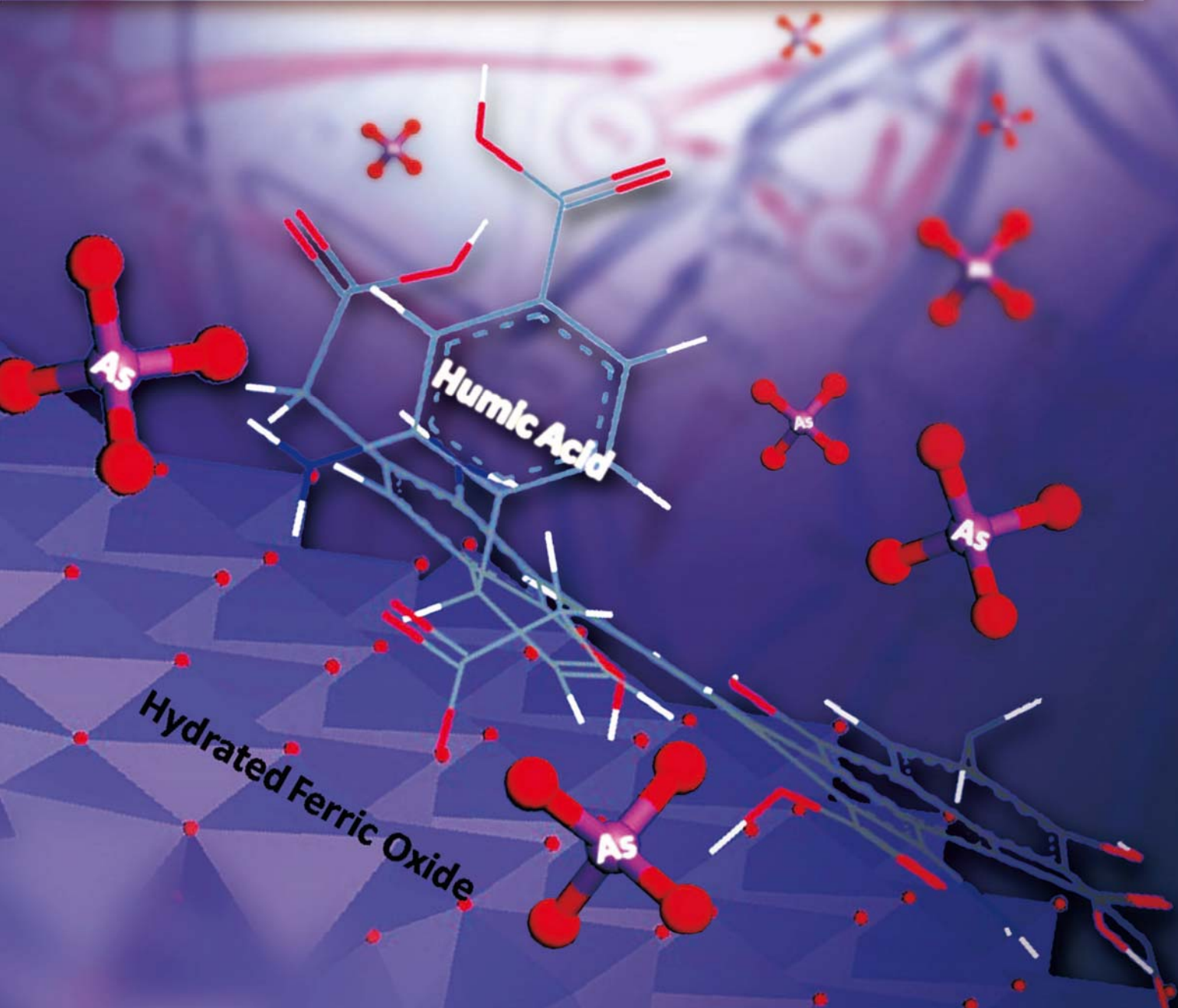


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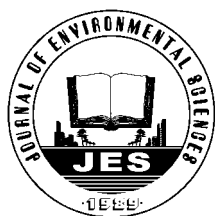
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## Alginate modifies the physiological impact of CeO<sub>2</sub> nanoparticles in corn seedlings cultivated in soil

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### ABSTRACT

Alginates are naturally occurring components of organic matter in natural soil whose effects on nanoparticle (NP) toxicity to plants is not well understood. In the present study, corn plants were grown for one month in soil spiked with 400 mg/kg CeO<sub>2</sub> NPs with various alginate concentrations. After one month of growth in the NPs impacted soil, plants were harvested and analyzed for Ce and mineral element concentrations. Chlorophyll concentration and heat shock protein 70, used as biomarkers for oxidative stress, were also evaluated. Results showed that, compared to CeO<sub>2</sub> NPs treatment, alginate at 10, 50, and 100 mg/kg increased Ce concentration in roots by approximately 46%, 38%, and 29% and by 115%, 45%, and 56% in shoots, respectively. CeO<sub>2</sub> NPs without alginate increased Mn accumulation in roots by 34% compared to control. CeO<sub>2</sub> NPs with low and medium alginate increased Mn by ca. 92% respect to NPs without alginate and by ca. 155% respect to control. CeO<sub>2</sub> NPs without/with alginate significantly increased accumulation of Fe and Al in roots. In addition, alginate at 50 mg/kg increased Zn accumulation in roots by 52% compared to control. In shoots, K increased at all NP treatments but the accumulation of other elements was not affected. Alginate enlarged the impact of CeO<sub>2</sub> NPs to corn plants by reducing chlorophyll *a* content and triggering overexpression of heat shock protein 70.

## Introduction

Cerium oxide nanoparticles (CeO<sub>2</sub> NPs) are widely used in applications such as catalyst, automotive industry, glass mirrors, plate glass, and ophthalmic lenses (Milt et al.,

2003; O'Neil et al., 2001). These NPs are among the 13 engineered nanomaterials in the list of priority for immediate testing by the Organization for Economic Cooperation and Development (OECD) (France et al., 2008; Project on Emerging Nanotechnologies, 2011). However, the environmental release of CeO<sub>2</sub> NPs from factories or applications, and their behavior and effects in the environment are not well known yet (Hoecke et al., 2009).

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Previous studies have shown that CeO<sub>2</sub> NPs are stable in soil at pH values of 7 to 9 (Cornelis et al., 2011). This suggests CeO<sub>2</sub> NPs will remain in soil for a long time. In addition, reports from recent investigations have shown a wide variety of plant responses after exposure to CeO<sub>2</sub> NPs. For instance, Schwabe (2013) found that CeO<sub>2</sub> NP treatments did not reduced the growth in pumpkin and wheat. However, Ma et al. (2010) reported that, at 2000 mg/L, nano-CeO<sub>2</sub> reduced root elongation in lettuce (*Lactuca sativa*). Van Hoecke et al. (2009) found that CeO<sub>2</sub> NPs, at concentrations as low as 2.6 and 5.4 mg/L, produced chronic toxicity to the unicellular alga *Pseudokirchneriella subcapitata*. Previous results from our research group have shown that CeO<sub>2</sub> NPs at 2000 mg/L reduced corn (*Zea mays*) and tomato (*Lycopersicon esculentum*) germination by 30% and cucumber (*Cucumis sativus*) germination by 20% (Lopez-Moreno et al., 2010). In a more recent study, we demonstrated that CeO<sub>2</sub> NPs are taken up and stored without change in maize roots (Zhao et al., 2012a). This previous study also revealed that the uptake of CeO<sub>2</sub> NPs by corn plants was affected by soil organic matter content and alginate surface coating (Zhao et al., 2012a). Alginates are naturally occurring polysaccharides (Chen and Elimelech, 2008; Kantar et al., 2008) that have been used to stabilize NPs for several applications (Li et al., 2008; Chico et al., 2009; Fayaz et al., 2009; dos Santos Silva et al., 2011). This suggests that excess of alginate can be released into the environment together with NPs, with unknown consequences for edible plants. Thus, more studies are needed to better understand the impact of CeO<sub>2</sub> NPs in plants, in environments where excess alginates could be present.

On the other hand, studies have shown that carbon-based nanoparticles such as single walled carbon nanotubes triggered reactive oxygen species (ROS) generation in *Arabidopsis* and rice (*Oryza sativa*) (Shen et al., 2010). In addition, multiwall carbon nanotubes have been found to induce gene expression of heat shock protein (HSP) 90 in tomato leaves and roots (Khodakovskaya et al., 2011). However, there are no reports on the effect of CeO<sub>2</sub> NPs on heat shock protein expression in plants.

A few studies have described the physiological impacts of rare earth elements (REE) in plants. For example, at concentration higher than 89 μmol/L, cerium affected the foliar chlorophyll content, nitrate reductase activity, shoot-root length and relative yield in cowpea plants (*Vigna unguiculata*) (Shyam and Aery, 2012). The authors suggested the effects could be produced by the substitution of Mg<sup>2+</sup> by Ce in chlorophyll synthesis. It has also been suggested that, due to their similar chemical characteristics, Eu, a REE, may compete with Ca for organic ligands (Shtangeeva and Ayrault, 2007). These studies suggest that REE elements can have serious impacts on the uptake of nutritional elements in food crops. However, to the authors' knowledge the impact of REE NPs on the uptake

of nutritional elements by plants has yet to be reported. The purposes of this work were to determine the effects of alginate on: (1) the transport of Ce within corn plants treated with CeO<sub>2</sub> NPs, (2) the uptake and transport of micro and macro nutrients, (3) the chlorophyll content, and (4) the expression of stress related heat shock protein 70. Maize was selected for this study because it is a crop widely cultivated throughout the world for direct and indirect consumption. In addition, 40% of the corn world's harvest is produced in the United States (FAO, 2009). In this study, corn plants were grown in soil spiked with CeO<sub>2</sub> NPs with various alginate concentrations for one month. After harvest, the concentration of Ce and many nutrient elements were determined by ICP-OES in the root and shoots tissues.

## 1 Materials and methods

### 1.1 Nanoparticles and soil characterization

CeO<sub>2</sub> NPs (Meliorum Technologies, USA) were obtained from the University of California Los Angeles Center for Environmental Implications of Nanotechnology (UC CEIN). The CeO<sub>2</sub> NPs had primary particle size of (8 ± 1) nm, hydrodynamic size of (1373 ± 32) nm, and zeta potential of -0.62 (±2.9) mV (25 mg/L in distilled water; measured with Zetasizer Nano-ZS 90, Malvern, Germany). The soil was collected from Texas Agri-Life Research Center at El Paso (Texas A&M University System, USA). The properties of the soil were introduced in previous article (Zhao et al., 2012b). The soil was air-dried and sieved through a 1 mm mesh prior to experimental use. Because this soil has very low organic matter content (native soil), a portion of this soil was mixed 1:1 (V/V) with a soil with high organic matter purchased from a nursery store (Scotts, premium potting soil, USA).

### 1.2 Corn cultivation in soil

Corn seeds were germinated and cultivated in Magenta boxes containing 400 g soil. Six corn kernels (Golden variety, Del Norte Seed Company, El Paso, TX, USA) were sown in the boxes with the following treatments: (1) control (no NPs, no alginate), (2) 400 mg/kg CeO<sub>2</sub> NPs (control for alginate), (3) 400 mg/kg CeO<sub>2</sub> NPs with 10 mg/kg alginate, (4) 400 mg/kg CeO<sub>2</sub> NPs with 50 mg/kg alginate; (5) 400 mg/kg CeO<sub>2</sub> NPs with 100 mg/kg alginate. Each treatment included 3 replicates. The soil was spiked with CeO<sub>2</sub> NP suspensions previously sonicated for 30 min.

Plants were grown for 30 days in a growth chamber (TC2 Microcontroller Environmental Growth Chambers, Chagrin Falls, OH, USA) at 25°C 16 hr light/8 hr dark cycle, 65% humidity, and 340 μmol/(m<sup>2</sup>·sec). The plants were irrigated as needed in order to keep the soil close

to field capacity. At harvest, the roots were thoroughly flushed with tap water followed by deionized water (DI). After washings, plants were oven dried at 60°C for 24 hr (Fisher Scientific, Isotemp Oven, UK) and processed for multiple element determination.

### 1.3 ICP-OES analysis

The dried samples (root and shoot tissues) were ground and microwave digested with a mixture of HNO<sub>3</sub> (65%) and H<sub>2</sub>O<sub>2</sub> (30%, V/V) (1:4) (CEM Corporation Mathews, NC, USA). The digest were analyzed by ICP-OES (PerkinElmer Optima 4300 DV, Shelton, CT, USA). Certified Reference Material (Peach leaves, NIST 1547, USA) was processed as sample and the recovery rate was (100.82 ± 0.48).

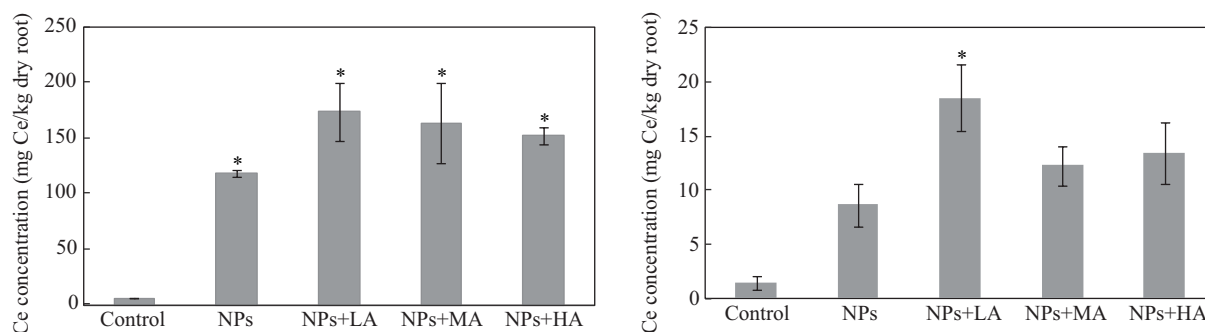
### 1.4 Chlorophyll content in corn leaves

Almost all stressors affect either directly or indirectly the photosynthesis performance and modify its optical and fluorescence properties. There is a long standing interest in the practical application of chlorophyll *a* (Chl-*a*) fluorescence as a rapid and sensitive bioindicator of plant stress in response to different chemical factors (Mallakin et al., 2002).

Fresh leaves were collected from one month-old corn plants to determine the chlorophyll concentration. Leaves were weighed and homogenized in 2 mL of 80% acetone in presence of MgCO<sub>3</sub> in a dark room at (25 ± 1)°C. The extracted solutions were centrifuged at 11,000 r/min for 10 min (Eppendorf 5417R, Hamburg, Germany). The chlorophyll content was determined by measuring the solution absorbance at 663 and 645 nm using a spectrophotometer (Thermo Spectronic GENESYS, Rochester, USA). Chlorophyll *a* and *b* concentrations ( $C_{\text{Chl-}a}$ ,  $C_{\text{Chl-}b}$ ) were calculated according to Eqs. (1) and (2) (Li et al., 1999):

$$C_{\text{Chl-}a} = 12.72A_{663} - 2.59A_{645} \quad (1)$$

$$C_{\text{Chl-}b} = 22.88A_{645} - 4.67A_{663} \quad (2)$$



**Fig. 1** Cerium concentration in (a) roots and (b) shoots of corn plants (Golden variety) grown for one month in a farm soil. The soil was treated with 400 mg/kg CeO<sub>2</sub> NPs and alginate at 10 (LA), 50 (MA) and 100 (HA) mg/kg soil. Error bars stand for standard deviation. \* Stands for statistic differences at  $p < 0.05$ . LA, MA, and HA represents low, medium, and high alginate.

### 1.5 Protein extraction from corn root and Western-blot analysis

Extraction of proteins from corn leaves was performed as previously described. Briefly, plant leaves were homogenized in liquid nitrogen until no more chunks were visible. Extraction buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 0.1% (W/V) Triton X-100, 1 mmol/L PMSF, 50 mmol/L leupeptin and 11 mmol/L 2-mercaptoethanol was subsequently added to the homogenized tissue at a ratio of 2 mL buffer per 1 g tissue and samples were grinded for another 10 min on ice. One mL of the supernatant was then transferred to 1.5 mL microcentrifuge tube and spun at 16,000 ×g for 30 min at 4°C. Supernatants containing total proteins were transferred to a new microcentrifuge tube and proteins were precipitated with cold acetone.

Twenty micrograms of protein were separated in 12% SDS-PAGE gels and wet transferred to nitrocellulose membrane (GE Healthcare Life Sciences, USA). Membranes were washed once in TBS-T buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20) and blocked for 1 hr at room temperature in TBS-T containing 5% nonfat milk. Membranes were then incubated at room temperature with the respective primary antibodies: rabbit anti-heat shock protein 70 (1:3000) (Agriser antibodies, Vannasa, Sweden) and mouse anti-actin (1:1000) (Agriser antibodies, Vannasa, Sweden) for 1 hr. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, USA) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (Santa Cruz Biotechnology, USA).

## 2 Results and discussion

### 2.1 Effect of alginate on Ce uptake

Cerium accumulation in roots and shoots of corn plants grown in soil treated with CeO<sub>2</sub> NPs with different alginate concentrations are shown in **Fig. 1**. Most of the Ce

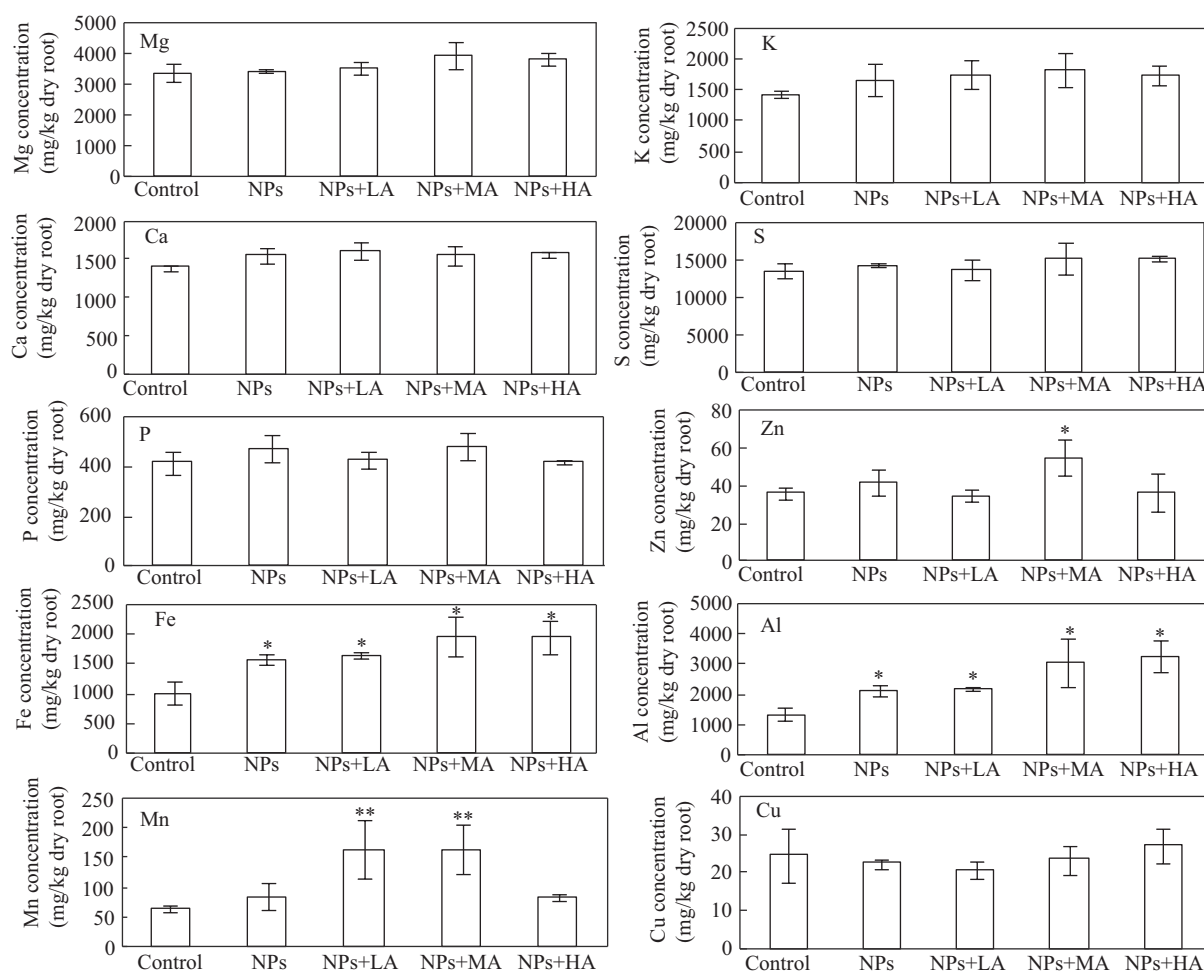
accumulated in corn root tissues (**Fig. 1a**). Plants grown with 400 mg/kg CeO<sub>2</sub> NPs without alginate, accumulated 118.3 and 8.6 mg/kg of Ce in roots and shoots, respectively. The presence of Ce in shoots indicates that the transportation of Ce from roots to the upper part of the plants occurred, though at low concentration. However, Ce uptake and translocation drastically increased in the presence of alginate. Compared to the CeO<sub>2</sub> NP treatments, alginate at 10, 50, and 100 mg/kg increased Ce in roots by approximately 46%, 38%, and 29% (**Fig. 1a**), and by 115%, 45%, and 56% in shoots, respectively (**Fig. 1b**). As previously reported (Zhao et al., 2012), CeO<sub>2</sub> undergo low biotransformation within corn tissues; this suggests that the increasing use of alginate as surface coating material could result in an enhanced risk for introducing CeO<sub>2</sub> NPs into the food chain.

It was noted that Ce accumulation in shoots was significantly higher at low alginate concentration (10 mg/kg). This suggests that in an eventual release of CeO<sub>2</sub> NPs, the higher risk of food contamination would occur in organic matter enriched soil. The mechanism involved in

the increase of Ce uptake and translocation by alginate is still unknown. However, our previous work showed that alginate surface coating increased the Ce translocation to shoots in corn plants grown in a soil with low organic matter content and treated with 400 mg/kg CeO<sub>2</sub> NPs. Sodium alginate has been associated with seed germination, shoot elongation, root growth, and flower production, among others in *Foeniculum vulgare* Mill (Sarfaraz et al., 2011; Hashmi et al., 2012). However, the mechanisms of these effects are still unknown.

## 2.2 Effect of alginate on nutrient elements accumulation in roots

The presence of CeO<sub>2</sub> NPs with/without alginate did not alter the uptake of macronutrients Mg, K, Ca, S, and P in one-month old corn roots. However, the uptake of Al and the micronutrients Fe, Mn, and Zn was increased (**Fig. 2**). Compared to control (no NPs), concentrations of Fe and Al were significantly higher in all NP treatments. For Al, the difference was significant at  $p \leq 0.023$ , but for Fe, the significance was only at  $p \leq 0.09$ . The accumulation



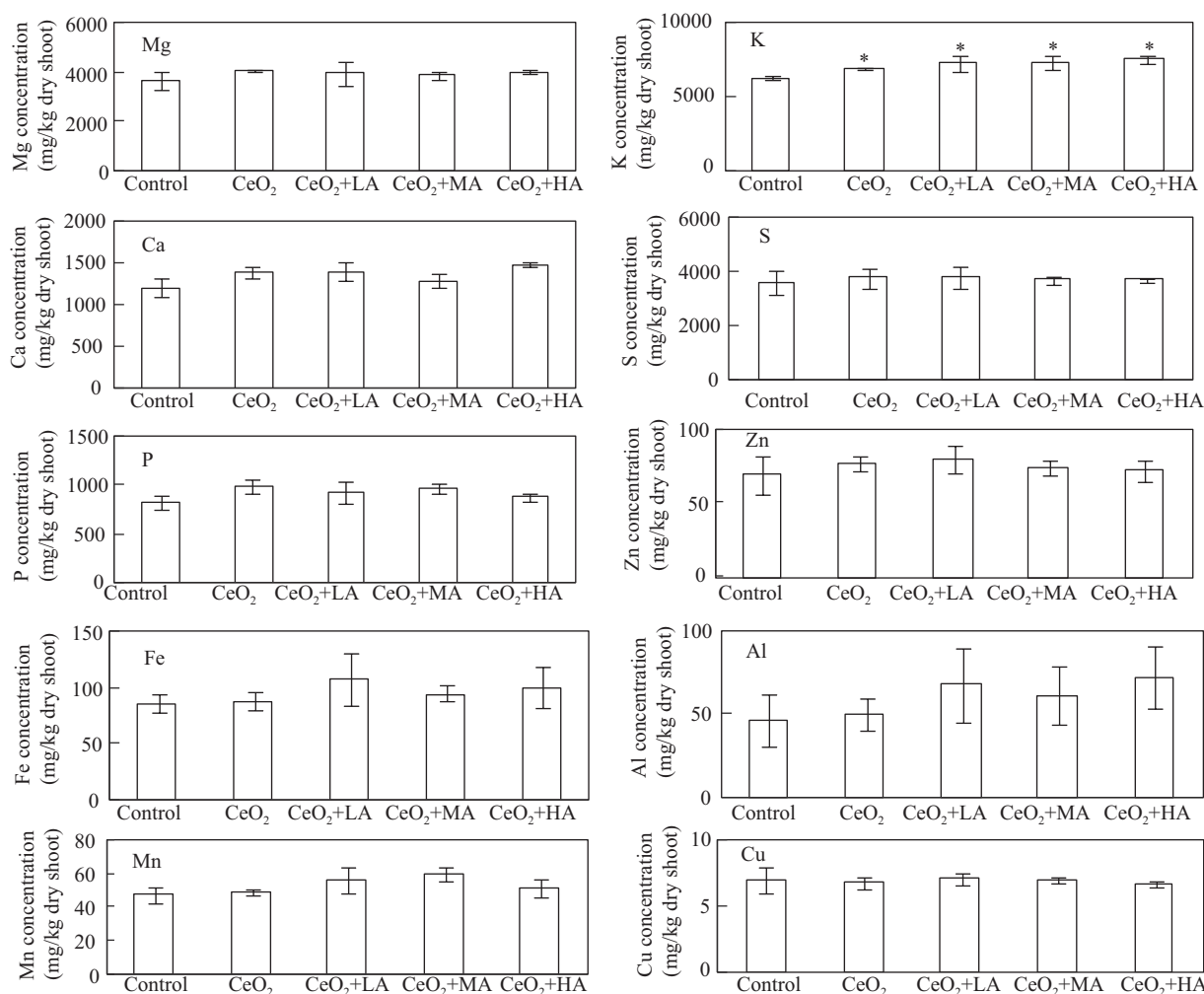
**Fig. 2** Micro and macro nutrient contents in roots of corn plants grown for one month in a farm soil. The soil was treated with CeO<sub>2</sub> NPs with different concentration of alginate. Error bars stand for standard deviation. \*\* Stands for statistic differences at  $p < 0.01$ ; \* stands for statistic differences at  $p < 0.05$ .

of both Fe and Al in roots was similar in all treatments. Moreover, compared to NPs alone and NPs-low alginate, the concentrations of both Fe and Al were significantly higher at medium and high alginate concentrations. It is very likely that the CeO<sub>2</sub> NPs were bound with Fe and Al oxides, which are widespread soil colloids. Previous results showed that Fe and Al are co-released from the soil column with ZnO NPs (Zhao et al., 2011). Manganese accumulation pattern was different. The addition of CeO<sub>2</sub> NPs without alginate increased Mn accumulation in roots by 34% compared to control (no NPs); but NPs-low alginate and NPs-medium alginate treatments increased the accumulation of Mn by 92% and 90% respect to NPs without alginate and 158% and 155% respect to control. These differences were significant at  $p \leq 0.05$ . Mannuronate and guluronate blocks of the alginate have the capability for Mn<sup>2+</sup> binding (Emmerichs et al., 2004). However, at high alginate treatment (100 mg/kg) the accumulation of Mn in roots was similar to the accumulation obtained with NPs alone. More experiments are needed to explain these results. In the case of Zn, the medium alginate treatment

(50 mg/kg) increased its accumulation in roots by 52% compared to control.

The increase of elements in roots by CeO<sub>2</sub> NPs may be due to the enhancement of soil cation exchange capacity (CEC) produced by the CeO<sub>2</sub> NPs. A small experiment was performed to determine if CeO<sub>2</sub> NPs increased soil CEC. Results showed that the CEC in soil spiked with 400 mg/kg CeO<sub>2</sub> NPs was 6× compared to control. Similar to soil natural colloids, e.g. kaolin and NOM, CeO<sub>2</sub> NPs have a large surface area, with negative surface charge ( $-22.8 \pm 4.5$  mV), that supplies many exchange sites for cation adsorption (Zhao et al., 2012).

The concentration of Na in roots of plants exposed to CeO<sub>2</sub> NPs alone (not shown) decreased by 10.5% compared to control. In the CeO<sub>2</sub> NP-alginate treatments, the reduction of Na in roots was higher (up to 16%). This could be associated with the increase in K absorption (Fig. 3). As a C<sub>4</sub> plant, corn needs Na to regenerate phosphoenolpyruvate, the substrate for carboxylation (Taiz and Zeiger, 1998). Thus, reduction of Na absorption could represent a toxicity pathway of CeO<sub>2</sub> NPs to corn plants.



**Fig. 3** Micro and macro nutrient contents in shoots of corn plants grown for one month in a farm soil. The soil was treated with CeO<sub>2</sub> NPs with different concentration of alginate. Error bars stand for standard deviation. \* Stands for statistic differences at  $p < 0.05$ .

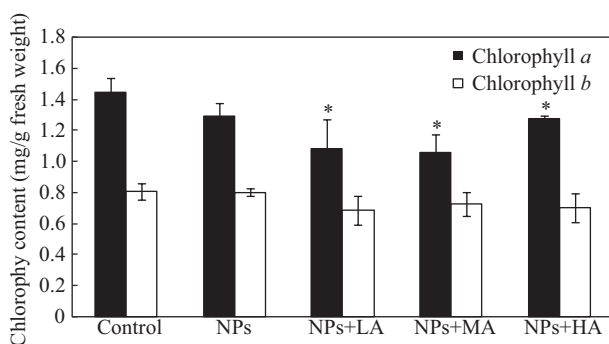


### 2.3 Effect of alginate on nutrient elements uptake in shoots

Concentration of macro and microelements in corn shoots treated with CeO<sub>2</sub> NPs and alginate are shown in Fig. 3. As one can see in this figure, only the concentration of K was significantly affected by the NP treatments ( $p \leq 0.016$ ). All the CeO<sub>2</sub> NP-containing treatments increased the concentration of K in corn shoots. However, there were no differences between the alginate and non-alginate treatments. An outward rectifying channel mediates potassium release into the xylem, which is controlled by the stress hormone abscisic acid (De Boer, 1999). Perhaps the presence of NPs up regulates the production of abscisic acid, which in turn, increases the uptake of K. The fact that higher concentrations of Al, Fe, and Mn in roots, compared to control, were not observed in shoots could indicate that these elements were bound to NPs and stuck on the surface of the roots. Divalent cations have shown to bind with alginate (Fatin-Rouge et al., 2006).

### 2.4 Chlorophyll changes

Chlorophyll and other leaf pigments are related to stress response (Mehta et al., 2010; Kopsell et al., 2011). To elucidate if the CeO<sub>2</sub> NPs coupled to alginate impacted the photosynthesis machinery in young corn plants (Golden variety), chlorophyll concentration in fresh leaves of one month-old plants was determined (Fig. 4). As seen in this figure, none of the treatments affected Chl-*b* fluorescence. Chl-*a* was not affected by CeO<sub>2</sub> NPs alone; however, the combination of CeO<sub>2</sub> NPs with alginate, at all concentrations, significantly reduced Chl-*a* fluorescence, compared to control. The CeO<sub>2</sub> NPs plus alginate at low (10 mg/kg) and medium (50 mg/kg) concentration reduced Chl-*a* fluorescence by 16.5% and 18.4%, respectively ( $p \leq 0.01$ , Fig. 4). Although the CeO<sub>2</sub> NPs in soil, by themselves or coupled to alginate, increased the concentration of some nutritional elements in corn roots (Fig. 2) and did not interfere with nutrient accumulation in shoots (Fig. 3), they showed toxicity to Chl-*a* fluorescence, which is

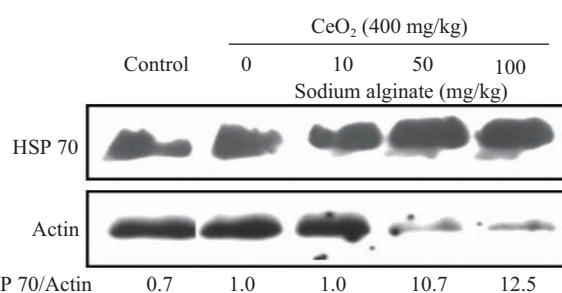


**Fig. 4** Content of Chlorophyll in corn leaves grown for one month in a farm soil. The soil was treated with CeO<sub>2</sub> NPs with different concentration of alginate. Error bars stand for standard deviation. \* Stands for statistic differences at  $p < 0.05$ .

an indicative of stress (Mehta et al., 2010; Kopsell et al., 2011). There exists the possibility that the CeO<sub>2</sub> NP treatments affected the concentration of nitrogen or silicon (not determined) in the corn plants, which are related to chlorophyll production or degradation (Zhao et al., 2005; Gottardi et al., 2012).

### 2.5 HSP 70 analysis

Heat-shock proteins are general stress related proteins involved in the protection, restoration, and degradation of damaged cell components, especially proteins, during most abiotic stresses (Parsell and Lindquist, 1994; Downs et al., 1999; Hamilton and Heckathorn, 2001). Western-blot analytical technique was used to determine the content of HSP 70 in fresh leaves of one month-old corn plants. As seen in Fig. 5, HSP 70 was apparently over produced in the CeO<sub>2</sub> NP treatments including medium and high amount of alginate. Khodakovskaya et al. (2011) discovered that multiwalled carbon nanotubes induce changes in gene expression in tomato leaves and roots, up-regulating the stress-related genes, such as heat shock protein 90. The present study showed that in corn plants, the HSP 70 increased expression in response to CeO<sub>2</sub> NPs-alginate effect. However, the specific functions or structures protected by HSPs remain unknown. Heckathorn et al. (2004) reported that the chloroplast small HSP, which function is to protect photosynthesis during heavy metal stress in corn leaves (*Zea mays*), were triggered by heavy metal. Previous studies have shown that most of the CeO<sub>2</sub> NPs taken by plants remain in the nanoparticulate form; thus, it is possible that some of the NPs present in corn leaves reached the chloroplast affecting the HSP 70 expression. More studies are needed in order to decipher these uncertainties.



**Fig. 5** Effects of CeO<sub>2</sub> NPs plus alginate on HSP 70 expression in corn leaves estimated by Western blot analyses. Equal amounts of total protein extracts (20  $\mu$ g) from leaves were loaded in each lane. Protein blots were probed with primary antibodies directed against HSP 70. Total tissue protein was fractionated by SDS-PAGE, transferred to membranes, and probed with HSP 70 antibodies. The expression level of HSP 70 was normalized to actin and fold of change was measured by densitometry.

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