Arch Environ Contam Toxicol (2010) 58:42–52
DOI 10.1007/s00244-009-9340-9

Responses of Antioxidant Systems After Exposition to Rare Earths and Their Role in Chilling Stress in Common Duckweed (*Lemna minor* L.): A Defensive Weapon or a Boomerang?

M. P. Ippolito · C. Fasciano · L. d'Aquino · M. Morgana · F. Tommasi

Received: 18 December 2008 / Accepted: 10 May 2009 / Published online: 7 June 2009

Abstract Extensive agriculture application of rare earth elements (REEs) in Far East countries might cause spreading of these metals in aquatic and terrestrial ecosystems, thus inducing a growing concern about their environmental impact. In this work the effects of a mix of different REE nitrate (RE) and of lanthanum nitrate (LA) on catalase and antioxidant systems involved in the ascorbate–glutathione cycle were investigated in common duckweed *Lemna minor* L. The results indicated that *L. minor* shows an overall good tolerance to the presence of REEs in the media. Treatments at concentrations up to 5 mM RE and 5 mM LA did not cause either visible symptoms on plants or significant effects on reactive oxygen species (ROS) production, chlorophyll content, and lipid peroxidation. Toxic effects were observed after 5 days of exposition to 10 mM RE and 10 mM LA. A remarkable increase in glutathione content as well as in enzymatic antioxidants was observed before the appearance of the stress symptoms in treated plants. Duckweed plants pretreated with RE and LA were also exposed to chilling stress to verify whether antioxidants variations induced by RE and LA improve plant resistance to the chilling stress. In pretreated plants, a decrease in ascorbate and glutathione redox state and in chlorophyll content and an increase in lipid peroxidation and ROS production levels were observed. The use of antioxidant levels as a stress marker for monitoring REE toxicity in aquatic ecosystems by means of common duckweed is discussed.

M. P. Ippolito · C. Fasciano · F. Tommasi (✉)
Department of Plant Biology and Pathology, University of Bari, Via Orabona 4, 70125 Bari, Italy
e-mail: tommasi@botanica.uniba.it

L. d'Aquino
ENEA Portici Research Center, Via Vecchio Macello, 80055 Portici (Napoli), Italy

M. Morgana
ENEA Trisaia Research Center, S. S. Jonica 106 Km 419, 5, 75026 Rotondella (Matera), Italy

Rare earth elements (REEs) include 15 elements in the periodic table [lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), lutetium (Lu)], also known as lanthanides, plus yttrium (Y) and scandium (Sc), that share chemical properties related to a similar external electronic configuration. La, Ce, Pr, Nd, Sm, and Eu are also indicated as light REEs because of their atomic mass lower than 153 amu, whereas Gd, Tb, Dy, Ho, Er, Tm, Yb, and Lu are also indicated as heavy REEs because of their atomic mass greater than 153 amu. REEs are naturally present in the environment and some, mainly La and Ce, are fairly abundant in nature despite their name, but they occur only in trace amounts in biological systems. The entry of REEs in the biological environment is increasing because of their utilization in agriculture in Far East countries to increase growth of plants and animals, thus inducing a growing concern about the possible environmental impact both in terrestrial and aquatic ecosystems. The application of about 5200 tons of REEs over millions of hectares of cultivated lands in China in 2002 has been reported by the China Rare Earth Information Center of Baotou (Inner Mongolia, China) (Anonymous 2003), but other authors estimated that 50–100 million tons of REE oxides entered the Chinese agricultural systems every year

(Xiong et al. 2001 as cited by Liang et al. 2005). In addition, REEs might also reach soil systems through animal dejections due to the use of lanthanides as a feed supplement to improve animal growth (He et al. 2001). REEs' effect on plants is still far from being clarified: Some authors report positive effects on crop production, such as faster development, greener foliage, larger roots, and better fruit color in different species (Hu et al. 2004), but negative effects, including disturbed mineral nutrition and seedling damages, have also been reported (d'Aquino et al. 2009; Hu et al. 2002). Furthermore, even though many steps forward have been made during the last decades in investigating physiological and biochemical effects induced by lanthanides on plant metabolism, yield, and quality of crops, the results of field trials and laboratory studies are still contradictory and sometimes inconsistent, and many questions about the mechanisms of REE effects, accumulation, and toxicity are still open, suggesting a more careful use of such elements in crop management. Accumulation in plants, both in underground and aerial parts, following REE application to soils has also been demonstrated (Xu et al. 2002; Zhang and Shan 2001) as well as in ectomycorrhizal fungi (Aruguete et al. 1998). Data from literature report total REE concentrations in soil surface up to 100–200 mg/kg (Liang et al. 2005; Tyler 2004; Wyttenbach et al. 1996; Xu et al. 2002; Zhang and Shan 2001), but accumulation in soils can take place following soil dressing with REE-enriched fertilizers or contamination phenomena, because of the overall low mobility of these elements in soils (Cao et al. 2000; Zhang and Shan 2001). Although some authors postulate that the use of REE enriched mineral or organic fertilizers, at the actual rate of application, will not cause a significant soil contamination in the near future (Liang et al. 2005; von Tucher and Schmidhalter 2005), these elements might affect the growth of important microorganisms in the soil microbial community (d'Aquino et al. 2004; Nardi et al. 2005). Despite their widespread and growing use in agriculture, little information is available about the effects of REE on aquatic organisms (Alibo and Nozaki 2004; Chua 1998; Weltje et al. 2002a, b). REEs have been detected in runoff and waste water and in aquatic ecosystems as a consequence of industrial and agricultural practices (Protano and Riccobono 2002), thus suggesting that lanthanides can reach the groundwater and migrate into rivers and lakes. From an ecotoxicological point of view, REEs have largely been considered of minor environmental concern because they are considered only slightly toxic to mammals (Haley 1979). Nevertheless, lanthanides interact with Ca-dependent biological systems and remarkably interfere with cell metabolism and could also have pathogenic potential (Ippolito et al. 2007; Wang et al. 1999). A stimulation of the antioxidant systems [i.e., molecules and enzymes involved in the reactions able to control reactive oxygen species (ROS) levels in plants] has been reported following REE treatment of plants (Ippolito et al. 2007; Jia et al. 2005; Zhang et al. 2003).

The antioxidant systems consist of enzymes (superoxide dismutase, catalase, peroxidases), redox metabolites such as ascorbate (ASC) and the tripeptide glutathione (GSH), and enzymes involved in their metabolism that control ROS production. ASC and GSH are characterized by a plethora of crucial roles in plant cell physiology (Noctor and Foyer 1998) and can protect plant cells against ROS-induced damages either by reacting directly with these species or taking part in some reactions such as those composing the ASC–GSH cycle, a pathway that is known to be carried out in the cytoplasm, in chloroplasts, and in mitochondria and peroxisomes and that is involved in ROS scavenging (Arora et al. 2002; Asada 1999). In this cycle, ascorbate peroxidase (APX) removes H_2O_2 using ASC as an electron donor and this reaction generates the ascorbic free radicals or monodehydroascorbate (MDHA) that can produce ASC and dehydroascorbate (DHA) or can be enzymatically reduced in ASC by monodehydroascorbate reductase (MDHAR) using NAD(P)H as a reducing equivalents donor. DHA can be reduced in ASC by dehydroascorbate reductase (DHAR) in a reaction that involves reduced GSH as an electron donor, generating oxidized GSH (GSSG), which is reconverted in its reduced form by NADPH-dependent glutathione reductase (GR).

Over the last years, interest has grown about the effects of REEs in increasing plant resistance to environmental stresses, as some works suggest that La^{3+}, at suitable concentrations, promotes higher resistance to drought stress (Diatloff et al. 1995) and alleviates injury to biological membranes caused by osmotic stress in wheat plants (Zeng et al. 1999). Some works report that La^{3+} reduces cellular damage induced by chilling in maize culture cells (Chen and Li 2001) and that La is also able to make wheat leaves less susceptible to oxidative stress by stimulating enzymes involved in ROS removal (Zhang et al. 2003). Although the increase in the activity of antioxidant enzymes has also been proposed as an explanation for beneficial effects induced by lanthanides on aged seed germination (Fashui 2002), the increase in antioxidant levels could also be interpreted only as an indicator of stress, as it is well known that many abiotic stressors induce an increase in antioxidant systems that might not be able to counteract the negative effects on plant growth and/or metabolism (Ippolito et al. 2007; Mittler 2002).

The aim of this work was to investigate the effect of treatments with La nitrate and a mixture of different REEs on antioxidant systems in common duckweed (*Lemna minor* L.), a floating macrophyte frequently used as model species in biomonitoring and in ecotoxicological studies

(Cascone et al. 2004; Ince et al. 1999). Because the chilling, defined as the range of temperatures between 0 and 15°C characterized by the absence of ice nucleation in the plant cell (Kocsy et al. 2001), could result in an increased generation of ROS in plants (Okuda et al. 1991; Prasad et al. 1994) and the antioxidants are involved in the tolerance responses (Prasad et al. 1994), we exposed plants pretreated with REEs and La to chilling with the aim to clarify the role of lanthanides in chilling stress tolerance.

Materials and Methods

Rare Earth Elements

A lanthanide nitrate form was used in this study because this is the elemental form present in REE-enriched fertilizers. In order to obtain a REE nitrate stock solution (RE) whose composition was somewhat similar to a REE-enriched fertilizer, a REE-enriched chloride mixture (supplied by Inner Mongolia Sanjili Rare Earth Materials Co. Ltd., Huhehaote, Inner Mongolia, China) was exposed to a concentrated nitric acid solution and kept at 70–80°C until fully dried, and the partially converted nitrate salt was dissolved in MilliQ water. Ion concentrations were determined by using an IRIS optical inductively coupled plasma (ICP) spectrometer (Thermo Jarrel-Ash, Waltham, MA, USA) and an ED40-GP40-LC30 ionic chromatographer (Dionex, Sunnyvale, CA, USA). Final concentrations were 100.07 mM La, 327.57 mM Ce, 25.76 mM Pr, 0.14 mM Nd, 0.006 mM Gd, pH 3; NO_3^-/Cl^- ratio 7:1. A La nitrate solution (LA) was prepared by dissolving a commercial reagent (≥ 98%; Prosintex, Settimo Milanese, Italy) in MilliQ water. A potassium nitrate (K) solution was prepared by dissolving a commercial reagent (≥99%; Sigma, St. Louis, MO, USA) in MilliQ water. Solutions were filtered, sterilized, and stored at room temperature.

Plant Material and Treatments

Lemna minor plants were maintained in 20-L-capacity trays and fed with Knop's solution under glasshouse conditions. Fresh Knop solution was added every 3 days to maintain the solution level. Four days before experiments were started, plants were placed in plastic Petri dishes containing MilliQ water, washed, and transferred in Knop solution at 24°C under white light (14-h photoperiod, 20 μmol/m^2/s) to acclimatize. Batches of plants (20 g) were washed and incubated for 2 and 5 days with 20 mL of 1, 5, and 10 mM La and 1, 5, and 10 mM RE. MilliQ water and 3, 15, and 30 mM K were used as controls, in order to evaluate the osmotic effect in addition to the lanthanides effect. Plant material (20 g) treated for 2 days 10 mM LA and RE and with 5 mM LA and RE for 5 days was washed with distilled water, transferred in 20 mL of MilliQ water, and incubated at 4°C for 24 h under white light (14-h photoperiod, 20 μmol/m^2/s) before being used for the following assays.

Total Chlorophyll Assay

Plant samples (2 g) were ground at 4°C with 8 vol acetone 80% (w/v) and the total chlorophyll content was determined as described by Harborne (1973) with minor modifications.

Biomass Yield Determination

Dry weights were gravimetrically estimated on the basis of fresh weight on 0.100-g plant samples using a Mettler infrared drier LP 16-M (Tommasi et al. 2001).

ROS Visualization and Quantitative Assay

The histochemical staining of H_2O_2 in duckweed leaves was performed as described by Paradiso et al. (2008) and the histochemical staining of the anion superoxide was performed as described by Song et al. (2005). The H_2O_2 production was estimated as H_2O_2 released in the incubation medium as reported by Bellicampi et al. (2000) on 1 mL of the incubation medium.

Lipid Peroxidation Assay

Plant material (2 g) was homogenized with 4 vol trichloroacetic acid 0.1% (w/v) and then centrifuged for 15 min at 12,000 *g*. The supernatant (1 mL) was mixed with 1 vol trichloroacetic acid 20% containing 0.5% (w/v) thiobarbituric acid. The level of lipid peroxidation was estimated as malondialdehyde (MDA) content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham (1996).

Ascorbate and Glutathione Pool Determination

Plant samples (2 g) were ground at 4°C with 3 vol metaphosphoric acid 5% (w/v) and centrifuged for 15 min at 20,000 *g* and the supernatant was used for ASC and GSH pool content determination as described by Zhang and Kirkham (1996).

Enzymatic Analysis

Plant samples (5 g) were homogenized at 4°C in 3 vol of 50 mM Tris-HCl, pH 7.8, containing 0.3 mM mannitol,

10 mM $MgCl_2$, 1 mM EDTA, and cysteine 0.05% (w/v) and the homogenate was centrifuged for 15 min at 25,000 *g*. The supernatant was dialyzed against 50 mM Tris-HCl, pH 7.8, and then used for spectrophotometric and electrophoretic analysis. The total protein content was determined as indicated by Bradford (1976), using bovine serum albumin as a standard. APX (EC 1.11.1.11), DHAR (EC 1.8.5.1), MDHAR (EC 1.6.5.4), GR (EC 1.6.4.2), and catalase (CAT) (EC 1.11.1.6) activity assays were performed as described by Paciolla et al. (2008). Native polyacrylamide gel electrophoresis (PAGE) of APX and DHAR were performed in accordance with the method of Tommasi et al. (2001). Native-PAGE of CAT was tested as reported by De Gara et al. (2003).

Statistical Analysis

All reported values are the average of five replicates ± SD and the differences were evaluated following the Student's *t*-test.

Results

Effect of Lanthanides on Plant Growth, ROS Production, Chlorophyll Content, and Lipid Peroxidation

Treatments with 1 mM and 5 mM LA and with 1 mM and 5 mM RE did not induced any visible symptom of suffering in treated plants or significant variation in ROS production, chlorophyll content, and lipid peroxidation, even after 5 days of exposure (data not shown). Following the 5-day exposure to 10 mM LA and 10 mM RE, chlorotic areas were observed on *L. minor* leaves associated with the reduction in total chlorophyll content and an increase in H_2O_2 production, as well as in lipid peroxidation (Table 1). No difference in dry weights was recorded in comparison to untreated plants (data not shown).

Effect of Lanthanides on Antioxidant Molecules and Enzymes

Treatments with 1 mM LA and 1 mM RE did not cause any significant effect in the levels of all the antioxidants analyzed, even after the longer exposures times. An increase in total GSH content, with an unchanged redox ratio GSH/GSH + GSSG, was revealed after 5 days of exposure to 5 mM LA and 5 mM RE, whereas the treatment with 10 mM LA and 10 mM RE led to an increase in the GSH pool starting at 2 days with a statistically significant reduction in its redox ratio at 5 days [i.e., a shift towards its oxidized forms (GSSG)] (Table 2). The total ASC content grew in treated plants compared to the control plants after 5 days of exposure to 10 mM LA and 10 mM RE, with a reduced ASC redox state (ASC/ASC + DHA) (i.e., a shift toward the DHA) (Table 3). Treatments with 5 mM LA and 5 mM RE induced a significant increment in APX activity at both exposure times, whereas DHAR, MDHAR, and GR enzymatic activity increased only after 5 days of exposure (Table 4).Treatments with 10 mM LA and 10 mM RE induced a relevant increase in the enzymes mentioned earlier starting at 2 days of exposure, as confirmed by the more intense bands in the electrophoretic profile of APX and DHAR (Fig. 1). Native-PAGE of APX showed a single band of activity after 10 mM LA and 10 mM RE treatments (Fig. 1a). The DHAR electrophoretic profile showed the presence of three bands in the control plants, which became more intense in the samples treated for 2 days with 10 mM RE and 10 mM LA, and with the appearance of two additional bands slightly evident (Fig. 1b).

Effect of Chilling on Plants Pretreated with REEs

Plant samples treated with LA and RE at concentration of 10 mM for 2 days were chosen to test the response of treated plants to chilling stress. Spectrophotometric analysis results indicated that pretreatments with LA and RE did not cause any significant change in either H_2O_2

Table 1 MDA concentration, H_2O_2 production and chlorophylls content in control plants (H_2O, K) and plants treated with LA and RE 10 mM for 5 days

Treatments	nmol MDA/g FW	H_2O_2 extracellular level (μM)	Total chlorophyll content (mg/g DW)
H_2O	9.18 ± 0.72	1.92 ± 0.22	0.77 ± 0.06
30 mM K	9.35 ± 0.8	2.13 ± 0.17	0.75 ± 0.04
10 mM LA	11.14 ± 0.58$^{\bullet\blacktriangle}$	3.17 ± 0.25$^{\bullet\bullet\blacktriangle}$	0.52 ± 0.07$^{\bullet\blacktriangle}$
10 mM RE	11.37 ± 0.66$^{\bullet\blacktriangle}$	3.21 ± 0.35$^{\bullet\bullet\blacktriangle}$	0.54 ± 0.08$^{\bullet\blacktriangle}$

Note: The results are given as the mean values of at least 5 experiments ± SD; • and •• indicate values that are significantly different compared to the control in water (with $p < 0.05$ and 0.01, respectively); ▲ indicates values significantly different respect to the control treated with K with $p < 0.05$

FW fresh weight, *DW* dry weight

Table 2 Total GSH content and GSH redox ratio content in control plants (H_2O, K) and plants treated with LA and RE at different concentrations

Treatments	Total GSH (μmoles/g DW)		GSH/GSH + GSSG	
	2 days	5 days	2 days	5 days
H_2O	17.99 ± 1.42	19.50 ± 1.29	0.98 ± 0.07	0.99 ± 0.07
3 mM K	18.46 ± 1.02	19.20 ± 0.82	0.97 ± 0.03	0.97 ± 0.02
1 mM LA	18.95 ± 0.96	19.45 ± 1.12	0.98 ± 0.06	0.98 ± 0.05
1 mM RE	1808 ± 1.56	20.20 ± 1.22	0.98 ± 0.05	0.97 ± 0.05
15 mM K	17.88 ± 0.99	22.79 ± 0.99	0.97 ± 0.05	0.98 ± 0.02
5 mM LA	18.15 ± 1.10	29.72 ± 1.11$^{\bullet\bullet\blacktriangle}$	0.98 ± 0.06	0.97 ± 0.05
5 mM RE	18.80 ± 1.22	28.71 ± 1.42$^{\bullet\bullet\blacktriangle}$	0.97 ± 0.04	0.96 ± 0.05
30 mM K	19.34 ± 1.05	21.28 ± 1.08	0.97 ± 0.05	0.97 ± 0.01
10 mM LA	24.85 ± 0.94$^{\bullet\blacktriangle}$	24.000 ± 1.12$^{\bullet\bullet\blacktriangle}$	0.95 ± 0.06	0.93 ± 0.01$^{\bullet\blacktriangle}$
10 mM RE	25.40 ± 1.00$^{\bullet\blacktriangle}$	27.20 ± 1.45$^{\bullet\bullet\blacktriangle\blacktriangle}$	0.96 ± 0.04	0.94 ± 0.01$^{\bullet}$

Note: The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01 respectively); ▲ and ▲▲ indicate values significantly different compared to the control treated with K ($p < 0.05$ and 0.01, respectively)

DW dry weight

Table 3 Total ASC content and ASC redox state in control plants (H_2O, K) and plants treated with LA and RE at different concentrations

Treatments	Total ASC (μmoles/g DW)		ASC/ASC + DHA	
	2 days	5 days	2 days	5 days
H_2O	11.88 ± 0.95	10.56 ± 0.73	0.76 ± 0.07	0.78 ± 0.05
3 mM K	11.05 ± 0.10	9.61 ± 0.80	0.77 ± 0.04	0.79 ± 0.02
1 mM LA	10.80 ± 0.83	9.89 ± 0.54	0.76 ± 0.06	0.77 ± 0.05
1 mM RE	11.45 ± 0.80	9.33 ± 0.90	0.77 ± 0.05	0.78 ± 0.05
15 mM K	10.97 ± 1.37	11.45 ± 0.80	0.74 ± 0.08	0.80 ± 0.09
5 mM LA	11.86 ± 1.25	12.76 ± 1.23	0.75 ± 0.10	0.79 ± 0.10
5 mM RE	12.26 ± 1.41	13.14 ± 1.44	0.76 ± 0.11	0.79 ± 0.11
30 mM K	12.75 ± 1.08	12.38 ± 1.78	0.74 ± 0.09	0.75 ± 0.04
10 mM LA	13.50 ± 1.23	34.69 ± 2.34$^{\bullet\bullet\blacktriangle\blacktriangle}$	0.73 ± 0.11	0.65 ± 0.07$^{\bullet\blacktriangle}$
10 mM RE	13.20 ± 1.42	42.66 ± 1.23$^{\bullet\bullet\blacktriangle\blacktriangle}$	0.74 ± 0.12	0.63 ± 0.08$^{\bullet\blacktriangle}$

Note: The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01, respectively); ▲ and ▲▲ indicate values significantly different compared to the control treated with K ($p < 0.05$ and 0.01, respectively)

DW dry weight

released from plants or in MDA levels compared to control plants (Fig. 2a and b). Following exposure to low temperatures, an increase in MDA and in extracellular H_2O_2 was observed (Fig. 2c and d). In the plants treated with 10 mM LA and 10 mM RE for 2 days and then exposed to cooling, the histochemical staining of both hydrogen peroxide and superoxide anion increased significantly, compared to non-pretreated plants subjected to chilling (Fig. 3a and b vs. Fig. 3c and d). In fact, the H_2O_2-dependent green fluorescence increased remarkably in the REE pretreated samples compared to the controls, as well as the blue color due to the NBT reaction with superoxide anion (Fig. 3a and b vs. Fig. 3c and d). The total chlorophyll content, which was similar in pretreated and control plants initially (Fig. 4a), then decreased as a consequence of the exposure to low temperatures (Fig. 4b). The exposure to the low temperatures in plants pretreated with LA and RE caused a sharp reduction in both the ASC and GSH total pool, with a statistically significant reduction in their redox state (Fig. 5). The same effect occurred also in APX, which showed a lower activity after chilling compared to the controls (Fig. 6a), as indicated also by the native-PAGE analysis for the pretreatment at the highest concentration (Fig. 6b). The CAT activity pattern was similar to that of APX, significantly higher than the controls in plants pretreated with LA and RE (Fig. 7a) and decreasing after

Table 4 Enzymatic activity of the cytosolic components of APX, DHAR, MDHAR, and GR in control plants (H_2O, K) and plants treated with lanthanides at different concentrations

Treatments	APX (U/mg prot)		DHAR (U/mg prot)		MDHR (U/mg prot)		GR (U/mg prot)	
	2 days	5 days	2 days	5 days	2 days	5 days	2 days	5 days
H_2O	696 ± 88	776 ± 49	32 ± 6	56 ± 7	429 ± 35	442 ± 44	303 ± 32	228 ± 50
3 mM K	650 ± 96	756 ± 59	33 ± 9	59 ± 8	423 ± 54	467 ± 28	271 ± 22	278 ± 14
1 mM LA	686 ± 55	735 ± 99	37 ± 4	51 ± 4	390 ± 27	430 ± 51	264 ± 56	250 ± 29
1 mM RE	688 ± 47	722 ± 103	35 ± 10	61 ± 5	434 ± 46	471 ± 30	314 ± 28	277 ± 41
15 mM K	607 ± 22	830 ± 21	30 ± 10	48 ± 12	450 ± 54	550 ± 40	321 ± 40	202 ± 43
5 mM LA	878 ± 25•*	1669 ± 39••**	35 ± 5	146 ± 8••**	525 ± 45	700 ± 41••**	378 ± 33	417 ± 39••**
5 mM RE	858 ± 24•*	1755 ± 38••**	36 ± 8	167 ± 11••**	550 ± 60	768 ± 34••**	386 ± 42	389 ± 42••**
30 mM K	721 ± 49	890 ± 51	30 ± 4	60 ± 7	523 ± 47	485 ± 35	347 ± 33	233 ± 33
10 mM LA	1085 ± 45•*	1210 ± 50•*	64 ± 5••*	164 ± 9••**	1081 ± 48••**	1324 ± 58••**	503 ± 41•*	388 ± 45••**
10 mM RE	1150 ± 40•*	1280 ± 45•*	75 ± 6••*	186 ± 8••**	1188 ± 38••**	1485 ± 63••**	521 ± 39•*	397 ± 28••**

Note: 1 U = 1 nanomole of substrate metabolized per minute. The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01, respectively); * and ** indicate values significantly different compared to the control treated with K ($p < 0.05$ and 0.01, respectively)

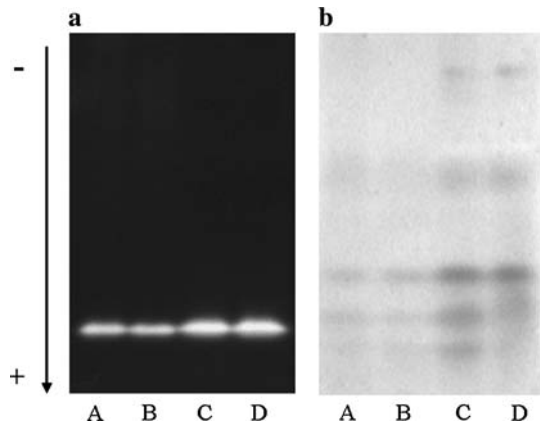

Fig. 1 Native-PAGE of the cytosolic isoenzymes of APX (**a**) and DHAR (**b**) in plants treated with water (A), 30 mM K (B), 10 mM LA, (C), and 10 mM RE, (D) for 2 days. One hundred micrograms of total proteins were loaded per each well

exposure to low temperatures (Fig. 7b), as also confirmed by electrophoretic analysis on the activity gel at the highest concentration (Fig. 7c and d). In contrast, the enzymatic activity of DHAR, MDHAR, and GR, as a consequence of the exposure to the low temperature for 24 h, remained at values similar to the controls (data not shown).

Discussion

Effect of Lanthanides on Plant Growth, ROS Production, Chlorophyll Content, and Lipid Peroxidation

Lemna minor has been extensively used as a model organism to study lanthanide toxicity in aquatic systems (Weltje 2002a), and although no effects were reported on *Lemna* growth at nanomolar concentrations, the bioaccumulation factor warrants carefully monitoring lanthanide emissions in water (Weltje 2002a). Our findings about chlorophyll content, dry weight, and ROS production suggest that *L. minor* plants tolerate millimolar concentrations of REEs and La nitrates, also following a few days of treatments, whereas toxic effects occur at the highest concentrations utilized and after longer exposures. Although REEs seem to be less toxic to *L. minor* plants than metals such as cadmium and mercury, which exert their toxicity toward duckweed at micromolar concentrations, and also less toxic than lead, which is tolerated by duckweed plants at concentrations up to 0.25 mM (Mohan and Hosetti 1997), our data on *L. minor* response to REEs indicate that these elements might represent a potential risk for the aquatic ecosystems. Little information are available on the actual REE concentrations in the aquatic environments; some data report the occurrence of micromolar concentrations in stream water (Protano and Riccobono 2002), but few data are available about the presence and the mobility of REEs in the effluents and their accumulation in aquatic ecosystems. Therefore, more studies are needed to monitor the risks of REE toxicity in waters, as already reported for the terrestrial environment (d'Aquino et al. 2009). The toxicity of REEs at millimolar concentrations observed in this study could be interpreted as a potential risk for the utilization of REE fertilizers in water cultures.

Effect of Lanthanides on Antioxidant Molecules and Enzymes

Indeed, data reported in this work show that a remarkable increase in some antioxidant systems was evident in

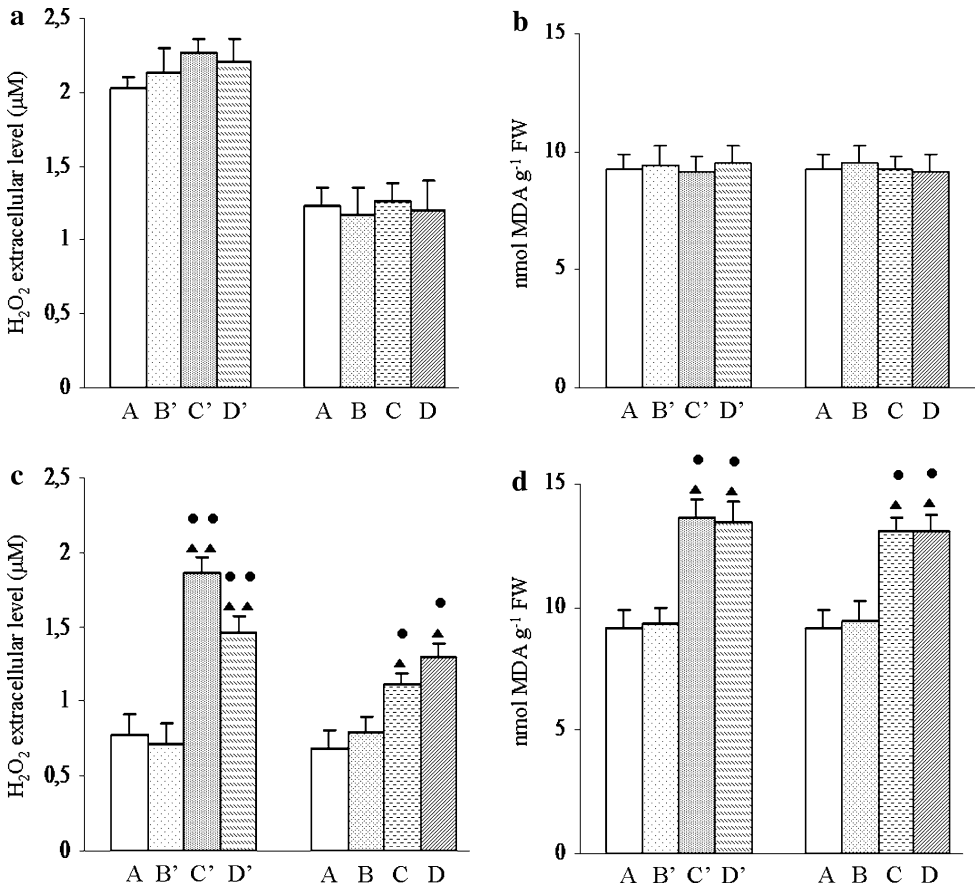

Fig. 2 H_2O_2 production (**a**) and MDA level (**b**) in duckweed plants pretreated with water (A), 15 mM K (B′), 5 mM LA (C′), and 5 mM RE (D′) for 5 days and with water (A), 30 mM K (B), 10 mM LA (C), and 10 mM RE (D) for 2 days; effect of chilling on H_2O_2 production (**c**) and MDA level (**d**) in plants pretreated with lanthanides as indicated and subjected to chilling (**d**). The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01, respectively); ▲ and ▲▲ indicate values significantly different compared to the control treated with K ($p < 0.05$ and 0.01, respectively)

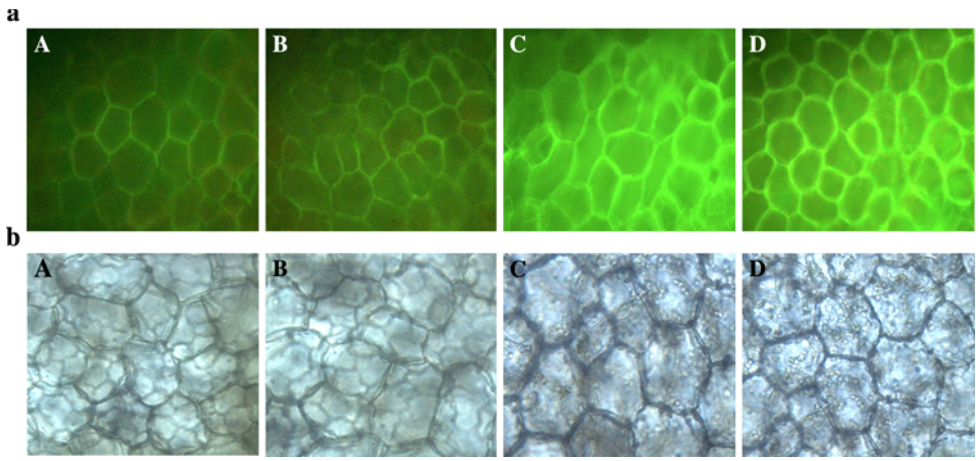

Fig. 3 Histochemical staining of hydrogen peroxide (**a**) and superoxide anion (**b**) in plants pretreated for 2 days with water (A), 30 mM K (B), 10 mM LA (C), and10 mM RE (D) and then exposed to chilling

duckweed plants before the appearance of lanthanide-induced toxicity symptoms. This increment could be interpreted as an early symptom of abiotic stress because the increase in antioxidants observed after REE treatments could be interpreted as a typical stress response finalized to counteract and control the ROS production, as already proposed (Mittler 2002). It is well known that oxidative stress and consequent increasing levels in antioxidants are induced by exposure to metals (Noctor and Foyer 1998). Moreover, the metals toxicity in terms of antioxidant responses (i.e., increase in the antioxidant levels) has been suggested as a marker of toxicity, as treatments of *L. minor* for 24 h at micromolar concentrations of copper were associated to an increase of the antioxidant enzymes GR, GSH peroxidase and CAT (Razinger et al. 2007).

Effect of Chilling on Plants Pretreated with REE

On the other hand, many data suggest that the increase of antioxidants induced by lanthanides is a mechanism to

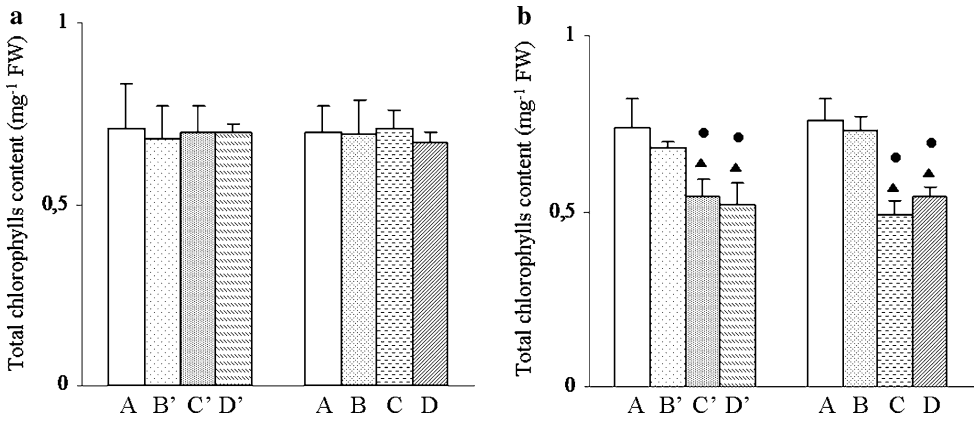

Fig. 4 Total chlorophylls content in plants pretreated with water (A), 15 mM K (B′), 5 mM LA (C′), and 5 mM RE (D′) for 5 days and with water (A), 30 mM K (B), 10 mM LA (C), and 10 mM RE (D) for 2 days (**a**); total chlorophyll content in plants pretreated as indicated and subjected to chilling (**b**). The results are given as the mean values of at least five experiments ± SD; • indicates values that are significantly different compared to the control in water with $p < 0.05$; ▲ indicates values significantly different compared to the control treated with K with $p < 0.05$

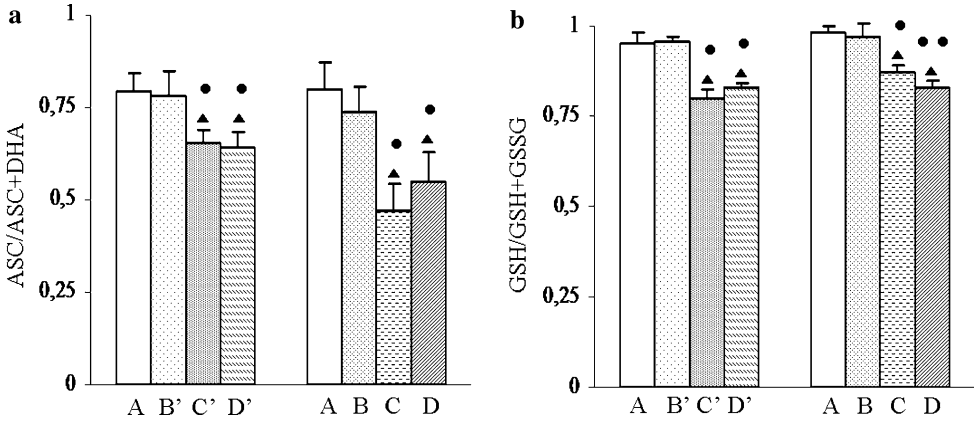

Fig 5 Effect of chilling on ASC (**a**) and GSH redox state (**b**) in plants pretreated with water (A), 15 mM K (B′), 5 mM LA (C′), and 5 mM RE (D′) for 5 days and with water (A), 30 mM K (B), 10 mM LA (C), and 10 mM RE (D) for 2 days and subjected to chilling. The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01, respectively); ▲ indicates values significantly different compared to the control treated with K with $p < 0.05$

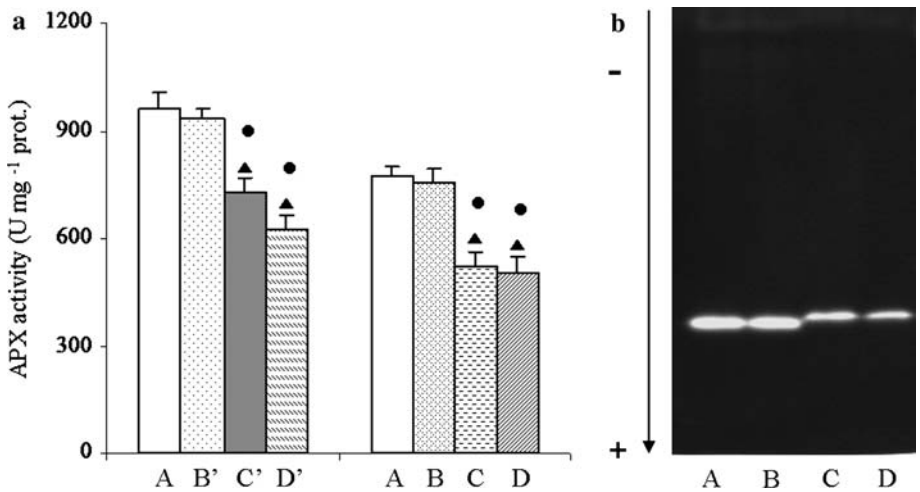

Fig. 6 Effect of chilling on the APX activity (**a**) and native-PAGE (**b**) in plants pretreated with water (A), 15 mM K (B′), 5 mM LA (C′), and 5 mM RE (D′) for 5 days and with water (A), 30 mM K (B), 10 mM LA (C), and 10 mM RE (D) for 2 days and subjected to chilling. The results are given as the mean values of at least five experiments ± SD; • indicates values significantly different compared to the control in water with $p < 0.05$; ▲ indicates values significantly different compared to the control treated with K with $p < 0.05$

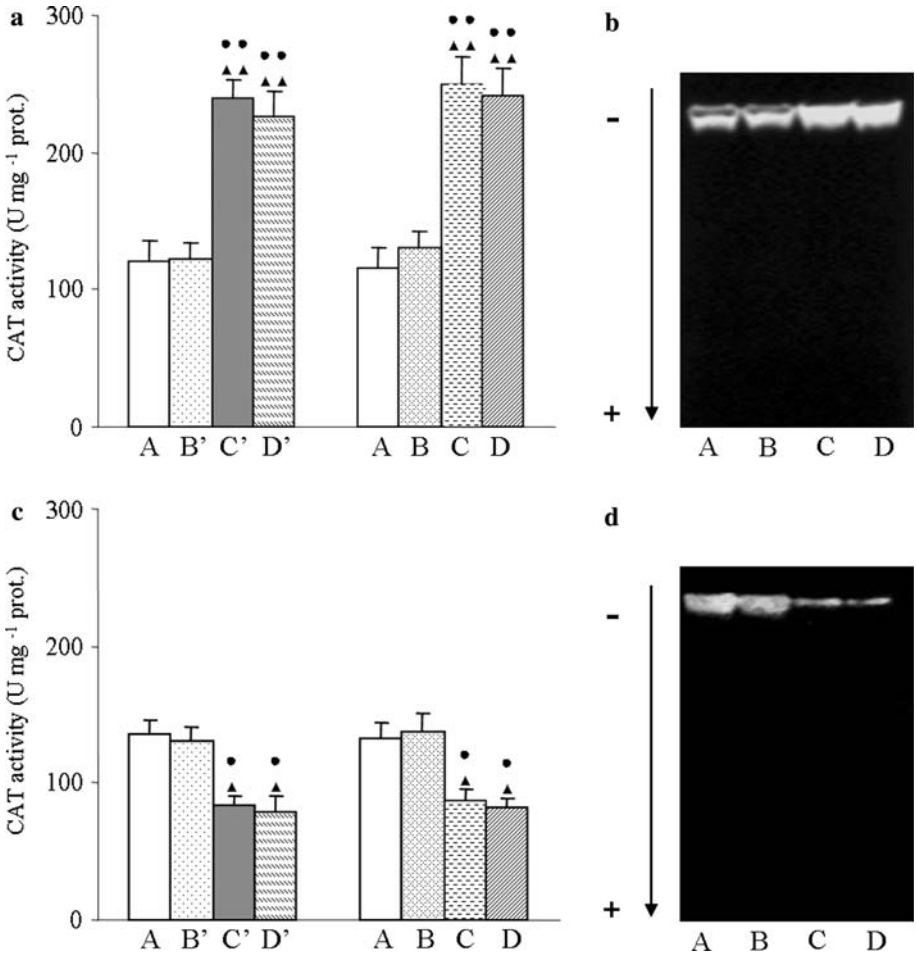

Fig. 7 CAT activity (**a**) and native-PAGE (**c**) in duckweed plants pretreated with water (A), 15 mM K (B′), 5 mM LA (C′), and 5 mM RE (D′) for 5 days and with water (A), 30 mM K (B), 10 mM LA (C), and 10 mM RE (D) for 2 days; CAT activity (**b**) and native-PAGE (**d**) in plants pretreated as indicated and subjected to chilling. The results are given as the mean values of at least five experiments ± SD; • and •• indicate values significantly different compared to the control in water ($p < 0.05$ and 0.01, respectively); ▲ and ▲▲ indicate values significantly different compared to the control treated with K ($p < 0.05$ and 0.01, respectively)

improve the responses of plants to the stress induced by chemicals (Jia et al. 2005). Our results indicate that although *L. minor* is tolerant to cold (Landolt 1986), pretreatments with lanthanides reduce its tolerance to chilling. Indeed, pretreatment of plants resulted in individuals being more susceptible to chilling than controls, as demonstrated by some stress indicator levels such as the lower total chlorophylls content, lipid peroxidation level, and ROS production. Therefore, the treatments with lanthanides did not increase plant ability in ROS quenching, in contrast with data already reported by Jia et al. (2005). Pretreated plants also showed a remarkable alteration in the redox state of both ASC and GSH, as well as in APX and CAT activities. The redox ratio of ASC and GSH contributes to the control of the redox state in different cell compartments that is essential for the integrity of cell structures and for proper function of various metabolic pathways (Kocsy et al. 2001). The GSH redox state resulted in being tightly dependent on the GR changes, whereas the ASC redox state was dependent on the variations of DHAR and MDHAR. In samples treated with 5 mM LA and 5 mM RE for 5 days and with 10 mM LA and 10 mM RE for 2 days, these enzymes showed a notably higher activity that contributed to maintain a GSH and ASC redox state similar to treated and control plants. On the contrary, in the plants pretreated with REEs, after chilling, a significant decrease both in GSH and ASC redox ratio was evident, as well as an increase in H_2O_2 production in the incubation medium. After chilling, the enzymatic antioxidants involved in the ASC–GSH cycle were no longer able to control the redox ratio of ASC and GSH; additionally, the main H_2O_2-removing enzymes, APX and CAT (Mittler 2002), did not regulate H_2O_2 levels properly. Particularly, the drop in CAT activity after chilling treatment can be considered a relevant sign of cellular dysfunction because CAT has been shown to be induced by low temperature and to be an essential enzyme for protecting mitochondria against chilling stress in maize (Prasad et al. 1994). These effects of REEs on chilling tolerance could be explained on the basis of the effects of La on membrane integrity and stabilization. La alters membrane structure and properties: it is known that this element blocks the ability of alfalfa to acclimate to cold, inhibiting cold-induced influx of calcium and thus resulting in the decreased expression of the cold-inducible Cas 15 gene (Monroy and Dhindsa 1995). It has also been reported that La induces cell membrane

perforation, and this effect is concentration dependent (Wang et al. 1999). In addition, treatments with La and H_2O_2 inhibit lipid peroxidation, whereas pretreatments with La alone enhance lipid peroxidation because La disturbs the orderly assembly of membrane, which becomes more sensitive to the oxidative stress (Wang et al. 1999). The similarity between lanthanides and calcium could explain the dose-dependent effect of REE activity/toxicity (Wang et al. 1999). The reduced tolerance to chilling can be explained as an altered physiological response to this stress. In fact, considering that it is well known that calcium and antioxidants are involved in stress tolerance (Mahajan and Tuteja 2005) and that La displaces calcium from extracellular binding sites of the plasma membrane (Hu et al. 2004), we suggest that lanthanides unbalance the antioxidant systems by altering calcium fluxes, so causing a reduction in tolerance to chilling, although more data are needed to support this hypothesis.

In conclusion, RE and LA treatments induced evident symptoms of toxicity only at the highest concentrations after the longest exposure times utilized. The increase in the antioxidants is a stress marker without any positive physiological role in the stress responses. The alteration in antioxidants before the appearance of toxicity symptoms could be utilized in monitoring REE toxicity in aquatic ecosystems, as suggested for other metals.

References

Alibo DS, Nozaki Y (2004) Dissolved rare earth elements in the eastern Indian Ocean: chemical tracers of the water masses. Deep-Sea Research Part I-Oceanograp Research Papers, vol 51. pp 559–576

Anonymous (2003) Chinese consumption continues to increase in 3 years. China Rare Earth Information Center News, vol 9. pp 2–3

Arora A, Sairam RK, Srivastava GC (2002) Oxidative stress and antioxidative system in plants. Curr Sci 82:1227–1238

Aruguete DM, Aldstadt JH, Mueller GM (1998) Accumulation of several heavy metals and lanthanides in mushrooms (*Agaricales*) from the Chicago region. Sci Total Environ 224:43–56. doi:10.1016/S0048-9697(98)00319-2

Asada K (1999) The water–water cycle in chloroplast: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50:601–639. doi:10.1146/annurev.arplant.50.1.601

Bellicampi D, Dipierro N, Salvi G, Cervone F, De Lorenzo G (2000) Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxine-regulated rolB gene expression in tobacco leaf explants. Plant Physiol 122:1379–1385. doi:10.1104/pp.122.4.1379

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Cao X, Wang X, Zhao G (2000) Assessment of the bioavailability of rare earths in soils by chemical fractionation and multiple regression analysis. Chemosphere 40:23–28. doi:10.1016/S0045-6535(99)00321-5

Cascone A, Forni C, Migliore L (2004) Flumequine uptake and the aquatic duckweed, *Lemna minor* L. Water Air Soil Pollut 156:241–249. doi:10.1023/B:WATE.0000036816.15999.53

Chen WP, Li PH (2001) Chilling induced Ca^{2+} overload enhances production of active oxygen species in maize (*Zea mays* L.) cultured cells: the effect of abscisic acid treatment. Plant Cell Environ 24:791–800 doi:10.1046/j.1365-3040.2001.00729.x

Chua U (1998) Bio-accumulation of environmental residues of rare earth elements in aquatic flora *Eichhornia crassipes* (Mart.) Solms in Guangdong Province of China. Sci Total Environ 214:79–85. doi:10.1016/S0048-9697(98)00055-2

d'Aquino L, Carboni MA, Woo SL, Morgana M, Nardi L, Lorito M (2004) Effect of rare earth application on the growth of *Trichoderma* spp and several plant pathogenic fungi. J Zhejiang Univ Sci 30:424

d'Aquino L, de Pinto MC, Nardi L, Morgana M, Tommasi F (2009) Effect of some light rare earth elements on seed germination, seedling growth and antioxidant metabolism in *Triticum durum*. Chemosphere 75:900–905. doi:10.1016/j.chemosphere.2009.01.026

De Gara L, de Pinto MC, Moliterni VMC, D'Egidio MG (2003) Redox regulation and storage processes during maturation in kernels of *Triticum durum*. J Exp Bot 54:249–258. doi:10.1093/jxb/54.381.249

Diatloff E, Smith FW, Asher CJ (1995) Rare earth elements and plant growth. I: Effects of lanthanum and cerium on root elongation of corn and mungbean. J Plant Nutr 18:1963–1976. doi:10.1080/01904169509365037

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

Haley TJ (1979) Toxicity. In: Schneider KA Jr, Eyring KA (eds) Handbook on the physics and chemistry of rare earths. North-Holland, Amsterdam, pp 553–585

Harborne J (1973) Phytochemical methods. Chapman & Hall, London

He ML, Ranz D, Rambeck WA (2001) Study on the performance enhancing effects of rare earth elements in growing and fattening pigs. J Anim Physiol Anim Nutr 85:263–270. doi:10.1046/j.1439-0396.2001.00327.x

Hu X, Ding Z, Chen Y, Wang X, Dai L (2002) Bioaccumulation of lanthanum and cerium and their effects on the growth of wheat (*Triticum aestivum* L.) seedlings. Chemosphere 48:621–629. doi:10.1016/S0045-6535(02)00109-1

Hu Z, Richter H, Sparovek G, Schnug E (2004) Physiological and biochemical effects of rare earth elements on plants and their agricultural significance: a review. J Plant Nutr 27:183–220. doi:10.1081/PLN-120027555

Ince NH, Dirilgen N, Apikyan IG, Tezcanli G, Üstün B (1999) Assessment of toxic interactions of heavy metals in binary mixtures: a statistical approach. Arch Environ Contam Toxicol 36:365–372. doi:10.1007/PL00006607

Ippolito MP, Paciolla C, d'Aquino L, Morgana M, Tommasi F (2007) Effect of rare earth elements on growth and antioxidant metabolism in *Lemna minor* L. Caryologia 60:125–128

Jia Y, Yongshen G, Fuli Z (2005) Resistence of $LaCl_3$ to oxidative stress induced by 2, 4-dichlorophenoxy. J Rare Earths 23:240–244

Kocsy G, Galiba G, Brunold C (2001) Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants. Physiol Plant 113:158–164. doi:10.1034/j.1399-3054.2001.1130202.x

Landolt E (1986) The family of Lemnaceae: a monographic study. In: Biosystematic investigations in the family of duckweeds (Lemnaceae). Veröffentlichungen des Geobotanischen Institutes der ETH, Stiftung Rübel, Zürich, pp 127–128

Liang T, Zhang S, Wang L, Kung H-T, Wang Y, Hu A, Ding S (2005) Environ biogeochemical behaviour of rare earth elements in soil–plant systems. Environ Geochem Health 27:302–311. doi:10.1007/s10653-004-5734-9

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158. doi:10.1016/j.abb.2005.10.018

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 9:405–410. doi:10.1016/S1360-1385(02)02312-9

Mohan BS, Hosetti BB (1997) Potential phytotoxicity of lead and cadmium to *Lemna minor* grown in sewage stabilization ponds. Environ Pollut 98:233–238. doi:10.1016/S0269-7491(97)00125-5

Monroy AF, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25%. Plant Cell 7:321–331

Nardi L, Carboni MA, Morgana M, Barbesti S, Errico S, Zoina A, d'Aquino L (2005) Effect of rare earth elements on the growth of *Agrobacterium* spp. and Rhizobium leguminosarum Frank. Cytometry 69:443–444

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279. doi:10.1146/annurev.arplant.49.1.249

Okuda T, Matsuda Y, Yamanaka A, Sagisaka S (1991) Abrupt increase in the level of hydrogen-peroxide in leaves of winter-wheat is caused by cold treatment. Plant Physiol 97:1265–1267. doi:10.1104/pp.97.3.1265

Paciolla C, Ippolito MP, Logrieco A, Mulè G, Dipierro N, Dipierro S (2008) A different trend of antioxidant defence responses makes tomato plants less susceptible to beauvericin than to T-2 mycotoxin phytotoxicity. Physiol Mol Plant Path 72:3–9. doi:10.1016/j.pmpp.2008.06.003

Paradiso A, Berardino R, de Pinto MC, Sanità di Toppi L, Storelli MM, Tommasi F, De Gara L (2008) Increase in ascorbate–glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. Plant Cell Physiol 49:362–374. doi:10.1093/pcp/pcn013

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6:65–74

Protano G, Riccobono F (2002) High contents of rare earth elements (REE) in stream waters of a Cu-Pb-Zn mining area. Environ Pollut 117:499–514. doi:10.1016/S0269-7491(01)00173-7

Razinger J, Dermastia M, Drinovec L, Drobne D, Zrimec A, Koce JD (2007) Antioxidative responses of duckweed (*Lemna minor* L.) to short-term copper exposure. Environ Sci Pollut Res 14:194–201. doi:10.1065/espr2006.11.364

Song XS, Hu WH, Mao WH, Joshua OO, Zhou YH, Yu JQ (2005) Response of ascorbate peroxidase isoenzymes and ascorbate regeneration system to abiotic stresses in *Cucumis sativus* L. Plant Physiol Biochem 43:1082–1088. doi:10.1016/j.plaphy.2005.11.003

Tommasi F, Paciolla C, de Pinto MC, De Gara L (2001) A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seed. J Exp Bot 52:1647–1654. doi:10.1093/jexbot/52.361.1647

Tyler G (2004) Rare earth elements in soil and plant systems: a review. Plant Soil 267:191–206. doi:10.1007/s11104-005-4888-2

von Tucher S, Schmidhalter U (2005) Lanthanum uptake from soil and nutrient solution and its effect on plant growth. J Plant Nutr Soil Sci 168:574–580. doi:10.1002/jpln.200520506

Wang K, Li R, Cheng Y, Zhu B (1999) Lanthanides—the future drugs? Coord Chem Rev 190:297–308. doi:10.1016/S0010-8545(99)00072-7

Weltje L, Brouwer AH, Verburg TG, Wolterbeek HT, de Goeij JJM (2002a) Accumulation and elimination of lanthanum by duckweed (*Lemna minor* L) as influenced by organism growth and lanthanum sorption to glass. Environ Toxicol Chem 21:1483–1489. doi:10.1897/1551-5028(2002)021<1483:AAEOLB>2.0.CO;2

Weltje L, Heidenreich H, Zhu W, Wolterbeek HT, Korhammer S, de Goeij JJM, Markert B (2002b) Lanthanide concentrations in freshwater plants and molluscs, related to those in surface water, pore water and sediment: a case study in the Netherlands. Sci Total Environ 286:191–214. doi:10.1016/S0048-9697(01)00978-0

Wyttenbach A, Tobler R, Furrer V (1996) The concentrations of rare earth elements in plants and in the adjacent soils. J Radioanal Nucl Chem 204:401–413. doi:10.1007/BF02060328

Xiong BK, Zhang SR, Guo BS, Zheng W (2001) Reviews of the use of REE-based fertilizer in the past of three decades. Symposium on the Use of REE in Agriculture of China. Baotou, People's Republic of China

Xu X, Zhu W, Wang Z, Witkamp GJ (2002) Distribution of rare earths and heavy metals in field-grown maize after application of rare earth-containing fertilizers. Sci Total Environ 293:97–105. doi:10.1016/S0048-9697(01)01150-0

Zeng FL, An Y, Zhang HT, Zhang MF (1999) The effects of La^{3+} on the peroxidation of membrane lipids in wheat seedling leaves under osmotic stress. Biol Trace Elem Res 69:141–150. doi:10.1007/BF02783865

Zhang J, Kirkham B (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytol 132:361–373. doi:10.1111/j.1469-8137.1996.tb01856.x

Zhang S, Shan X (2001) Speciation of rare earth elements in soil and accumulation by wheat with rare earth fertilizer application. Environ Pollut 112:395–405. doi:10.1016/S0269-7491(00)00143-3

Zhang L, Zeng F, Xiao R (2003) Effect of lanthanum ions (La^{3+}) on the reactive oxygen species scavenging enzymes in wheat leaves. Biol Trace Elem Res 91:243–255. doi:10.1385/BTER:91:3:243