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# Effect of some light rare earth elements on seed germination, seedling growth and antioxidant metabolism in *Triticum durum*

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# ABSTRACT

Rare earth elements (REEs) enriched fertilizers have been commonly used in China since the 1980s, thus inducing a growing concern about their environmental impact in agriculture. In this work, the effect of some light REEs nitrate mixture and La<sup>3+</sup> nitrate on seed germination, seedling growth and antioxidant metabolism in Triticum durum was investigated with the aim of clarifying the potential benefits or damages of REEs on plants. Seed pre-soaking for 8 h with La<sup>3+</sup> and REEs nitrate inhibited seed germination at low concentrations (0.01 mM and 0.1 mM), while pre-soaking for 2 and 4 h already inhibited seed germination when higher concentrations (1 mM and 10 mM) of La<sup>3+</sup> and REEs nitrate were used. La<sup>3+</sup> and REEs nitrate treatment also affected seedling growth. Root growth was enhanced and inhibited at low and high concentrations, respectively. Shoot growth was inhibited by La<sup>3+</sup> and REEs nitrate at all tested concentrations after 12 d of treatments. Enzymatic and non enzymatic antioxidants were differently affected by  $La^{3+}$  and REEs nitrate and their behaviour changed also depending on the plant organ. In roots  $La^{3+}$ and REEs nitrate treatments induced an increase in ascorbate (ASC) and glutathione (GSH) contents. In shoots only La<sup>3+</sup> nitrate induced an increase in the ASC content whereas GSH decreased following both La<sup>3+</sup> and REEs nitrate treatments. An increase in ASC peroxidase activity was observed in shoots and roots, while catalase did not change in roots and slightly decreased in shoots. The possible role of the increase in some antioxidants as indicators of stress caused by lanthanide treatments is discussed.

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# 1. Introduction

Rare earth elements (REEs) include 15 elements in the periodic table, also known as lanthanides, plus yttrium and scandium, that share chemical properties related to a similar external electronic configuration. With few exceptions, their valence state is 3+. REEs are today used mainly in industrial production of technological devices and China provides most of the worldwide REEs supply. These elements are naturally present in the environment and some of them (mainly  $La^{3+}$  and  $Ce^{3+}$ ) are fairly abundant in nature, but they occur only in trace amounts in biological systems. Although beneficial effects of REEs on crops have not been clearly demonstrated, REEs enriched fertilizers have been currently used in China since the 1980s for soil and leaf dressing of crops to enhance seed germination, root growth, chlorophyll content, plant resistance (Hu et al., 2004). REEs enriched fertilizers are reported to be a mixture of REEs, mainly as their nitrates, obtained by extracting REEs from their ores using nitric acid (Xu et al., 2002). The application of about 3.400 tons REEs over millions hectares of cultivated lands

in 2001 was reported in China (Source: China Rare Earth Information Center, Baotou, Inner Mongolia, China) while 50-100 million tons of REEs oxides entered the agricultural systems in 2001 according to Liang at al. (2005 and references therein). In addition, REEs may reach soil system through animal dejections following the use of lanthanides to improve animal growth (He et al., 2001). Positive effects on crop production following treatments with REEs are largely reported in Chinese literature (Hu et al., 2004) and a number of physiological responses have also been reported in different plant species (Fashui et al., 2000; Chen et al., 2001; Hu et al., 2002a, 2004). However, results of field trials and laboratory studies are still contradictory and results depend on the plant species, physiological stage and type of treatments (Diatloff et al., 1995a,b,c; He and Loh, 2000; von Tucher and Schmidhalter, 2005). It has also been reported that La<sup>3+</sup> promotes plant tolerance to environmental stresses by increasing antioxidant systems (Zhang et al., 2003), i.e. molecules and enzymes constituting the network of reactions able to control reactive oxygen species (ROS) levels in the stress responses (Arora et al., 2002). Consistently, Fashui et al. (2000) reported that La<sup>3+</sup> treatment of aged Oryza sativa L. seeds contrasts seed aging by promoting catalase, superoxide dismutase and peroxidase activity.



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The aim of this work was to study the effects of treatment with either  $La^{3+}$  nitrate and with a mix of REEs nitrate on seed germination, seedling growth and antioxidant metabolism of *Triticum durum* in order to clarify the potential benefits or damages of REEs to plants.

#### 2. Materials and methods

# 2.1. Chemicals

In order to obtain a REEs mixture similar to REEs based fertilizers, a light REEs nitrate stock solution ( $La^{3+}$  100.07 mM,  $Ce^{3+}$  327.57 mM,  $Pr^{3+}$  25.76 mM,  $Nd^{3+}$  0.14 mM,  $Gd^{3+}$  0.006 mM, pH 3,  $NO_3^{-}/Cl^{-}$  7:1) was prepared exposing a REEs enriched chloride mixture (supplied by *Inner Mongolia Sanjili Rare Earth Materials* Co. Ltd.) to concentrate nitric acid solution, stored at 70–80 °C until full drying, then dissolving the partially converted nitrate salt in MilliQ water. Ion concentrations were determined using a IRIS optical ICP spectrometer (Thermo Jarrel-Ash, Waltham, MA, USA) and a ED40-GP40-LC30 ionic chromatographer (Dionex, Sunnyvale, CA, USA).  $La^{3+}$  nitrate solution was prepared by dissolving a commercial reagent ( $\geq$  98%, Prosintex, Settimo Milanese, Italia) in MilliQ water. Solutions were filter sterilized and stored at room temperature.

#### 2.2. Seed germination test

Seeds of *T. durum* Desf. var. "Trinacria" were soaked in either 0.01 mM, 0.1 mM, 1 mM and 10 mM  $La^{3+}$  nitrate and 0.01 mM, 0.1 mM, 1 mM and 10 mM REEs nitrate solutions for 2, 4 and 8 h. Control seeds were soaked in MilliQ water for 2, 4 and 8 h. In order to evaluate osmotic effect in addition to lanthanides effect, seeds were also soaked in 0.03 mM, 0.3 mM, 3 mM and 30 mM potassium nitrate for 2, 4 and 8 h. After soaking, seeds were placed in Petri dishes (40 seeds/dish) onto a wet germination paper and allowed to germinate at +20 °C, 90% RH in the dark. The number of germinated seeds was scored after 2 and 6 d. All tests were repeated five times.

## 2.3. Seedling growth test

Seeds of *T. durum* were surface sterilized with 2% sodium hypochlorite for 5 min, rinsed with sterile MilliQ water and then placed in evenly spaced rows (30 seeds/paper) onto adhesive tape between two germination papers  $50 \times 50$  cm wide. Paper rolls were placed in plastic trays with their bottom immersed in either 0.01 mM, 0.1 mM, 1 mM and 10 mM La<sup>3+</sup> nitrate and 0.01 mM, 0.1 mM, 3 mM and 30 mM potassium nitrate were used for germination of control seeds. Germination papers were placed at +20 °C, 90% RH in the dark. Shoot and root lengths were scored after 6, 9 and 12 d. All tests were repeated five times.

## 2.4. Mitotic index

Root apexes 1–2 mm wide were cut from seedlings after 12 d long treatment with La<sup>3+</sup> and REEs nitrate solutions. Mitotic index in root apexes was determined as reported in Paradiso et al. (2008).

## 2.5. Antioxidant assays

Antioxidant assays were carried out on roots and shoots of etiolated plants after 12 d treatment with 1 mM La<sup>3+</sup> nitrate, 1 mM REEs nitrate, 3 mM potassium nitrate and MilliQ water. For ascorbate (ASC) and glutathione (GSH) determination 0.5 g of roots or shoots were homogenized in 5% metaphosphoric acid at 4 °C 1:6 (w/v) in order to obtain deproteinized extracts. Homogenates were centrifuged at 20000g for 15 min and the supernatants were collected and used for the determination of ASC and GSH level as reported by de Pinto et al. (1999). For the determination of the activity of ASC-GSH cycle enzymes 0.5 g of roots or shoots were grounded to a fine powder in a mortar in liquid nitrogen and mixed to 50 mM Tris-HCl pH 7.5, 0.05% cysteine and 0.1% bovine serum albumin with a 1:3 ratio (w/v). Homogenates were centrifuged for 15 min at 20000g and the supernatants were used for enzymatic determinations. Cytosolic ASC peroxidase (APX - EC 1.11.1.11), dehydroascorbate reductase (DHAR - EC 1.8.5.1) monodehydroascorbate reductase (MDHAR - EC 1.6.5.4), glutathione reductase (GR - EC 1.6.4.2) were assayed as described in Tommasi et al. (2001). Catalase (CAT – EC 1.11.1.6) activity assay and native polyacrylamide gel electrophoresis (PAGE) were performed as reported by De Gara et al. (2003). APX Native-PAGE was performed on the cytosolic fraction as reported by Tommasi et al. (2006). Protein assay was performed following the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. All experiments were repeated five times.

#### 2.6. Statistical analysis

Experimental data were analyzed with a repeated measures analysis of variance (ANOVA) and a nested design. Means separation was obtained with LSD test.

## 3. Results

100

80

60

40

20

п

KNO<sub>2</sub> RE

2 hours

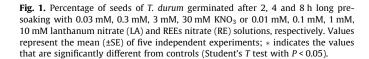
]0mM

(%)

Serminability

## 3.1. Seed germination

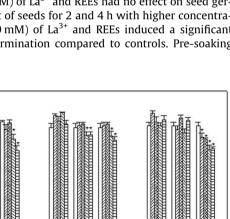
Pre-soaking of seeds for 2 and 4 h with low concentrations (0.01 mM and 0.1 mM) of  $La^{3+}$  and REEs had no effect on seed germination. Treatment of seeds for 2 and 4 h with higher concentrations (1 mM and 10 mM) of  $La^{3+}$  and REEs induced a significant decrease of seed germination compared to controls. Pre-soaking



KNO, RE

4 hours

1 or 3 mM 10 or 30 mM



LA

KNO, RE

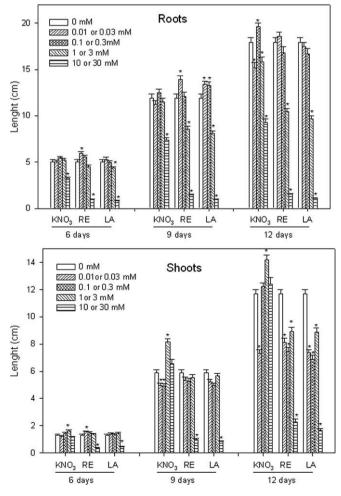
2001 or 0.3 mM

8 hours

of seeds for 8 h with  $La^{3+}$  at all tested concentrations inhibited seed germination; 8 h long pre-soaking with REEs did not affect significantly germination. Treatments with all tested concentrations of potassium nitrate for all pre-soaking time did not induce significant variations in seed germination compared to MilliQ water control (Fig. 1). No effects were observed on the average time of germination neither due to the pre-soaking time nor to the different concentrations of  $La^{3+}$  and REEs. A reduction of root length was observed at the end of the experiment for both  $La^{3+}$  and REEs treatments, especially when tested concentration was 10 mM. In this condition a decrease of mitotic index in the root tissues was also observed (data not shown).

# 3.2. Seedling growth

Effects of La<sup>3+</sup>, REEs and potassium nitrate treatments on seedling growth are reported in Fig. 2. In some cases, root growth was slightly enhanced by La<sup>3+</sup>, REEs and potassium treatments at low concentrations and after 6, 9 d long treatments, compared to water. Higher concentrations (1 mM La<sup>3+</sup>, 1 mM REEs and 30 mM potassium nitrate) reduced root growth after 6, 9 and 12 d of treatments. 10 mM La<sup>3+</sup> and REEs inhibited root growth in all treatments. Shoot growth was differently affected by La<sup>3+</sup>, REEs and potassium treatments. Potassium nitrate at concentrations of



**Fig. 2.** Root and shoot length of *T. durum* etiolated plants measured after 6, 9 and 12 d of treatment with 0.03 mM, 0.3 mM, 3 mM, 30 mM KNO<sub>3</sub> or 0.01 mM, 0.1 mM, 1 mM, 10 mM lanthanum nitrate (LA) and REEs nitrate (RE) solutions, respectively. Values represent the mean ( $\pm$ SE) of five independent experiments; \*, indicates the values that are significantly different from controls (Student's *T* test with *P* < 0.05).

3 mM enhanced shoot length, but growth with potassium nitrate was also reduced after 9 d with 0.03 and 0.3 mM and after 12 d with 0.03 mM. After 6, 9 and 12 d,  $La^{3+}$  and REEs caused a reduction of shoot length at a concentration of 10 mM. After 12 d, shoot growth was also reduced by 0.1 and 1 mM  $La^{3+}$  and REE. Apart from growth inhibition, seedlings treated with 10 mM  $La^{3+}$  and REEs showed tip browning and thickening compared to controls (data not shown).

# 3.3. Mitotic index

In order to verify whether the reduction in root growth observed after lanthanides treatments was due to a decrease in cell division, mitotic index was calculated in seedling root tips after 12 d long treatments with different concentrations of La<sup>3+</sup>, REEs and potassium nitrate (Table 1). Mitotic index was 21.53% in samples treated with water, while treatment with 0.03 mM, 3 mM and 30 mM potassium nitrate reduced the mitotic index to 5.53%, 4.70% and 2.60%, respectively. After treatment with 0.01 mM La<sup>3+</sup> and REEs, mitotic index was 9.91% and 7.44%, respectively. Samples treated with 1 mM and 10 mM La<sup>3+</sup> showed a decrease of the mitotic index to 1.90% and 0.69%, respectively. In these samples, dividing cells were in prophase, without other mitotic figures, with chromatin condensation and absence of nucleoli. In samples treated with 1 mM REEs mitotic index decreased to 1.90% and at 10 mM REEs it was not possible to determine mitotic index because of the absence of dividing cells.

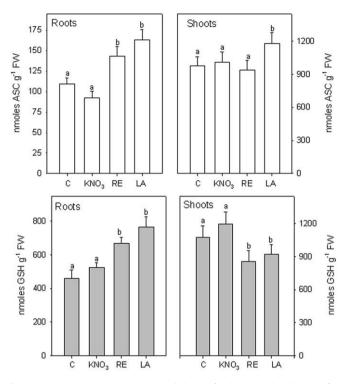
# 3.4. Antioxidant metabolism

Antioxidant assays were performed on seedlings collected after 12 d long treatment with 1 mM La<sup>3+</sup> and 1 mM REEs because at this concentrations the effects on root and shoot length were evident without a complete inhibition of the growth. Assays were carried out also on samples treated with 3 mM potassium nitrate in order to point out any osmotic effect in addition to the effects induced by lanthanides. Compared to water, total ASC content increased both in roots and shoots after the treatment with La<sup>3+</sup>, while REEs treatments induced an increase in total ASC content only in the roots (Fig. 3). La<sup>3+</sup> and REEs treatments induced an increased in total GSH (oxidized + reduced forms) in the roots, whereas a decrease was observed in the shoots (Fig. 3). ASC and GSH redox state were not affected neither in roots nor in shoots (data not shown). No significant variations in ASC and GSH contents were induced by potassium nitrate treatment neither in roots nor in shoots (Fig. 3). APX activity significantly increased in roots and shoots after La<sup>3+</sup> and REEs treatments compared to water and potassium treated controls (Fig. 4). These results were also confirmed by

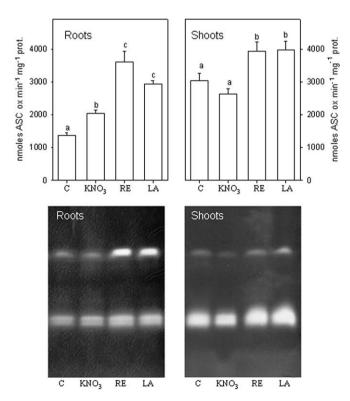
Table 1

Mitotic index in root tips of *T. durum* seedlings after 12 d of treatment with different concentrations of KNO<sub>3</sub>, lanthanum nitrate (LA) and REEs nitrate (RE) solutions. Control indicates samples treated with MilliQ water. Values represent the mean ( $\pm$ SE) of five independent experiments. Different letters represent values which are statistically different (by one-way Anova test).

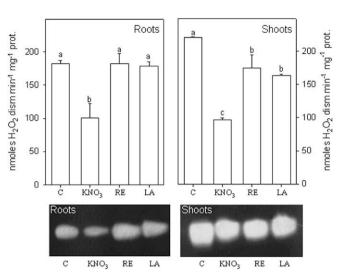
Treatments	Mitotic index (%)
Control	$21.53 \pm 4.44^{a}$
KNO3 0.03 mM	5.53 ± 0.81 <sup>c</sup>
KNO <sub>3</sub> 3 mM	$4.70 \pm 0.70^{\circ}$
KNO3 30 mM	$2.60 \pm 0.62^{d}$
RE 0.01 mM	$7.44 \pm 1.86^{d}$
RE 1 mM	$1.90 \pm 0.32^{d}$
RE 10 mM	nd
LA 0.01 mM	$9.91 \pm 0.81^{b}$
LA 1 mM	$1.90 \pm 0.80^{\rm d}$
LA 10 mM	$0.69 \pm 2.22^{d}$



**Fig. 3.** ASC and GSH contents in roots and shoots of *T. durum* etiolated plants after 12 d of treatment with water (C), 3 mM KNO<sub>3</sub> or 1 mM lanthanum nitrate (LA) and REEs nitrate (RE), respectively. Values represent the mean ( $\pm$ SE) of five independent experiments. Different letters represent values which are statistically different (by one-way Anova test).



**Fig. 4.** APX activity (above) and native-PAGE (below) of roots (left) and shoots (right) of *T. durum* etiolated plants after 12 d of treatment with water (C), 3 mM KNO<sub>3</sub> or 1 mM lanthanum nitrate (LA) and REEs nitrate (RE) respectively. Values represent the mean ( $\pm$ SE) of five independent experiments. Different letters represent values which are statistically different (by one-way Anova test).

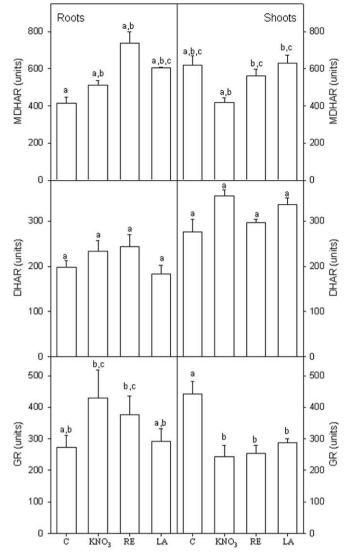


**Fig. 5.** CAT activity (above) and native-PAGE (below) of roots (left) and shoots (right) of *T. durum* etiolated plants after 12 d of treatment with water (C), 3 mM KNO<sub>3</sub> or 1 mM lanthanum nitrate (LA) and REEs nitrate (RE), respectively. Values represent the mean ( $\pm$ SE) of five independent experiments. Different letters represent values which are statistically different (by one-way Anova test).

native-PAGE. Three bands of APX activity in roots and shoots of all tested samples were detected. REEs and La<sup>3+</sup> treatments induced an increase in the intensity of the band with lower mobility in the roots and of the two bands with greater mobility in the shoots (Fig. 4). Potassium nitrate treatment induced a decrease of CAT activity in both shoots and roots, while La<sup>3+</sup> and REEs treatments induced a slightly decrease in CAT activity only in shoots (Fig. 5). Native-PAGE analysis confirmed that a single band with CAT activity was present in all tested samples, with minor intensity only in samples treated with potassium nitrate (Fig. 5). Enzymes involved in the reduction of the oxidized forms of the ASC, MDHAR and DHAR, were not affected by treatments with La<sup>3+</sup> and REEs, since in all cases differences were not significant compared to controls (Fig. 6). La<sup>3+</sup> and REEs treatments induced a decrease of the GR activity only in the shoots compared to the water control, probably due to the osmotic effect since it was evident also after potassium nitrate treatment.

## 4. Discussion

Many papers report yield improvement, dose-dependent acceleration of germination and an increment in root volume and aminoacid content associated with REEs application in wheat (Hu et al., 2004 and references therein). Beneficial effects of La<sup>3+</sup> and Ce<sup>3+</sup> nitrate treatments on germination and growth of aged rice seeds have also been reported (Fashui, 2002; Fashui et al., 2003). On the other hand, Hu et al. (2002a) reported that La<sup>3+</sup> and Ce<sup>3+</sup> inhibit root elongation and reduce dry weight of roots and shoots in Triticum aestivum L. seedlings. Moreover, toxic effects of La<sup>3+</sup> and Ce<sup>3+</sup> on root growth in corn and mungbean (Diatloff et al., 1995a,b,c), as well as an inhibition of root elongation in barley (van Stevenick et al., 1976) have been reported. Our results indicate that seed pre-soaking for 2 and 4 h with La<sup>3+</sup> and a with a mixture of different REEs at low concentrations has no effects on the germination of T. durum seeds while greater concentrations, especially when La<sup>3+</sup> was used, clearly inhibit seed germination. A clear dose-dependent effect of REEs mixture and La<sup>3+</sup> on *T. durum* root growth is evident since low and high concentrations stimulate and inhibit, respectively, root length. A decrease in shoot length also occurs after 12 d of treatment with La<sup>3+</sup> and REE nitrate at



**Fig. 6.** Activities of MDHAR, DHAR and GR in roots (left panels) and shoots (right panels) of *T. durum* etiolated plants after 12 d of treatment with water (C), 3 mM KNO<sub>3</sub> or 1 mM lanthanum nitrate (LA) and REEs nitrate (RE), respectively. MDHAR units = nmoles NADH ox min<sup>-1</sup> mg<sup>-1</sup> prot; DHAR units = nmoles DHA red min<sup>-1</sup> mg<sup>-1</sup> prot. GR units = nmoles NADPH ox min<sup>-1</sup> mg<sup>-1</sup> prot. Values represent the mean (±SE) of five independent experiments. Different letters represent values which are statistically different (by one-way Anova test).

all tested concentrations. This is in accordance with the results of Hu et al. (2002b), who reported that La<sup>3+</sup> and Ce<sup>3+</sup> may interfere with nutrient uptake inhibiting growth of seedlings in T. aestivum. Mitotic index is correlated to frequency of cell divisions and is an important parameter to determine the rate of root growth due to cell proliferation. Our results indicated that potassium nitrate treatments inhibited root growth at all tested concentration, and this is in accordance with results already reported for T. aestivum (Williams, 1968). Compared to inhibitory effects induced by potassium nitrate, effects on root growth were more severe in samples treated with either REEs mix and La<sup>3+</sup> nitrate but only at higher concentrations. REEs and La<sup>3+</sup> nitrate at high (1 mM and 10 mM) and low (0.1 mM) concentrations seem to reinforce and alleviate, respectively, the inhibitory effect of potassium nitrate. The complete inhibition of mitosis associated with the 10 mM REEs treatment agrees with the hypothesis of a synergistic inhibitory effect of some, if not all, REEs that are present in the mixture used for the tests.

Data from literature report total REEs concentration in soil surface up to  $100-200 \text{ mg kg}^{-1}$  (Tyler, 2004; Liang et al., 2005), but accumulation in soils can take place following soil dressing with REEs enriched fertilizers or contamination phenomena, due to the overall low mobility of these elements in soils (Cao et al., 2000). Therefore, although the low mobility of REEs associated with their high adsorption to soils seem to lead to very low solution concentrations and some Authors postulate that the use of REEs enriched mineral fertilizers or REEs containing organic fertilizers at the actual rate of application will not cause a significant soil contamination in the next future (Liang et al., 2005; von Tucher and Schmidhalter, 2005), it is worth noting that only a few information are so far available about the effect of these elements on microbiological components of soils (d'Aquino et al., 2004; Nardi et al., 2005), that may synergistically interact with plants in the absorption process of REEs from the soil. Also the increase in some antioxidants observed in our experimental conditions should led to a careful use of REEs as fertilizers. It is known that antioxidants, such as CAT, superoxide dismutase, peroxidase, ASC, GSH and the enzymes of the ASC-GSH cycle are involved in ROS detoxification (Mittler, 2002) and play a major role in stress tolerance and in the protection of cells from damage induced by ROS. Involvement of lanthanides in the regulation of antioxidant systems has already been reported (Zhang et al., 2003; Jia et al., 2005; Ippolito et al., 2007) and 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 et al., 2000; Fashui, 2002). Our results indicate that La<sup>3+</sup> and REEs induce an increase in the two main soluble antioxidants ASC and GSH levels in the roots, while only La<sup>3+</sup> is able to induce an increase in the ASC content in shoots. Both La<sup>3+</sup> and REEs induce a GSH decrease in shoots showing that the GSH metabolism is mostly negatively affected by treatments in this part of the seedlings. The enzymes catalyzing the reactions involved in ASC and GSH recycling remain substantially unchanged. APX and CAT show a different behaviour after lanthanide treatments: CAT activity does not change in the roots and decrease slightly in the shoots: on the opposite, treatments with  $La^{3+}$  and REEs mainly stimulate APX activity both in roots and shoots. APX has a pivotal role in the plant responses to stresses (Mittler, 2002). The increase in APX as well as in ASC content may be interpreted as a defence mechanism against stress caused in T. durum by REEs supply. Our results are in accordance with data reported by Shi et al. (2005) that show that low concentrations of  $La^{3+}$ (0.002-0.02 mM) promote plant growth in cucumber seedlings but do not affect activities of antioxidant enzymes, while higher concentrations (0.2-2 mM) stimulate these activities but inhibit plant growth and induce an increase in stress markers. The effects of lanthanides on stress responses are sometimes questionable because they are strictly related to the experimental conditions. Pang et al. (2002) reported that treatment of *T. aestivum* seedlings with La<sup>3+</sup> induces an increase in superoxide dismutase and CAT activities improving plant resistance to a moderate lead stress. The increase in some antioxidant defences however is not always sufficient to prevent tissue damages as reported for lanthanides application in Lemna minor plants (Ippolito et al., 2007). Therefore, the increase in antioxidant levels in T. durum could be interpreted as an indicator of stress caused by REEs, since it is well known that many abiotic stresses induce an increase in the antioxidant systems that may not be able to counteract the negative effects on plant growth and/or metabolism (Mittler, 2002; Ippolito et al., 2007).

In conclusion, the dose and time dependent negative effect of lanthanides on seed germination and seedling growth observed in our experimental conditions should lead to a more careful use of such elements in crop management.

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