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Exogenous Ca^{2+} alleviates waterlogging-caused damages to pepper

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Abstract

Ca^{2+} has been considered as a necessary ion for alleviation of stress-induced damages in plants. We investigated effects of exogenous Ca^{2+} on waterlogging-induced damage to pepper and its underlying mechanisms. Pepper seedlings under stress were treated by spraying of 10 mM $CaCl_2$. Applying exogenous Ca^{2+} increased the biomass of pepper leaves and roots, improved photosynthetic characteristics, membrane permeability, root activity, osmotic substance contents, antioxidant enzyme and alcohol dehydrogenase activities, while it reduced lactate dehydrogenase activity. It maintained hydroxide radical contents and activities of malate dehydrogenase and succinate dehydrogenase relatively high. Our results suggested that applying exogenous Ca^{2+} could regulate osmotic substance contents, antioxidant system activity, root respiration, and metabolism, and subsequently alleviate waterlogging-induced damages to pepper plants.

Additional key words: calcium; *Capsicum annuum*; respiratory; waterlogging.

Introduction

One third of the global area is considered potentially suitable for arable agriculture. However, only 10% of the lands are farm lands. This is because abiotic stress, such as waterlogging, drought, low temperature, *etc*., affects crop production (Wang *et al*. 2007). Among abiotic stresses, waterlogging stress is very critical for determining the agricultural production. Waterlogging refers to an excessive amount of water in soil, which is above optimal plant requirement. It occures in vast regions throughout the world, especially, in low-lying rained areas and/or high rainfull environments. Responses of plants to soil waterlogging include extremely complex physiological and biochemical changes and are regulated by genetic mechanisms (Neill and Burnett 1999, Egert and Tevini 2002, França *et al*. 2000). Different plants, even different varieties of the same plant, are significantly different in their abilities to tolerate waterlogging and differ also in their underlying mechanisms (Pistelli *et al*. 2012, Le Provost *et al*. 2012). Plants utilize multiple ways to generate reactive oxygen species (ROS) and increase a malondialdehyde (MDA) content (Wu *et al*. 2003). Normally, there is an equilibrium between production and scavenging of ROS in plant cells. However, under waterlogging conditions, this equilibrium is disrupted, triggering membrane lipid peroxidation (McCord and Fridovich 1969, 1978). In the long evolutionary process, both enzymatic (antioxidant enzymes) and nonenzymatic (osmolytes) systems have been formed to clear ROS and protect plant cells from ROS-induced damages (Lima *et al*. 2002).

Ca^{2+}, as an abundant element necessary for plant growth, can bridge phosphate salts and esters on the cell surface with carboxyl group of proteins to stabilize the structures of biomembranes and maintain the selective ion-absorbing function of cell membrane. In addition, Ca^{2+}

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Abbreviations: ADH – alcohol dehydrogenase; CAM – calmodulin; Car – carotenoids; CaT – Ca^{2+} treatment group; CAT – catalase; Chl – chlorophyll; CK – control; DM – dry mass; DCPIP – 2,6-dichlorophenol-indophenol; E – transpiration rate; F_0 – minimal fluorescence yield at the dark adapted-state; F_0' – minimal fluorescence yield at the light-adapted state; F_m – maximal fluorescence yield at the dark-adapted state; F_m' – maximal fluorescence yield at the light-adapted state; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; GR – glutathione reductase; g_s – stomatal conductance; LDH – lactate dehydrogenase; MDA – malondialdehyde; MDH – malate dehydrogenase; P_N – net photosynthetic rate; POD – peroxidase; q_P – photochemical quenching coefficient; ROS – reactive oxygen species; SD – standard deviation; SDH – succinate dehydrogenase; SOD – superoxide dismutase; TTC – triphenyl tetrazolium chloride; TTF – trityl hydrazone; WL – waterlogging group; WUE – water-use efficiency; Φ_{PSII} – effective quantum yield of PSII photochemistry.
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could couple extracellular signals with the intracellular second messengers of physiological responses. When certain signals reach cells, the permeability of cell membranes to Ca^{2+} transiently increases, leading to an elevated intracellular Ca^{2+} concentration. These Ca^{2+} then binds to calmodulin (CAM), forming a Ca-CAM complex, which activates many key enzymes, such as phospholipase, NAD kinase, Ca^{2+}-ATPase, thereby enabling cells to produce corresponding physiological responses (Xiong *et al*. 2002). Studies have shown that Ca^{2+} can enhance the efficiency of carboxylation activity, and increase membrane ATPase activity and photosynthetic pigment contents, thereby keeping photosynthesis at a high rate (Chen 1998). Ca^{2+} treatment can maintain a high photosynthetic rate in soybean leaves under waterlogging (Yang *et al*. 1995), improve photosynthetic rate of muskmelon leaves under hypoxic stress (Gao *et al*. 2005a), and enhance photosynthesis of rice under salt stress (Zhu *et al*. 2004). Moreover, Ca^{2+} treatment can improve antioxidant enzyme activities and unsaturation degree of cell membrane lipids in plants, alter polyamine metabolism and respiration process of plant roots, as well as enhance cold resistance of eggplants (Gao *et al*. 2002), heat resistance of maize plants (Gong and Li 1995), and hypoxia tolerance of cucumber and melon seedlings (Gao *et al*. 2002, 2005a,b; Hu *et al*. 2006, 2007).

Pepper is native to tropical regions of Latin America. It has the highest level of vitamin C among vegetables. In addition, it is also rich in vitamin B, carotene as well as calcium, iron, and other mineral substances. Moreover, pepper can stimulate oral mucosa, enhance gastric motility, increase appetite, promote digestion, alleviate abdominal and chest pain, inhibit diarrhea, kill abdominal and gastric parasites, control heart and coronary artery diseases, among other medicinal properties. It is a shallow-rooted plant with strong root respiration and oxygen consumption (Peng *et al*. 2010). Preliminary studies have indicated that although pepper shows a strong drought tolerance (Ou *et al*. 2012), it possessses a weak resistance to waterlogging (Ou *et al*. 2011). In this work, we studied the effects of Ca^{2+} treatment on the activity of photosynthetic apparatus as assessed by biomass, photosynthetic parameters, and other related physiological and biochemical indices, with the aim to provide a theoretical basis for alleviating waterlogging-induced damages.

Materials and methods

Plants and treatments: Pepper (*Capsicum annuum L.* variety 5901) was provided by the Hunan Provincial Institute of Vegetables. Seeds were disinfected with 0.1% mercuric chloride for 5 min, rinsed with distilled water, and soaked in water for 24 h for germination. When the seedlings reached a five-leaf stage, they were transplanted into pots with a diameter of 30 cm, and grown further in a glasshouse. When the seedlings grew up to the six-leaf stage, the seedlings with similar characteristics were selected and assigned in a control group (CK), waterlogging group (WL), and Ca^{2+} treatment group (CaT) with nine seedlings per group. The seedlings in the CK group were not treated with waterflooding. By contrast, the seedlings in the WL and CaT groups were subjected to waterlogging treatment by soaking in water 2 cm above the soils. In addition, seedlings in CaT were also sprayed daily with 10 ml of 10 mM $CaCl_2$ solution per seedling continuously for 10 d. Each treatment had three replicates. Based on the Huaihua Weather Network (http://www.hhqx.com/), during the treatment period, the temperature was 26.9–27.8°C. The average temperature in the glasshouse was 27 ± 5°C during the day and 20 ± 2°C at night, and the plants were grown under 16 h of light/8 h of dark with natural light. The seedlings were collected and subjected to measurements and analysis after 10 days of the CaT treatment.

Biomass determination: After each treatment, the aerial and roots parts of the plants were washed with deionized water, and weighted to obtain the fresh mass (FM). Afterwards, they were dried at 105°C for 30 min and then at 75°C to constant mass, and measured for dry mass (DM).

Pigment content was determined according to Arnon (1949). All leaves were fully developed and selected from the second to the fourth leaves of the plants. Fresh leaves (2.0 g) were homogenized using quartz sand, $CaCO_3$, and 3 ml of 100% acetone, and then extracted with 80% acetone. After centrifugation at 2,500 rpm for 2 min, the absorbance of the supernatant was measured at 663, 645, and 470 nm by a spectrophotometer *(Ruili UV-2100*, Beijing, China). Contents of chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) were calculated using the equations:

Chl $a = 13.95\ OD_{665} - 6.88\ OD_{649}$;

Chl $b = 24.96\ OD_{649} - 7.32\ OD_{665}$;

Car = (1,000 OD_{470} – 2.05 Chl *a* – 114.8 Chl *b*)/245.

Gas exchange and Chl fluorescence parameters were monitored by *LI-6400XT* portable photosynthesis system (*LI-COR*, USA) during 09:00–11:00 h of Beijing time. Gas exchange was parameterized using the protocol described by Li *et al*. (2007). The net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) were measured at the irradiance of 1,000 μmol(photon) m^{-2} s^{-1}, temperature of 30°C ± 0.5°C, and the natural CO_2 concentration. Water-use efficiency (WUE) was calculated according to $WUE = P_N/E$.

Chl fluorescence parameters were measured according to the protocol described by Genty *et al*. (1989). The minimal fluorescence yield of the dark-adapted state (F_o) and the maximal fluorescence yield of the dark-adapted

state (F_m) were recorded after dark adaptation for 20 min. Then the minimal fluorescence yield of the light-adapted state (F_0') and the maximal fluorescence yield of the light-adapted state (F_m') were measured after 1-h irradiation. The maximal quantum yield of PSII photochemistry (F_v/F_m), photochemical quenching coefficient (q_P), and the effective quantum yield of PSII photochemistry (Φ_{PSII}) were calculated as $F_v/F_m = (F_m - F_o)/F_m$; $q_P = (F_m' - F_s)/(F_m' - F_o')$; $\Phi_{PSII} = (F_m' - F_t)/F_m'$.

Root activity was measured by triphenyl tetrazolium chloride (TTC) method. Roots were collected and washed. Their surfaces were dried carefully with absorbent papers. Root tips (0.5 g) was cut into 1-cm pieces, and incubated with 10 ml of mixture (1:1, v/v) of 1% TTC solution and 0.l M phosphate buffer (pH 7.0) at 37°C for 1 h in dark and then mixed with 2 ml of l M sulfuric acid. The blank assays was performed as followings: firstly, treating the root tips with sulfuric acid, then incubating the roots with TTC solution and phosphate buffer. The roots were then collected and grounded with 3 − 5 ml of ethyl acetate and quartz sand in a mortar after removal of moisture in order to extract trityl hydrazone (TTF). The remaining roots were further extracted with ethyl acetate for three times. All the red extractions were collected, transferred into a new tube and diluted with ethyl acetate to 10 ml. Absorption of the diluted extraction was determined spectrophotometrically at 485 nm (*Ruili UV-2100*, Beijing, China). The blank solution was prepared similarly without adding root. Reduced TTC amount [mg] was obtained from the standard curve and its intensity in root tips was calculated as follows: TTC reduction intensity [mg g^{-1} h^{-1}] = reduced TTC amount per g(FM) per h, where FM was fresh root mass [g], and h was the incubation time.

Membrane permeability: In brief, after being washed twice with deionized water, 0.1 g of plant leaves or roots were cut into l-cm pieces and placed into a tube containing 10 ml of deionized water. After marking the water value, the tube was incubated at 25°C for 1 h with frequent shaking. At that time, the conductivity C_1 was determined by conductivity meter (*HI 98309*, Hanna, Mauritius). Then the tube was boiled at 100°C for 15 min. After cooled to room temperature, distilled water was added to the tube till water reached the original mark, then the conductivity C_2 was measured. Thus, the membrane permeability was expressed as the following: relative electrolyte leakage = $(C_1 - C_0)/(C_2 - C_0) \times 100\%$, where C_0 is the conductivity of distilled water.

Enzyme activity assay: The activities of superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.8.1.7), lactate dehydrogenase (LDH, EC 1.1.1.27), alcohol dehydrogenase (ADH, EC 1.1.1.1), succinate dehydrogenase (SDH, EC 1.3.5.1), and malate dehydrogenase (MDH, EC 1.1.1.37) were respectively assayed by corresponding determination kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China). Leaves without midrib (0.1 g) were thoroughly ground with a cold mortar and pestle in an ice bath. The grinding medium was 4 ml of 0.9% (w/v) normal saline solution with homogenizing glass beads. The homogenate was centrifuged at 2,500 rmp for 10 min at 4°C. The supernatant, referred to as the crude enzyme extract, was used for determination. The absorbance of the reaction mixture was determined by using a spectrophotometer (*Ruili UV-2100*, Beijing, China). SOD activity was determined at 550 nm as 50% hydroxylamine inhibition at 37°C and expressed in U per mg of total protein. POD activity was determined at 420 nm and calculated per mg of protein in U, representing 1 μg(guaiacol) per min at 37°C. CAT activity was measured as a decrease in absorbance at 405 nm and calculated per mg of protein in U representing 1 μmol(H_2O_2 consumption) per second at 37°C. GR activity was measured at 340 nm and calculated per g(protein) in U, which represented the amount of enzyme that produced 1 mmol of NAPDH per min at 37°C. LDH activity was measuerd at 440 nm and calculated per g(protein) in U, which represented the amount of enzyme that produced 1 mmol(pyruvic acid) per min at 37°C. ADH activity was determined at 340 nm and calculated per mg of protein in U, which represented the amount of enzyme that formed 1 nmol(acetaldehyde) per min at 37°C. MDH activity was assayed at 340 nm and calculated per mg of protein in U, which represented the amount of enzyme that used 1 μmol(tetramethylbenzidine) per min at 37°C. SDH activity was measured by the decrease in absorbance at 600 nm, corresponding to the reduction of 2,6-dichlorphenol-indophenol (DCPIP).

Proline determination: Fresh leaves were washed and dried with paper towel. The leaves (0.5 g) were cut into pieces, mixed with 5 ml of 3% (w/v) sulfosalicylic acid solution, and boiled for 10 min under shaking. The homogenate was filtered after cooling down, 2 ml of the filtered extract was mixed with 2 ml of acetic acid and 2 ml of acid ninhydrin, and boiled for 30 min. After cooling down, 4 ml of toluene was added into the tube and fully oscillated. The upper red solution was collected and its absorption at 520 nm was measured (*Ruili UV-2100*, Beijing, China) using toluene as reference. Proline content was obtained from the standard curve and was calculated as [μg g^{-1}(FM)].

Total soluble sugar contents were measured with the anthrone colorimetric method. The leaves (0.1 g) were placed in a ground glass weighing bottle, mixed with 20 ml of distilled water, and extracted twice in boiling water for 30 min. The extract was filtered into a 50 ml volumetric flask, and the weighing bottle and residues were washed repeatedly before being filled to a constant volume. Sample extract (0.5 ml) was mixed thoroughly with 1.5 ml of distilled water, 0.5 ml of anthrone ethyl acetate solution,

and 5 ml of concentrated sulfuric acid, before being immediately placed in boiling water bath for 1 min. The heated sample extract was cooled to room temperature to determine the absorbance at 630 nm (*Ruili UV-2100*, Beijing, China). The total soluble sugar contents were calculated from the standard curve: soluble sugar [mg g^{-1}(FM)] = [(sucrose content from standard curve (μg) × 10^{-3} × total extract volume (ml)]/[(measurement volume (ml) ×FM of the sample (g)].

Hydroxide radicals (·OH) amount and MDA content were respectively assayed by corresponding determination kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China). The leaves without midrib (0.1 g) were thoroughly ground with a cold mortar and pestle in an ice bath. The grinding medium was 4 ml of 0.9% (w/v) normal saline solution the same as above, plus homogenizing glass beads. The homogenate was centrifuged at 2,500 rpm for 10 min at 25°C. The supernatant referred to as crude enzyme extract, was used for a determination. The absorbance of the reaction mixture was determined at 550 nm by using a spectrophotometer (*Ruili UV-2100*, Beijing, China), ·OH amount was calculated as 1 mmol of H_2O_2 decrease expressed in U per mg of total protein per min at 37°C. The MDA concentration was determined spectrophotometrically using the thiobarbituric acid method. The values of thiobarbituric were estimated at 532 nm against a blank consisting of 5% cold trichloroacetic acid mixed with 1% thiobarbituric acid. We used malondialdehyde bis (dimethyl acetal) as a standard. Concentrations of lipid peroxidation compounds were expressed as nmol mg^{-1}(protein).

Statistical analysis: All experimental results were presented as mean ± standard deviation (SD) of six replicates. One-way analysis of variance (*ANOVA*) and *Duncan*'s test at a significance values of p<5% were performed by *SPSS 16.0* softwave (*SPSS*, Chicago, IL, USA).

Results

Biomass: FM and DM of pepper leaves and roots were significantly affected under WL. FM of leaves and roots indicated 35.5 and 53.4% decrease, respectively, and DM of leaves and roots indicated 41.7 and 47.1% decrease, respectively, compared with the CK conditions. However, CaT significantly reversed these declines. FM of leaves and roots exhibited 40.8 and 33.3% increase, respectively, and DM of leaves and roots were 57.1 and 77.8% higher, respectively, compared with the WL treatment. The DM of leaves and roots after CaT was not significantly different from that of the CK group (Table 1).

Photosynthetic characteristics: Pigment contents and Chl fluorescence parameters decreased during the WL and CaT treatments. Compared with the CK conditions, Chl *a*, Chl *b*, and Car subjected to WL decreased by 49.5, 56.1, and 31.8%, respectively. However, these declines were significantly alleviated by CaT. Chl *a*, Chl *b*, and Car exhibited 24.9, 14.7 and 13.3% increase after the CaT treatment compared to WL (Fig. 1*A*,*B*,*C*).

Compared with the CK conditions, P_N, g_s, *E,* and WUE showed 97.1, 94.8, 92.4, and 61.6% decrease under the WL treatment, while these declines were significantly alleviated by the CaT treatment. P_N, g_s, *E*, and WUE exhibited 233.9, 133.3, 71.0, and 95.8% increase in the CaT treatment compared to WL (Table 2).

Compared with the CK conditions, F_v/F_m, q_P, and Φ_{PSII} indicated 49.6, 63.5, and 53.4% decrease under the WL treatment, while these reductions were significantly alleviated by CaT. F_v/F_m, q_P, and Φ_{PSII} showed 27.3,43.3, and 28.7% increase after CaT compared to WL (Fig. 1*D*,*E*,*F*).

Membrane permeability: After WL, the electrolyte leakage rates significantly increased in pepper leaves and roots and CaT significantly attenuated such enhancements. The electrolyte leakage rates of leaves and roots reached only 73.0 and 65.3%, respectively, after the CaT treatment in comparison with the plants of the WL group. The data suggested that CaT could alleviate damages from WL on pepper membranes (Table 3).

Root activity: The root activity of the WL and CaT plants significantly decreased. However, the decrease was 56.9% in the plants of the CaT group; it was obviously smaller reduction than that of 72.6% in the plants of the WL group (Table 3).

Table 1. Biomass of leaves and roots under different treatments based on one-way *ANOVA*. Each value is the mean ± SD, n = 6. The effects are significant at the value of p<0.05 with a one-way *ANOVA*, the same is below. FM – fresh mass; DM – dry mass.

Treatment	Leaf FM [g]	Root FM [g]	Leaf DM [g]	Root DM [g]
Control	0.76 ± 0.08^a	1.74 ± 0.12^a	0.12 ± 0.01^a	0.17 ± 0.02^a
Waterlogging	0.49 ± 0.05^c	0.81 ± 0.09^c	0.07 ± 0.01^b	0.09 ± 0.01^b
Ca^{2+}+ waterlogging	0.69 ± 0.05^b	1.08 ± 0.07^b	0.11 ± 0.01^a	0.16 ± 0.01^a

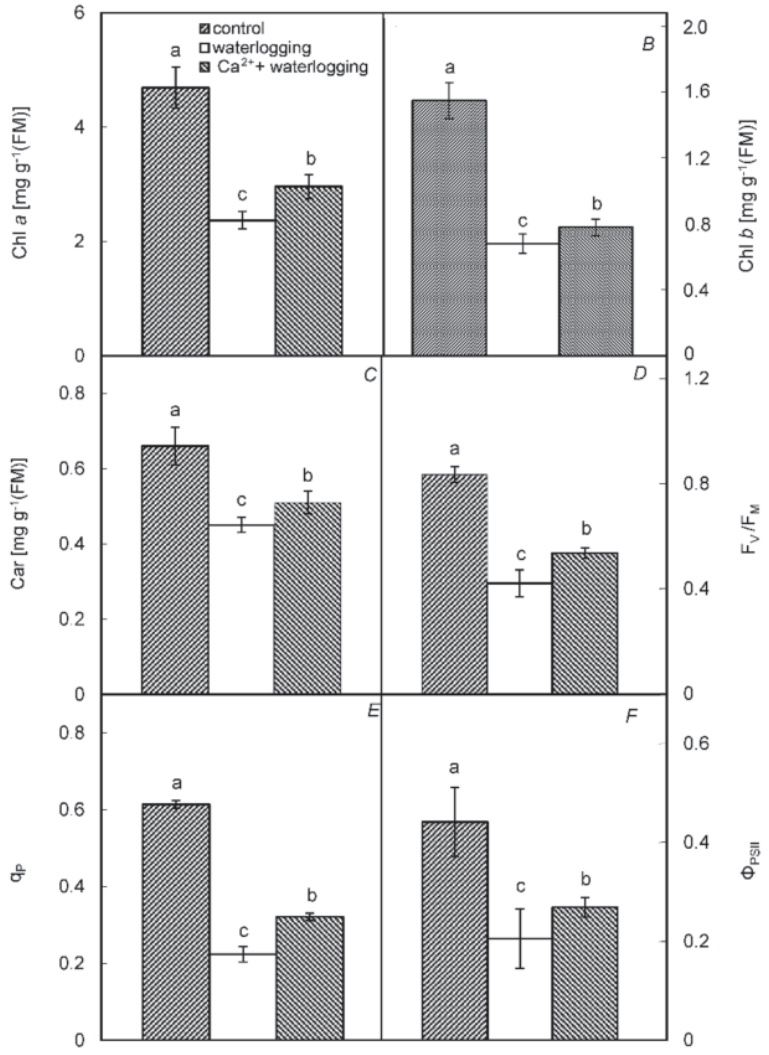

Fig. 1. Chlorophyll (Chl) *a* (*A*), Chl *b* (*B*), carotenoids (Car) (*C*), maximal quantum yield of PSII photochemistry (F_v/F_m) (*D*), photochemical quenching (q_P) (*E*), and effective quantum yield of PSII photochemistry (Φ_{PSII}) in pepper leaves under different treatments. All values are means ± SD, $n = 6$. *Different letters* denote significant differences ($p<0.05$).

Table 2. Gas-exchange parameters of pepper seedlings under different treatments based on one-way *ANOVA*. Each value is the mean ± SD, $n = 6$. The effects are significant at the value of $p<0.05$ with a one-way *ANOVA*, P_N – net photosynthetic rate; g_s – stomatal conductance; E – transpiration rate; WUE – water-use efficiency.

Treatment	P_N [μmol m^{-2} s^{-1}]	g_s [mol(H_2O) m^{-2} s^{-1}]	E [mmol(H_2O) m^{-2} s^{-1}]	WUE [mol(CO_2) mol^{-1}(H_2O)]
control	20.18 ± 1.52^a	0.58 ± 0.05^a	4.08 ± 0.26^a	4.95 ± 0.38^a
waterlogging	0.59 ± 0.03^c	0.03 ± 0.002^c	0.31 ± 0.08^c	1.90 ± 0.14^c
Ca^{2+}+waterlogging	1.97 ± 0.12^b	0.07 ± 0.001^b	0.53 ± 0.15^b	3.72 ± 0.31^b

Table 3. Electrolyte leakage and root activity of pepper under different treatments based on one-way *ANOVA*. Each value is the mean ± SD, $n = 6$. The effects are significant at the value of $p<0.05$.

Treatment	Electrolyte leakage [%]		Root activity [mg g^{-1} h^{-2}]
	Leaves	Roots	
control	17.01 ± 1.87^c	28.82 ± 2.99^c	86.54 ± 6.87^a
waterlogging	72.58 ± 6.75^a	81.25 ± 7.85^a	23.75 ± 3.54^c
Ca^{2+}+waterlogging	52.95 ± 4.63^b	53.05 ± 5.68^b	37.28 ± 3.61^b

Osmotic regulating substances: Under WL, proline and soluble sugar contents in pepper leaves significantly increased by 20.6 and 67.9% , respectively, in the CaT group, and 7.8 and 34.9%, respectively, in the WL group. The results showed that CaT was beneficial to plant growth (Fig. 2*A*,*B*).

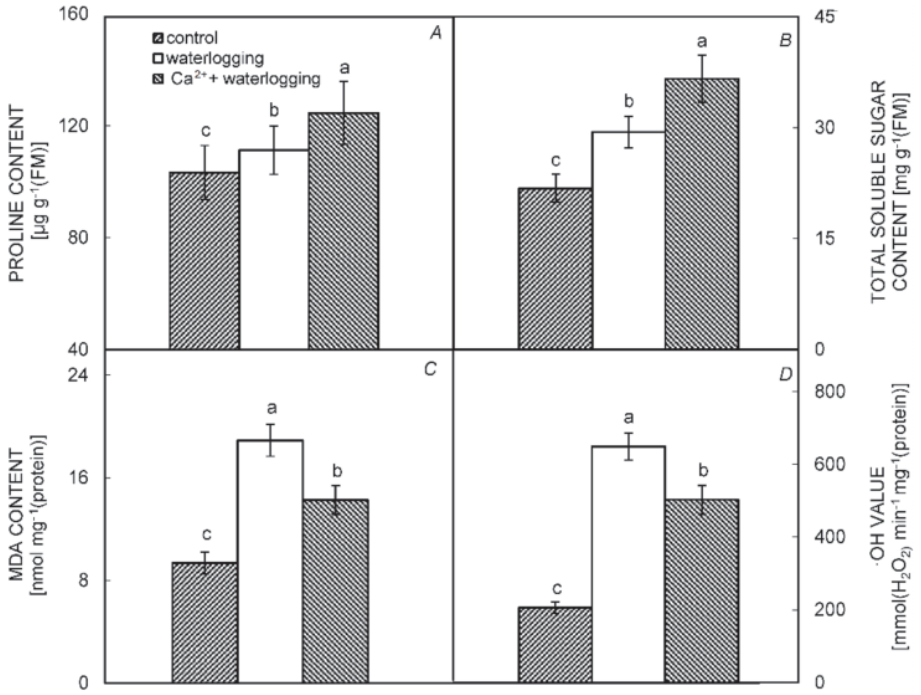

Fig. 2. Proline (*A*), total soluble sugar (*B*), malondialdehyde (MDA) (*C*), and hydroxide radicals (·OH) (*D*) of pepper leaves under different treatments. All values are means ± SD, $n = 6$. *Different letters* denote significant differences ($p<0.05$).

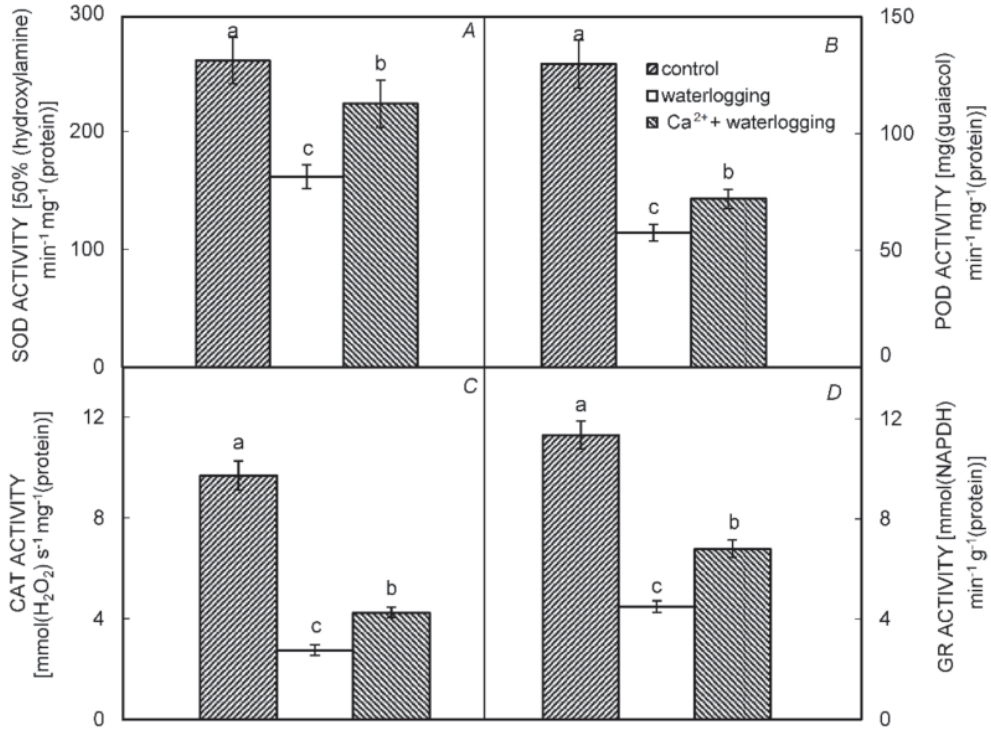

Fig. 3. Enzyme activities of superoxide dismutase (SOD) (*A*), peroxidase (POD) (*B*), catalase (CAT) (*C*), and glutathione reductase (GR) (*D*) of pepper leaves under different treatments. All values are means ± SD, $n = 6$. *Different letters* denote significant differences ($p<0.05$).

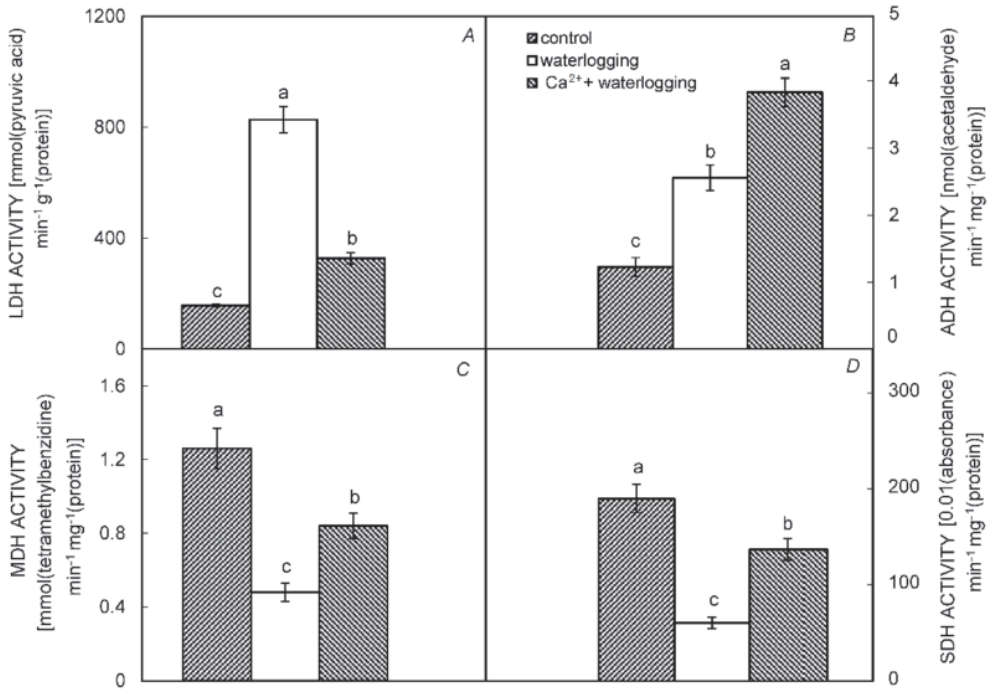

Fig. 4. Enzyme activities of lactate dehydrogenase (LDH) (*A*), alcohol dehydrogenase (ADH) (*B*), malate dehydrogenase (MDH) (*C*), and succinate dehydrogenase (SDH) (*D*) of pepper leaves under different treatments. All values are means ± SD, $n = 6$. *Different letters* denote significant differences ($p<0.05$).

Metabolites: Under WL, the MDA content and ·OH amount significantly increased, but these increases were much smaller in the CaT group, only about 75.3 and 77.5%, respectively, of those in the WL group (Fig. 2*C*,*D*).

Antioxidant enzyme system: Under WL, activities of antioxidant enzymes decreased. However, CaT increased the activities of antioxidant enzymes such as SOD, POD, CAT, and GR by 38.3, 25.1, 54.7, and 51.1%, respectively, compared with those in the WL group (Fig. 3).

Respiratory metabolism: Under WL, LDH activity showed an rising trend in both WL and CaT groups, and the latter was only 39.6% of the former one. In addition, ADH activity was significantly higher in both WL and CaT groups compared with that of the CK group. The ADH activity in plants of the CaT group was obviously higher than those of the WL group (Fig. 4*A*,*B*).

MDH and SDH activities were significantly reduced in the WL and CaT plants compared with those in the CK group, but the decreases in the CaT group were only 1.8- and 2.3-fold of those in the WL group, respectively (Fig. 4*C*,*D*).

Discussion

Our results suggested that CaT increased the biomass of pepper leaves and roots, photosynthetic parameters, membrane permeability, root activity, osmotic substance contents, antioxidant enzyme activities, and ADH activity, while it decreased the LDH activity, thus maintaining hydroxide radicals (·OH) amounts and the activities of malate dehydrogenase (MDH) and SDH relatively high.

Waterlogging sets in motion a serie of physical, chemical, and biological processes that profoundly influence the quality of soil as a medium for plant growth. The soil pore space is totally water-filled, and gas exchange between soil and atmosphere is virtually eliminated. Thus, waterlogged plants have a stunted growth compared to normal well-aerated plants. Several researches revealed that plant height, root length and activity, leaf area, and plant biomass were drastically reduced after the waterlogging treatment (Issarakraisila *et al*. 2007, Zhang *et al*. 2009, Broughton *et al*. 2015). Our results showed that FM and DM of pepper leaves and roots, along with root activity, declined in the WL group, but the CaT treatment significantly reversed this trend. This result indicated that exogenous calcium promoted the absorption of nutrients and moisture, eased the consumption of plant nutrients, and reduced the waterlogging-induced inhibition of plant growth.

Chl content is an important factor reflecting photosynthetic capacity. The plant accumulates a lot of ROS under flooding, which speeded up Chl degradation. Decrease in the Chl content under waterlogging had been reported in wheat and maize (Prasad *et al*. 2004, Kuai *et*

al. 2014); it was consistent with our result. The decrease of the Chl content led to a significant drop of the photosynthetic rate. Other researches reported that waterlogging made P_N, g_s, E, WUE, F_v/F_m, q_P, and Φ_{PSII} significantly reduced (Liang *et al*. 2009, Ren *et al*. 2015). Our results showed that the gas exchange and Chl parameters of the pepper seedlings were both lowered. In addition, we found that these decreases were significantly alleviated by the CaT treatment. These results indicated that Ca^{2+} could protect photosynthetic pigments from degradation by modulating antioxidant systems and scavenging reactive oxygen, delay the rate of Chl degradation, and thus improve the stability of photosynthetic pigments.

Membrane permeability is one of the important physiological indexes for an evaluation of plant suffering under stressful conditions. Wu *et al* (2000) showed that membrane permeability in leaves of blueberry decreased under waterlogging treatment, and the membrane permeability of a less flooding-tolerant cultivar was significantly higher than that of the higher flooding-tolerant cultivar, which was consistent also with the findings in leaves of loquat (Pan *et al*., 1993) and soybean (Han 1999). Our results showed that the electrolyte leakage rates of the pepper leaves and roots significantly increased under flooding and the CaT treatment significantly attenuated these increases. It indicated that waterlogging stress caused plant damage by changing the membrane permeability, while Ca^{2+} decreased the plasma permeability by adjusting the osmotic pressure. Thus, Ca^{2+} played a pozitive regulatory role in the pepper seedlings, and reduced the degree of suffering under stressful conditions.

Soluble sugars, soluble proteins, and free amino acids are important osmotic stress-regulating substances in plants and enhancements of their amounts favor plant resistance to external stress. Studies showed that increasing Ca^{2+} supply could promote sugar synthesis and soluble sugar contents in plants (Wang *et al*. 2009, Zhang *et al*. 2009, Li *et al*. 2010, Zhang *et al*. 2011, Deng *et al*. 2012, Zhu *et al*. 2012). Calcium could also enhance soluble protein and free amino acids contents in plant tissues (Duan *et al*. 1997, Wang *et al*. 1999, Zhou *et al*. 2008). Our results showed that soluble sugar and proline contents increased in pepper in both WL and CaT groups, and the latter was significantly higher than the former one, suggesting that spraying exogenous Ca^{2+} could promote the synthesis of soluble sugars and proline, thereby increasing the tolerance of peppers to waterlogging.

MDA contents and ·OH amounts had been associated with lipid peroxidation *via* an increased generation of ROS, and thus its quantification had been suggested as a general indicator for waterlogging tolerance (Wu *et al*. 2003). We demonstrated that the MDA content and ·OH amount significantly increased under waterlogging condition, but these increases were much smaller after the exogenous Ca^{2+} treatment. This fully demonstrated that the pepper seedlings after the CaT treatment could maintain a higher active oxygen metabolism, thus improving free-radical scavenging ability and reducing damage to plant cells due to plasma membrane peroxidation.

The ability of plants to scavenge the toxic effects of active oxygen seems to be a very important determinant of their tolerance to waterlogging stress. Antioxidants are in the first line of defense against free-radical damage. There are various antioxidants and metabolites involved in the scavenging of active oxygen in plants; their activation is known to increase upon exposure to oxidative stress (Tanaka 1994, Gong *et al*. 1998). The former research indicated that the activities of SOD, CAT, POD, and GR increased in order to reduce the degree of damage under stressful conditions. Fan *et al.* (2008) showed that antioxidant enzymes increased at the beginning and decreased later under high temperature or drought conditions. Pei *et al.* (2013) showed that the activities of SOD, POD, CAT increased and then decreased with the continuing drought stress. Our results showed that the activities of antioxidant enzymes decreased under waterlogging, while CaT increased the activities of antioxidant enzymes, such as SOD, POD, CAT, and GR by 38.3, 25.1, 54.7, and 51.1%, respectively, compared with those in the WL group, which was consistent with the findings in tomato (Li *et al*. 2009), strawberry (Christou *et al*. 2014), sugarcane (Boaretto *et al*. 2014), and other crops. It confirmed that Ca^{2+} could alleviate waterlogging damage to cell membrane by improving the activities of antioxidant enzymes and protecting photosynthetic apparatus, especially PSI from active oxygen.

Wang *et al.* (2002) showed that at the early stages of hypoxia, Ca^{2+} was required for activation and expression of MDH and SDH genes, and Ca^{2+} inhibitors could suppress the increase in intracellular Ca^{2+} concentration, thereby inhibiting the expression of MDH and SDH genes. Exogenous Ca^{2+} could eliminate the inhibition, improve the normal expression of anaerobic protein genes, and enhance the resistance of plant seedlings (Subbaish *et al*. 2003). In this study, we found that aerobic respiration and metabolism ability of the pepper seedlings decreased as indicated by the significantly decreased MDH and SDH activities under waterlogging condition, However, the Ca^{2+} treatment enabled the pepper seedlings to maintain higher MDH and SDH activities and an aerobic respiration capacity, which was one of the reasons why the waterlogging resistance was enhanced by this treatment. Meanwhile, the roots under waterlogging increased their LDH and ADH activities, which was consistent with the findings in tomato (Guo *et al*. 1999), lettuce (Ismond *et al*. 2003), *Arabidopsis* (Fukao *et al*. 2003), and other crops. Perata and Alpi (1993) showed that the real reason for plant damage was acetaldehyde rather than ethanol and increased ADH activity could reduce the damages caused by accumulation of acetaldehyde and lactic acid. Compared with the pepper plants in the WL group, the plants under CaT exhibited the lower LDH activity and higher ADH activity, suggesting that under waterlogging,

lactate metabolism significantly increased, and the plants accumulated more lactic acid, leading to reduced tolerance to waterlogging. By contrast, the pepper plants in the CaT group accumulated less lactic acid but enhanced an ethanol-fermentation capacity, thus avoiding damages from accumulation of lactic acid and acetaldehyde and improving the tolerance to waterlogging.

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