Plant Soil (2009) 323:285–294
DOI 10.1007/s11104-009-9937-9

REGULAR ARTICLE

Cerium under calcium deficiency—influence on the antioxidative defense system in spinach plants

Liu Chao · Cao Weiqian · Lu Yun · Huang Hao · Chen Liang · Liu Xiaoqing · Hong Fashui

Received: 6 June 2008 / Accepted: 11 February 2009 / Published online: 3 March 2009

Abstract The influence of calcium-deficiency and cerium addition on the antioxidative defense system in spinach leaves was investigated. It was found that spinach cultivated in calcium-deficiency media developed distinct calcium deficiency symptoms and the plant growth was significantly inhibited as expected. While cerium-treated spinach remained green and expanded, and plant growth was improved. Calcium deprivation in spinach also increased the permeability of plasma membrane, malondialdehyde as a degradation product of lipid peroxidation, reactive oxygen species such as superoxide radicals, hydrogen peoxide, and decreased activities of the antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione content. However, cerium treatment cultivated in calcium-deficiency media decreased the permeability of plasma membrane, malondialdehyde and reactive oxygen species, and increased activities of the antioxidative defense system. This is viewed as evidence for cerium addition to calcium-deficiency media in the spinach plants could substitute calcium and enhance oxidative stress-resistance.

Keywords Cerium · Calcium-deficiency · Antioxidative defense system · Spinach

Responsible Editor: Jian Feng Ma.

Liu Chao and Cao Weiqian contributed equally to this work.

L. Chao · C. Weiqian · L. Yun · H. Hao · C. Liang · L. Xiaoqing · H. Fashui (✉)
Medical College, Soochow University,
Suzhou 215123, Peoples Republic of China
e-mail: Hongfsh@hotmail.com

L. Chao · H. Fashui
College of Chemistry, Chemical Engineering and Materials Science, Soochow University,
Suzhou 215123, China

As one of essential nutrient macroelements for plant growth, calcium plays very important roles in keeping of plant cell structure, resistance of adversity stresses (such as oxidative, drought, chilling and fungal intrusion et al.) and signal transduction (Chaney et al. 2008; Wu 2003; Buchanan et al. 2002). And it is well known that chemical property of lanthanide series elements (Ln^{3+}) is similar with Ca^{2+}. For example, the radius of Ce^{3+} is 1.01–1.20 Å when its coordination number is 6–9, while Ca^{2+} is in the same coordination numbers and its radius is 1.00–1.18 Å. Therefore, Ce^{3+} could enter organism and occupy a Ca^{2+} position, thus led to biological function alteration (Ni 2002). On the one hand, however, charge and potential energy in Ln^{3+} are higher than Ca^{2+}, the bound stability of Ln^{3+}-compounds is also higher than that of Ca^{2+}-compounds. Ln^{3+} could not only occupy a Ca^{2+} position, but also substitute for bound Ca^{2+}, thus Ln^{3+} are known as"supercalcium". On the other hand, Ln are located on the IIIB of element periodic table, their outmost shell is $6s^25s^25p^6$, their suboutmost shell is all filled, as 5d is either empty or only

one electron left. When they combined with other elements, they always lost two s electrons of outmost shell and one d electron of sub-outmost shell, thus made their natural atomic value (+3). 4f shell of Ln^{3+} is regularly filled from Ce^{3+} to Lu^{3+}. Except for La^{3+}, Lu^{3+} without 4f electron in the 4f shell, 4f electron of other Ln^{3+} is discretionarily distributed in seven 4f electron orbits, which causes a series of light, magnetism and other catalysis effects (Ni 2002).

The mechanism study of lanthanide as rare earth fertilizer to plants has been continuously active in China since 1980's. Hong et al. soaked rice seeds or aged rice seeds with lanthanum (La^{3+}) and cerium (Ce^{3+}) solution to study their effects on germination rate and discovered that two kinds of rare earths have similar chemical properties, but the optimum concentration differed a lot. The optimum concentration in La^{3+} treatment was 500 μM while the optimum concentration in Ce^{3+} was 10 μM (Hong et al. 2000a; Hong et al. 2000b; Hong et al. 2000c; Hong 2002a; Hong et al. 2003a) . We also worked on the germination rate of aged spinach seeds, the results showed that the most effective concentration for La^{3+} and neodymium (Nd^{3+}) was 400 μM, and it displayed inhibition up to 800 μM; the optimum concentration for Ce^{3+} was 16 μM, and after the concentration exceeded 50 μM it represented significant inhibition (Liu et al. 2004). Moreover, the effect of Ce^{3+} on the promotion of rice and spinach growth was the most obvious, and their disease-resistance ability remarkably enhanced. The results showed that no matter the aged rice seeds or spinach seeds, $LaCl_3$, $CeCl_3$ and $NdCl_3$ treatments could enhance the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Moreover, the three rare earth element treatments could decrease $O_2^{\cdot -}$ and malondialdehyde (MDA) contents and reduce cell membrane permeability of aged seeds of rice and spinach. Among three rare earth elements, Ce^{3+} treatment enhanced vigor of aged seeds and repaired cell membrane structure most significantly (Hong et al. 2000a; Hong et al. 2000b; Hong et al. 2000c; Hong 2002a; Hong et al. 2003a; Liu et al. 2004). The reason might be the difference of their structure of electron shell. However the electron quantity of 4 f electron shell also affect the atomic value. There are 0, 2 and 4 electrons in 4 f shell for La, Ce and Nd, respectively. Moreover, Ce not only has the atomic value of +3 but also can be oxidated to +4 values while La and Nd have no alterable valence (Ni 2002). Those facts indicated that the significant differences between reinforcing effects of La, Ce and Nd on seeds vigor, and the optimum treat concentration had closely related to 4f electron shell of structures and the characteristics of alterable valence of Ln.

Dong et al. studied relationship between $La^{3+}$and Ca^{2+} in cucumber plants, suggesting that calcium deprivation caused chlorosis and wilting of cucumber young leaves and brown spot of old leaves, and plant was short; while $LaCl_3$ treatment cultivated in calcium-deficiency media could decrease calcium-deficiency symptoms and improve plant growth (Dong et al. 1993). We hypothesize that calcium-deficiency symptoms might be related to oxidative damage in plant cell, and the disappearance of the symptoms caused by $LaCl_3$ might be related to the enhancement of antioxidative stress of plants. But these need further study.

The oxidative damage caused by calcium deprivation in spinach and antioxidative stress in $CeCl_3$-treated spinach grown in calcium-deficiency conditions were studied in the paper. The results showed that calcium deprivation in spinach plants increased plasma membrane permeability, MDA and reactive oxygen species (ROS), and decreased activities of the antioxidant enzymes and glutathione content. However, cerium treatment cultivated in calcium-deficiency media decreased plasma membrane permeability, MDA and ROS, and increased activities of the antioxidative defense systems, suggesting that on cerium added to calcium-deficiency media in the spinach plants could substitute for calcium and increase oxidative stress-resistance.

Materials and methods

Material treatment and culture

Experimental material was *Spinacia oleracea*. The seeds were purchased from a local seed company. The seeds were carefully selected and planted in a perlite-containing pot and placed in porcelain dishes, which were respectively added with following culture solutions: 1) (modified Hoagland nutrient solution containing 6 mM K as KNO_3, 4 mM Ca as $Ca(NO_3)_2$ and 1 mM Mg as $MgSO_4$, 16 mM N, 2 mM P as NaH_2PO_4, 1 mM S, 50 μM Cl, 25 μM B

as H_3BO_3, 2 μM Mn as $MnCl_2$, 2 μM Zn as $ZnSO_4$, 0.5 μM Cu as $CuSO_4$, and 0.5 μM Mo as H_2MoO_4, 20 μM Fe as NaFeDTPA (pH=5.8). (2) Calcium-deficiency Hoagland's nutrient solution without Ca $(NO_3)_2$ as described in ref (Li 1999), NH_4NO_3 was supplied to adjust nitrogen concentration. These were placed in a glasshouse under sunlight (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of light intensity) for four weeks. When spinach seedlings were at the age of two leaves, 15 μM $CeCl_3$ or $CaCl_2$ solution were sprayed on the leaves respectively, and deionized water for control, the same treatments were replicated every 5 days. Thus made 5 groups: (1)Hoagland's solution; (2)Hoagland's solution + 15 μM cerium chloride treatment; (3)Ca^{2+}-deficiency Hoagland's solution; (4) Ca^{2+}-deficiency Hoagland's solution+ 15 μM cerium chloride treatment, (5) Ca^{2+}-deficiency Hoagland's solution+ 15 μM calcium chloride treatment.

Assay of physiological and biochemical indexes

Plant weight

The fresh weight and dry weight of spinach were weighted at 28 days after planting.

Plasma membrane permeability of spinach leaves

Plasma membrane permeability was measured by a DDS—11 electric conductivity instrument (DDS-11A**,** Lengpu Inc., Shanghai) as described previously (Li 1999), samples were placed in a beaker containing 420 ml of deionized distilled water and allowed to equilibrate for 6 h at room temperature. Electrical conductivity of the solution was measured before and after incubation. Samples were then placed in boiling water 1 min, cooled to room temperature, and the electrical conductivity measured again. Plasma membrane relative permeability%=electrical conductivity in respective treatment/ electrical conductivity in total destructive plasma membrane.

Lipid peroxide (MDA) of spinach leaves

The level of lipid peroxidation of spinach leaves was measured as 2-thiobarbituric acid-reactive metabolites (TBA), mainly MDA, following the modified method of Heath and Packer (Heath and Packer 1968). Frozen samples were homogenized in a pre-chilled mortar and pestle with two volumes of ice cold 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 min at 15, 000×*g*. Assay mixture containing 1 ml aliquot of supernatant and 2 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA was heated to 95 °C for 30 min and then rapidly cooled in an ice-bath. After centrifugation (10, 000×*g* for 10 min at 4 °C), the supernatant absorbance (532 nm) was read and values corresponding to non-specific absorption (600 nm) were subtracted. MDA concentration was calculated using its extinction coefficient (155 $mM^{-1} \cdot cm^{-1}$).

ROS of spinach leaves

Superoxide ion ($O_2^{\cdot -}$) was measured as described by Able et al. (Able et al. 1998) , by monitoring the reduction of 3'-[1-[phenylamino-carbonyl]-3,4-tetrzolium] -bis(4-methoxy-6-nitro) benzenessulfonic acid hydrate (XTT) in the presence of $O_2^{\cdot -}$, with some modifications. The spinach leaves were homogenized in prechilled mortar and pestle with an ice-cold isolation buffer containing 400 $mmol.L^{-1}$ sucrose, 10 $mmol.L^{-1}$ NaCl, and 20 mmol $.L^{-1}$ Tris–HCl buffer (pH 7.8). The slurry was filtered through five layers of cheesecloth and the chloroplasts were sedimented at 3000 $r.min^{-1}$ for 5 min at 4°C to prepare chloroplasts (Arnon 1949). The chloroplasts were homogenized with 2 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 5, 000×g for 10 min. The reaction mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 7.5), 20 μg chloroplast supernatant proteins, and 0.5 mM sodium, XTT. The reaction of XTT was determined at 470 nm for 5 min. Correction were made for the background absorbance in the presence of 50 units of SOD. The production rate of $O_2^{\cdot -}$ was calculated using an extinction coefficient of 2.16×10^4 $M^{-1}.cm^{-1}$

H_2O_2 were extracted according to Wang and Luo (Wang and Luo 1990)*;* the chloroplasts were homogenized in 3 ml of ice-cold acetone. The homogenate was centrifuged at 30,000×*g* for 10 min and the supernatant was used for assays of the contents of H_2O_2. The reaction mixture contained 0.1 ml of 5% $Ti(SO_4)_2$, 0.2 ml of ammonia solution, and 1 ml of the H_2O_2 extract, which was centrifuged at 30,000×*g* for 10 min. The precipitate was repeatedly washed with

acetone until pigments were completely removed; it was then dissolved with 5 ml of 2 M H_2SO_4, and the contents of H_2O_2 was measured at 415 nm.

Antioxidant enzyme activity of spinach leaves

Spinach leaves were homogenized in 1 ml of ice-cold 50 mM sodium phosphate (pH 7.0) that contained 1% polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged at 30,000×*g* for 30 min and the supernatant was used for assays of the actives of SOD, CAT, ascobate peroxidase (APX) and guaiacol peroxidase (GPX).

The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 100 μM EDTA, and 200 μl of the enzyme extract. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan (Ginnopolitis and Rice 1977). One unit was defined as the amount required inhibiting the reduction of NBT to NBT formazan in the presence of superoxide by 50%.

The activity of CAT was determined by measuring the rate of disappearance of H_2O_2 at 240 nm. Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 12.5 mM H_2O_2, and 200 μl of enzyme extract (Prasad 1997). One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 240 nm.

APX activity was assayed using the method described by Reuveni et al. (Reuveni et al. 1992). A reaction mixture consisting of 100 μl supernatant, 17 mM H_2O_2 (450 μl), and 25 mM ascorbate (450 μl) was then assayed for 3 min at 290 nm. Activity was measured as disappearance of ascorbate. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 290 nm.

GPX activity was also measured using the method described by Reuveni et al. (Reuveni et al. 1992). A reaction mixture consisting of supernatant (100 μl), 17 mM H_2O_2 (450 μl), and 2% guaiacol (450 μl) was then assayed for 3 min at 510 nm. Activity was measured as appearance of tetraguaiacol. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} at 510 nm.

Reduced glutathione of spinach leaves

In order to perform the reduced glutathione assay, spinach leaves homogenized as described above. However, supernatants were not diluted fivefold as described in the case of the antioxidant enzyme assays. Reduced glutathione content was estimated using the method of Hissin and Hilf (Hissin and Hilf 1976). The reaction mixture contained 100 μl of supernatant, 100 μl *o*-phthaldehyde (1 mg ml^{-1}), and 1.8 ml phosphate buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0). Fluorometry was performed using a F-4500 fluorometer (Hitachi Co., Japan) with excitation at 350 nm and emission at 420 nm.

Results

Growth of spinach

As shown in Fig. 1 (photo 1, 2), cerium could greatly improve spinach growth. The spinach leaf area treated by cerium grown in Hoagland's solution was larger than that of control. The single fresh and dry weights of the cerium-treated groups were enhanced by 31.83% and 40.98% compared to the control, respectively (see Fig. 2, column 1, 2).

From Fig. 1 (photo 3), young leaves of spinach plants developed Ca^{2+}-deficiency symptoms such as chlorosis and wilting, plants were short in Ca^{2+}-deficiency conditions. It seems low concentration of

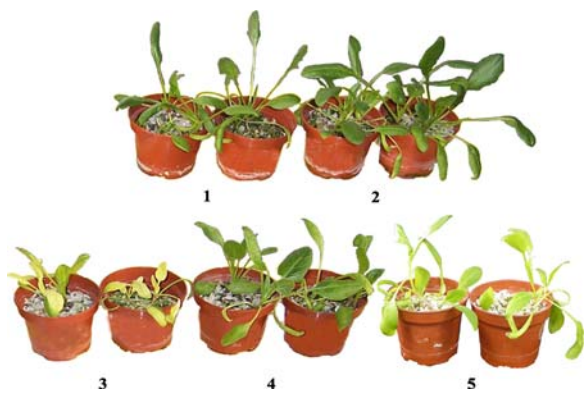

Fig. 1 Effect of Ce3+ on growth of spinach cultivated in Ca2+-deficiency media. 1. Hoagland's solution(control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4. Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+Ca^{2+}

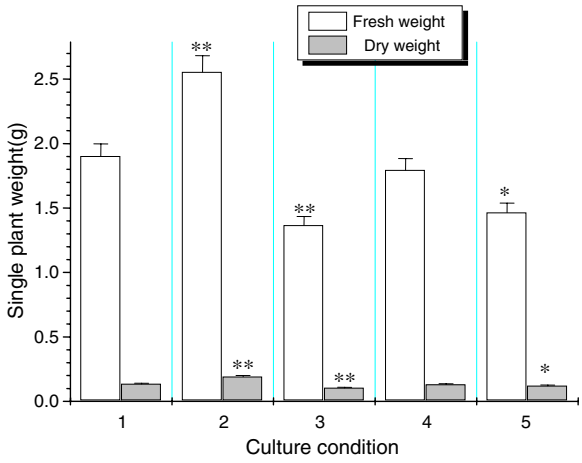

Fig. 2 Effect of Ce3+on spinach plant weights cultivated in Ca2+-deficiency media. 1. Hoagland's solution(control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4. Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

Ca^{2+} treatment didn't improve the spinach growth effetely, the plant in Ca^{2+}-deficiency Hoagland's solution+Ca^{2+} group still showed to be Ca^{2+}-deficient (photo 5). While all leaves treated by cerium grown in Ca^{2+}-deficiency media kept green and expanded, and plants grown vividly (see photo 4). We can see from Fig. 2 (column 3) that the fresh and dry weights of single plant cultivated in Ca^{2+}-deficiency conditions were much lower than those grown in Hoagland's solution, suggesting 28.22% and 22.56% reduction, respectively; and Ce^{3+} and Ca^{2+} treatment grown in Ca^{2+}-deficiency media suggested 5.72%,22.93% and 3.42%,11.23% reduction (Fig. 2, column 4, 5), respectively. Ce^{3+} treatment enhance the growth of Ca^{2+}-deficient spinach better than the same concentration of Ca^{2+}.

Plasma membrane permeability and lipid peroxide level of cell of spinach

Figure 3 shows the relative permeability of plasma membrane of spinach cell cultivated in various conditions. Under Hoagland's solution, the membrane permeability of spinach treated by Ce^{3+} was reduced by 7.92%. And the membrane permeability grown in Ca^{2+}-deficiency media was increased by 66.55% and in Ca^{2+}-deficiency Hoagland's+Ce^{3+} media rose by 17.19%, as compared to culture with Hoagland's solution, respectively. While Ca^{2+}-deficiency Hoagland's+Ca^{2+} was 32.53% higher than the control.

Lipid peroxide level is a very important index of cell to valuate the damage of membrane structure. It can be seen in Fig. 4 that MDA content of spinach cells grown in Ca^{2+}-deficiency conditions was 78.46% higher than that grown in Hoagland's solution, Ce^{3+} and Ca^{2+} treatment in Ca^{2+}-deficiency media was 16.92% and 43.03% higher than that grown in Hoagland's solution, respectively. However, MDA content of spinach treated by Ce^{3+} grown in Hoagland's solution was reduced by 18.46%. The changes of MDA content are consistent with the membrane permeability mentioned above.

ROS accumulation of spinach

The effects of various culture media on the production rate of $O_2^{\cdot-}$ and H_2O_2 in spinach are shown in Fig. 5. It can be seen that ROS grown in Ca^{2+}-deficiency conditions rose sharply, i.e. $O_2^{\cdot-}$ and H_2O_2 generating rates increased by 66.76% and 126.39% as compared to grown in Hoagland conditions, respectively, suggesting that exposure to

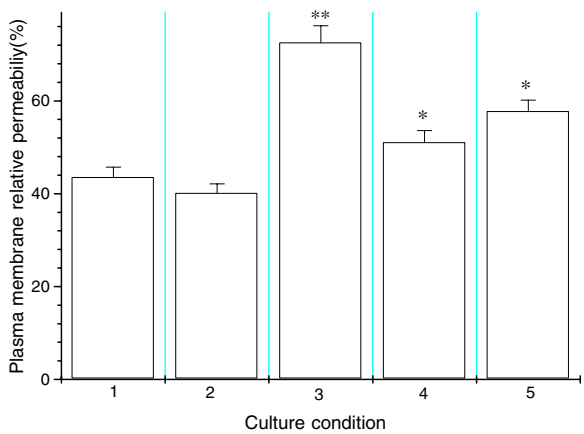

Fig. 3 Effect of Ce3+ on plasma membrane relative permeability of cell of spinach leaves cultivated in Ca2+-deficiency media. 1 .Hoagland's solution (control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4.Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

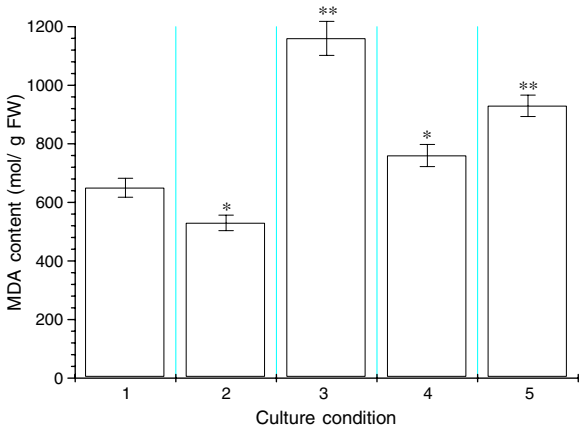

Fig. 4 Effect of Ce3+on MDA content in spinach cultivated in Ca2+-deficiency media. 1.Hoagland's solution; 2. Hoagland's solution + Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4. Ca^{2+}-deficiency Hoagland's solution+ Ce^{3+}. 5. Ca^{2+}-deficiency Hoagland's solution+Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

Ca^{2+}-deficiency media caused a strong oxidative stress in spinach cell. Ca^{2+} treatments better the ROS accumulation scarcely, while Ce^{3+} treatments significantly decreases oxidative damage, i.e. $O_2^{\cdot -}$ and H_2O_2 generating rates grown in Ca^{2+}-deficiency

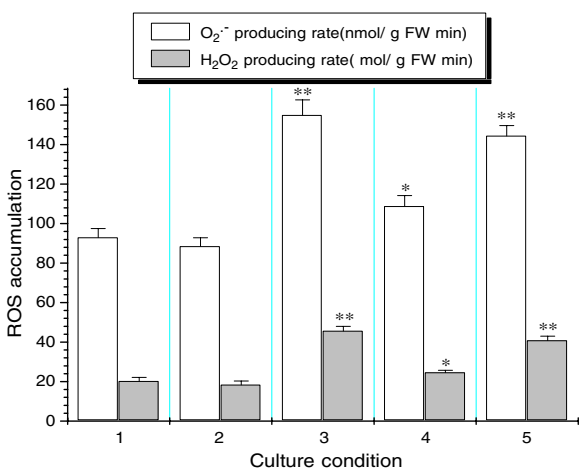

Fig. 5 The Effects of Ce3+ on ROS accumulation of spinach cultivated in Ca2+-deficiency media. 1.Hoagland's solution(control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4. Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+ Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

conditions showed 17.16% and 21.78% enhancement. It is also found $O_2^{\cdot -}$ and H_2O_2 generating rates in Hoagland's media treated by Ce^{3+} suggeste 4.77% and 9.03% reduction, respectively.

Antioxidant defense of spinach

It can be seen in Fig. 6. that the activities of SOD, CAT, APX and GPX enzymes of spinach cultivated in Ca^{2+}-deficiency media were decreased by 46.54%, 53.35%, 38.13% and 47.54% as compared to culture of Hoagland's solution, respectively. The enzymes activities by low concentration Ca^{2+} treatments in Ca^{2+}-deficiency media are little improved., while Ce^{3+} -treated spinach grown in Ca^{2+}-deficient media were 16.16%, 8.38%, 16.71% and 12.00% lower than those grown in Hoagland's solution, respectively, which is much better than Ca^{2+}-deficient group. However the activities of the antioxidant enzymes from Ce^{3+}- treated groups grown in Hoagland's media were 34.91%, 32.83%, 13.21% and 16.22% higher than the control.

Figure 7 shows the ratios of GSH to GSSGG of spinach cultivated in Ca^{2+}-deficiency media. The ratios of GSH to GSSGG in Ca^{2+}-deficiency Hoag-

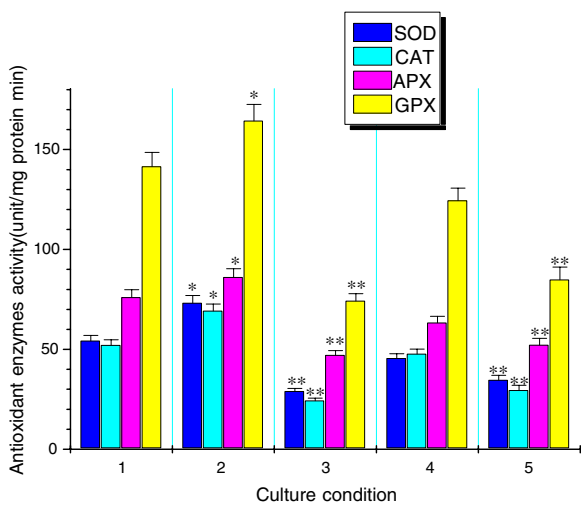

Fig. 6 The Effects of Ce3+ on the activities of antioxidant enzymes of spinach cultivated in Ca2+-deficiency media. 1. Hoagland's solution (control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4.Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

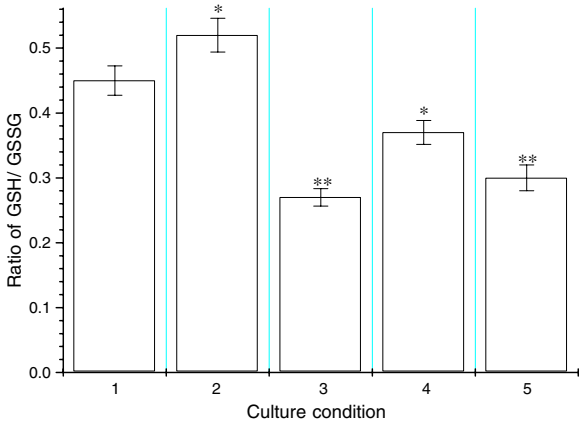

Fig. 7 The Effects of Ce3+ on the ratio of GSH/GSSG of spinach cultivated in Ca2+-deficiency media. 1.Hoagland's solution (control); 2. Hoagland's solution+Ce^{3+}; 3. Ca^{2+}-deficiency Hoagland's solution; 4. Ca^{2+}-deficiency Hoagland's solution+Ce^{3+}; 5. Ca^{2+}-deficiency Hoagland's solution+ Ca^{2+}. Bars marked with an * and double* were different from the others in that panel at the 5% confidence level and at the 1% confidence level, respectively. Bars indicate mean and error bars are SE (n=5)

land's group decreased by 40.00%, while the ratio in Ca^{2+}-deficiency group treated by Ce^{3+} decreased by 17.78% compared with the control. But the same concentration of Ca^{2+} treatment gives a 33.33% reduction compared with the control. However, the ratio in Hoagland's solution group treated by Ce^{3+} increased 15.56% compared with the control. It indicated that Ca^{2+}-deficiency accelerated reduced glutathione (GSH) to be oxidized to oxidized glutathione (GSSG) and Ce^{3+} treatment alleviated the oxidation of GSH caused by Ca^{2+}-deficiency in spinach.

Discussion

The effects of cerium on reduction of oxidative stress of spinach and growth of plant grown in calcium-deficiency conditions were investigated. The question was raised whether oxidative stress of calcium deprivation can occur and whether oxidative damage can be decreased by cerium treatment in spinach cell grown in calcium-deficiency conditions.

The experimental results showed that calcium deprivation caused young leaves turning chlorosis and wilting, and a significant reduction of spinach plant weight as well (Figs. 1 and 2), implying that the cell water content was absent, which was related to reduction of water absorbance and keep of cell. It had been proved that calcium could increase drought-resistance of plants (Hong et al. 1996; Lu et al. 1999; Chen 1998). Cerium treatment with low concentration could decrease the calcium-deficiency symptoms of spinach plants (Figs. 1 and 2), suggesting that cerium could increase water absorbance and keep of spinach cell. Dong et al. proved that cucumber plants grown in calcium-deficiency media were short, young leaves developed chlorosis and wilting, old leaves displayed brown spots. While La^{3+} added to calcium-deficiency media could decrease chlorosis and wilting of young leaves, plant fresh weight increased by19% (Dong et al. 1993), which is consistent with our results. However, Dong et al. didn't study mechanism of La^{3+} on decreasing the calcium-deficiency symptoms of cucumber plants.

For increasing plant resistance, calcium occupies an important position in keep of stability and integrality of cell membrane, in direct or indirect regulation of various metabolisms (Wu 2003; Buchanan et al. 2002). The reduction of water content of cell was closely related to the damage of plasma membrane structure. Due to calcium deprivation, the obvious enhancements of MDA content and permeability of plasma membrane of spinach cell were observed in the study (Figs. 3, 4), implying that plasma membrane structure of spinach cell was damaged, solute and water in cell were easily lost, which led to the reduction of water content of cell and the wilting of spinach young leaves. Hong et al. (2000b, 2000c, 2002a) had proved that La, Ce, Nd could repaire membrane structure of aged seed cell (Hong et al. 2000b, 2000c; Hong 2002a; Liu et al. 2004). The reduction of MDA content and permeability of plasma membrane of spinach cell were observed by cerium treatment grown in calcium-deficiency media, suggesting that cerium could repaire damaged membrane structure grown in calcium-deficiency conditions, thus increase water content of cell and avoid young leaves wilting.

Oxidative stress, resulting from the generation of ROS, is a common phenomenon in many stress responses, such as drought, cold shock, photoinhibition and hypo-osmotic stress. ROS generation leads to cellular damage and ultimately to cell death, primarily through damage to the photosystem II reaction centre

and to membrane lipids (Chris and Robert 2000). The results showed that the obvious accumulation of ROS such as $O_2^{\cdot-}$, H_2O_2 occurred in spinach cell grown in calcium-deficiency media (Fig. 5), it was consistent with the significant enhancement of lipid peroxide level, implying that calcium deprivation caused the oxidative stress in spinach cell, thus conduced to the damage of plasma membrane structure. However, cerium treatment could decrease ROS accumulation (Fig. 5), showing that cerium could remove ROS and increase the oxidative stress-resistance of spinach. It was proved that the increase of the aged seed (rice, spinach) vigor by La, Ce, Nd pretreatment was closely related to ROS removal, and Ce treatment was better (Hong et al. 2000b; Hong et al. 2000c; Hong 2002a; Liu et al. 2004). Numerous studies have shown that calcium and ROS exhibit important signalling functions in responses to both biotic and abiotic stresses, implying that they might be central components controlling cross-tolerance, at least at the cellular level (Chris and Robert 2000). Due to calcium deprivation, the controlling cross-tolerance was impaired in spinach cell, and cerium might resume the controlling cross-tolerance.

The generation of oxidative stress in plant cell is due to an imbalance between ROS and their removal makes macromolecules and membranes damaged, thus leads to the reduction of plant growth. The control plant possesses its own active antioxidant defense systems (antioxidative enzymes such as SOD, CAT, APX and GPX, as well as non-enzymatic antioxidants such as glutathione, ascorbate, guaiacol and carotenoids et al.) through which production and removal of ROS is in balance. SOD can convert $O_2^{\cdot-}$ into H_2O_2 and O_2; moreover, CAT, APX and GPX can reduce H_2O_2 into H_2O and O_2 (Lin et al. 1988) . Therefore, SOD, CAT, APX and GPX can keep a low level of ROS and prevent ROS toxicity and protect cells (John and Scandalios 1993). In the experiments, we observed that the activities of SOD, CAT, APX and GPX were significantly inhibited and the content of glutathione was decreased (Figs. 6, 7), ROS was greatly accumulated in spinach grown in calcium deficiency media, suggesting that exposure to calcium deficiency media caused an imbalance between ROS and their removal in spinach cell. It is well known that GSH can directly interact with and detoxify oxygen free radicals and thus contribute significantly to nonenzymatic ROS scavenging. We also found that the ratio of GSH to GSSG in spinach caused by calcium deficiency was significantly decreased (Fig. 7). The depletion of GSH was associated with increases in ROS and MDA, suggesting that spinach grown in calcium deficiency media was using up antioxidative defences to prevent oxidative stress. Michaela et al. (2002) reported that calcium deficiency significantly influenced the antioxidative defense system in tomato plants (Michaela et al. 2002). Gibson et al. (2002) indicated that oxidative stress increased internal calcium stores and reduced a key mitochondrial enzyme (Gibson et al. 2002). However, our study proved that cerium treatment could significantly increase the activities of SOD, CAT, APX, GPX and the content of glutathione (Figs. 6, 7), and led to the reduction of ROS accumulation of spinach plants. Zhou et al. (2000) also proved that antioxidative stress of cucumber was increased by neodymium treatment (Zhou and Wei 2000). Our previous researches demonstrated that La, Ce and Nd treatments increased the activities of antioxidative enzymes, which led to ROS removal in aged seeds of rice and spinach (Hong et al. 2000b; Hong et al. 2000c; Hong 2002a; Hong et al. 2003a; Liu et al. 2004). On the other hand, cerium treatment caused the reduction of ROS, which related to direct removal of $O_2^{\cdot-}$, it had been proved by Wang et al. (Wang et al. 1997, 2000). The mechanism was alleged to be: Ce^{3+} can reduce $O_2^{\cdot-}$ to H_2O_2 and oxidize itself to Ce^{4+} and Ce^{4+} can oxidize $O_2^{\cdot-}$ to O_2 while it reduces itself to Ce^{3+}.

As mentioned above, calcium-deficient plants depicted acute abnormalities in many fields, which was consistent with previous studies (Singh and Sharma, 1972). Since the normal concentration of Ca in nutrient solution is up to 4 mM, it was obviously to found 15 μM Ca treatment is far from adequate. The spinach grown in Ca^{2+}-deficient Hoagland's solution+ Ca^{2+} solutions still showed Ca^{2+}-deficient symptoms and the antioxidative defense system was hardly improved. However, 15 μM Ce treatments could partly recover the Ca^{2+}-deficient condition, especially for the antioxidative defense system. It strongly suggested that cerium could take place of some biological functions of calcium and have better effects for its special physical and chemical properties. For similar chemical properties as Ca^{2+}, it was believed that Ce^{3+} could enter organism and occupy a Ca^{2+} position (Mulqueen et al. 1985; Ni 2002) and act different roles as

interchangeability of Ca^{2+} in plants. We had found that Ce and some other lanthanide ion (La, Nd) could bind to chlorophyll, D1/D2/Cytb559 and Rubisco to change the biological functions (Hong et al. 2002b; Hong et al. 2003b; Liu et al. 2006). In our recent research, Ce could also directly bind to the Ca-depleted PSII (The paper was not contributed). It was another example for the substitution function of Ce on Ca though the biological function might be different. The improvement of antioxidative defensive system might be only one aspects of Ce^{3+} substitution to Ca^{2+}.

In conclusion, calcium-deficiency conditions applied to spinach cultures caused an oxidative stress status in cell monitored by an increase in ROS accumulation. The enhancement of lipids peroxide and permeability of plasma membrane of spinach cell grown in calcium-deficiency media suggested an oxidative attack that was activated by a reduction of antioxidative defense mechanism measured by analyzing the activity of SOD, CAT, APX and GPX enzymes, as well as antioxidants such as glutathione content. As the antioxidative response of cell was reduced in spinach grown in a calcium-deficiency media, it caused a significant reduction of spinach plant weight, young leaves turning chlorosis and wilting. However, cerium treatment grown in calcium-deficiency conditions decreased the permeability of plasma membrane, MDA and ROS, and increased activities of the antioxidative defense system, and improved spinach growth, thus cerium treatment could help spinach recover from calcium-deficient conditions, espically on the antioxidative defensive system in the text. However, since Ce^{3+} has similar chemical properties as Ca^{2+} and calcium plays various roles in plants, cerium might easially bind to different functional components in plants as "super-calcium". The mechanism of cerium's special physiological function might be complicated and need further studies.

Acknowledgements This work was supported by the National Natural Science Foundation of China (grant no. 30800068) and by the Jiangsu Province Universities Natural Science Foundation (grant no. 06KJB180094).

References

Able AJ, Guest DI, Sutherland MW (1998) Use of a new tetrazolium-based assay to study the prodution of superoxide radicals by tobacco cell cultures challenged with avirulent zoopspores of *Phytophthora parasitca* var *nicotianae*. Plant Physiol 117:491–499 doi:10.1104/pp.117.2.491

Arnon DI (1949) Copper enzymes in isolated chloroplasts polyphenol oxidase in beta lgaris. Plant Physiol 24:1 doi:10.1104/pp.24.1.1

Buchanan BB, Gruissem W, Johones RL (2002) Biochemistry and Molecular Biology of Plants. Science Press, American Society of Plant Physiology, Beijing, pp 962–9781

Chaney LR, Chen KY, Li YM, Scott JA, Baker JMA (2008) Effects of calcium on nickel tolerance and accumulation in Alyssum species and cabbage grown in nutrient solution. Plant Soil 311:131–140 doi:10.1007/s11104-008-9664-7

Chen LH (1998) Effects of calcium treatment on physiological properties of cotton seedlings under drought stress. Chin Bull Bot 15(6):70–72

Chris B, Robert F (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant 5(6):241–246 doi:10.1016/S1360-1385(00)01628-9

Dong B, Wu ZM, Tang XK (1993) Effects of $LaCl_3$ on physiological activity of cucumber roots under calcium-deficiency conditions. J Rare Earths 11(1):65–68

Gibson GE, Zhang H, Xu H, Park LCH, Jeitner TM (2002) Oxidative stress increases internal calcium stores and reduces a key mitochondrial enzyme. Biochim Biophys Acta 1586:177–189

Ginnopolitis CN, Rice SK (1977) Superoxide dismutase purification and quantitative relationship with water soluble protein in seedling. Plant Physiol 59:315–318 doi:10.1104/pp.59.2.315

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198 doi:10.1016/0003-9861(68)90654-1

Hissin PJ, Hilf RA (1976) Fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74:214–226 doi:10.1016/0003-2697(76)90326-2

Hong FS, Dong ZJ, Ma CC (1996) Effects of Ca^{2+}, PEG on enzymes activities of wheat seedlings under drought stress. Acta Agronomica sinica 22:101–106 (in Chinese)

Hong FS, Fang NF, Zhao GW (2000a) Physiological effects of cerium nitrate on seed germination of rice. Acta Agronomica sinica 26(1):7–12 (in Chinese)

Hong FS, Wei ZG, Zhao GW (2000b) Effect of lanthanum on aged seed germination of rice. Biol Trace Elem Res 75:205–213 doi:10.1385/BTER:75:1-3:205

Hong FS, Wei ZG, Zhao GW (2000c) Study on mechanism of lanthanum nitrate effected on the vigor of aged rice seed. J Rare Earths 18(3):220–225

Hong FS (2002a) Study on the mechanism of cerium nitrate effects on germination of aged rice seed. Biol Trace Elem Res 87(1–3):191–200 doi:10.1385/BTER:87:1-3:191

Hong FS, Wei Z, Zhao G (2002b) Mechanism of lanthanum effect on chlorophyll of spinach. Science in china. Ser.C 45(2):166–176

Hong FS, Wang L, Liu C (2003a) Study of lanthanum on seeds germination and growth of rice. Biol Trace Elem Res 94 (3):273–286 doi:10.1385/BTER:94:3:273

Hong FS, Wang XF, Liu C, Su GX, Song WP, Wu K, Tao Y, Zhao GW (2003b) Effect of Ce^{3+} on spectral characteristic

of D1/D2/Cytb559 Complex from Spinach. Science in china. Ser.B 46:42–50

John G, Scandalios JG (1993) Oxygen stress and superoxide dismutase. Plant Physiol 101:7–12

Li ZG (1999) Plasma membrane permeability of plant. In: Tang ZC (ed) Experimental Guide of Modern Plant Physiology. Science Press, Beijing, pp 75, 302–303 (in Chinese)

Lin ZF, Li SS, Lin GZ, Guo JY (1988) Relation between H_2O_2 accumulation and membrane lipid peroxidation in aging leaves and chloroplasts. Acta Phytophysiol Sinica 14 (1):16–22 (in Chinese)

Liu C, Hong FS, Zheng L, Wang ZG, Tang P (2004) Effects of rare earth elements on vigor enhancement of aged spinach seeds. J Rare Earths 22(4):547–552

Liu C, Hong FS, Wu K, Ma HB, Zhang XG, Hong CJ, Wu C, Gao FQ, Yang F, Zheng L, Wang XF, Liu T, Xie YN, Xu JH, Li ZR (2006) Effect of Nd^{3+} ion on carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase of spinach. Biochem Biophys Res Commun 342(1):36–43 doi:10.1016/j.bbrc.2006.01.105

Lu SY, Li YC, Guo ZF, Li BS, Li MQ (1999) Enhancement of drought resistance of rice seedling by calcium. Chin J Rice Sci 13(3):161–164

Mulqueen P, Tingey JM, Horrocks WD Jr (1985) Characterization of lanthanide (III) ion binding to calmodulin using luminescence spectroscopy. Biochemistry 24:6639–6645 doi:10.1021/bi00344a051

Michaela SE, Roland H, Georg N (2002) Calcium deficiency — Influence on the antioxidative defense system in tomato plants. J Plant Physiol 159(7):733–742 doi:10.1078/0176-1617-0621

Ni JZ (2002) Rare Earth Bioinorganic Chemistry. Science Press, Beijing, pp 2–24 (in Chinese)

Prasad TK (1997) Role of catalase in inducing chilling tolerance in pre-emergent maize seedlings. Plant Physiol 114:1369–1376

Reuveni R, Shimoni M, Karchi Z, Kuc J (1992) Peroxidase activity as a biochemical marker for resistance of muskmelon *(Cucumis melo)* to seudoperno spora cubensis. Phytopathology 82:749–753 doi:10.1094/Phyto-82-749

Singh OS, Sharma KV (1972) Alterations in growth and metabolism of Potato plants by calcium deficiency. Plant Soil 36:363–369 doi:10.1007/BF01373490

Wang AG, Luo GH (1990) Relationship between superoxide free radicals of plant and hydroxyl-ammonia quantitative reaction. Plant Physiol Commun 26(6):55–59 (in Chinese)

Wang JS, Guo CR, Cheng YX (1997) Mechanism of cerium ion clearing superoxide radical. J The Chin Rare Earth Soc 15(2):151–154 (in Chinese)

Wang CX, Liu YL, Li FM, Wang ZJ, Liu XG, Peng A (2000) Restrain effect of rare earth nitrate on superoxide ion generation. J The Chin Rare Earth Soc 18(3):286–288 (in Chinese)

Wu WH (2003) Plant Physiology. Science Press, Beijing, p 93 (in Chinese)

Zhou XB, Wei YZ (2000) Effect of neodymium on membrane damage and peroxidation process of calcium absent cole. J The Chin Rare Earth Soc 18(1):63–66 (in Chinese)