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ORIGINAL ARTICLE

Ce^{3+} induces flavonoids accumulation by regulation of pigments, ions, chlorophyll fluorescence and antioxidant enzymes in suspension cells of *Ginkgo biloba* L.

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Abstract Rare earth elements, a type of abiotic elicitor, were used to investigate the shift in the mechanism of flavonoid accumulation from primary metabolites in Ginkgo suspension cell culture. The changes in flavonoids, mineral ions, photosynthetic pigments, chlorophyll fluorescence and antioxidant enzymes with varying Ce^{3+} ($Ce(NO_3)_3$) doses (0.01–5.0 mM) were studied. Low doses of Ce^{3+} (0.01–0.1 mM) improved cell growth and the highest increased dry weight was 2.67 g L^{-1} medium at 0.1 mM Ce^{3+} dose. Moderate doses (0.5–1.0 mM) limited cell growth and initiated a self-protective mechanism through modulation of the pigments, ions and flavonoid content, chlorophyll fluorescence and antioxidant enzymes, while high doses (2.0–5.0 mM) inhibited the cell growth and even caused cell mortality. The chlorophyll content, K^+, Zn^{2+} and growth mass peaked at 0.1 mM Ce^{3+}. The *Fv*/*Fm*, and *Fv*′/*Fm*′ values, and the amounts of P, Ca^{2+}, Fe^{2+} and Zn^{2+} reached a maximum at 0.5 mM Ce^{3+} while the flavonoids content was highest at 1.0 mM Ce^{3+} (11.1 mg L^{-1} medium). The antioxidant enzyme activities were high from 0.05 to 0.5 mM Ce^{3+}. Moreover the changes of chlorophyll fluorescence images of the suspension cells were consistent with the cell growth, relative electrical conductivity (REC) and malondialdehyde (MDA) content with increasing Ce^{3+} doses. Our findings suggest doses of 0.1–0.5 mM Ce^{3+} in Ginkgo cells shifted the metabolism from primary to secondary processes, diverting the metabolism from growth to defense related pathways as a result of the flavonoids accumulation. The high ratio of Car/Chl *t*, high Car content and 0.5–1.0 mM Ce^{3+} were beneficial for the accumulation of flavonol glycosides in Ginkgo suspension cells.

Keywords Flavonoids · Pigments · Chlorophyll fluorescence · Mineral ions · Ce^{3+} doses · Ginkgo suspension cell culture

Abbreviations

Chl	Chlorophyll
Chl *a*/*b*	Chlorophyll *a*/*b* ratio
Car	Carotenoid
Chl t/Car	Rate of total chlorophyll to Carotenoid
PSII	Photosystem II
F_v/F_m	Maximum PSII photochemical efficiency
Fv′/*Fm*′	Photochemical efficiency of PSII in the light
q^p	Photochemical quenching
NPQ	Non-photochemical quenching
NAA	Naphthaleneacetic acid
KT	Kinetin
REC	Relative electrical conductivity
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
POD	Guaiacol peroxidase
PAL	Phenylalanine ammonia-lyase
ROS	Reactive oxygen species

Introduction

Flavonoid biosynthesis is closely linked to environmental induction and stress, and is up-regulated in response to a wide range of stimulators including biotic and abiotic

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stresses (Agati et al. 2012). Abiotic elicitors include high salinity, drought stress, heavy metals, rare earth elements (REEs), ozone, and ultraviolet irradiation, etc. Methyl jasmonate (MJ), Salicylic acid (SA), KCl, NaCl, fungal endophytes and UV-B irradiation have been tested as inducers in Ginkgo cell culture for production of bilobalide, ginkgolides and flavonoids (Kang et al. 2009; Hao et al. 2009; Chen et al. 2014).

Rare earth elements (REEs) are generic terms representing 17 chemical elements which include 15 lanthanide series elements such as lanthanum (La), cerium (Ce), plus scandium (Sc) and yttrium (Y) (Wang et al. 2012). REEs have been applied as micro-fertilizers to improve plant growth and yield in China (Fitriyanto et al. 2011). Cerium and lanthanum are two major kinds of rare earth elements which have been used to induce the secondary metabolites production in plant cell culture as an elicitor (Table 1).

A few researchers have reported the positive or negative effects of REEs on plant physiological metabolism depending on the dosage, time, plant species, growth conditions, and specific REEs used (Xu and Chen 2011; Wu et al. 2001; Chu et al. 2014). The toxicity and damage of surplus REEs on plant growth may be as a result of REEs ions from the cell wall moving into the intracellular space through Ca channels, leading to a blockage of Ca^{2+} uptake (Liu et al. 2009). Therefore, REEs were recognized as Ca channel antagonists and are known to inhibit Ca^{2+}-dependent enzymes or other proteins (Shtangeeva and Ayrault 2007). Additionally, the phospholipase A2 activation involved in Ce^{4+}-induced taxol production was speculated to be associated with a JA-dependent signaling pathway. Furthermore, Ce^{4+} induced programmed cell death (PCD) in *Taxus cuspidate* cells was observed (Yang et al. 2008). In the Ce^{4+} induced cellular apoptosis signaling pathway and taxol biosynthesis, phospholipase D (PLD) and the rapid accumulation of PA may be required for the initial MAPK activity in *Taxus cuspidata* suspension cultures (Yang et al. 2009).

Despite the available literature focused on the yield and the mechanism of action of REEs on taxol biosynthesis in *Taxus* cell cultures, the underlying mechanisms of REEs on Ginkgo flavonoid metabolism has not been explored. Only the accumulation of flavonoids and growth in Ginkgo cell culture were studied, there is no data on the correlation of flavonoids and primary metabolism in Ginkgo cell culture under cerium induction. Therefore, an in-depth study into the mechanism of the shift between primary and flavonoid metabolism is needed in Ginkgo cell culture in the presence of cerium.

The aim of this work was to investigate the relationship of flavonol glycoside production with photosynthetic pigment, chlorophyll fluorescence, mineral content, and antioxidant systems in the presence of varying cerium nitrate doses in suspension Ginkgo cells. The results should help to improve our understanding of how the mechanism of REEs increases the accumulation of flavonol glycosides, and be beneficial for in-depth analysis of the biosynthetic pathways of flavonol glycosides in suspension cell culture.

Materials and methods

Suspension cell culture and cerium nitrate treatment

The cell lines were attained from young leaves of *Ginkgo biloba* L. cv., 'Da Fozhi', and subcultured by diluting 50 mL into 100 mL fresh medium in 250 ml flasks every 12 days in liquid MS medium (no agar) containing

Table 1 The effect of REEs on secondary metabolites produced in plant tissue and cell culture

Plant	In vitro culture types	REEs Types	REEs amount used	Metabolites	Yield Folds	Reference
Taxus cuspidate	Suspension cell	Ce^{4+}	1.0 mM	Taxol	3.5	Yuan et al. (2002a)
Tetrastigma hemsleyanum	Suspension cell	Ce^{3+}	0.1 mM	Flavonoids	1.5	Peng et al. (2013)
		Nd^{3+}	0.1 mM	Flavonoids	1.5	
Saussurea medusa	Callus	Ce^{3+}+ La^{3+}	0.05 mM	Flavonoids	2.0	Yuan et al. (2002b)
Cistanche deserticola	Suspension cell	La:Ce:Pr:Sm	255:175:3:1 (M/M)	Phenylethanoid glycosides	1.7	Ouyang et al. (2003)
Catharanthus roseus	Suspension cell	Ce^{3+}		Ajmalicine	11.0	Zhao et al. (2000)
			50 mg L^{-1}	Catharanthine	30.0	
		Nd^{3+}		Ajmalicine	10.8	
Crocus sativus	Callus	La^{3+} + Ce^{3+}	60 + 20 (μM)	Crocin	7.1	Chen et al. (2004)
Ginkgo biloba	Callus	Ce^{4+} or La^{3+}	0.1 mg L^{-1}	Terpene lactones	2.1	Cui et al. (2002)
Ginkgo biloba	Callus	La^{3+}	1.0 mg L^{-1}	Flavonoids	1.3	Chen (2005)

30 g L^{-1} sucrose, 1.0 mg L^{-1} NAA (Naphthaleneacetic acid) and 0.5 mg L^{-1} KT (Kinetin) on a rotary shaker at 100 rpm (Chen et al. 2014). Eight cerium nitrate ($Ce(NO_3)_3$, Sinopharm Chem. Reag. Co., Ltd, Shanghai, China) filter sterilized doses including 0 (control), 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 mM Ce^{3+} (all final concentrations) were added into the medium on the 6th day after inoculation, and the cells were harvested 7 days after the addition of cerium nitrate. All plant growth regulators were filter sterilized via a syringe filter (0.22 micrometer) then added into the autoclaved medium and the pH of the medium was adjusted to 5.8. Cultures were incubated at 25 ± 1 °C with a 14 h photoperiod using 55 μmol m^{-2} s^{-1} fluorescent illumination.

Cell growth analysis

Cell growth was primarily measured by cell fresh weight (FW) and dry weight (DW). Half of the fresh cells were weighed and stored at −70 °C for the active compound assay, and the other half was dried at 60 °C for 12 h until a constant weight (DW) was obtained for determination of flavonoids and mineral elements. The FW and DW were calculated as shown below:

$$\text{FW } (\Delta FW, \text{g L}^{-1}\text{medium}) = FW_1 - FW_2;$$

$$\text{DW } (\Delta DW, \text{g L}^{-1}\text{medium}) = DW_1 - DW_2$$

here, FW_1 (DW_1) is final fresh (dry) weight of cells; FW_2 (DW_2) is initial fresh (dry) weight of cells i.e. the weight before addition of cerium nitrate into culture medium.

Flavonoids contents and phenylalanine ammonia-lyase (PAL) activity

The flavonoid extraction was performed using the method of Hao et al. (2009). HPLC was performed according to Chen et al. (2014) on an Agilent 1200 HPLC (Agilent Inc., USA). The analytical column was a C18 column (ODS, 4.6 × 250 mm, 5.0 μm), while the injection volume was 10 μL. The mobile phase consisted of methanol and 0.4 % phosphoric acid in H_2O (55:45, v/v) with a flow rate of 1.0 mL/min. The column temperature was 30 °C and the wavelength was monitored at 360 nm.

The three flavonol glycosides (quercetin, kaempferol, isorhamnetin) (μg g^{-1} DW) contents and total flavonoids content (μg g^{-1} DW) or flavonoids yield (mg DW L^{-1} medium) were calculated as shown of Chen et al. (2014).

PAL (EC 4.3.1.5) activity was determined by the method of Koukol and Conn (1961). PAL activity was measured at 290 nm based on the production of cinnamic acid.

Photosynthetic pigment contents and imaging chlorophyll fluorescence

The chlorophyll (*a* and *b*) absorbance was measured at 645 and 663 nm and carotenoids were determined at 470 nm according to Lichtenthaler (1987). Chlorophyll fluorescence measurements were performed according to the method of Osorio et al. (2013) by using a chlorophyll fluorescence imager (CF Imager, Technologica, UK). The maximal quantum yield of PSII photochemistry (*Fv*/*Fm*), the photochemical efficiency of PSII in the light (*Fv*′/*Fm*′), the photochemical quenching coefficient q^P (*Fq*′/*Fv*′), and the non-photochemical quenching coefficient NPQ (*Fm*/*Fm*′−1) were calculated using the software supplied by the imager manufacturer.

Ion contents

According to the method of Maria et al. (2012), the dry sample (200 mg) was digested with 0.1 M HNO_3 in a closed microwave system. The concentration of ions was analyzed using an ICS-900 (Dionex, Sunnyvale, CA, USA).

Antioxidant enzymes, membrane permeability and lipid peroxidation

The enzyme extract method was performed by the method of (Chen et al. 2014). The Superoxide dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6) and POD (guaiacol peroxidase, EC1.11.1.7) activities were assayed according to the methods of Dong et al. (2010), respectively. One unit (U) of SOD was defined as inhibiting 50 % of NBT reduction at 560 nm. One unit (U) of CAT or POD was defined as 1 μmol or 1 nmol H_2O_2 decomposed in 1 min, respectively.

The relative electrical conductivity (REC) and the malondialdehyde (MDA) were measured according to Chen et al. (2014). The absorbance of the supernatant was monitored at 532 and 600 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA concentration.

Statistical analysis

Each treatment had three replications (flasks) within each experiment and the independent experiment repeated two times. The analytical assays data were collected from six replicates (n = n = 3). The data were expressed as mean values ± SD (standard deviation). The data were analyzed using one-way ANOVA and the data generated used SPSS version 17.0 (SPSS Inc., IL, USA) analysis and Duncan's multiple-range test (DMRT). Associations were also

examined by simple correlation analysis using SPSS version 17.0.

Results

Cell growth response to varying Ce^{3+} doses

Figure 1 shows the effects of Ce^{3+} doses on cell growth after adding cerium nitrate into the medium for the 7th day. We select this day to perform all experiments, since it is well-known that a high total flavonoids production happens at approximately 1 week after elicitors' supplementation (Peng and He 2013; Wang et al. 2015; Hao et al. 2009). The same tendency were found for the cell ΔFW and ΔDW after adding Ce^{3+}, while cell growth (ΔFW and ΔDW) was significantly enhanced from 0.01 to 0.1 mM Ce^{3+} and gradually decreased from 0.5–5.0 mM Ce^{3+} treatments compared with the control. The ΔFW at Ce^{3+} doses <2.0 mM and ΔDW at Ce^{3+} doses ≤ 2.0 mM were lower than the control values and showed the suspension cell growth was limited. The most significant increases in cell ΔFW and ΔDW were observed at 0.1 mM, at 87.5 and 100 % higher than those of the control cells, respectively.

Flavonoid content and PAL activity responses to varying Ce^{3+} doses

The effects on Ginkgo suspension cells subjected to elevated levels of Ce^{3+} on the total amounts of flavonoids, three flavonol glycosides (quercetin, kaempferol and isorhamnetin) and PAL were determined and are shown in Fig. 2. The concentration of these components increased as the Ce^{3+} dose significantly increased from 0.01 to 1.0 mM, then decreased from 2.0 to 5.0 mM, but all Ce^{3+} treatments induced flavonoid accumulation more than that of the control (Fig. 2a–d). The maximum values observed at 1.0 mM Ce^{3+} were 1.04, 2.76, 1.97, and 2.06 folds (1.49 mg g^{-1} DW) higher for quercetin, kaempferol, isorhamnetin and total flavonoids content compared with the control ($p < 0.05$), respectively. The next highest amounts of these were found at 0.5 mM Ce^{3+} treatment (Fig. 2a–d). By comparison, flavonoids yield were only increased by 0.39 fold or 0.09 fold at 5.0 mM Ce^{3+} treatment, respectively (Fig. 2e). The PAL activity showed a similar trend as the flavonoids yields, with the highest activity observed at 0.5 mM Ce^{3+} treatment (Fig. 2f).

Photosynthetic pigment content response to varying Ce^{3+}doses

Suspension cell chlorophyll concentrations (Chl *a*, Chl *b*, Chl *t* (Chl *t* = Chl *a* + Chl *b*), Chl *a/b*, carotenoids (Car) and Car/Chl *t* are shown in Fig. 3. Exposure to 0.01–0.1 mM Ce^{3+} caused an increase in the contents of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*), and total chlorophyll (Chl *t*) while a gradual decrease was observed for treatments at 0.5–5.0 mM Ce^{3+}. The Chl *b* content was particularly reduced at these Ce^{3+} levels compared with the control. The maximum values of Chl *t* was observed at 0.1 and 5.0 mM Ce^{3+} at 34.1 and 147.6 % higher than that of the control, respectively ($p < 0.05$). There was no significant difference in the Chl *t*, Chl *a*, and Chl *b* content between 0.05 and 0.1 mM Ce^{3+} treatments ($p > 0.05$) (Fig. 3a–c). In contrast, little change in the carotenoid (Car) content was observed at 0.01 and 0.1 mM, while a significant enhancement was observed at 0.5–2.0 mM Ce^{3+} treatment compared with the control, and the peak value

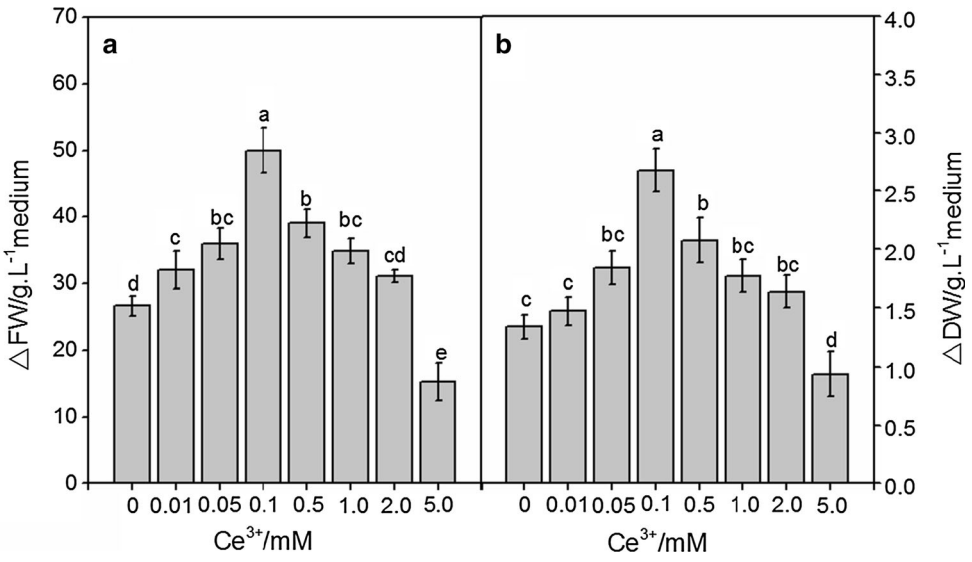

Fig. 1 Effects of Ce^{3+}doses on Ginkgo suspension cell of ΔFW and ΔDW (in 1L media) subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (standard deviation, n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.05$) among treatments according to the Duncan's multiple range test

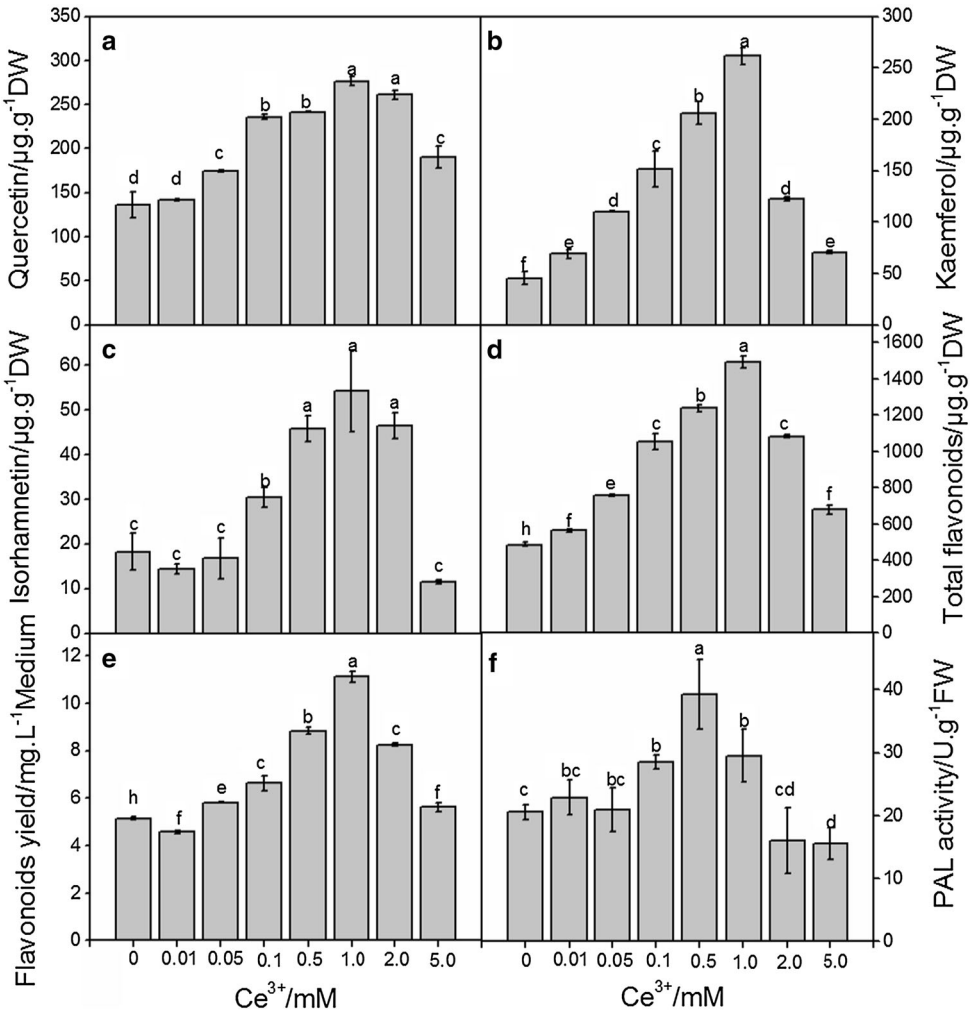

Fig. 2 Quercetin, kaemferol, isorhamnetin (**a**–**c**) and total flavonoids content (**d**) and flavonoids yield (**e**) and PAL activity (**f**) in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (standard deviation, n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.01$) among treatments according to the Duncan's multiple range test

was examined at 0.5 mM Ce^{3+}. No significant difference in Car content was observed between 0.01 and 5.0 mM Ce^{3+} treatment ($p > 0.05$) (Fig. 3e).

The Chl *a*/*b* and Car/Chl *t* ratios both decreased in the presence of 0.01–0.1 mM Ce^{3+} and were lower than the control, while they significantly increased from 0.5 to 2.0 mM Ce^{3+}, especially at 0.5 mM Ce^{3+}. The maximum ratios of Chl *a*/*b* occurred at 0.5 mM Ce^{3+} where it was 183.1 % of the control, and at 5.0 mM Ce^{3+} treatment where it was 107.4 % of the control (Fig. 3d), while the Car/Chl *t* ratio at 0.5 mM was 145.4 % of the control ($p < 0.05$) (Fig. 3f). There were no significant differences among the 0.01–0.1 mM Ce^{3+} treatments and between the 0.5–2.0 mM Ce^{3+} treatments for Car/Chl *t* ratios.

Chlorophyll fluorescence response to varying Ce^{3+} doses

The maximum quantum yield of photosystem-II (*Fv*/*Fm*), the photochemical efficiency of PSII in the light (*Fv*′/*Fm*′), the non-photochemical quenching coefficient (q^P) and the non-photochemical quenching (NPQ) were monitored by using a chlorophyll fluorescence imager (Fig. 4). Similar trends in the *Fv*/*Fm* and *Fv*′/*Fm*′ values were observed (Fig. 4a, b). At doses of $Ce^{3+} \leq 0.1$ mM, both *Fv*/*Fm* and *Fv*′/*Fm*′ were higher than the control but were not significantly different between them ($p > 0.05$), however at doses of over 0.1 mM Ce^{3+}, these values began to decrease and became lower than the control. For both parameters, the highest values were obtained at 0.1 mM Ce^{3+} treatment. In contrast, the lowest values were obtained at 5.0 mM Ce^{3+} treatment, where the *Fv*/*Fm* value decreased by 32.9 % and the *Fv*′/*Fm*′ value decreased by 41.1 % compared with the control, respectively. Conversely, the q^P value was not significantly different between 0.01 and 0.1 mM Ce^{3+} compared with the control, while it was significantly increased from 0.5 to 5.0 mM Ce^{3+}. The highest q^P value was obtained at 5.0 mM Ce^{3+} where it was increased by 34.2 % compared with the control (Fig. 4c). The same trend was observed for the NPQ value from 0.01 to 0.1 mM

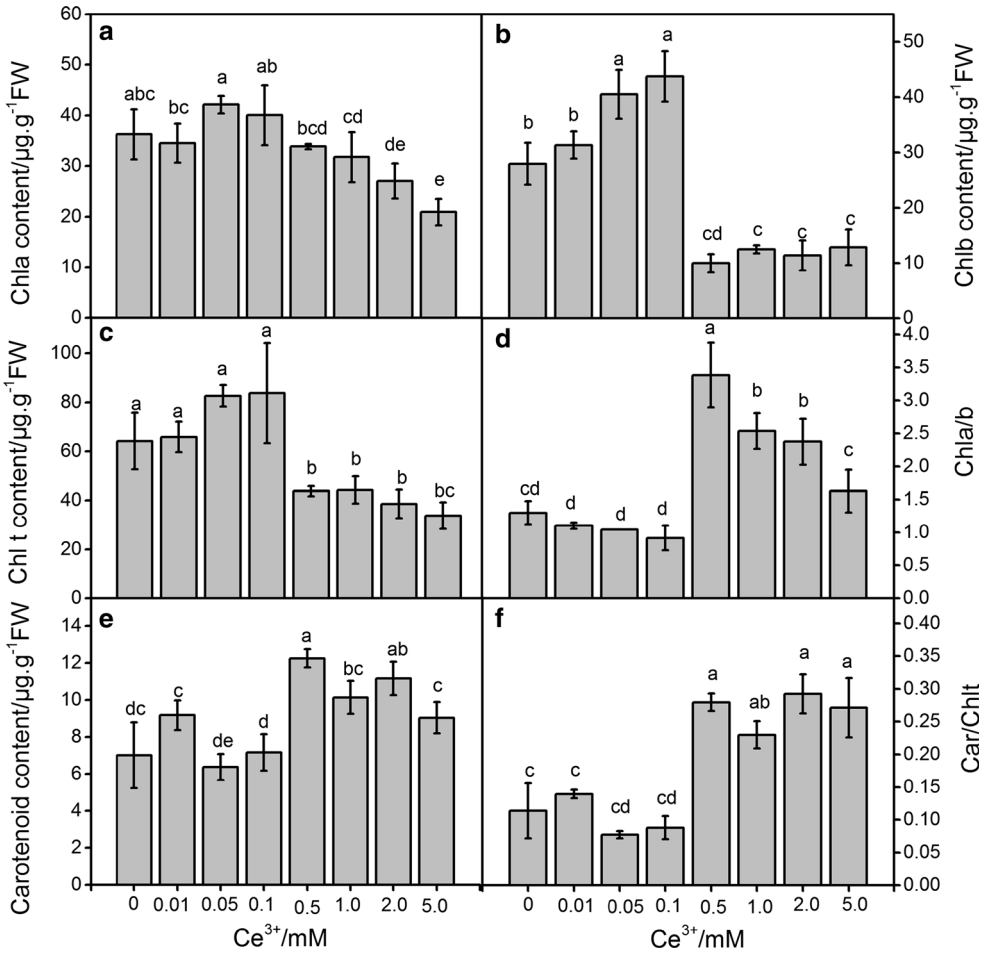

Fig. 3 The chlorophyll *a* and *b*, total chlorophyll content *Chl t* (*Chl t* = *Chl a* + *Chl b*) (**a**–**c**), ratio *Chl a/b* (**d**), carotenoid content (*Car*) (**e**) and ratio of carotenoid/total chlorophyll (*Car*/*Chl t*) (**f**) in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.05$) among treatments according to the Duncan's multiple range test

Ce^{3+} although no significant difference was found compared with the control ($p > 0.05$). However, the NPQ value was higher (54.8 %) than the control at 0.1–0.5 mM Ce^{3+} (Fig. 4d).

No significant difference were observed in the color of the cell *Fv*/*Fm* and *Fv*′/*Fm*′ images at 0.01 M $Ce(NO_3)_3$ treatment compared with the control, however the color of these two images from 0.5 to 1.0 mM $Ce(NO_3)_3$ were deeper in red than the control (Fig. 5). The color of the *Fv*′/*Fm*′ images at 5.0 mM $Ce(NO_3)_3$ was observed to contain only a small amount of red area and a large amount of blue area (Fig. 5). These changes were in accordance with the cell color changes (Fig. 6).

Ion content response to varying Ce^{3+} doses

The cerium dose effects on the absorption of mineral elements in the suspension cell medium after treatment are shown in Fig. 7. Potassium (K) absorption was substantially higher in the suspension cells exposed to between 0.01 and 0.5 mM Ce^{3+} comparison with the control. No differences were observed between the control and 5.0 Ce^{3+} dose ($p > 0.05$). The highest absorption of K was found at 0.1 mM Ce^{3+} (Fig. 7a). Phosphorus (P) absorption was also higher than the control for all Ce^{3+} treatments. At 0.5 mM Ce^{3+}, the absorption of P was 134.8 % more than the control. There was no significant difference with the control between the Ce^{3+} treatments except at 0.5 mM (Fig. 7b). The highest level of Mg ion was observed at 0.1 mM Ce^{3+} treatment and the lowest level was at 5.0 mM treatment ($p < 0.05$), while no significant differences were found at other Ce^{3+} treatments, compared with the control ($p > 0.05$) (Fig. 7c). The calcium (Ca) content were significantly reduced and lower than that of the control under the Ce^{3+} treatments except 0.1 and 0.5 mM. The 0.1 and 0.5 mM Ce^{3+} treatments could maintained the Ca level in Ginkgo cells and no significant differences were found between 0.1 and 0.5 mM and control treatments ($p > 0.05$) (Fig. 7d).

As shown in Fig. 7e–g, the Ce^{3+} doses differentially affected the accumulation of microelements (Fe, Zn, Cu) in Ginkgo suspension cells. In this study, Ce^{3+} treatment increased Zinc (Zn) accumulation from 0.05 to 1.0 mM while no significant difference was noted for the other

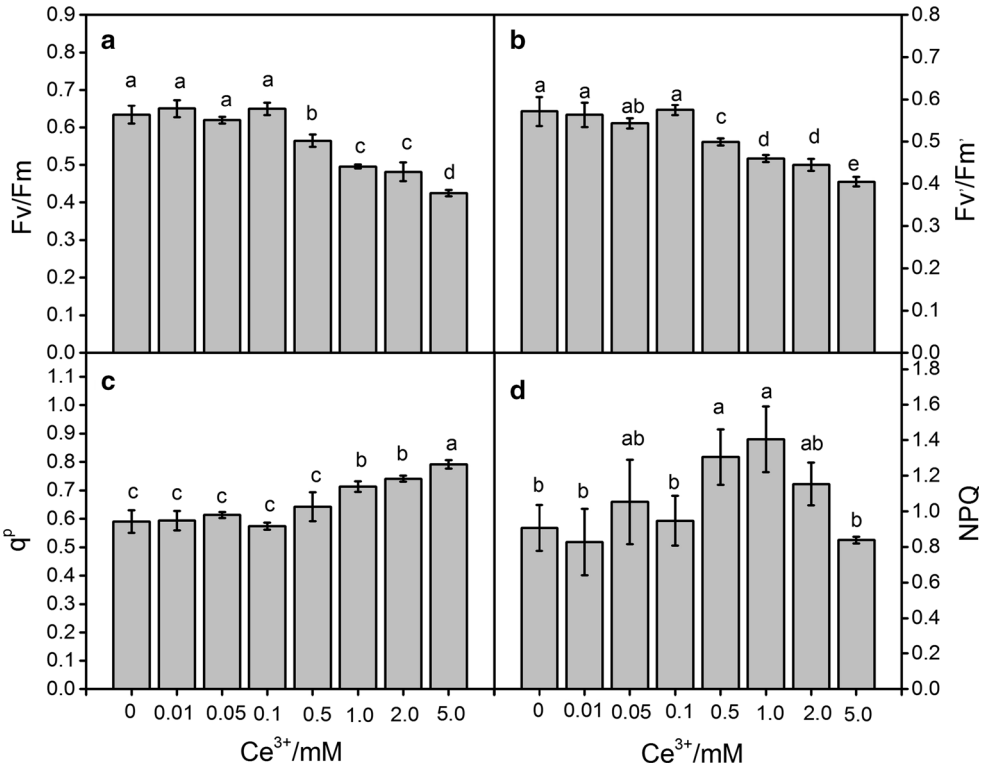

Fig. 4 Chlorophyll fluorescence images of maximum PSII photochemical efficiency (*Fv/Fm*) (**a**), photochemical efficiency of PSII in the light (*Fv′/Fm′*) (**b**), photochemical quenching (q^p) (**c**), and non-photochemical quenching (NPQ) (**d**) in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.01$) among treatments according to the Duncan's multiple range test

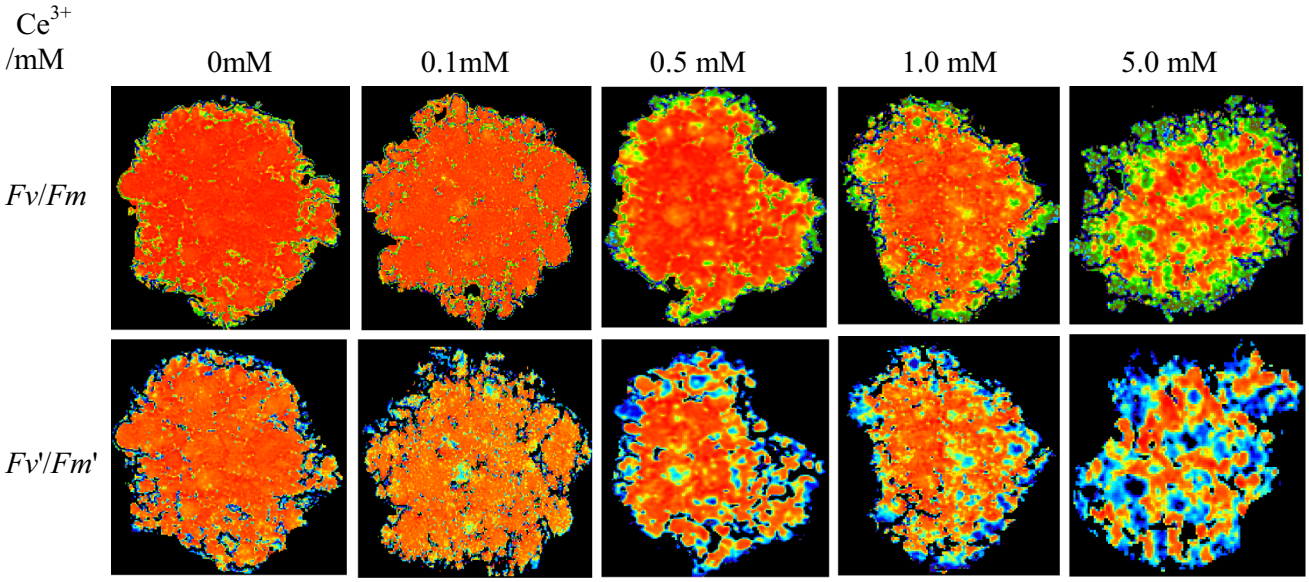

Fig. 5 Chlorophyll fluorescence images of *Ginkgo* suspension cells subjected to cerium nitrate elicitation for 7 days

treatments compared with the control. Interestingly, at a low Ce^{3+} dose (0.01 mM), the Zn content in the cells was similar to the control and as the high Ce^{3+} doses (2.0–5.0 mM). There was a significant increase of Zn at 0.1 mM (104.6 %) and 0.5 mM (102.4 %) Ce^{3+} in the suspension cells compared with the control (Fig. 7e). Fe accumulation was significantly reduced in the cells ($p < 0.05$) at all Ce^{3+} dose treatments. The lowest reduction of Fe content was found at 0.5 mM Ce^{3+} (only 21.1 % of Fe was reduced), whereas reduction of Fe was 70.8 % at 5.0 mM Ce^{3+} compared with the control (Fig. 7f). Similar results were observed for copper (Cu). The Ce^{3+} treatments

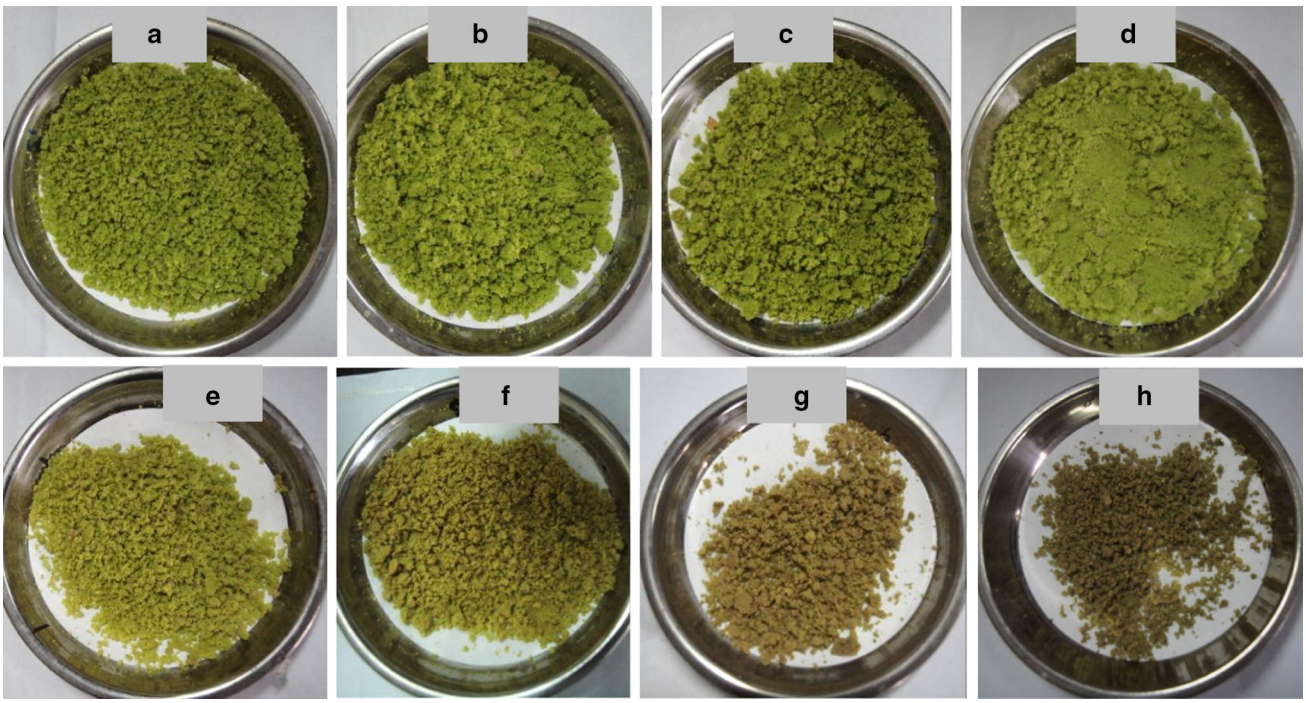

Fig. 6 *Ginkgo biloba* L. suspension cells subjected to cerium nitrate elicitation for 7 days. *Note* **a–h**: 0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 mM $Ce(NO_3)_3$

reduced Cu accumulation in this study. However, there was no significant difference between 0.1, 0.5, 1.0 mM Ce^{3+} treatments and the control. Cu accumulation only decreased by 17.6 % at 0.1 mM Ce^{3+} treatment whereas it was reduced by 74.5 % at 5.0 mM Ce^{3+} treatment (Fig. 7g). The percent of increased or decreased of mineral element contents compared to control was appeared in Table 2.

Response of antioxidant enzyme activities to varying Ce^{3+} doses

The activities of the major ROS (reactive oxygen species) scavenging enzymes, the antioxidant enzymes SOD, POD and CAT, after Ce^{3+} treatment are shown in Fig. 8. An increase in SOD activity was observed from 0.05 to 0.5 mM Ce^{3+} doses in Ginkgo cells while no significant changes were observed for other Ce^{3+} treatments ($p > 0.05$) (Fig. 8a). However, the CAT activities of the suspension cells for 0.1,1.0 and 2.0 mM Ce^{3+} dose treatments were significantly higher than those of the control. The cells exposed to 0.5 mM Ce^{3+} treatment showed an enhancement of over 94 % in CAT activity (Fig. 8b). In contrast, the POD activity significantly increased from 0.05 to 5.0 mM Ce^{3+} in Ginkgo suspension cells where the highest increase over the control was 139 % at 0.05 mM Ce^{3+}. No change in the POD activity was observed at a low level (0.01 mM) of Ce^{3+} (Fig. 8c).

Membrane potential, lipid peroxidation response to varying Ce^{3+}doses

Application of varying Ce^{3+} doses to Ginkgo suspension cells significantly enhanced the membrane permeability and the membrane deterioration as reflected by the increased relative conductivity (REC) and MDA content. As shown in Fig. 9a, the REC of the Ginkgo cells did not significantly increase in the presence of 0.01–0.5 mM Ce^{3+} compared with the control. However the REC sharply increased in the presence of 1.0–5.0 mM Ce^{3+} where it was 291 % higher than the control at 5.0 mM Ce^{3+}. Ce^{3+} treatment also caused an increase in the MDA content in suspension cells, however, the magnitude of the increase was slightly higher at doses from 0.01 to 0.5 mM while it sharply increased at doses from 1.0 to 5.0 mM. The MDA content at 5.0 mM Ce^{3+} treatment was 51.7 % larger than the control (Fig. 9b).

Discussion

Our present research showed varying Ce^{3+} dose treatments significantly changed suspension cell growth, chlorophyll content, chlorophyll fluorescence, ion content and flavonol glycosides in *Ginkgo biloba* L. Low doses (0.01–0.1 mM) had a positive effect and improved cell growth. The maximum increase of dry and fresh weight

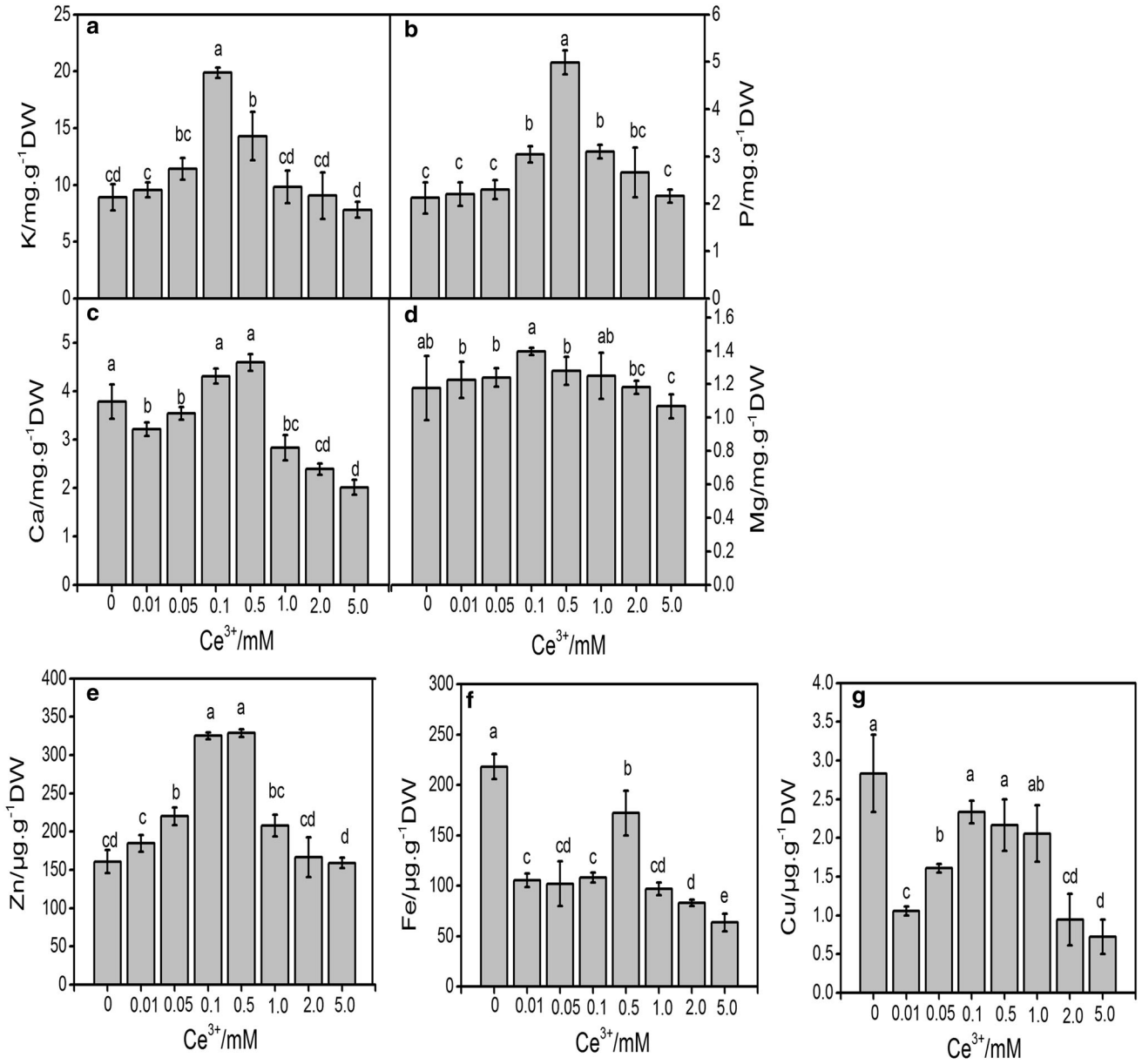

Fig. 7 Mineral elements (K, P, Ca, Mg, Zn, Fe, Cu) (**a**–**g**) in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.05$) among treatments according to the Duncan's multiple range test

was found at 0.1 mM Ce treatment. The moderate dose (0.5–1.0 mM) treatments limited the cell growth, while the high dose (2.0–5.0) mM treatments inhibited the cell growth or even caused cell mortality especially at 5.0 mM (Fig. 1). These findings were verified by the photos of the suspension cells (Fig. 6), and images of the chlorophyll fluorescence (Fig. 5) to Ce^{3+} treatment. Similar results have also been obtained by others (Xu and Chen 2011; Huang et al. 2010; dosSantos et al. 2014). These results indicated the positive or negative effects of REEs on suspension cell physiological metabolism mainly depended on the dosage of Ce^{3+}. In this work, treatment with less than 0.5 mM Ce^{3+} had positive effects on Ginkgo suspension cell growth.

REEs such as La^{3+}, Ce^{3+}, Nd^{3+}, and Y^{3+} have been used to stimulate taxol, flavonoid, crocin, and catharanthine production in *Taxus, Tetrastigma hemsleyanum, Saussurea medusa, Crocus sativus* cells and callus (Table 1). In the present work, moderate doses of Ce^{3+} (0.5–1.0 mM) significantly increased the accumulation of total flavonoids, and three flavonol glycosides in the Ginkgo suspension cells, whereas the low or high doses (such as 0.01–0.05 and

Table 2 The effect of Ce^{3+} doses on mineral element levels in *Ginkgo biloba* suspension cell culture

Ce^{3+}/mM	K	P	Ca	Mg	Fe	Zn	Cu
0.00	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.01	7.5	3.8	−25.9	4.2	−51.7	14.8	−62.7
0.05	28.4	8.2	−18.4	5.5	−53.2	36.9	−43.2
0.1	123.6	43.5	−0.6	18.7	−50.4	102.4	−17.6
0.5	60.7	134.8	5.9	8.8	−21.1	104.6	−23.5
1.0	110.5	46.0	−34.8	6.3	−55.6	29.3	−27.5
2.0	1.9	25.2	−44.9	0.4	−61.9	3.5	−66.7
5.0	−12.2	1.8	−53.6	−9.3	−70.8	−1.1	−74.5

The date in this table means the percent of increased or decreased of one mineral element content after adding Ce^{3+} to medium after 7 days compared to control

That is increased or decreased (%) = (The content of Ce^{3+} treatment − the content of control) × 100/the content of control

5.0 mM) resulted in no obvious increases in the above mentioned compounds compared with the control. PAL is a key branch point enzyme which regulates phenylalanine biosynthesis into phenolic compounds (Dong et al. 2010). The maximum PAL activity was also found at moderate doses of Ce^{3+} (0.5–1.0 mM) and was consistent with the accumulation of flavonoids in Ginkgo suspension cells. These results indicated that stimulation with Ce^{3+} switched primary metabolism to secondary metabolism and doses in the range of 0.1–0.5 mM Ce^{3+} may initiate a shift from primary to secondary metabolism in Ginkgo cells.

Appropriate doses of Ce increases the chloroplast pigment content, and maintain the structure of the chloroplast, while a high dose of Ce reduces these parameters (Wang et al. 2012; Maksimović et al. 2014). A similar result was found in the present work where 0.01–0.1 mM Ce^{3+} treatments gradually increased the chlorophyll *a* and *b*, while doses higher than 0.1 mM Ce^{3+} (0.5–5.0 mM) gradually decreased the content of these pigments. Unlike the changes observed for chlorophyll, significant increases in carotenoids were found at 0.5–2.0 mM Ce^{3+} treatments. Moreover, the Chl *a*/*b* ratio increased about one fold in the presence of 0.5–5.0 mM Ce^{3+} and the Car/Chl *t* ratio showed the same trend, where at higher doses (0.5–5.0 mM Ce^{3+}), the ratio increased about two folds compared to that at lower (0.01–0.1 mM Ce^{3+}). These results suggest that higher doses of Ce (0.5–5.0 mM) primarily disturbed Chl *b* biosynthesis while it stimulated the biosynthesis of Car. Furthermore, the high ratio of Car/Chl *t* and high Car content were beneficial to the accumulation of flavonol glycosides (Fig. 3). These results were strongly supported by the chlorophyll fluorescence results and similar to the results of Osorio et al. (2013).

Chlorophyll fluorescence has been used as a sensitive tool to diagnose the damage caused by environmental stresses (Zhang et al. 2014). Imaging of chlorophyll fluorescence provides an ideal screening platform which allows multiple plants and sample heterogeneity to be monitored under identical conditions at the same time (Murchie and Lawson 2013). In present work, the effect of Ce on chlorophyll fluorescence parameters in Ginkgo cells was dependent on the Ce dose. When the Ce^{3+} doses were greater than 0.1 mM (0.5–5.0), the *Fv*/*Fm* and *Fv*′/*Fm*′ ratios decreased while the q^P and NPQ (0.5–1.0 mM) values increased in Ginkgo suspension cells (Fig. 4). The *Fv*/*Fm* ratio is thought to represent the electron capacity captured in the PSII reaction center. The decrease in the

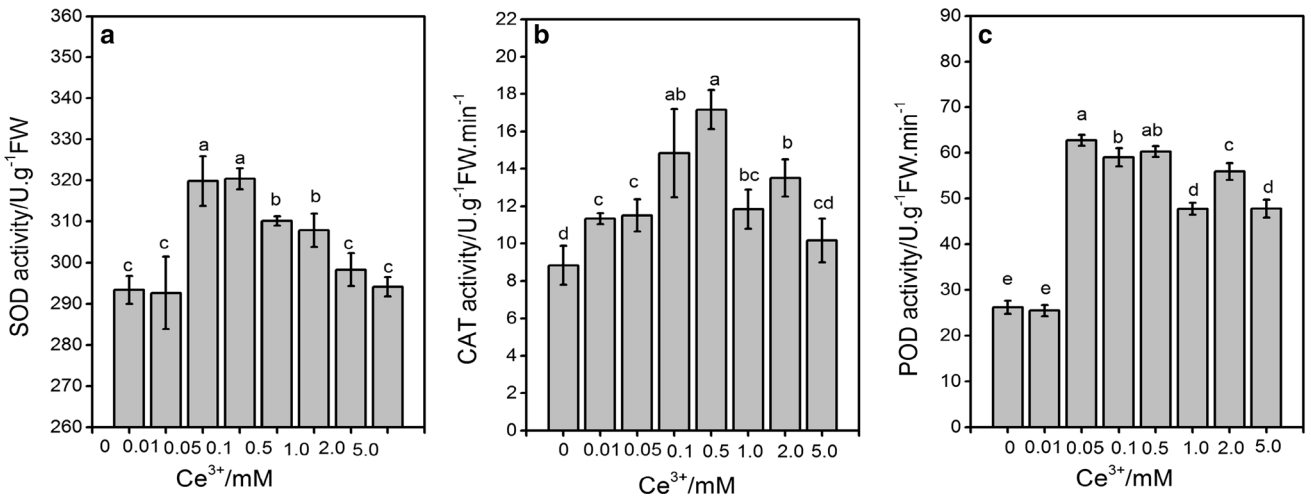

Fig. 8 The antioxidant enzymes of SOD (**a**), CAT (**b**) and POD (**c**) activities in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.05$) among treatments according to the Duncan's multiple range test

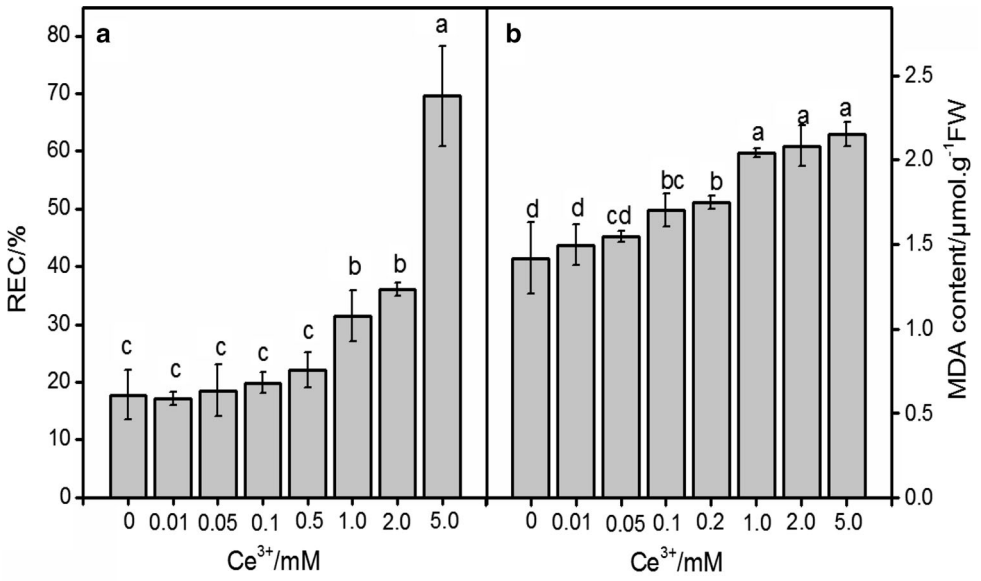

Fig. 9 The relative conductivity (REC) (**a**) and MDA (**b**) content in Ginkgo suspension cells subjected to cerium nitrate elicitation for 7 days. Values are mean ± SD (n = 3). Means followed by different letters above the *bars* indicate significant differences ($p < 0.01$) among treatments according to the Duncan's multiple range test

Fv/*Fm* and *Fv*′/*Fm*′ ratios may work to protect the PSII and the integrity of the thylakoid membrane in the presence of high doses of Ce. The increases of the q^P and NPQ values may be caused by carotenoid accumulation from moderate doses of Ce treatments, while xanthophylls play a critical part in the NPQ value (Adams III et al. 1999). These results indicated Ginkgo suspension cells contained a self photoprotective regulatory mechanism at moderate doses of Ce (0.5–1.0 mM). These results were also in agreement with the findings of chlorophyll fluorescence in rice (Zhang et al. 2014; Chen et al. 2014).

Low dose of REEs (Ce and La) could increase the uptake of mineral elements, and be beneficial to photosynthesis in plants, while high doses of REEs could limit their uptake (Peralta-Videa et al. 2014). Interestingly, we found the accumulation of K and P was significant increased at 0.1 and 0.5 mM Ce^{3+} treatments, respectively, while the other Ce dose treatments did not significantly promote the accumulation of these elements compared with the control (Fig. 7a, b). These findings were similar to the work of Corral-Diaz et al. (2014). The increase of K uptake was believed to have been brought about because REEs stimulated the synthesis of Abscisic acid (ABA) which then increases the uptake of K (Zhao et al. 2014). These results suggested the effect of Ce on these mineral nutrients required a dose threshold, and lower or higher doses of Ce treatment had no significant effects on the accumulation of these elements.

Mg, Ca, Zn, Fe and Cu are fundamental in the photosynthesis process and the biosynthesis of pigments. Hong et al. (2005) found REEs were mainly bound to chlorophyll and located in the chloroplast membrane as well as in the thylakoid. REEs (Ce^{3+} or La^{3+}) can enter the chlorophyll and replace the Mg^{2+} ion, then form Ce-chlorophyll or La-chlorophyll in the spinach chlorophyll molecule under conditions deficient in Mg (Ze et al. 2009). However, the accumulation of Mg was not significantly dependent on the dose of Ce in Ginkgo cells compared with the control in this work. It seems probable that Ce^{3+} may act as a substitute for Mg^{2+} only under conditions of Mg deficiency in cells (Fig. 7d).

Calcium is a secondary messenger that relates to biotic and abiotic environmental stressors, hormone biosynthesis, and development in plants (Peralta-Videa et al. 2014). It was known that Ce could fully displace Ca because the ionic radius of Ce was similar to that of Ca in biological systems (Paoli et al. 2014). Calcium accumulation was significantly reduced in Ginkgo cells in the presence of varying Ce^{3+} doses except from 0.1 to 0.5 mM ($p < 0.05$) (Fig. 7c). Thus, improper doses of $Ce(NO_3)_3$ might interfere with the function of Ca channels leading to a blockage of Ca^{2+} uptake in Ginkgo cells.

Fe and Cu accumulations were significantly reduced in Ginkgo cells at all Ce doses (Fig. 7f, g), while Zn accumulation was significantly increased in the presence of 0.01–2.0 mM Ce^{3+} (Fig. 7e). Iron is utilized in electron transport or stored complexed with ferritin and copper ion is an element of plastocyanin in chloroplasts (Grusak et al. 1999). However, there is an ion antagonism between zinc ion and the Fe or Cu ion, and an increase of Zn^{2+} might displace Fe^{2+} or Cu $^{2+}$ from their binding sites (Corral-Diaz et al. 2014), therefore causing decreases in the accumulation of Fe and Cu. Additionally, low concentrations of Fe and Cu compared with the control could also indicate that these elements are bound to Ce and stuck on the cell wall (Zhao et al. 2014). However, the chlorophyll

Table 3 The correlation between total flavonoids content and other parameters under varying Ce^{3+} doses treatment (Pearson Correlation)

	K	P	Ca	Mg	Fe	Zn	Cu	Chla	Chlb	Chlt	**Car**	**Car/Chlt**	**TF**	PAL	SOD	POD	CAT	REC	MDA	*Fv/Fm*	*Fv'/Fm'*	q^p	NPQ	ΔGW
	0.762**																							
Ca	0.183	0.202																						
Mg	0.535**	0.493**	0.262																					
Fe	0.020	0.119	0.594**	0.140																				
Zn	0.756**	0.874**	0.291	0.465*	0.167																			
Cu	0.374*	0.260	0.697**	0.452*	0.626**	0.445*																		
Chl*a*	0.515**	0.543**	0.201	0.551**	0.362*	0.403*	0.577**																	
Chl*b*	0.464*	0.321	-0.316	0.352*	0.073	0.198	0.247	0.741**																
Chl*t*	0.520**	0.424*	-0.115	0.519**	0.193	0.282	0.370*	0.874**	0.950**															
Car	-0.064	0.065	0.284	0.015	0-.112	0.098	-0.163	-0.358*	-0.757**	-0.641**														
Car/Chlt	**-0.320**	**-0.228**	**0.143**	**-0.284**	**-0.249**	**-0.101**	**-0.364***	**-0.755****	**-0.942****	**-0.916****	**0.834****													
TF	**0.298**	**0.382***	**0.428***	**0.227**	**-0.186**	**0.453***	**0.128**	**-0.062**	**-0.425***	**-0.304**	**0.523****	**0.448***												
PAL	**0.538****	**0.698****	**0.590****	**0.347***	**0.312**	**0.732****	**0.439***	**0.256**	**-0.110**	**0.019**	**0.394***	**0.103**	**0.616****											
SOD	0.633**	0.674**	0.062	0.377*	-0.076	0.668**	0.306	0.557**	0.456*	0.516**	-0.213	**-0.365***	**0.448***	**0.432***										
POD	0.445*	0.476**	-0.041	0.165	-0.324	0.545**	-0.042	0.084	-0.041	-0.002	0.162	**0.200**	**0.587****	**0.304**	0.729**									
CAT	0.559**	0.767**	0.300	0.260	0.029	0.772**	0.161	0.210	-0.132	-0.008	0.456*	**0.228**	**0.706****	**0.746****	0.539**	0.576**								
REC	-0.365*	-0.515**	-0.289	-0.311	-0.524**	-0.352*	-0.523**	-0.795**	-0.516**	-0.622**	0.192	**0.571****	**0.004**	**-0.362***	-0.325	0.113	-0.260							
MDA	-0.222	-0.297	-0.119	-0.238	-0.545**	-0.142	-0.456*	-0.711**	-0.645**	-0.678**	0.445*	**0.709****	**0.517****	**-0.089**	-0.065	0.387*	0.147	0.750**						
Fv/Fm	0.442*	0.469*	0.028	0.349*	0.494**	0.296	0.436*	0.787**	0.795**	0.820**	-0.491**	**-0.821****	**-0.352***	**0.217**	0.271	-0.251	0.069	-0.844**	-0.860**					
Fv'/Fm'	0.424*	0.422*	0.069	0.381*	0.542**	0.276	0.459*	0.752**	0.790**	0.813**	-0.562**	**-0.843****	**-0.356***	**0.182**	0.240	-0.279	0.011	-0.809**	-0.841**	0.981**				
q^p	-0.506**	-0.528**	-0.146	-0.341	-0.497**	-0.358*	-0.446*	-0.726**	-0.731**	-0.754**	0.472**	**0.763****	**0.266**	**-0.287**	-0.310	0.202	-0.086	0.825**	0.827**	-0.950**	-0.951**			
ΔGW	**0.422***	**0.720****	**0.475***	**0.307**	**0.299**	**0.637****	**0.280**	**0.193**	**0.609****	**0.328**	**-0.168**	**0.009**	**0.473***	**0.494***	**0.745****	**0.523****	**-0.107**	**-0.491***	**-0.107**	**0.129**	**-0.249**	**0.597****	**0.794****	
ΔFW	**0.600****	**0.846****	**0.446***	**0.424***	**0.313**	**0.753****	**0.407***	**-0.070**	**0.559****	**0.537****	**0.101**	**0.263**	**0.299**	**0.610****	**0.789****	**0.451***	**-0.274**	**-0.667****	**-0.204**	**0.374**	**-0.480***	**0.504***	**0.832****	**0.937****

* means significant difference at $p < 0.05$ level and ** means significant difference at $p < 0.01$ level. TF: total flavonoids content

and carotenoid amounts did not decrease with a reduction in the Fe^{2+} or Cu^{2+} concentration in Ginkgo cells from 0.01 to 0.5 mM $Ce(NO_3)_3$ treatment, likely as a result of an increasing Zn^{2+} concentration.

High dose of REEs may cause the generation of ROS and lead to oxidative stress (Rico et al. 2013) in plant cells. Antioxidant enzymes (SOD, CAT, POD) play an important role in the defense mechanisms of plants. SOD dismutates $O_2^{\cdot -}$ to H_2O_2 while CAT and POD catalyze the conversion of H_2O_2 to H_2O (Tian et al. 2015; Chen et al. 2014). In the present work, the 0.05–0.1, 0.1–2.0 and 0.05–5.0 mM Ce^{3+} doses were all observed to increase the activities of SOD, CAT, and POD in Ginkgo cells relative to the control, respectively. This indicated the doses of 0.05–0.5 mM Ce^{3+} were the sensitivity and key levels, in these levels Ginkgo cells maybe divert their metabolism from growth to defense by higher antioxidant enzymes activities. These results were also in agreement with the work of others (Corral-Diaz et al. 2014; Rico et al. 2013).

The lipid peroxidation (MDA) and electrolyte leakage (REC) were greatly enhanced at 1.0–5.0 mM Ce^{3+} doses in Ginkgo cells. The results suggest that the decreased activities of SOD, and CAT at higher Ce doses (>1.0 mM) were inefficient to remove $O_2^{\cdot -}$ and H_2O_2 leading to enhanced membrane damage. Furthermore, the higher the concentration of Ce treatment, the less effective the enzymes were at removing ROS and the greater the damage to the cells.

Additionally, the associations between the total flavonoid content and the P, Ca^{2+}, Zn^{2+}, and Car amounts, and the Car/Chl *t* ratio, the SOD, CAT, POD, PAL activities, ΔGW were significantly positive ($p < 0.01$ or 0.05), but were negatively correlated with Chl *b*, *Fv*/*Fm*, *Fv*′/*Fm*′ respectively. However, the correlation of Car/Chl *t* with flavonoid, Car, and REC, MDA, q^p was significantly positive ($p < 0.01$ or 0.05) (Table 3) while its correlation with Cu, Chl *a*, Chl *b*, total Chl *t* content, *Fv*/*Fm*, and *Fv*′/*Fm*′ is negative, respectively. Importantly, PAL activity was positively correlation with six mineral elements content (not including Fe), Car, total flavonoids, SOD, CAT, ΔGW, and ΔFW. While ΔGW was positively correlation with K, P, Ca, Zn, Chl *b*, total flavonoids, SOD, POD, q^p, NPQ (Table 3). These correlations suggest that flavonoid biosynthesis is closely related to photosynthetic pigments, mineral ions and antioxidant enzymes. The increase of the Car/Chl *t* ratio, P, Ca^{2+} and Zn^{2+} content, and the decrease of Chl content, *Fv*/*Fm*, and *Fv*′/*Fm*′ by REEs could lead to an increase in the accumulation of flavonoids. The changes in the Car/Chl *t* ratio, Ca^{2+}, Zn^{2+} and Car concentrations, and chlorophyll fluorescence in Ginkgo suspension cells were likely indicators that the metabolism was shifting from primary to secondary processes (e.g. as a result of the flavonoids accumulation) (Table 3).

In summary, 0.1 mM $Ce(NO_3)_3$ promoted the Ginkgo cell growth by increasing the Chl and *Fv*/*Fm* value, ions content, while 0.5–1.0 mM $Ce(NO_3)_3$ limited the cell growth and promoted flavonoid accumulation and antioxidant enzyme activities. These observed changes in flavonoids and antioxidant enzyme activities might be a self–protection mechanism to alleviate the environment stress in Ginkgo cells. Induction of 1.0 mM $Ce(NO_3)_3$ may be beneficial for in vitro production of flavonol glycosides in Ginkgo suspension cell culture.

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