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Research Report

Seed Germination, Seedling Growth and Antioxidant System Responses in Cucumber Exposed to $Ca(NO_3)_2$

Huaifu Fan, Ling Ding, Yanli Xu, and Changxia Du*

The Key Laboratory for Quality Improvement of Agricultural Products of Zhejiang Province, School of Agriculture and Food Science, Zhejiang Agriculture & Forestry University, Lin'an, Zhejiang, 311300, P. R. China

*Corresponding author: changxiadu@zafu.edu.cn

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Abstract. This study investigated the effects of varying calcium nitrate ($Ca(NO_3)_2$) supply on seed germination, seedling growth, and antioxidant responses during cucumber seed germination. Five and 20 mM $Ca(NO_3)_2$ stimulated seed germination, while 10 and 40 mM $Ca(NO_3)_2$ inhibited it. Germinating seed weight was clearly promoted by 5 mM $Ca(NO_3)_2$, but decreased under 40 mM $Ca(NO_3)_2$. Ten or 20 mM $Ca(NO_3)_2$ caused no marked change. Addition of 10 or 40 mM $Ca(NO_3)_2$ increased the activity of many enzymes in germinating seeds, such as superoxide dismutases (SOD), peroxidases (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). On the other hand, 5 and 20 mM $Ca(NO_3)_2$ markedly decreased CAT activity. Among all the treatments, only 10 mM $Ca(NO_3)_2$ increased malondialdehyde content. Similarly, the production rate of $O_2^{\cdot-}$ was only higher in 20 mM $Ca(NO_3)_2$. Compared with the control (0 mM $Ca(NO_3)_2$), protein content significantly increased in all treatments except for 20 mM $Ca(NO_3)_2$. Calcium nitrate strongly inhibited the growth of seedlings, and damaged leaf and root microstructure. The inhibition and damage were more severe as the $Ca(NO_3)_2$ concentration increased. Calcium nitrate promoted the accumulation of photosynthetic pigment, but led to a decrease in chlorophyll *a/b*. These results suggest that the effect of different $Ca(NO_3)_2$ levels on seed germination was variable, while the inhibition effect on seedling growth enhanced with increase of $Ca(NO_3)_2$ concentration. This effect is closely associated with Ca^{2+} and NO_3^- concentration, antioxidant enzyme activity, and the different growth and development stages of cucumber.

***Additional key words*:** antioxidantive enzymes, *Cucumis sativus*, germination rate, biomass production, secondary salinization

Introduction

Soil salinity increasingly limits horticultural production in arid and semi-arid regions (Ebert et al., 2002). About 800 million hectares of land all around the world (almost 6% of world's total area) are adversely affected by salt, which reduces crop growth, dry matter accumulation and yield (Kim et al., 2014). The majority of these soils are naturally saline, but recently a noticeable proportion of cultivated land is also being affected by secondary salinization (Yuan et al., 2012), due to long-term, heavy fertilization and irrigation with saline water. Salt accumulation is also induced by heavy application of fertilizer in greenhouse conditions. In Northern China, secondary salinization has been an important factor limiting the plastic film greenhouse crop production. Because nitrogen nutrition plays a significant role in both crop yield and quality, it is very common to over-apply nitrogen fertilizer in efforts to obtain high quality and maximum yields in greenhouse vegetable production systems (He et al., 2007). Other major reasons for excessive ion accumulation in the soil solution are the lack of rainfall leaching, intensive farming, and strong surface evaporation in protected cultivation. Salt concentration increases in the upper soil layer due to high evaporation water losses, which exceed irrigation and precipitation. According to Tong and Chen (1991), the high level of $Ca(NO_3)_2$ accumulation was one of the main reasons for soil salinity in greenhouses. Yuan et al. (2013) also reported that salt stress in plant cells is primarily caused by a combination of osmotic and ionic stress resulting from high concentrations of Ca^{2+} (over 60% of total cations) and NO_3^- (67-76% of total anions) in secondary salinization soil.

Soil salinization causes increased production of reactive

oxygen species (ROS), which can severely damage the cellular apparatus of the plant (Kim et al., 2014). ROS are highly toxic and can cause membrane lipid peroxidation, DNA damage, protein oxidation, and enzyme inhibition (Tanou et al., 2009), all of which contribute to cell death. To remove ROS and maintain redox homeostasis, plants have developed a complex array of antioxidant defense systems against oxidative damage resulting from high levels of ROS. These include superoxide dismutases (SOD), catalase (CAT), peroxidases (POD), glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), low molecular mass antioxidants ascorbate (AsA), glutathione (GSH), and compatible solutes such as betaines and proline (Foyer and Noctor, 2005; Veljovic-Jovanovic et al., 2006; Huang and Song, 2013).

The effects of many environmental stresses on plants have been researched by determining the changes in the activities of such antioxidant enzymes. To study how the plant antioxidant defense systems respond to salt stress can provide valuable information for improving stress tolerance. Previous studies have shown that excessive $Ca(NO_3)_2$ can cause severe oxidative damage and metabolic disorder, leading to reduced biomass production in cucumber (Yuan et al., 2012), eggplant (Wei et al., 2009) and tomato seedlings (Zhang et al., 2008). Limited information is currently available in the literature regarding the effect of different $Ca(NO_3)_2$ levels on overall plant growth and development. Cucumber is an important vegetable crop grown worldwide and highly sensitive to salinity, especially during germination and the seedling stage. Thus, it is important for cucumber cultivation to study the effects of $Ca(NO_3)_2$, and to determine safe $Ca(NO_3)_2$ concentrations. In this study we investigated cucumber seed germination, changes in enzymes involved in antioxidant system during seed germination, and seedling growth in response to different $Ca(NO_3)_2$ levels.

Materials and Methods

Plant Material and Treatments

Seeds of cucumber (*Cucumis sativus* L. cv. Jinyou No.1) were pre-soaked with sterilized distilled water for 6 hours before germination. The soaked seeds were germinated in Petri dishes (9 cm diameter × 1.5 cm depth) containing two layers of Whatman No. 1 filter paper moistened with 5 ml of treatment solution and incubated at 29 ± 1°C in the dark. In this study, we conducted three separate experiments.

Firstly, we investigated the effect of different $Ca(NO_3)_2$ levels on seed germination. Several treatments were performed: 0, 5, 10, 20 and 40 mM $Ca(NO_3)_2$. Germination rate was recorded at 24, 36, 48, 60 and 72 h.

In order to study the effects of $Ca(NO_3)_2$ on ROS metabolism during seed germination, five treatments were performed at 0, 5, 10, 20, 40 mM $Ca(NO_3)_2$. The germinating seeds were tested after 60 h of treatment and used for further measurements.

To check the effects of $Ca(NO_3)_2$ on seedling growth, five treatments were performed at 0, 5, 10, 20, 40 mM $Ca(NO_3)_2$. First, the seeds were germinated exposed to these five concentrations. After 24 h, the germinating seeds were sown in plastic pots (8 cm tall and 10 cm diameter) filled with washed quartz sand. The plants were watered with the corresponding treatment solution every day until the first true leaf appeared. Then, the seedlings were watered with the corresponding treatment solution +1/2 Hoagland solution. After 48 days, 30 plants per treatment were collected for determination of plant growth and microstructure.

Germination Rate

Germination was recorded and germination rates were expressed as a percentage, according to Fan et al. (2013). Percentage of seeds with radicles breaking through testa (%RBT) > 2 mm, ratio of the length of hypocotyls and radicles to the seed length (RHRS) ≧ 1/2 were investigated and recorded, respectively.

Determination of Weight of Germinating Seeds

After 12 and 72 h of germination, excess water on the surface of the seeds was blotted with a paper towel, and seeds were weighted.

Assay of Antioxidant Enzyme Activity

For the enzyme assays, germinating seeds (seed coat was peeled) were ground with ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% PVP. The homogenates were centrifuged at 4°C at 12,000 × *g* for 20 min and the resulting supernatant was used for spectrophotometric determination of enzyme activity and protein content. All steps in the preparation of the enzyme extract were carried out at 4°C. SOD activity was estimated according to Giannopotitis and Ries (1977). POD activity was assayed according to Egley et al. (1983), with modifications: the reaction mixture in a total volume of 2 ml contained 25 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.05% guaiacol (2-ethoxyphenol), 1.0 mM H_2O_2 and 100 µl enzymes extract. The increase in absorbance due to oxidation of guaiacol was measured at 470 nm. APX activity was determined according to Nakano and Asada (1981). CAT activity was measured according to Cakmak and Marschner (1992).

GR activity was measured as the decrease in A_{340} due to oxidation of NADPH according to Foyer and Halliwell (1976). MDHAR activity was determined according to Hossain et

al. (1984) with minor modifications. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM NADH, 5 mM ascorbic acid, 0.15 U ascorbate oxidase, and 30 μl of enzymes extract. The activity was calculated from the change in absorbance at A_{340} for 2 min when the extinction coefficient was 6.2 $mM^{-1} \cdot cm^{-1}$. DHAR activity was determined following Nakano and Asada (1981) with minor modifications. The reaction mixture contained 50 mM KH_2PO_4 buffer (pH 7.0), 50 mM GSH, 10 mM DHA, and 50 μl of enzyme extract. The activity was calculated from the change in absorbance at A_{265} for 2 min when the extinction coefficient was 14 $mM^{-1} \cdot cm^{-1}$. All spectrophotometric analyses were conducted on a Shimadzu (UV-2550) spectrophotometer.

Determination of $O_2^{\cdot-}$ Production Rate, Protein, Photosynthetic Pigment and Malondialdehyde (MDA) Content

The rate of $O_2^{\cdot-}$ production was measured according to Elstner and Heupel (1976) with some modifications, by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\cdot-}$. One gram of sample was homogenized with 3 ml of 65 mM phosphate buffer (pH 7.8), 0.1 ml 10 mM hydroxylamine hydrochloride, and 1 ml supernatant. After incubation at 25°C for 20 min, the same volume of ethyl ether was added and samples centrifuged at 1,500 × *g* for 5 min. A standard curve with NO_2^- was used to calculate the rate of $O_2^{\cdot-}$ production from the chemical reaction of $O_2^{\cdot-}$ and hydroxylamine. Photosynthetic pigments were extracted from leaves with a 10 ml mixture consisting of acetone:ethanol = 1:1 (v/v) incubated overnight. The extract was centrifuged at 4,000 × *g* for 15 min, and photosynthetic pigments were determined spectrophotometrically at 440, 662 and 644 nm, according to Yu et al. (2002). Protein content was determined according to Bradford (1976) utilizing bovine serum albumin as standard. MDA content was measured by the thiobarbituric acid reaction method (Heath and Packer, 1968).

Determination of Growth and Water Content of Shoot and Root

Plant height was measured from cotyledons to the apical point with a ruler with accuracy of 1 mm. Stem thickness was measured at a consistent point 1 cm below cotyledons with a vernier caliper with an accuracy of 0.05 mm. The total leaf area, root length, number of root tips, root surface area and volume were measured with an Expression 1680 scanner (Epson, Sydney, Australia) and image analysis software (WinRHIZO, Regent Instruments Inc., Quebec, Canada). For the determination of fresh weight, shoots and roots were separated and weighed after being washed with sterile distilled water. Dry weight was determined after oven drying the samples at 80°C to a constant weight.

Microstructure of Leaf and Root

The leaves were cut into pieces of ~0.5 cm^2 and roots tips were cut into segments of 1-1.5 cm. The cut leaves and roots were then fixed immediately in formalin-acetic acidalcohol (FAA), dehydrated in an alcohol series according to Zheng (1979). After being embedded in paraffin, 10 μm sections were cut from the samples using a Leica RM 2235 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Samples were stained with safranin O and fast green FCF, and observed and photographed under an OLYMPUS BX53 fluorescence microscope (Olympus, Tokyo, Japan).

Statistical Analysis

All data presented are mean values. All experiments were conducted using at least three replicates. All data were statistically analyzed with SAS software (SAS Institute Inc., Cary, NC, USA) using Duncan's multiple range test at a 0.05 level of significance.

Results

The Effect of $Ca(NO_3)_2$ on Cucumber Seed Germination Rate and Weight

As shown in Fig. 1A, at 24 h after treatment plants treated with 5 or 20 mM $Ca(NO_3)_2$ showed significantly higher %RBT than the control (0 mM $Ca(NO_3)_2$). In contrast, 10 and 40 mM $Ca(NO_3)_2$ treatment inhibited cucumber seed germination. The %RBT under 10 and 40 mM $Ca(NO_3)_2$ were 53.06% and 4.08%, respectively. There were no significant differences in %RBT between seeds treated with different concentrations of $Ca(NO_3)_2$ for 36 h or 72 h. Treatment with 10 mM $Ca(NO_3)_2$ resulted in the lowest %RBT during this period.

Similarly, exposure to $Ca(NO_3)_2$ also affected the RHRS (Fig. 1B). At 24 h, the RHRS was zero under all treatments tested. At 36 h of treatment, the RHRS of 5 mM $Ca(NO_3)_2$ treatment was significantly higher than the control (0 mM $Ca(NO_3)_2$). Treatment with concentrations above 5 mM $Ca(NO_3)_2$ drastically decreased the RHRS, which was zero under 10 and 40 mM $Ca(NO_3)_2$. At 48 h of treatment, the RHRS under 5 and 20 mM $Ca(NO_3)_2$ were significantly higher than that of the control. The RHRS under 10 and 40 mM $Ca(NO_3)_2$ remained lower than the value at 0 mM $Ca(NO_3)_2$, with the RHRS under 10 mM $Ca(NO_3)_2$ clearly lower than under any other treatments. After 60 h of treatment, the RHRS under 5 and 20 mM $Ca(NO_3)_2$ were significantly higher than the control. The RHRS of seedlings treated with 10 mM $Ca(NO_3)_2$ was not significantly different than the control, while the RHRS of seedlings treated with 40 mM $Ca(NO_3)_2$ was significant lower than the control. After 72 h of treatment, all the concentrations tested had no clear difference in RHRS

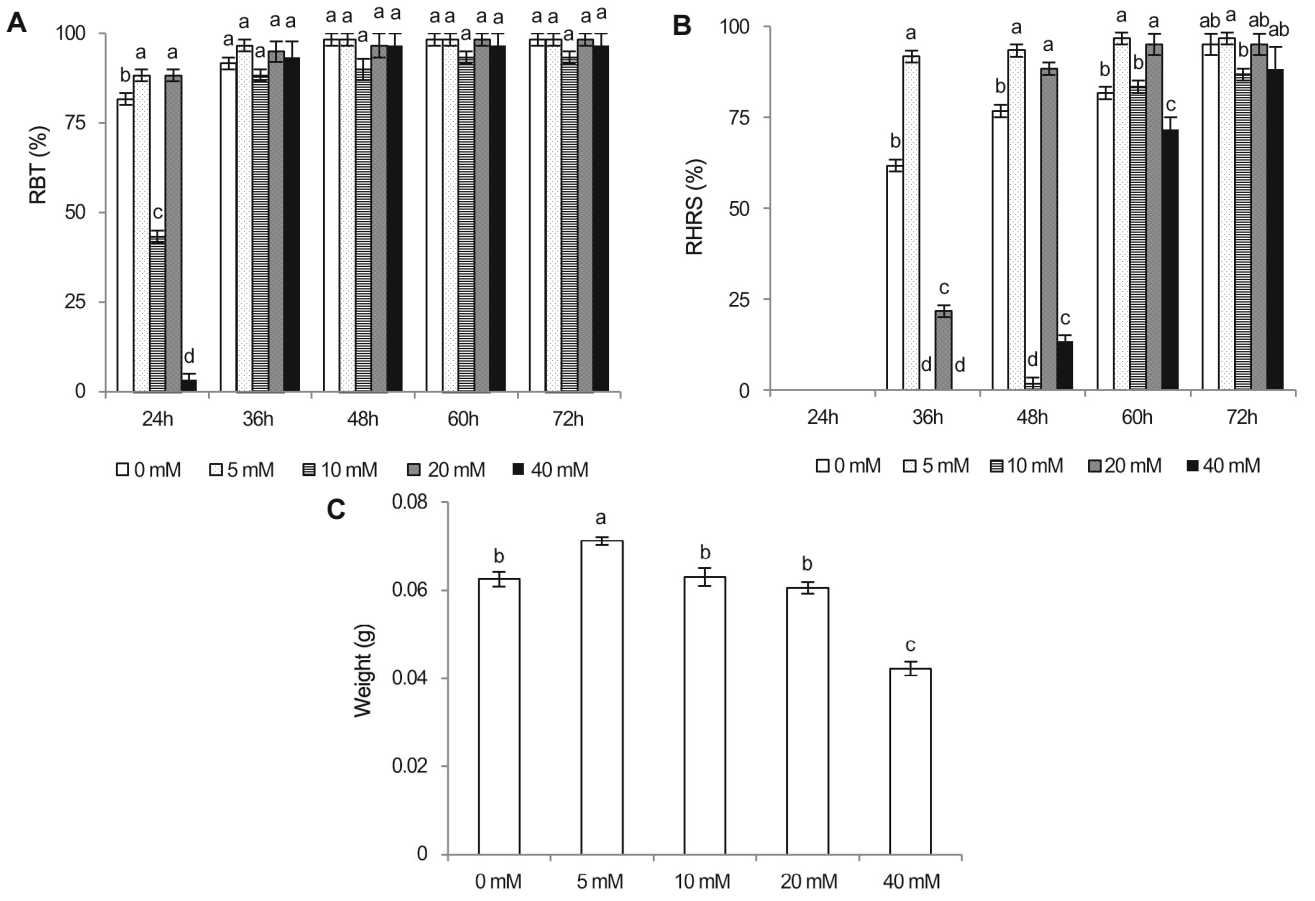

Fig. 1. Effect of different concentrations of $Ca(NO_3)_2$ on %RBT (A), RHRS (B) and seedling weight (C) during cucumber seed germination. Values represent the mean ± SE (n = 3). Letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

relative to the control. However, the RHRS of plants treated with 10 mM $Ca(NO_3)_2$ was significantly lower than that of 5 mM $Ca(NO_3)_2$.

As shown in Fig. 1C, treatment with low concentrations of $Ca(NO_3)_2$ promoted weight increase during seed germination, whereas treatment with high concentrations restrained it. The increase in weight after 5 mM $Ca(NO_3)_2$ treatment was significantly higher than that of the control, while the weight of seeds treated with 10 and 20 mM $Ca(NO_3)_2$ had no significant difference relative to the control. When the $Ca(NO_3)_2$ levels exceeded 20 mM, the increase of weight was significantly lower than the control.

The Effect of $Ca(NO_3)_2$ on Cucumber Seed Enzyme Activity During Germination

Compared to the control, SOD activity significantly increased in the presence of $Ca(NO_3)_2$ (Fig. 2A). On average, SOD activities in seedlings treated with 5, 10, 20, or 40 mM $Ca(NO_3)_2$ were 141.55%, 213.57%, 170.91% and 227.42% of the activity in control plants. Clear differences were observed among all treatments, except for 10 and 40 mM $Ca(NO_3)_2$. POD activity exhibited a similar trend (Fig. 2B). However, POD activity under 5 mM $Ca(NO_3)_2$ treatment indistinguishable from the control.

Five and 20 mM $Ca(NO_3)_2$ treatments resulted in significant decreases in CAT activity. By contrast, CAT activity increased under 10 and 40 mM $Ca(NO_3)_2$ treatments. Moreover, the difference was clear between the 40 mM $Ca(NO_3)_2$ and 0 mM $Ca(NO_3)_2$ treatments (Fig. 2C).

APX activity had no clear change under 5 mM $Ca(NO_3)_2$ treatment. Treatment with concentrations higher than 5 mM $Ca(NO_3)_2$ led to an increase in APX activity. APX activities under 10, 20, 40 mM $Ca(NO_3)_2$ treatment were 229.56%, 161.56% and 204.08% of the activity in control seedlings (Fig. 2D).

GR activities under 10 and 40 mM $Ca(NO_3)_2$ treatment were significant higher than the activity in control seedlings. No clear difference was observed between the control, 10 and 40 mM $Ca(NO_3)_2$ treatments. Five and 20 mM $Ca(NO_3)_2$ treatments were not significantly different from the control (Fig. 2E).

DHAR activity increased under 5, 10, and 40 mM $Ca(NO_3)_2$ treatments. A clear difference was observed between 0 and 10 mM $Ca(NO_3)_2$ treatment. However, 20 mM $Ca(NO_3)_2$

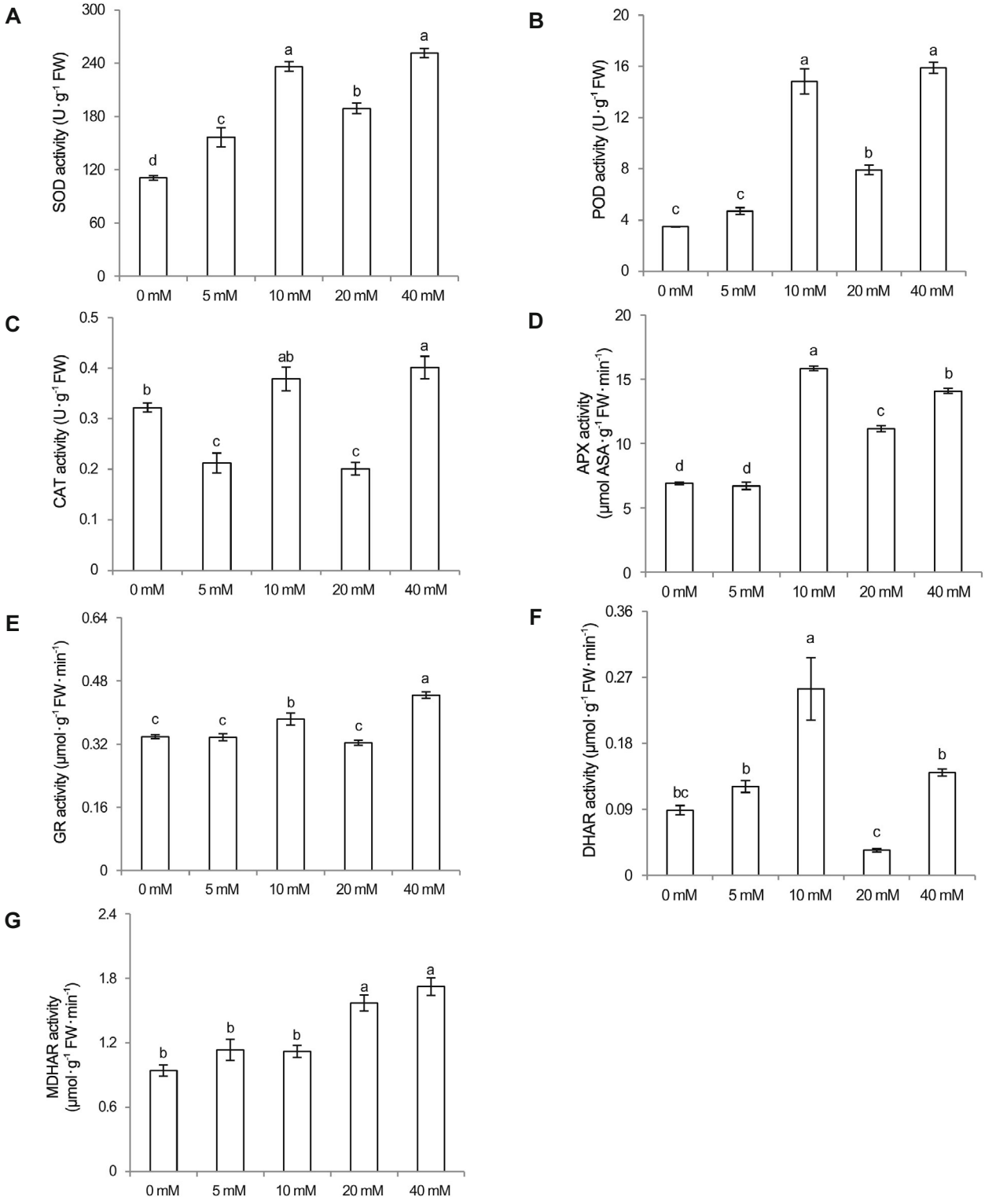

Fig. 2. Effect of different concentrations of $Ca(NO_3)_2$ on SOD (A), POD (B), CAT (C), APX (D), GR(E), DHAR(F) and MDHAR (G) activities in cucumber seeds during germination after 60 h of treatment. Values represent the mean ± SE (n = 3). Letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

treatment restrained the DHAR activity and was only 38.56% of the activity in control seedlings (Fig. 2F). $Ca(NO_3)_2$ treatment caused an increase in MDHAR activity. MDHAR activities under 20 and 40 mM $Ca(NO_3)_2$ treatments were significantly different from the control, while 5 and 10 mM $Ca(NO_3)_2$ were not (Fig. 2G).

The Effect of $Ca(NO_3)_2$ Treatment on Cucumber Seed $O_2^{\cdot-}$ Production Rate, MDA and Protein Content During Germination

The variation in MDA content is shown in Fig. 3A. Five, 20, and 40 mM $Ca(NO_3)_2$ treatments had no clear effect on MDA content, and were 103.54%, 90.27%, and 110.62% of the control, respectively. Ten mM $Ca(NO_3)_2$ treatment significantly increased MDA content.

Under $Ca(NO_3)_2$ treatment, $O_2^{\cdot-}$ production rate in seeds increased among of all the treatment groups. The $O_2^{\cdot-}$ production rate under 20 mM $Ca(NO_3)_2$ treatment was the highest. Compared with the control, 5, 10, and 40 mM $Ca(NO_3)_2$ treatments had no clear difference (Fig. 3B).

Results in Fig. 3C demonstrate that protein content was significantly increased under 5, 10, and 40 mM $Ca(NO_3)_2$ treatments. The protein content under 10 mM $Ca(NO_3)_2$ treatment was highest among all the treatments. Compared with the control, the protein content under 20 mM $Ca(NO_3)_2$ treatment had no clear difference.

The Effect of $Ca(NO_3)_2$ Treatment on Cucumber Seedling Growth

The germinating seeds were not able to grow into seedlings under 40 mM $Ca(NO_3)_2$. As shown in Figure 4A, fresh weight of shoot and root all were restrained significantly. As the $Ca(NO_3)_2$ concentration increases, the weight decrease becomes more pronounced. However, the fresh weight of shoot and root in seedlings supplied with 20 mM $Ca(NO_3)_2$ were all markedly lower than the 5 mM $Ca(NO_3)_2$ treatment. Dry matter production exhibited the same trend as fresh weight (Fig. 4B).

$Ca(NO_3)_2$ treatment led to a decrease in water content in shoots. Ten and 20 mM $Ca(NO_3)_2$ treatments were clearly lower than the control. Twenty mM $Ca(NO_3)_2$ treatment was also significantly different from 5 and 10 mM $Ca(NO_3)_2$. On the contrary, water content in roots increased under $Ca(NO_3)_2$ treatment. Ten and 20 mM $Ca(NO_3)_2$ treatment were significant higher than the control. There was no significant difference between 5 mM $Ca(NO_3)_2$ and the control (Fig. 4C).

Plant height significantly decreased as the $Ca(NO_3)_2$ concentration increased, and all the treatments had clear differences with each other. Plant height under 20 mM $Ca(NO_3)_2$ treatments was only 14.45% of the control (Fig. 4D). Stem diameter and total leaf area presented the same trend as plant height (Fig. 4E, F).

The total root length also markedly decreased under

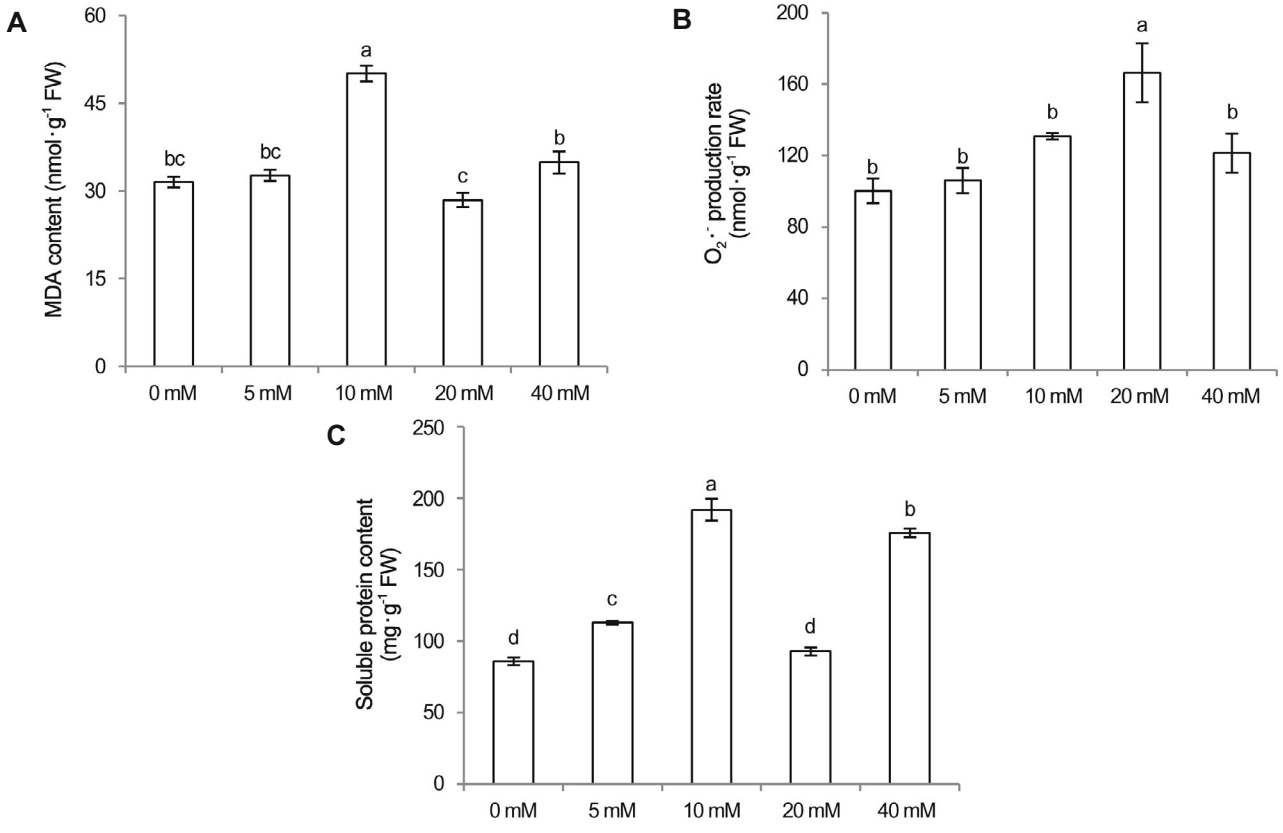

Fig. 3. Effect of different concentrations of $Ca(NO_3)_2$ on MDA content (A), $O_2^{\cdot-}$ production rate (B) and protein content (C) in cucumber seeds during germination after 60 h of treatment. Values represent the mean ± SE (n = 3). Letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

$Ca(NO_3)_2$ treatment (Fig. 4G). Ten and 20 mM $Ca(NO_3)_2$ treatment were significantly lower than 5 mM $Ca(NO_3)_2$. However, there was no significant difference between 10 and 20 mM $Ca(NO_3)_2$. The total number of root tips presented a similar trend (Fig. 4H). $Ca(NO_3)_2$ treatment significantly decreased the total root surface area (Fig. 4I). Five and 20 mM $Ca(NO_3)_2$ treatment were significantly different. In contrast, there was no significant difference between 10 and 20 mM $Ca(NO_3)_2$, or 10 and 5 mM $Ca(NO_3)_2$. Under 5 and 20 mM $Ca(NO_3)_2$ treatment, the total root volume decreased, and was 84.18% and 81.73% of the control, respectively. Ten mM $Ca(NO_3)_2$ treatment increased the total root volume, and was significantly different from 20 mM $Ca(NO_3)_2$ treatment (Fig. 4J).

Under treatment with different concentrations of $Ca(NO_3)_2$, the content of chlorophyll *a*, chlorophyll *b*, and the ratio of chlorophyll *a* + *b* all significantly increased. In contrast, no significant difference in carotenoid content was observed among the different $Ca(NO_3)_2$ treatments. Five and 10 mM $Ca(NO_3)_2$ treatment showed no clear difference in photosynthetic pigment. $Ca(NO_3)_2$ treatment clearly decreased the ratios of chlorophyll *a/b*, which were 89.31%, 88.36% and 81.45% of the control under 5, 10, and 20 mM $Ca(NO_3)_2$ treatment, respectively (Table 1).

Microstructure of Leaves and Roots

Under $Ca(NO_3)_2$ treatment leaf thickness increased, especially under treatment with 20 mM $Ca(NO_3)_2$, and part of the epidermis became abnormal (Fig. 5A-D). The palisade tissue and spongy tissue became loose and the number of cells per unit area decreased (Data not shown).

$Ca(NO_3)_2$ caused remarkable structural changes in roots. $Ca(NO_3)_2$ treatment partly destroyed the epidermis, which was very clear under 20 mM $Ca(NO_3)_2$ treatment (Fig. 5B-g, h). Under 10 or 20 mM $Ca(NO_3)_2$ treatment, the primary vascular tissue was damaged and some ruptures were observed in these tissues (Fig. 5B-e, f, g, h).

Discussion

Salinity greatly affects seed germination (Misra and Dwivedi, 2004), leading to a reduction in germination rate and a delay in the initiation of germination and seedling establishment (Almansouri et al., 2001). However, little evidence has been gathered on the effects $Ca(NO_3)_2$ on seed germination. In this study, we report that addition of 5 mM $Ca(NO_3)_2$ stimulated cucumber seed germination during the whole treatment period. Twenty mM $Ca(NO_3)_2$ also increased %RBT after 24 h and RHRS after 36 h of treatment, while 10 and 40 mM $Ca(NO_3)_2$ inhibited seed germination. There was no clear pattern on seed germination as the $Ca(NO_3)_2$ concentration increased. This observation may be closely associated with Ca^{2+} and NO_3^- concentrations, and their positive or negative impact on plant physiology in different levels. It is well-known that Ca^{2+} is not only a main component of plant cells, but also acts as secondary messenger to regulate plant responses to environmental changes. Appropriate Ca^{2+} concentration may decrease stress; however, excess Ca^{2+} may inhibit the absorption of coupling factors such as Fe, Mn, Cu, and Zn ions. Salt-induced nutrient deficiency has been reported by many researchers. Generally, plant growth decreases under a nitrogen supply exceeding 10 mM, a value considered to be the threshold of toxicity for some species (Sánchez et al., 2004). Excessive nitrogen fertilization not only inhibits biological nitrogen fixation, but also causes osmotic stress, in which ROS are produced. Therefore, in different concentrations of $Ca(NO_3)_2$, seed germination exhibited different tendencies. Nelson et al. (2003) also showed that Ca content in tulip declined with increasing $Ca(NO_3)_2$ levels in nutrient solution, suggesting an inhibition of Ca uptake at high $Ca(NO_3)_2$ concentrations.

It has been well documented that stress tolerance in plants is associated with their ability to remove ROS. A transient generation of ROS is said to be the trigger for systematic acquired resistance, otherwise called “oxidative burst” (Park et al., 1998). Higher nitrate levels in soil or nutrient solution will cause osmotic stress, which can cause oxidative damage and induce ROS. Excess ROS can trigger peroxidative reactions and cause major damage to essential macromolecules, such as proteins involved in photosynthesis and in the systems of photosynthetic membranes (Foyer et al., 1994). In this experiment, $O_2^{\cdot-}$ production rate increased under $Ca(NO_3)_2$ stress, especially under 20 mM $Ca(NO_3)_2$. MDA is formed by the reaction of ROS with lipid molecules of tissues and its content is often used as an indicator of the extent of lipid peroxidation. MDA is also toxic for biomacromolecules. Under 10 mM $Ca(NO_3)_2$, MDA content increased significantly in germinating seeds. Antioxidant enzymes are an important pathway of ROS detoxification. Addition of 10 or 40 mM $Ca(NO_3)_2$ stimulated the activity of all antioxidant enzymes tested, including SOD, POD, CAT, APX, GR, DHAR, and MDHAR. While 5 mM $Ca(NO_3)_2$ significantly decreased CAT activity, 20 mM $Ca(NO_3)_2$ significantly decreased CAT and DHAR activity. By comparing the germination rate and the activity of antioxidant enzymes, in our study we observed a negative correlation between germination rate and CAT activity, which is likely to be also involved in many other physiological and biochemical mechanisms. It is well known that CAT catalyses a redox reaction in which dismutation of hydrogen peroxide generates water and oxygen, and that CAT is the key enzyme for scavenging hydrogen peroxide in plant cells. However, to our knowledge, the exact mechanism is still unclear and merrits further studies.

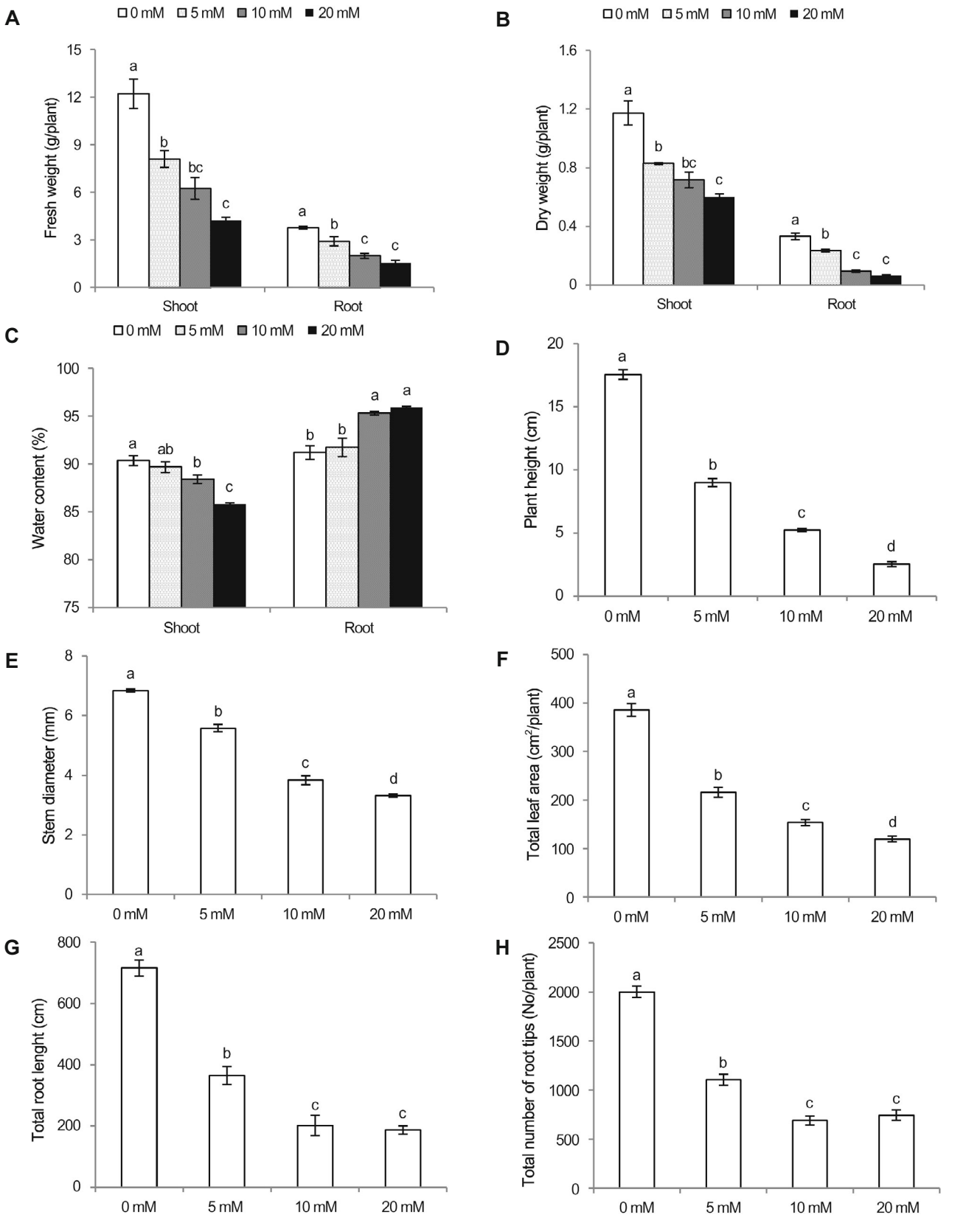

Fig. 4. Effect of different concentrations of $Ca(NO_3)_2$ on cucumber seedling growth 48 d after sowing. Values represent the mean ± SE (n = 3). Letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

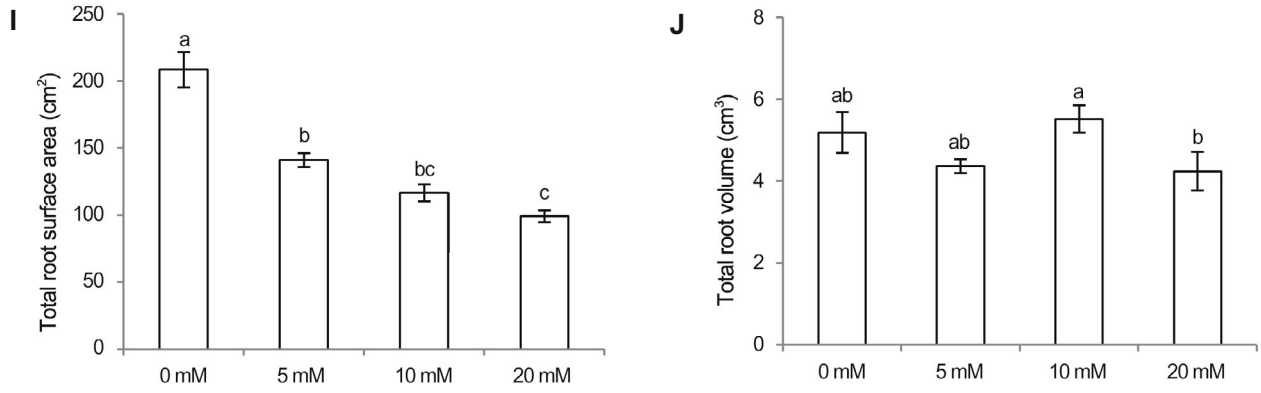

Fig. 4. Continued.

Table 1. Effect of different concentrations of $Ca(NO_3)_2$ on the accumulation of photosynthetic pigment in cucumber seedlings. Values represent the mean ± SE (n = 3). Letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests

Treatment	Chl *a* (mg·g^{-1} FW)	Chl *b* (mg·g^{-1} FW)	Car (mg·g^{-1} FW)	Chl *a*+*b* (mg·g^{-1} FW)	Chl *a*/*b*
0 mM	1.68±0.04 c	0.53±0.14 c	0.61±0.02 b	2.21±0.06 c	3.18±0.01 a
5 mM	3.66±0.05 b	1.29±0.01 b	1.23±0.02 a	4.96±0.06 b	2.84±0.01 b
10 mM	3.69±0.13 b	1.31±0.07 b	1.26±0.02 a	5.00±0.20 b	2.81±0.04 b
20 mM	4.15±0.23 a	1.61±0.13 a	1.28±0.02 a	5.75±0.36 a	2.59±0.08 c

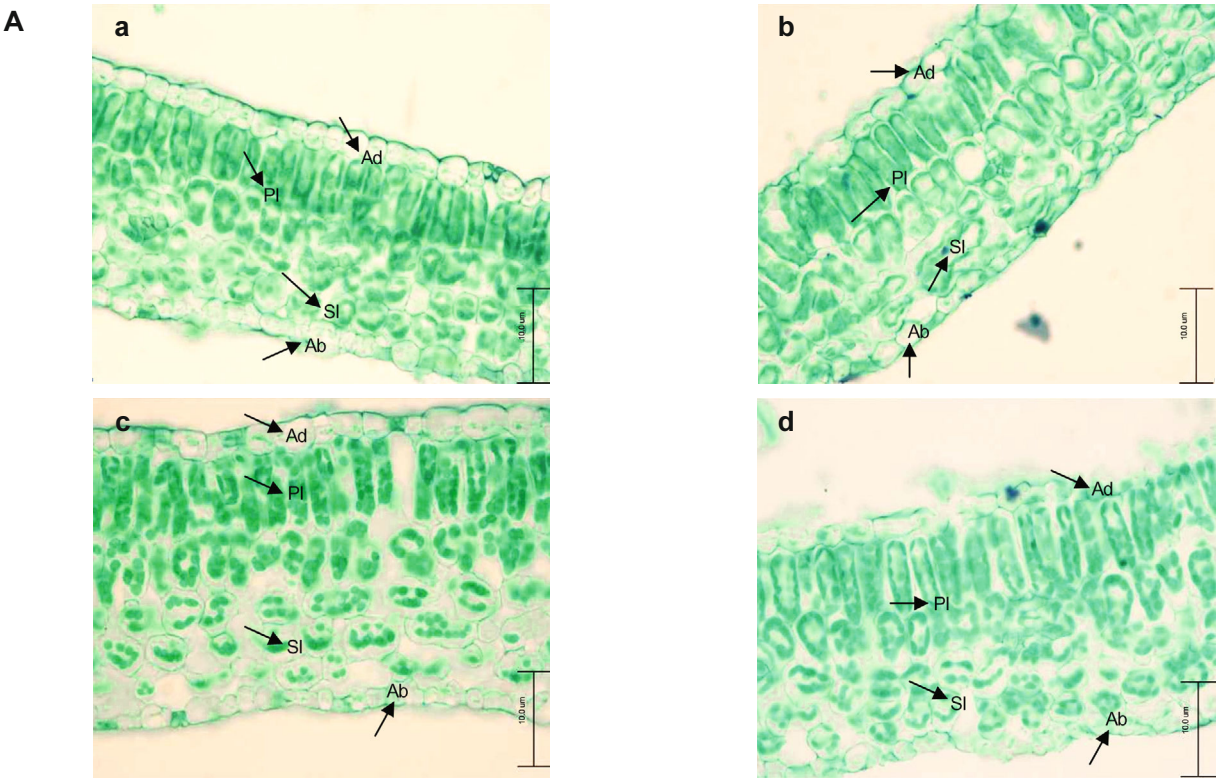

Fig. 5. Effect of different concentrations of $Ca(NO_3)_2$ on the micro-structure of leaves in cucumber seedlings (A) [Ad: upper epidermis; Ab: lower epidermis; Pl: palisade tissue; Sl: spongy tissue. a: 0 mM $Ca(NO_3)_2$; b: 5 mM $Ca(NO_3)_2$; c: 10 mM $Ca(NO_3)_2$; d: 20 mM $Ca(NO_3)_2$] and roots (B) [a, c, e and g: entire structure of root tips; b, d, f and h: primary vascular tissue structure of root. a, b: 0 mM $Ca(NO_3)_2$ (control); c, d: 5 mM $Ca(NO_3)_2$; e, f: 10 mM $Ca(NO_3)_2$; g, h: 20 mM $Ca(NO_3)_2$. Arrows indicate the change of structure compared to the control (0 mM $Ca(NO_3)_2$).

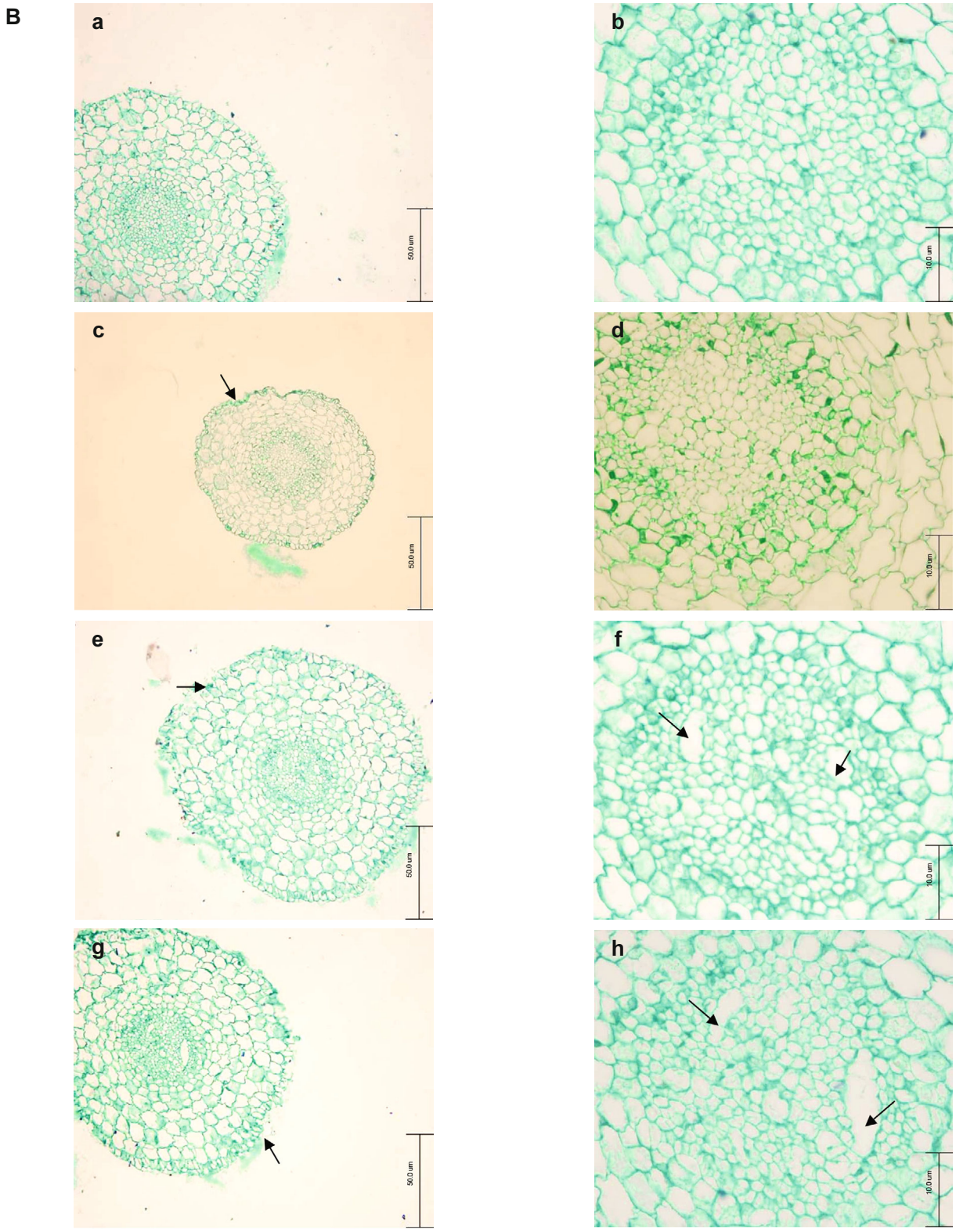

Fig. 5. Continued.

The detrimental effects of high salinity on plants can be observed at the whole plant level, as plant death or decreases in productivity. Most plant species show reduced growth, smaller leaves and stunted root systems when exposed to high nitrogen stress, and in severe cases this leads to the death of the plant. Cucumber seeds could not grow into seedlings under 40 mM $Ca(NO_3)_2$ in this study. Plants exposed to high nitrate levels will absorb excessive NO_3^-, which can

increase the pH around the roots due to the efflux of HCO_3^- or OH^- from the roots. This pH increase can considerably reduce Fe availability. In the current experiment, our results clearly show that 5-20 mM $Ca(NO_3)_2$ caused a decrease in growth of cucumber seedlings, and significantly reduced plant fresh and dry weight, which could suppress the normal functions of plants and block nutrient absorption. $Ca(NO_3)_2$ treatment resulted in the increase of total chlorophyll, chlorophyll *a* and chlorophyll *b* content. We observed that $Ca(NO_3)_2$ treatment darkened the green color of leaves, which also indicates an increase in the total chlorophyll content. At the same time, chlorophyll *a/b* was found to decline under $Ca(NO_3)_2$ stress (Table 1), which further affected the normal photosynthesis of seedlings. This decline may be responsible for the inhibition of plant growth under $Ca(NO_3)_2$.

It is well known that salinity can affect many processes in plant life cycles, including inhibitory effects on cell division and mitotic activity and chromosomal abnormalities in root tip cells. In our study, we also found that $Ca(NO_3)_2$ decreased root length and the number of root tips. Similarly, Forde and Lorenzo (2001) reported lateral root development was inhibited by NO_3^- accumulation in *Arabidopsis* shoots. Mathematical modeling using available experimental data showed that root-to-shoot ratio decreased as N concentration in plants increased (Levin et al., 1989). Accumulation of nitrate in shoots is a key factor in biomass allocation between shoots and roots in plants (Scheible et al., 1997). It was interesting that $Ca(NO_3)_2$ resulted in decrease of shoot water content and increase of root water content. There are major constraints for plant growth on saline substrates: (1) "drought stress" related to low water potential of the rooting medium; (2) ion toxicity associated with excessive uptake; (3) nutrient imbalance as a result of lower uptake, shoot transport and impaired internal distribution of minerals (Greenway and Munns, 1980). Our results indicate that, in this study, plant growth inhibition was not due to drought stress.

NaCl stress causes extreme damage to the microstructure of leaves and roots. However, few studies have reported on the harmful effects of $Ca(NO_3)_2$ on plant microstructure. Our results indicate that $Ca(NO_3)_2$ also causes a change in microstructure in higher plants, which further affects photosynthesis and water absorption and transport. In addition, $Ca(NO_3)_2$ promoted the thickening of leaves. Some ruptures were found in roots, and the whole osmosis system of roots was destroyed, which may be one of the reasons why water content in roots under $Ca(NO_3)_2$ treatment was higher than that in the control.

The present study has shown that different concentrations of $Ca(NO_3)_2$ affect cucumber seed germination to different extents without a clear pattern, which may be related to antioxidant systems. The activities of antioxidant enzymes are affected by Ca^{2+} and NO_3^- to different extents. Concentrations higher than 5 mM $Ca(NO_3)_2$ suppressed cucumber seedling growth under long-term treatment. The mechanisms behind these phenomena remain unknown. These matters are currently being investigated from the viewpoints of biochemistry and molecular biology.

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