on nutrients contents in many plants, few studies were found to explore the effects in mung bean sprouts.

In order to clarify the effects of NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on nutritional quality and phytic acid degradation in mung bean sprouts, the nutrients compounds (total phenols, ascorbic acid, soluble sugar and free amino acid), phytic acid degradation, inositol phosphate content, phytase activity and related genes expressions under NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments in mung bean sprouts were investigated.

MATERIALS AND METHODS

Materials and chemicals

Mung bean seeds (*Phaseolus radiantus* L. cv. Qindou 20) were supplied by Jiangsu Academy of Agricultural Science and stored at -20°C. Standard samples of inositol triphosphates (IP3), tetraphosphates (IP4), pentaphosphates (IP5), hexphosphate (IP6) and ascorbic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade. They were all bought from Shanghai Institute Biochemistry (Shanghai, China).

Cultivation condition

Mung bean seeds were washed and steeped in distilled water for 4 h at 30°C after surface-sterilization with 5% (v/v) sodium hypochlorite, and then they were put into the sprouters (BX-801, Beixin Hardware Electrical Factory, Zhejiang, China) to be germinated at 30°C in darkness. Chemical solutions containing 1.6 mM NaCl, 6 mM $CaCl_2$, and 1.6 mM NaCl plus 6 mM $CaCl_2$ were sprayed on the mung bean seeds every 1 h, respectively. The spraying solutions were renewed every 24 h and the control sprouts were sprayed with distilled water. The germination was conducted for 4 days and samples were collected every 2 days in liquid nitrogen for further experiments. The spraying condition has been optimized previously.

Measurement of sprout length and sprout weight

Sprout length and sprout weight were determined using a caliper and analytical balance on different sampling schedules, respectively.

Measurement of total phenols, ascorbic acid, soluble sugar and free amino acid

Total phenols content was determined according to the method of Lin & Tang (Lin et al., 2007) with minor modifications. The phenols compounds (0.5 g) were extracted with 5 mL of 50% methanol by grinding with ice-bath. The mixture was centrifuged at 10,000×g for 15 min. One mL of the supernatant was reacted with 1 mL of 0.2 mol/L Folin–Ciocalteu reagent and 2 mL of 2% Na_2CO_3 for 2 h in darkness. Total phenolic content was determined by reading the absorbance at 765 nm with a UV-Vis spectrophotometer (UV-2802, Unico Instrument Co. Ltd., Shanghai, China). Gallic acid was used as the standard. The results were expressed as micrograms of gallic acid equivalent (GAE) per sprout.

Ascorbic acid content was determined according to Volden et al. (Volden et al., 2009). Five hundred milligrams of fresh mung bean sprouts were ground with 5 mL of 1.0% (w/v) oxalic acid with ice-bath. After centrifuged at 10,000×g for 10 min and filtered through a 0.45 mm Millipore (Darmstadt, Germany), the extracted sample (20 µL) was analyzed using an Agilent 1200 HPLC system (Agilent Technologies Co. Ltd., Santa Clara, CA, USA) using a ZORBAX SB-C18 (5 mm particle size, 4.6×250 mm; Agilent Technologies Co. Ltd.) at 254 nm. The mobile phase consisted of 5% v/v methanol/water (0.1% oxalic acid in water) at a flow rate of 0.8 mL/min. Ascorbic acid was quantified by external calibration and results were as ng/sprout.

Soluble sugar content was determined by the method of Buysse and Merckx (Buysse et al., 1993). Five hundred milligrams of fresh mung bean sprouts were ground with 5 mL of distilled water, and then extracted in boiling water for 1 h. After filtrating, adding distilled water up to 25 mL. The 0.1 mL of extracting solution was mixed with 0.9 mL of distilled water, 0.5 mL of Anthrone-Ethyl acetate reagent and 5 mL of sulfuric acid. After reacted in boiling water for 1 min then cooled to the room temperature the soluble sugar content was determined by reading the absorbance at 630 nm with a UV-Vis spectrophotometer (UV-2802, Unico Instrument Co. Ltd., Shanghai, China). Sucrose was used as the standard. The results were expressed as mg/sprout.

Free amino acid content was determined following the method of Moor and Stein (Moore et al., 1948). Five hundred milligrams of fresh mung bean sprouts were ground with 5 mL of 0.2 mM sodium acetate buffer (pH=5.4). The mixture was centrifuged at 10,000×g for 15 min. One µL of the supernatant reacted with 1.95 mL of distilled water, 3 mL of ninhydrin solution and 0.1 mL of 10% Vc solution (m/v), and then put into boiling water for 15 min. After adding 60% ethanol (v/v) up to 20 mL, the free amino acid content was determined by reading the absorbance at 570 nm with a UV-Vis spectrophotometer (UV-2802, Unico Instrument Co. Ltd., Shanghai, China). Leucine was used as the standard. The results were expressed as µg/sprout.

Determination of tri-, tetra -, penta- and hexphophates (IP3, IP4, IP5 and IP6) content

The inositol phosphates contents were determined by HPLC according to the methods of Lehrfeld (1994) and Burbano et al. (1995) with some modifications. The mung bean sprouts were frozen to dryness and then extracted with 20 mL of 0.5 M HCl with vortex for 2 h at room temperature. The filtrate was evaporated to dryness under a vacuum after being centrifuged and filtered through MF-Millipore (0.22 mm), and then dissolved in 15 mL of 25 mM HCl. The dissolution was passed through a strong anion-exchange (SAX) column which was linked to a vacuum manifold (Supelco, Bellafonte, PA, USA). The loaded SAX column was washed successively with 15 mL of 25 mM HCl. The resin bound inositol polyphosphates were then eluted with 10 mL portions of 2 M HCl. The eluent was evaporated to dryness and dissolved with the mobile phase (pH=4.3) which consisted of 0.05M formic acid: Methanol 49:51 and 1.5 mL/100 mL of TBA-OH were added. The IP3, IP4, IP5 and IP6 were determined by ion-pair C18 reverse-phase HPLC with refractive index. The flow rate was 0.4 mL/min.

Determination of inorganic phosphorus content

Inorganic phosphate was determined according to the method of Chen et al. (2004), dried samples were milled and extracted in 15 mL of 0.2 M HCl in 30°C for 2 h. The 0.5 mL of the extract was mixed with 1 mL ferric solution (0.4 mM ammonium iron (III) sulfate dissolved in 0.2 M HCl). Then the mixtures were heated in a boiling water bath for 30 min. Once the tubes reached room temperature, 2 mL of 2-2'-Bipyridine solution was added. The absorbance was measured at 660 nm and the result was expressed as nmol/sprout.

Phytase activity assay

Phytase was extracted and the activity determined as the description of Hegeman et al. (2001) with minor modifications. Mung bean sprouts (1.0 g) were ground with 5 mL cool sodium acetate buffer (50 mM sodium acetate, pH 5.5). After centrifugation, the supernatant was used for enzyme activity analysis. Free inorganic phosphorus determination was measured according to Chiera et al. (Chiera et al., 2004). One phytase unit corresponded to 1 mM inorganic phosphorus formed per minute. The activity is expressed as units per fresh sprout.

Phytic acid degradation related genes expression assay

Total RNA from mung bean sprouts after 2 and 4-day germination was isolated using an E.Z.N.A.™ Plant RNA Kit (Omega, Norcross, GA, USA; R6827-01). Five mung bean sprouts were grounded in a mortar and pestle with liquid nitrogen, and 0.2 g of powdered sprouts was used to extract total RNA. Approximately 2 μg of total RNA was used as a template for the first-strand cDNA synthesis, which was performed with a RT-PCR Kit (TaKaRa, Shiga, Japan: DR027S). The PCR amplification was performed using TaKaRa *Ex Taq*™ polymerase for the target genes and *β-Actin*. For qRT-PCR analysis, the sequence-specific primers used were shown in Table 1. Relative real-time PCR was performed in a total volume of 20 μL including 2 μL of cDNA solution, 0.4 μL each of 10 μM forward and reverse primer, 10 μL SYBR Premix Ex Taq (5 U/μL; TaKaRa) and 7.2 μL sterile H_2O. The amplification reactions were carried out in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Samples for qRT-PCR were run in three biological replicates with three technical replicates.

Statistical analyses

Experimental data were expressed as the mean ± standard deviation (SD) with three replications (n=3). SPSS 18.0 (SPSS Inc., Chicago, IL) was applied for significant difference tests. Mean of separations was performed by Duncan's multiple-range tests. A difference at $p<0.05$ was considered to be statistically significant.

RESULTS AND ANALYSIS

Sprout length and sprout weight of mung bean sprouts

Compared with the control, the sprout length increased significantly under NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments ($p<0.05$), and the NaCl-$CaCl_2$ treated sprouts length exhibited the longest, which was 1.72-fold of the control on the 4th day (Fig. 1a). NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments all significantly increased the weight of mung bean sprouts, which was 1.30-fold, 1.49- fold and 1.61-fold of the control (Fig. 1b).

Total phenols, ascorbic acid, soluble sugar and free amino acid content of mung bean sprouts

Total phenols content increased with germination time. Moreover, NaCl spraying treatment showed the highest

Table 1: Primers used in this study

Gene	Primer name	Primer sequences (5'→3')
ALP	Sense	TCCTGAGATTACTGAGGCTGTTG
	Ant-sense	CTCAGCCCTTCATTCATTTTAGC
MIPP	Sense	CTGTT ACAAGT GAAAGCCGTGC
	Ant-sense	GAA AAGACCAC ACGCTTGAT T
PY	Sense	ATGGGA CTATTGGGG AAGGTTTA
	Ant-sense	CCCCAG CATTGAAA GAGTAGTAT
PAP	Sense	GGT GATGGAGG AAACAAAGAAG
	Ant-sense	CCTG TGCCAACTCCA GAAAG
Actin	Sense	CGAAGTTCTGTTCCAGCCAT
	Ant-sense	CTACTCGGTGCCAATGCTGT

content of total phenols. No significant difference was found in mung bean sprouts among the $CaCl_2$, NaCl-$CaCl_2$ and the control (Fig. 2a). A different trend was observed in the content of ascorbic acid. Its content in $CaCl_2$ and NaCl-$CaCl_2$ treated sprouts was significantly higher than that in the control, which was 2.09-fold and 1.92-fold of the control, respectively, after 4-day germination (Fig. 2b). The results showed a remarkable increase in soluble sugar content along with the germination time. The three treatments all had significant effect on increasing soluble sugar content in sprouts. Especially, NaCl-$CaCl_2$ performed best which was followed by $CaCl_2$ and NaCl (Fig. 2c). As shown in Fig. 2d, free amino acid content had the similar trend to soluble sugar content in sprouts. A significant difference was found on free amino acid content among the three treatments and the control in 4-day-old sprouts. Compared with the control, the free amino acid content under NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments increased by 34.81%, 62.04% and 142.21%, respectively.

Changes of phytic acid, IP5, IP4, IP3 and inorganic phosphorus acid content

Phytic acid was the major form of inositol phosphate in mung bean seeds, whose content decreased along with the germination time. The content of phytic acid reduced significantly under NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments with the reduction of 20.01%, 53.87% and 77.06% respectively in 4-day-old germinated mung bean. The lowest phytic acid content was found in NaCl-$CaCl_2$ treatment (Fig. 3a) ($p < 0.05$). The content of lower inositol phosphate (IP5, IP4 and IP3) content increased

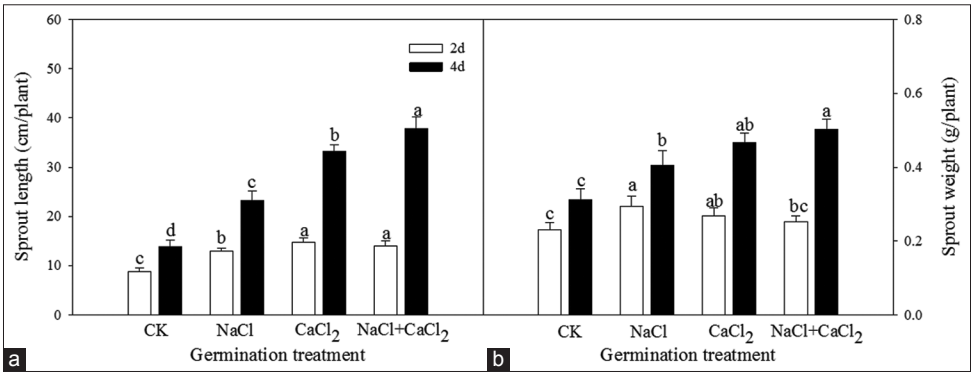

Fig 1. Change of sprout length (a) and sprout weight (b) with CK (distilled water), NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on 2nd and 4th day. Different letters indicate significant difference for each treatment in the same day ($p < 0.05$).

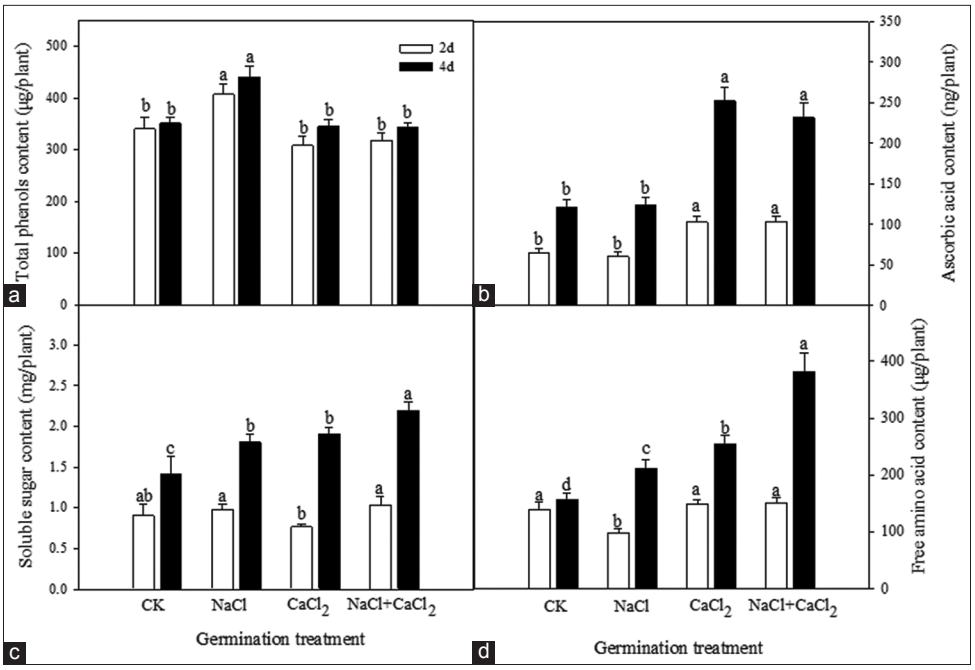

Fig 2. Change of total phenols content (a), ascorbic acid content (b), soluble sugar content (c) and free amino acid content (d) in mung bean sprouts with CK (distilled water), NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on 2nd and 4th day. Different letters indicate significant difference for each treatment in the same day ($p < 0.05$).

significantly along with the germination ($p<0.05$). As shown, three treatments significantly enhanced the content of IP5 ($p<0.05$), $CaCl_2$ and NaCl-$CaCl_2$ had better effect than NaCl, and No noticeable difference was found between $CaCl_2$ and NaCl-$CaCl_2$ (Fig. 3b). Differently, three treatments showed similar effect on increasing IP3 and IP4 content. In the germination, IP3 and IP4 content increased gradually. The content of IP3 and IP4 in sprouts under $CaCl_2$ and NaCl-$CaCl_2$ treatments increased significantly compared with the control, which increased by 68.24% to 103.33% on the 4th day (Fig. 3c, Fig. 3d). With the resolving of phytic acid, inorganic phosphorus content increased. As shown in Fig. 3e, the changing trend of inorganic phosphorus content was contrary to that of phytic acid. The NaCl-$CaCl_2$ treatment enhanced the inorganic phosphorus content best, followed by $CaCl_2$ and NaCl, and remarkable difference was found on the content of inorganic phosphorus content in 4-day-old germinated sprouts compared with the control ($p < 0.05$).

Changes of phytase activity during the germination under NaCl-$CaCl_2$ treatment

A decrease was found on the phytase activity with the growth time of the sprouts. On the 2nd day, the activity of phytase under three treatments enhanced significantly compared with the control ($p < 0.05$); meanwhile, $CaCl_2$ and NaCl-$CaCl_2$ had the better effect on phytase activity increasing than NaCl. The activity of phytase in NaCl-$CaCl_2$ treatment showed the highest on the 4th day (Fig. 4).

Gene expressions related to phytic acid degradation

ALP (alkaline phosphatase), *MIPP* (multiple inositol polyphosphate phosphatase), *PY* (phytase) and *PAP* (purple acid phosphatase) expressions in mung bean sprouts were investigated based on qRT-PCR analysis (Fig. 5). On the 2nd day, NaCl-$CaCl_2$ treatment significantly raised the expressions of *APL*, *MIPP*, *PY* and *PAP* ($p < 0.05$), which were 1.95-, 2.69-, 6.43- and 6.78- fold of the control, respectively. Meanwhile, $CaCl_2$ also raised the expressions of these genes; their expression was lower than NaCl-$CaCl_2$

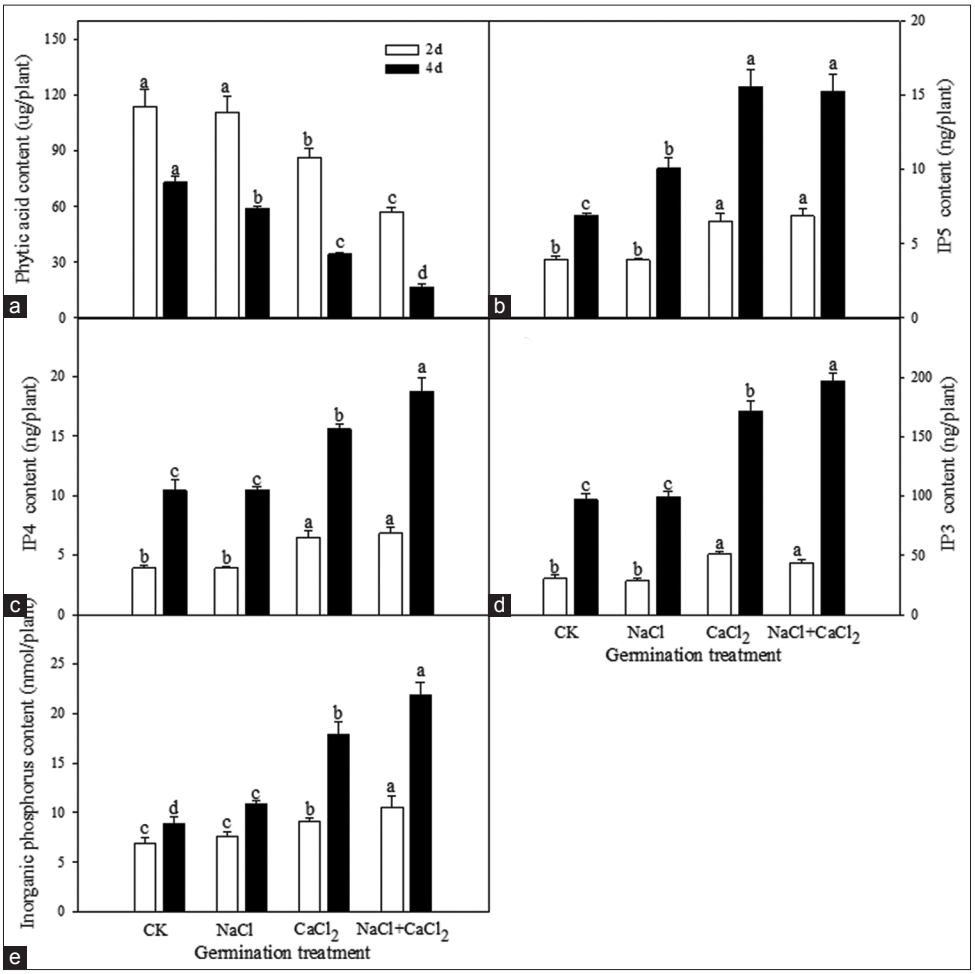

Fig 3. Change of phytic acid content (a), IP5 content (b), IP4 content (c), IP3 content (d) and inorganic phosphorus acid content (e) in mung bean sprouts with CK (distilled water), NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on 2nd and 4th day. Different letters indicate significant difference for each treatment in the same day ($p < 0.05$).

except ALP, which was 1.43-, 1.04-, 1.04- and 4.45- fold higher than the control. However, NaCl treatment only increased *PY* and *PAP* expressions. In addition, after 4-day germination, the expressions of these genes were down-regulated or showed insignificant changes compared with the control.

DISCUSSION

Applications of NaCl, $CaCl_2$ and NaCl-$CaCl_2$ to mung bean sprouts led to a significant increase in sprout length and weight. Previous studies have reported the similar biomass-promoting effect of NaCl (Guo et al., 2014) and $CaCl_2$ (Jain et al., 2011). In this study, the biomass was facilitated most by NaCl-$CaCl_2$ which may be due to the additive effect of combination. In the present study, total phenols in the mung bean sprouts were significantly increased only in NaCl treatment. This might be resulted from the stressful condition caused by NaCl because phenols act as antioxidants to protect plant organs from the stressful conditions (Ksouri et al., 2008). While after NaCl-$CaCl_2$ treatment, total phenols content decreased. This is probably due to NaCl stress being in remission by $CaCl_2$. This phenomenon was similar to the study of Yang et al. (Yang et al., 2015) who found that $CaCl_2$ treatment could remit the stress caused by Zn^{2+}. Ascorbic acid, like total phenols, was one of the antioxidants in plants. Ascorbic acid content and the activities of its regenerating enzymes in plants are also highly related to environmental influence (Davey et al., 2000). Hence, its content remarkably increased under $CaCl_2$, and NaCl-$CaCl_2$ treatment which probably was mainly caused by $CaCl_2$ in our study (Fig. 2b). Soluble sugar and free amino acids were disassembled from macromolecule carbohydrate and protein. Their content increased with the germination time. The NaCl-$CaCl_2$ treatment behaved more prominently than the other two treatments. That may due to the synergistic effect of NaCl and $CaCl_2$ (El-Samad, 1993). In summary, NaCl-$CaCl_2$ treatment could improve the nutritional quality of mung bean sprouts by increasing the biomass, some functional component content and promoting the macromolecular degradation.

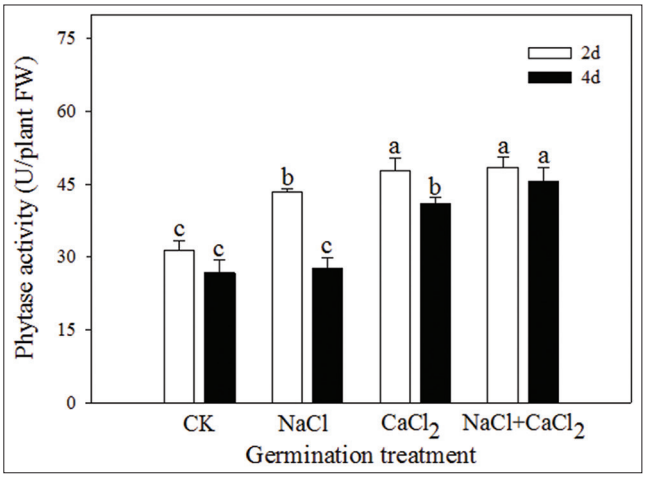

Fig 4. Change of phytase activity of mung bean sprouts with CK (distilled water), NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on 2nd and 4th day. Different letters indicate significant difference for each treatment in the same day ($p < 0.05$).

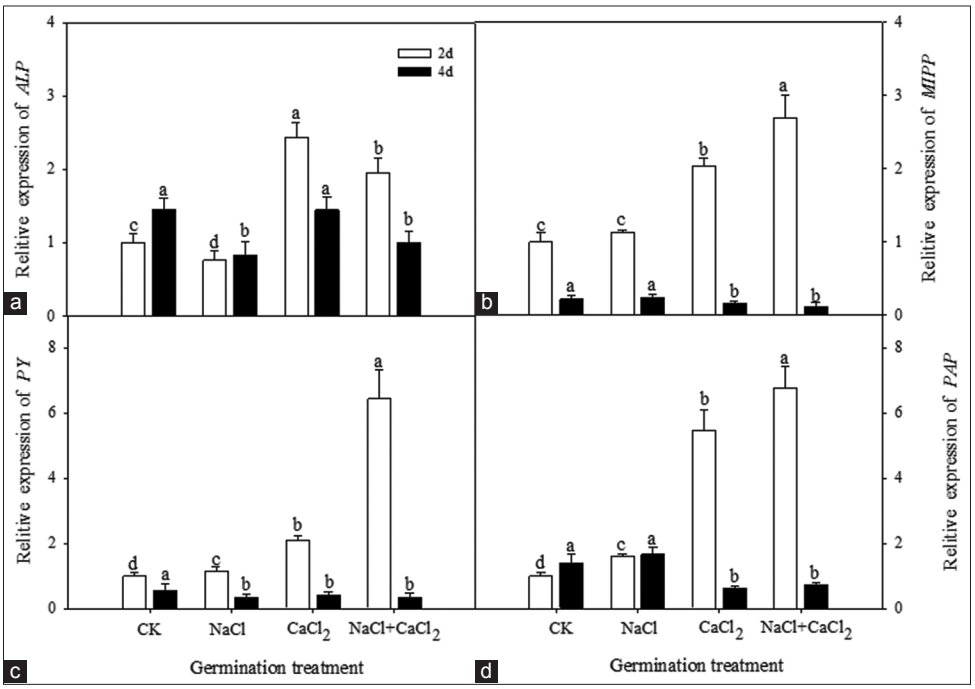

Fig 5. Change of relative expressions of *ALP* (a), *MIPP* (b), *PY* (c) and *PAP* (d) in mung bean sprouts with CK (distilled water), NaCl, $CaCl_2$ and NaCl-$CaCl_2$ treatments on 2nd and 4th day. Different letters indicate significant difference for each treatment in the same day ($p < 0.05$).

As Egli et al. (Egli et al., 2002) found, phytic acid, as an anti-nutritional factor, its content decreased during the germination. The applications of NaCl, $CaCl_2$, and NaCl-$CaCl_2$ all stimulated the degradation of phytic acid and NaCl-$CaCl_2$ showed the more significant impact. The reason may be due to the higher phytase activity (Fig. 4) and higher expressions of *ALP*, *MIPP*, *PA* and *PAP* (Fig. 5). The increase in phytase activity under NaCl might result from the adverse situation caused by NaCl (Li et al., 2008). Meanwhile, the high level of Ca^{2+} can induce expressions of genes and regulate related metabolic pathway in order to respond to the adversity of the stress (Reddy et al., 2004). Hence, the results of genes expressions related to phytic acid degradation and the change of phytase activity under NaCl-$CaCl_2$ in the present study were probably because of the accrued response to the NaCl stress conducted by extraneous Ca^{2+}. The content of inorganic phosphate was predominantly increased with the decrease of phytic acid content during germination. Obviously, the most content of inorganic phosphate occurred with the phytic acid degradation and sprouts growth. This could suggest that the phytic acid was broken into inorganic phosphate for the growth of sprouts (Muzquiz et al., 1997). The IP5, IP4 and IP3 were the products from the stepwise degradation of phytic acid (IP6) (Hayakawa et al., 1990). Lower inositol phosphate plays a vital role in regulating cell proliferation, apoptosis, cell migration, endocytosis, and cell differentiation as signal transduction (Vucenik et al., 2006), besides, it can also be considered to be functional material due to its anticancer (Zhou et al., 2012) and antioxidant abilities. Centeno et al. (Centeno et al., 2001) also found that the content of lower inositol phosphate increased with germination time. In the present study, the content of IP3 was higher than IP4 and IP5 content, because phytic acid mainly degraded into IP3 during hydrolysis (Thavarajah et al., 2009). Three treatments all enhanced the IP5 content remarkably, while IP3 and IP4 content only significantly raised under $CaCl_2$ and NaCl-$CaCl_2$ treatment compared with the control and NaCl-$CaCl_2$ performed better. This phenomenon may be due to the higher phytase activity and level of the phytic acid degradation under NaCl-$CaCl_2$. These suggested that NaCl and $CaCl_2$ could enhance phytase activity and phytic acid degradation. As a result, the nutritional and fuctional qualities were enhanced, because the anti-nutritional factor (phytic acid) degraded and fuctional materials (IP5, IP4 and IP3) increased.

CONCLUSIONS

The growth profiles, ascorbic acid, soluble sugar and free amino acid contents were enhanced most under NaCl-$CaCl_2$ treatment. Simultaneously, phytic acid degraded most because of the higher phytase activity and related genes expression. The level of lower inositol phosphates including IP3, IP4 and IP5 was the highest under NaCl-$CaCl_2$ treatment. So that, NaCl-$CaCl_2$ treatment was an effective way to increase nutritional and functional qualities of mung bean sprouts.

ACKNOWLEDGEMENTS

We are grateful for the financial support from National Natural Science Foundation of China (31471596).

Conflict of interest

The authors declare no conflict of interest.

Author's contributions

All authors contributed extensively to the work presented in this paper. X. Yan performed the field experimentation, date analysis and the first draft of the manuscript; P. Wang and T. Zhou participated in the field experimentation and revised the paper; R. Yang planned and designed the study conceptualization and critically revised the paper; Z. Gu was the project manager.

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