Effects of exogenous Ca^{2+} on photosynthetic characteristics and fruit quality of pepper under waterlogging stress

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ABSTRACT

Pepper is one of the most important vegetable crops in China. Consumers in China are increasingly demanding high quality pepper (*Capsicum annuum* L.) fruit. As in flood crops, natural disasters, such as flooding, might not only affect pepper yield, but also significantly impact fruit quality. Thus, it is necessary to study the change in fruit quality under waterlogging stress and seek an improved method. Pepper '5901' was the experimental material; we investigated the effects of spraying exogenous Ca^{2+} on the photosynthetic characteristics and quality of pepper plants under waterlogging stress. We found that waterlogging significantly reduced the photosynthesis rate (P_N), stomatal conductance (g_s), fruit length, individual fruit diameter, individual fruit fresh weight, and soluble protein, soluble sugar, free amino acid, P, Fe, vitamin C (Vc), and vitamin E (Ve) contents. It significantly decreased the expression levels of 1-aminocyclopropane-1-carboxylate oxidase 1 (*ACO-1*), catalase (*CAT-2*), and pyruvate kinase (*PK*) genes, and it significantly increased intracellular CO_2 concentration, capsaicin content, and the expression level of the alcohol dehydrogenase-1 (*ADH-1*) gene. The parenchymatous cells in the root system and stem were loosely arranged, the degree of lignification was deepened, leaves became thinner, and palisade tissue thickness was reduced. Exogenous Ca^{2+} significantly enhanced capsaicin content, alleviated the extent of the reduction in the abovementioned physiological parameters, and maintained epithelial cell shape in the mesophyll layer. It maintained the intactness of the cell wall in the root system, cell membrane, and pulp cavity; it also reduced cell injury and stimulated the expression levels of *ACO-1*, *ADH-1*, *CAT-2*, and *PK* genes. Compared to the normal control, expression levels of these genes increased by 52.03%, 99.48%, 40.78%, and 125.67%, respectively. The present study indicates that spraying exogenous Ca^{2+} can effectively alleviate injury to pepper plants caused by waterlogging stress and improve the quality of pepper fruit.

Key words: *Capsicum annuum*, fruit inclusions, gene expression, waterlogging stress.

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INTRODUCTION

In recent years, environmental problems have become increasingly serious. The continuing deterioration of climate conditions has led to the significant increase of extreme climate and natural disaster occurrences. Waterlogging disasters have become important hurdles for agricultural development in the world. For example, approximately 16% of soils have environmental limitations because they are too wet, and insurance indemnities for crop losses due to excess water (excluding flood) are second only to drought in the United States (Boyer, 1982). Waterlogging frequently occurs in most areas of the irrigated rice-wheat rotation systems used throughout East, South, and Southeast Asian countries, including Pakistan, India, Nepal, Bangladesh, and China (Samad et al., 2001). Waterlogging disasters have caused erosion and the loss of a large quantity of some mineral elements and important intermediate products around plant root systems; they have also generated toxic substances, such as ethanol and acetaldehyde, via anaerobic respiration that intoxicates plants (Xu et al., 2013; 2016). Furthermore, waterlogging disasters have also affected the quality of crops. For example, they have significantly reduced the content of soluble sugars, sucrose, and free amino acids in wheat grains (Zheng et al., 2010), and crude proteins in maize grains (Ren et al., 2013), which significantly change their nutritional quality.

As the second messenger coupling extracellular signals and intracellular physiological responses, Ca^{2+} is actively involved in plant responses to external environmental changes and adaptation; it is therefore an important factor for plants to resist/tolerate external environmental stresses. Since calmodulin (CaM) was first discovered in 1976, many studies have been conducted on its biological roles (Montoro et al., 2000; Kulkarni and Chavan, 2014). Calmodulin plays important roles in regulating the tolerance/resistance of many crops to environmental stresses, such as waterlogging stress, and in the alleviation of plant injury caused by environmental stress (Rentel and Knight, 2004; Jaleel et al., 2007). Moreover, Ca^{2+} can also effectively improve the quality of crop products/fruit. For example, it increases soluble sugar and vitamin C (Vc) contents in nectarine fruit (Li et al., 2010a) and increases fatty oil and protein contents in peanut (Zhou et al., 2008). Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops in China. Its annual cultivation area is greater than 13 000 km². It has a relatively strong drought tolerance/resistance ability (Ou et al., 2012), but its waterlogging tolerance ability is much weaker (Ou et al., 2011). During the pepper growth seasons, heavy rainfall and waterlogging

disaster events frequently occur. Approximately 60% to 70% of pepper plants in China are cultivated on open lands. Frequent waterlogging disasters seriously affect the nutritional quality of pepper cultivated on open land during the spring and summer seasons. While the effects of exogenous Ca^{2+} on pepper under waterlogging stress have been studied (Zhang et al., 2009; Yang et al., 2016), these studies mainly focused on physiological and biochemical characteristics in the seedling stage. Few studies have been conducted on the effects of Ca^{2+} on the nutritional quality of pepper fruit. Given that nutritional quality is one of the most important factors determining the general quality of pepper fruit, the present study aimed to explain the mechanisms by which Ca^{2+} alleviates injury induced by waterlogging stress. The study analyzes the effects of Ca^{2+} on changes in the photosynthetic characteristics of pepper plants and nutritional quality of pepper fruit under waterlogging stress to provide the theoretical basis to improve the quality of pepper fruit.

MATERIALS AND METHODS

The experiments were conducted in the light incubator at the Vegetable Research Institute of Hunan Province, Hunan Academy of Agricultural Sciences (Changsha, Hunan, China). The daytime temperature was $28 \pm 2^\circ\text{C}$ and the nighttime temperature was $18 \pm 2^\circ\text{C}$ with 60% RH. After the seeds germinated, seedlings were cultivated in a plug. When the seedlings grew to the five-leaf and one bud stage, the well-grown seedlings with the same growth status were transplanted to nursery cups (9×9 cm). One plant was cultivated in each cup, which was filled with soil to 1 cm from the edge. Soil proportions were as follows: field soil (60%), compost (20%), and river mud (20%).

Pepper (*Capsicum annuum* L.) ‘5901’ was the experimental material; at the six-leaf and one bud stage, seedlings were treated as (a) Waterlogging only: two-cup method was applied, that is, the nursery cup was put in a larger 15×40 cm cup and six small cups were randomly put in the larger cup. The water surface was maintained 2 cm above the top surface of the nursery cup and considered as waterlogging stress (Waterlogging). The water was renewed at a fixed time, and (b) Waterlogging stress plus spraying exogenous Ca^{2+} (Waterlogging + Ca^{2+}). The exogenous Ca^{2+} source was CaCl_2 and Ca^{2+} spray concentration was 10 mmol L^{-1} . The entire leaf area of each seedling plant was sprayed with the Ca^{2+} solution until drops fell from the leaf surface. Pepper plants were sprayed

with a Ca^{2+} solution 2 d before waterlogging stress and consecutively for 15 d. Routine water management was used as the control. Plants were watered on a fixed schedule to maintain soil water content at 75% field water-holding capacity. There were 12 nursery cups for each treatment group and the experiment was triplicated. The waterlogging treatment was 10 d. For each treatment, pepper roots, leaves, and fruit at the 35th d after bearing fruit were collected and saved for subsequent measurements.

Fruit phenotype

Ten fruit from each treatment group were randomly selected. Their fresh weights were measured with an electronic balance (MP200B; Cany Precision Instruments, Shanghai, China). The pedicel diameters of pepper fruit and the length of individual fruit were measured with a Vernier caliper.

Gas exchange analysis

Gas exchange was determined between 09:00 and 11:00 h (Beijing time) with a portable photosynthesis system (Li-6400 LI-COR, Lincoln, Nebraska, USA). The net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured at the irradiance of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $32 \pm 0.5^\circ\text{C}$. Six plants were randomly selected from each treatment, which was triplicated.

Light microscopy

Leaves and roots were fixed in a formalin-acetic acid-alcohol (FAA) solution (37% formalin:glacial acetic acid:50% ethanol = 5:5:90). Samples were initially vacuum-infiltrated at room temperature for 2 h and then preserved in FAA at room temperature. Samples were pre-stained with Ehrlich’s hematoxylin, dehydrated in a graded ethanol series, embedded in paraffin, and then sectioned (8 mm thickness) with a rotary microtome (Leica RM2016; Leica Microsystems, Wetzlar, Germany). All the sections were photographed with a light microscope (Olympus BH60, Olympus Optical Co., Tokyo, Japan) equipped with a charge-coupled device.

Gene expression

A total RNA isolation kit (TAKARA, Dalian, Liaoning, China) was used to isolate total RNA from pepper seedling root systems. The quality of the isolated total RNA was double-checked by 1% agarose gel electrophoresis and with a UV spectrophotometer. The well-qualified total RNA

Table 1. Primer sequence.

Primers	Forward sequence	Reverse sequence
ACO-1	5'-TTTGTGGTGAGAGATGCCG-3'	5'-TTTGGCTACCCACACTTCC-3'
ADH-1	5'-TACCGGAGAATGTGGGGAGT-3'	5'-GAATCATCCCTCCTCGCTCG-3'
CAT-2	5'-CCGGTGTTCAGACTCCTGTC-3'	5'-AAGAGCGTGGACCATGTGAG-3'
PK	5'-AGTGAACCTAAACCTTCCATT-3'	5'-GGTGCCGTTTGTGGACTA-3'
Ca-actin	5'-ATCCCTCCACCTCTTCACTCTC-3'	5'-GCCTTAACCATTCCTGTTCCATTATC-3'

ACO-1: 1-Aminocyclopropane-1-carboxylate oxidase 1 gene; ADH-1: alcohol dehydrogenase-1 gene; CAT-2: catalase gene; PK: pyruvate kinase gene; Ca-actin: actin gene.

samples, that is, intact RNA samples with the 260/280 ratio greater than 1.8, were used to conduct reverse transcription with a reverse transcription kit (TAKARA) to synthesize cDNA. Based on the known gene sequences, primers specifically targeting *ACO-1*, *CAT-2*, *PK*, *ADH-1*, and actin genes and corresponding probes were designed with Primer Premier 5.0. The primer sequences are shown in Table 1. Fluorescent real-time quantitative PCR (RT-qPCR) was conducted with a fluorescent real-time quantitative PCR system (Life Technologies Applied Biosystems, Foster City, California, USA). The reaction system consisted of 10 μL 2 $\times$ All-in-One qPCR mix, 2.0 μL each up-stream and down-stream primers (0.4 $\mu\text{mol L}^{-1}$), 2 μL template cDNA, and 0.4 μL 50 $\times$ Rox reference dye brought to final 20 μL with double distilled water. Quantitative RT-qPCR was programed as pre-denaturation at 95 $^{\circ}\text{C}$ for 10 min, denaturation at 95 $^{\circ}\text{C}$ for 10 s, annealing at 60 $^{\circ}\text{C}$ for 20 s, and extension at 72 $^{\circ}\text{C}$ for 15 s for 45 cycles. Actin gene was used as the internal reference gene. Each sample was triplicated. At the end of the reaction, the relative quantitation of expression level of each gene was calculated according to the $2^{-\Delta\text{Ct}}$ method.

Fruit inclusion and trace element content

Fruit inclusion contents, including total soluble sugar and soluble protein, were measured by the anthrone colorimetric method and G-250 Coomassie brilliant blue colorimetric method. Capsaicin content was determined with a capsaicin kit (Beacon Analytical Systems, Saco, Maine, USA). Total amino acid, Ca, Fe, Vc, and vitamin E (Ve) contents were assayed with their corresponding determination kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All experimental results were displayed as mean $\pm$ standard deviation (SD) of six replicates. One-way ANOVA and Duncan's test at a significance value of $p < 5\%$ were performed with SPSS 16.0 software (SPSS, Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Fruit phenotype

Under waterlogging stress, the length, diameter, and fresh weight of individual pepper fruit were all significantly lower than the control group with reductions of 16.44%, 27.89%, and 42.03%, respectively. The decrease in the

Waterlogging + Ca^{2+} group was less for all the above parameters. Fruit length, individual fruit diameter, and individual fruit fresh weight decreased by 14.20%, 18.00%, and 19.25%, respectively, compared to the control group. A nonsignificant difference in fruit length was observed between the Waterlogging and Waterlogging + Ca^{2+} groups, which increased by only 2.67% in the waterlogging stress group. However, individual fruit diameter and fresh weight were significantly higher in the Waterlogging + Ca^{2+} group than in the Waterlogging group, which increased by 13.71% and 39.29%, respectively (Table 2).

Photosynthetic characteristics

Under waterlogging stress, P_N , g_s , and respiration rate of pepper plants in both the Waterlogging and Waterlogging + Ca^{2+} groups all decreased to different extents, while the intracellular CO_2 concentration increased to different extents. In the Waterlogging group, pepper plant P_N , g_s , and respiration rate decreased by 60.71%, 51.37%, and 59.23%, respectively, and intracellular CO_2 increased by 45.45% compared to the control group. In the Waterlogging + Ca^{2+} group, pepper plant P_N , g_s , and respiration rate decreased by 44.99%, 24.14%, and 34.62%, and the intracellular CO_2 concentration increased by 26.98% compared to the control groups. In the Waterlogging + Ca^{2+} group, these parameters increased by 40.00%, 55.99%, and 60.38%, and the intracellular CO_2 concentration decreased by 12.77% compared to the Waterlogging group (Table 3).

Changes in ultrastructure

Under waterlogging stress, the parenchyma cells in the cuticular layer of both pepper plant root system and stem were significantly enlarged, intercellular space obviously increased, and xylem increased. The proportions of cuticular layer and xylem were significantly higher than in the control group. In the Waterlogging + Ca^{2+} group, the proportions of cuticular layer and xylem were significantly lower than in the Waterlogging group. The proportions of cuticular layer and xylem in the root system decreased by 7.3% and

Table 2. Effects of pepper fruit phenotype under different treatments.

Treatment	Fruit length	Fruit diameter	Fresh fruit weight
	cm	mm	g
Control	21.55 $\pm$ 0.15a	17.50 $\pm$ 0.33a	13.61 $\pm$ 0.86a
Waterlogging	18.01 $\pm$ 0.23b	12.62 $\pm$ 0.25c	7.89 $\pm$ 0.24c
Ca^{2+} + waterlogging	18.49 $\pm$ 0.09b	14.35 $\pm$ 0.12b	10.99 $\pm$ 0.69b

Each value is the mean $\pm$ standard deviation (SD, $n = 6$). The effects are significant at $p < 0.05$ with a one-way ANOVA.

Table 3. Effects of pepper photosynthetic characteristics under different treatments.

Treatment	P_N	g_s	E	C_i
	$\mu\text{mol m}^{-2} \text{s}^{-1}$	$\mu\text{mol}(\text{H}_2\text{O}) \text{m}^{-2} \text{s}^{-1}$	$\text{mmol m}^{-2} \text{s}^{-1}$	$\mu\text{mol m}^{-2} \text{s}^{-1}$
Control	6.49 $\pm$ 0.25a	93.58 $\pm$ 2.55a	1.30 $\pm$ 0.06a	245.67 $\pm$ 4.48c
Waterlogging	2.55 $\pm$ 0.10c	45.51 $\pm$ 5.21c	0.53 $\pm$ 0.03c	357.33 $\pm$ 11.09a
Ca^{2+} + waterlogging	3.57 $\pm$ 0.05b	70.99 $\pm$ 3.75b	0.85 $\pm$ 0.09b	311.67 $\pm$ 5.61b

Each value is the mean $\pm$ standard deviation (SD, $n = 6$). The effects are significant at $p < 0.05$ with a one-way ANOVA.

P_N : Net photosynthetic rate; g_s : stomatal conductance; E: transpiration rate; C_i : intercellular CO_2 concentration.

6.1%, respectively, compared to the Waterlogging group. The proportions of cuticular layer and xylem in pepper stem in the Waterlogging + Ca^{2+} group decreased by 10.7% and 15.3%, respectively, compared to the Waterlogging group. When comparing to the control group, the mesophyllous parenchyma cells of pepper plants in the Waterlogging group became irregular and a few parenchyma cells were fragmented, leaves became thinner, and the differentiation of palisade mesophyll tissue and spongy tissue was not obvious. The thickness of the palisade mesophyll tissue significantly decreased. The ratio between palisade mesophyll tissue thickness and leaf thickness decreased by 16.9% compared to the control group. The irregular arrangement of palisade mesophyll cells was observed. Intracellular spaces became larger and larger. After spraying Ca^{2+} , the shape of the lower epidermis cells in palisade mesophyll tissue became mellow and full, leaves became thicker, palisade mesophyll tissue thickened, and the ratio between palisade mesophyll tissue thickness and leaf thickness increased. The cells in spongy tissue were regularly arranged (Figure 1).

Gene expression

Results of the fluorescent RT-qPCR assay indicated that the expression levels of *ACO-1*, *CAT-2*, and *PK* genes in the Waterlogging group all significantly decreased by 56.86%, 84.42%, and 70.58%, respectively. In the

Figure 1. Root, stem, and leaf microstructure crosscutting map of pepper under different treatments.

1-3: Control, Waterlogging, and Waterlogging + Ca^{2+} groups, respectively; A-D: root, stem, vein, and mesophyll microstructure microphotographs, respectively.

P: Pith; X: xylem; c: cortical cells; Pa: fence organization.

Waterlogging + Ca^{2+} group, the expression levels of these genes increased by 52.03%, 40.78%, and 125.67%, respectively, compared to the control group; they increased by 252.43%, 803.83%, and 667.14%, respectively, compared to the Waterlogging group. The *ADH* expression levels under flood stress increased to different extents compared to the control group. The *ADH* expression levels in the Waterlogging group increased by 19.62%, while it increased by 99.48% in the Waterlogging + Ca^{2+} group compared to the control group. The extent of the increase was significantly larger than for the Waterlogging group (Figure 2).

Changes in fruit inclusion and capsaicin contents

Under waterlogging stress, soluble protein, soluble sugar, and free amino acid contents in the Waterlogging and Waterlogging + Ca^{2+} groups all decreased to different extents. Soluble protein, soluble sugar, and free amino acid contents in the Waterlogging group decreased by 14.03%, 40.97%, and 49.06%, respectively, while those in the Waterlogging + Ca^{2+} group decreased by 10.39%, 19.26%, and 33.99%, respectively, compared to the control group. Under waterlogging stress, capsaicin contents increased to different extents. The extent of the increase in the Waterlogging + Ca^{2+} group was larger (27.90%) compared to the control group (Figure 3).

Changes in the contents of some trace mineral elements and vitamins

Under waterlogging stress, mineral element and Fe contents greatly changed. In the Waterlogging group, P and Fe contents in pepper fruit decreased by 16.39% and 51.46%, while in the Waterlogging + Ca^{2+} group, the reduction was 0.96% and 31.69%. Under waterlogging stress, Vc and Ve contents in pepper fruit greatly changed. The contents of Vc and Ve in pepper fruit in the Waterlogging group were lower, by 58.63% and 48.43% compared to the control group. The Vc and Ve contents in the Waterlogging + Ca^{2+} group were between the control and waterlogging stress group; these values decreased by 34.51% and 24.16% compared to the control group, but they increased by 58.32% and 47.06% compared to the Waterlogging group (Figure 4).

Stomatal resistance and non-stomatal resistance are two factors that can reduce plant photosynthesis. As an essential element required for plant growth and development, Ca^{2+} is not only the intracellular second messenger, but it is also the main structural component of the cell wall, cell membranes, and membrane-bound proteins. It can alleviate plant injury caused by environmental stresses (Song et al., 2007). The study by Assche and Clijsters (1990) indicated that within a certain concentration range, Ca^{2+} can increase plant leaf water content, maintain chloroplast membrane structure stability, and enhance ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and phosphoenolpyruvate carboxylase (PEPC) activity, which

Figure 2. Effects of related gene expression of pepper under different treatments.

Different letters denote significant differences ($p < 0.05$). Mean $\pm$ SM represents standard errors, $n = 6$.
 ACO-1: 1-Aminocyclopropane-1-carboxylate oxidase 1 gene; CAT-2: catalase gene; PK: pyruvate kinase gene; ADH-1: alcohol dehydrogenase-1 gene.

Figure 3. Effects of pepper inclusions and capsaicin under different treatments.

Different letters indicate significant differences ($p < 0.05$). Mean $\pm$ SM represents standard errors, $n = 6$.

increases CO₂ carboxylation efficiency. Ai et al. (2006) found that exogenous Ca²⁺ could alleviate the negative effects of weak light and sub-suitable temperature on cucumber photosynthesis by maintaining a relatively high CO₂ assimilation rate and light utilization efficiency. Zhu et al. (2004) reported that exogenous Ca²⁺ alleviated the extent of reduced P_N and g_s in rice leaves under salinity stress and maintained relatively high photosynthesis efficiency. In the present study, we found that both the Waterlogging and Waterlogging + Ca²⁺ treatments reduced P_N and g_s and

increased intracellular CO₂ concentration, indicating that waterlogging-induced reduction in P_N is the result of non-stomatal resistance: it is caused by reduced activity of the photosynthetic apparatus, which in turn, reduces P_N. Furthermore, adding Ca²⁺ significantly increased pepper plant P_N and g_s and reduced intracellular CO₂ concentration compared to the Waterlogging group, indicating that Ca²⁺ can reduce non-stomatal resistance caused by waterlogging stress; it can increase the photosynthetic apparatus activity, and thus increase P_N, g_s, and transpiration rate.

Figure 4. Effects of some trace elements and vitamins in pepper under different treatments.

Under waterlogging stress, the cell walls of epithelial layer cells of the root system thicken and they are heavily cornified because of the resistance to high osmotic pressure. The enzymatic protective system is destroyed by oxidation caused by oxidative free radicals and the cell membrane is destroyed by lipid peroxidation, which leads to wave-shaped epithelial cells, a few fragmented cells, destroyed pulp cavity to a different extent, and increased pore space to increase water-transporting capacity (Broughton et al., 2015). Therefore, the proportion between the pericycle and total root thickness is significantly increased and the degree of lignification deepened. These changes are all favorable for rapid water transportation. In the present study, we found that pepper leaves became thinner, palisade tissue thickness was reduced, and the ratio between palisade tissue thickness and spongy tissue thickness significantly decreased under waterlogging stress. This is consistent with findings by Qu et al. (1999), who found that waterlogging stress altered the apple ultrastructure. The mesophyll cells in palisade tissue contain a large number of chloroplasts, which is the major site of photosynthesis to produce organic substances. The reduced chloroplast number and chlorophyll content led to reduced photosynthetic capacity and the leaves became yellow. The results of the present study indicate that parenchymatous cells in the pepper plant root system of the Waterlogging + Ca²⁺ group were more closely arranged, the intracellular space was reduced, the degree of lignification decreased, and the pulp cavity in the stem was maintained intact. The proportion between the pericycle and total root thickness was significantly reduced. The shape of epithelial and endothelial cells in the mesophyll tissue became mellow and full, leaves thickened, and the ratio between mesophyll tissue thickness and spongy tissue thickness somehow

increased. These structural changes indicate that exogenous Ca²⁺ can effectively alleviate injury to the pepper plant root system and leaves caused by waterlogging stress.

The expression of important genes plays a decisive role during plant growth and development. The changes in agronomic trials as well as plant physiological and biochemical parameters under environmental stresses are all related to the differential gene expression. The study in oak trees by Dat and Parent (2012) indicated that there existed differential gene expression related to plant morphological structure, physiological and biochemical parameters, metabolism, and signal transduction under waterlogging stress. A key enzyme in ethylene biosynthesis pathway is *ACO-1*, which catalyzes the oxidation of 1-aminocyclopropanecarboxylic acid to form ethylene. The formation of ethylene is involved in regulating the formation of adventitious roots and aerenchyma in plant tissues (Iwamoto et al., 2010). A main limiting enzyme in glycolysis is *PK*; it catalyzes the conversion of phosphoenolpyruvic acid and ADP to ATP and pyruvate, providing energy for organisms. Under hypoxia conditions, *ADH-1* is the anaerobic respiration/fermentation terminal, it can convert acetaldehyde to ethanol and produce a limited amount of energy. During the early stage of soybean growth and development, waterlogging stress could significantly induce the expression of *ADH-1* genes (Nanjo et al., 2010; Komatsu et al., 2010). Under environmental stresses, catalase-2 plays an important role in eliminating reactive oxygen species (ROS). The results of the present study indicated that the expression levels of *ACO 1*, and *PK* were all significantly reduced, whereas the expression levels of *ADH 1* significantly increased under the waterlogging stress condition. In the Waterlogging + Ca²⁺ group, the

expression levels of *ACO 1*, and *PK* were significantly higher than in the Waterlogging group. These results indicate that waterlogging stress stimulates the differential gene expression related to plant stress response, inhibits normal pepper plant metabolism, and damages cellular structures. Treatment with Ca^{2+} can effectively enhance the expression levels of metabolism-related genes under waterlogging stress to maintain the energy supply for pepper seedling growth and thus enhance plant tolerance/resistance to environmental stresses, including waterlogging stress.

The osmotic pressure-regulated substances and mineral trace elements are not only important osmotic regulatory substances but also important nutritional substances for plants to resist environmental stresses. Their contents directly affect their nutritional quality (Singh et al., 1984). Studies in pepper (Zheng et al., 2012), wheat (Dong et al., 1998), and Chinese cabbage (Wang et al., 2013) found that the accumulation of osmotic-regulatory substances caused by prolonged waterlogging stress displayed a trend that initially increased and then decreased. Spraying exogenous Ca^{2+} significantly increased soluble sugar contents in *Ficus hirta* fruit (Wang et al., 2009) and both soluble sugar and soluble solid contents in sweet cherry (Zhu et al., 2008). Luo et al. (2012) found that waterlogging stress stimulated Fe accumulation in the root system of *Aegiceras corniculatum*, but it inhibited the accumulation of Ca^{2+} , Fe in leaves, and Ca^{2+} in the root system. The present study indicated that in the Waterlogging + Ca^{2+} group, soluble protein, soluble sugar, and free amino acid contents increased to different extents compared to the Waterlogging group, indicating that Ca^{2+} helped to reduce osmotic-regulatory substances; therefore, the effects of increasing the ability of the plant to resist/tolerate waterlogging stress was achieved and nutritional quality was improved. Moreover, in the Waterlogging group, P and Fe contents significantly decreased, while the extent of the reduction in P and Fe contents was smaller in the Waterlogging + Ca^{2+} group. This may be because Ca^{2+} can enhance the ability of pepper plants to resist waterlogging stress, maintain a stronger absorption ability function, and the normal operation of intracellular nutritional transport system; therefore, pepper plants can absorb more P and Fe from the soil and transport them to the fruit where they accumulate.

Vitamins are a group of small molecules that are essential for maintaining animal growth and development. Vitamin C is one of the most important vitamins in human nutrition. External environmental stresses affect vitamin biosynthesis and accumulation (Guo et al., 2006; Qiu et al., 2006). Pepper is rich in both Vc and Ve, and its Vc content is ranked first among vegetables. Furthermore, only pepper contains capsaicin. In the present study, we found that the contents of both Vc and Ve significantly decreased under waterlogging stress, whereas capsaicin content significantly increased, indicating that environmental stresses, such as waterlogging stress, can reduce the contents of nutritional substances, such as Vc and Ve, and increase capsaicin

content. In this way, pepper plants can resist environmental stresses. This finding concurs with Li et al. (2010b), who found that abscisic acid (ABA) could enhance the resistance of pepper plants to Cd stress. Furthermore, the present study also found that the extent of the reduction in Vc and Ve contents was smaller in the Waterlogging + Ca^{2+} group than the Waterlogging group. Capsaicin content was higher in the Waterlogging + Ca^{2+} group than the Waterlogging group, indicating that Ca^{2+} can stimulate Vc and Ve biosynthesis in pepper fruit. The increase of capsaicin was higher in the Waterlogging group than the Waterlogging + Ca^{2+} group. This may be related to the activation by Ca^{2+} of gene expression related to secondary metabolism pathways, leading to the metabolism switch toward synthesis and accumulation of more capsaicin.

CONCLUSIONS

The present study indicates that Ca^{2+} can affect the changes in the ultrastructures of the root system, stem, and leaves and cause changes in expression levels of the related genes in pepper plants under waterlogging stress. Exogenous Ca^{2+} can effectively alleviate the reduction of photosynthetic capacity and osmotic substances by waterlogging stress. At the same time, it can enhance the absorption of mineral elements and the biosynthesis and accumulation of vitamins and capsaicin, which affect the enhancing ability of waterlogging-resistance and improve the quality of pepper fruit.

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