Physiological and biochemical responses of sunflower (*Helianthus annuus* L.) exposed to nano-CeO₂ and excess boron: Modulation of boron phytotoxicity

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Abstract

Little is known about the interaction of nanoparticles (NPs) with soil constituents and their effects in plants. Boron (B), an essential micronutrient that reduces crop production at both deficiency and excess, has not been investigated with respect to its interaction with cerium oxide NPs (nano-CeO₂). Considering conflicting results on the nano-CeO₂ toxicity and protective role as antioxidant, their possible modulation on B toxicity in sunflower (*Helianthus annuus* L.) was investigated. Sunflower was cultivated for 30 days in garden pots containing original or B-spiked soil amended with nano-CeO₂ at 0-800 mg kg⁻¹. At harvest, Ce and B concentrations in tissues, biomass, and activities of stress enzymes in leaves were determined. Results showed that in the original soil, Ce accumulated mainly in roots, with little translocation to stems and leaves, while reduced root Ce was observed in plants from B-spiked soil. In the original soil, higher levels of nano-CeO₂ reduced plant B concentration. Although morphological effects were not visible, changes in biomass and oxidative stress response were observed. Sunflower leaves from B-spiked soil showed visible symptoms of B toxicity, such as necrosis and chlorosis in old leaves, as well as an increase of superoxide dismutase (SOD) activity. However, at high nano-CeO₂ level, SOD activity decreased reaching values similar to that of the control. This study has shown that nano-CeO₂ reduced both the B nutritional status of sunflower in original soil and the B phytotoxicity in B-spiked soil.

Key words: *Helianthus annuus*, Nano-CeO₂, Ce uptake, Boron interaction, Growth parameters, Oxidative stress, Toxicity symptoms

Graphical Abstract

Highlights

- First report on boron nutritional status of sunflower grown in CeO₂ NP amended soil
- Cerium concentration in roots was reduced in boron-spiked soil
- Boron nutritional status was reduced at high nanoparticle concentration
- Low oxidation and balance effects between pro- and anti-oxidants in leaves

Introduction

The increasing production and use of engineered nanoparticles (NPs) in consumer products and other applications, such as environmental remediation, agriculture, and pharmacology, lead to their release in the environment. Once in the soil or water, they may be taken up by living organisms, enter into the food chain, persist in different environmental matrixes for extended periods, cause ecotoxicological hazards, and bioaccumulate in the food chain (Rico et al. 2011, Baalousha et al. 2016). Thus, it is important to recognize the consequences of the use of nanomaterials and their interaction with soil components, water, and living organisms. Soil represents an important sink for NPs, since their accumulation could occur after the end user application of nano-enabled products, their presence in fertilizers, plant protection products, and soil amendments (Servin et al. 2016). Moreover, edible plants are considered the point of entry for their bioaccumulation in the food chain.

Cerium oxide nanoparticles (CeO_2 NPs, nano- CeO_2) are used in a variety of applications comprising solid oxide fuel cells, environmental remediation, mechanical polishing, sensing, catalysis, and biomedicine (Andreescu et al. 2014). The soil-plant system has been exposed to CeO_2 NPs, showing effects in the status of nutrients and physiological processes. Gui et al. (2015) reported a significant increase of nitrate content and disruption of the activity of superoxide dismutase (SOD), peroxidase (POD), and malondialdehyde (MDA) in *Lactuca sativa* exposed to 100 and 1000 mg kg^{-1} nano- CeO_2 in soil, respectively. Rico et al. (2013a,b,c and 2015) reported that nano- CeO_2 produced a significant modification in the antioxidant system of rice (*Oryza sativa*) and a negative impact on rice grain quality. In addition, previous studies have shown that plants take up and store nano- CeO_2 in different tissues (Lopez-Moreno et al. 2010, Zhang et al. 2012, Hernandez-Viezcas et al. 2013). Later investigations suggested that nano- CeO_2 may release Ce^{3+} on the root surface, which uptakes both the Ce^{3+} ions and the nano- CeO_2 (Gui et al. 2015, Ma et al. 2015, Schwabe et al. 2015).

Recent studies have shown conflicting results on the biological impact of nano- CeO_2 , varying from toxicity to protective effects, due to their role as antioxidant/free-radical scavenger (Rico et al. 2015). Most of the exciting and harmful properties of nano- CeO_2 derive from the switching between Ce^{3+} and Ce^{4+} oxidation states of NPs and their surface adsorption properties (Andreescu et al. 2014). It has been stated that smaller CeO_2 NPs have higher surface-to-volume ratio and number of defects in their crystalline structure, which have been considered the main factors responsible for increasing the $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio (Loschen et al. 2008). The switching reaction between Ce cations has been associated with the SOD and catalase (CAT) mimetic activities of

1 nano-CeO₂ (Celardo et al. 2011). The chemical reduction of superoxide requires high Ce³⁺/Ce⁴⁺
2 ratio, due to the ability of Ce³⁺ to get oxidized producing peroxide, a reaction that corresponds to
3 the reduction of superoxide by SOD, hence termed SOD-mimetic. On the other hand, low Ce³⁺/Ce⁴⁺
4 ratio favors the process of oxidation of H₂O₂ by reacting with Ce⁴⁺, producing reduced Ce and
5 molecular O₂, a reaction similar to that of CAT. The spontaneous switching from the oxidized to the
6 reduced state could produce a mechanism of regeneration of nano-CeO₂, which could work as an
7 ideal antioxidant, scavenging two types of reactive oxygen species (ROS): (O₂^{•-} and H₂O₂).
8 However, it is still not clear whether, and in which conditions, nano-CeO₂ promote ROS generation
9 or ROS scavenging.

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11 Boron (B) is an essential micronutrient characterized by a narrow range between deficiency
12 and excess, both producing toxic symptoms that affect crop production (da Silva Leonardo et al.
13 2016). Although B requirement varies among crops, the optimum B content for most crops is 20-
14 100 mg kg⁻¹. Its excess can result in plant toxicity, while its deficiency causes large losses in crop
15 production and crop quality.

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17 Our previous studies have shown that the excess of B from contaminated soil causes
18 oxidative stress in sunflower (*Helianthus annuus* L.) (Giansoldati et al. 2012, Tassi et al. 2011). On
19 the other hand, B deficiency in plants affects vegetative and reproductive growth, resulting in
20 inhibition of cell expansion, death of meristems, reduced fertility, and oxidative stress. Studies in B-
21 deficient sunflower have shown ROS generation and changes in antioxidant enzyme activities,
22 resulting in oxidative damage (Cakmak and Ronheld 1997, El-Shintinawy 1999).

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24 Sunflower seed oil represents one of the largest source of vegetable oil. Sunflower is used as
25 forage and sunflower oil has several industrial applications, e.g. soap and detergents (Myers 2002).
26 However, sunflower is one of the most sensitive plants to the deficiency and excess of B (Blamey et
27 al. 1997, Asad et al. 2002).

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29 This study was performed with sunflower plants cultivated in farm soil, spiked or unspiked
30 with B, to evaluate the effects of nano-CeO₂ amendment on plant B homeostasis and phytotoxicity.
31 Considering the conflicting results on the biological impact of nano-CeO₂, i.e. toxicity and
32 protective role as antioxidant and free-radical scavenger, the possible modulation of B toxicity was
33 also investigated. In addition to evaluating Ce and B accumulation in the different plant tissues, the
34 activity of stress enzymes was also analyzed. To our knowledge, this is the first report showing the
35 implications of nano-CeO₂ on B nutritional status of sunflower plants.

36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 **2. Materials and methods**

2.1. *CeO₂ nanoparticles characteristics*

The nano-CeO₂ used in this study were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN), through The University of Texas at El Paso, United States. These NPs were previously characterized as having a primary size of 8 ± 1 nm, hydrodynamic size of 1373 ± 32 nm, and zeta potential of -0.62 ± 2.9 mV (pH=5.8) (Keller et al. 2010, Zhao et al. 2012). Size and zeta potential of NPs suspended in deionized water at 25 mg L⁻¹ were measured using a Malvern Zetasizer (Nano-ZS 90, Malvern) with three determinations per sample, consisting of 12 runs per determination.

2.2. *Soil collection and characterization*

Top soil (20-30 cm) was collected from an agricultural area in Tuscany, Italy; air-dried, sieved (0-2 mm), and homogenized for characterization and preparation of pot trials. The soil properties [pH, cation exchange capacity (CEC), organic matter (OM), electrical conductivity (EC), texture (clay, silt, and sand fractions)], and essential element contents (N, P, K, Ca, Mg, B) were determined according to the procedure reported in Methods of Soil Analysis (SSSA, 1996). In short, the pH was determined using a glass electrode in a suspension with soil/water ratio of 1:2.5 w/v; CEC using barium acetate; OM by oxidation with potassium dichromate (Walkley-Black method), and texture by the pipette method, using sodium hexametaphosphate as a dispersant. Boron analysis in soil was performed after digestion with a mixture of H₂O₂ and HNO₃ (v/v, 1:2.5). The digestion was carried out in polytetrafluoroethylene-tetra-fluoromethoxil (PTFE-TFM) pressure digestion vessels in a microwave oven (FKV-ETHOS 900) according to the method 3051-A (US-EPA 1995). Digested soil samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Liberty Axial) and results were expressed as the mean of four replicates on a dry weight basis.

2.3. *Set up of pot trials and plant analysis*

A portion of the collected soil was spiked with an aqueous solution of B to simulate a contamination level of about 20 mg/kg of B available to plants. A soil paste formed with addition of the B solution was air dried and occasionally mixed until the excess of water evaporated. The mixing process was repeated every 10 days for about one month for equilibration and to obtain a uniform distribution of the element. The original control soil was also submitted to the same wet-and-dry procedure with only water, to maintain the same conditions.

A portion of both B-spiked and native (unspiked) soil was treated with the nano-CeO₂ suspensions. Suspensions of nano-CeO₂ were obtained by adding the required amount of powder to

1 ultra-pure milli-Q water (18 MΩ·cm, Merck Millipore) in order to obtain the nominal
2 concentrations of 100, 200, 400, and 800 mg kg⁻¹ soil. The NP suspensions were prepared by
3 sonication for 30 min. in continuous mode with an output power of 80W (Sonifier 250, Branson).
4 Suspensions were immediately added to the soil by mixing with a mechanical mixer for about 10
5 min and left to equilibrate for 24 h. To ensure comparable conditions, portions of control and B-
6 spiked soils not treated with the NPs were exposed to the same procedure. To avoid
7 misunderstanding on terminology, the term ‘spiked’ refers to B-spiked soil and the term ‘treated’ to
8 soil amended with NP suspensions, spiked or not with B.

14 Plastic pots (10 cm height, 12 cm diameter) were filled with 300 g (DW) of: *i*) control soil
15 (any additive); *ii*) B-spiked soil; *iii*) treated soil; and *iv*) B-spiked and treated soil and sown with
16 four seeds (Paola variety) per pot. Each treatment was replicated four times (total of 40 planted
17 pots). Sunflower plants grew for about 35 d in controlled growth chamber conditions (day/night
18 photoperiod 16/8h, light intensity of 300 μmol m⁻² s⁻¹, day/night temperature of 25/20°C and
19 constant humidity of 70%). Pots were watered with tap water whenever needed. At the end of the
20 growth period, plants were collected, severed into roots, stems, and leaves, and exhaustively washed
21 with tap water and rinsed with deionized water. In order to eliminate any further soil particle, roots
22 also received an ulterior cleaning treatment with sonication in pulse mode and output power of 20W
23 for about 5 min in a water-bath.

32 A sub-sample of bulked healthy leaves was frozen in liquid N₂ immediately after collection
33 and stored at -70°C for further analysis of antioxidant enzymes. The remaining plant tissues were
34 oven dried at 40°C until constant weight, ground to fine powder, acid digested, and analyzed for Ce
35 and B concentration by using ICP-OES.

42 2.4. *Determination of antioxidant enzymatic activity in sunflower plants*

44 Frozen leaves (0.2 g FW) were ground under liquid nitrogen and homogenized in 2 ml of
45 extraction buffer, consisting of 50 mM sodium phosphate buffer (pH 7.0), 1 mM
46 ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl-fluoride, 1 mM ascorbic acid
47 and 2% polyvinylpolypyrrolidone (PVPP). Plant extracts were centrifuged at 20,000 × g for 30 min
48 at 4° C and the supernatants were used to measure the enzymatic activity. The SOD (EC 1.15.1.1)
49 activity was assayed by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium
50 (NBT), according to the method of Beyer and Fridovich (1987). In this assay, one unit of SOD is
51 defined as the amount required to inhibit the photoreduction of NBT by 50%. The CAT (EC
52 1.11.1.6) activity was measured according to Aebi (1974), by monitoring the decomposition of
53 H₂O₂. The activity of this enzyme was calculated by measuring the decrease in absorbance at 240
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nm for 1 minute ($\epsilon = 0.04 \text{ mM}^{-1} \times \text{cm}^{-1}$). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by following the decrease in absorbance at 290 nm ($\epsilon = 2.7 \text{ mM}^{-1} \times \text{cm}^{-1}$) due to ascorbate oxidation (Nakano and Asada 1981). Glutathione reductase (GR, EC 1.6.4.2) activity was determined by following the decrease in absorbance at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1} \times \text{cm}^{-1}$) due to the glutathione-dependent NADPH oxidation, according to the modified method of Foyer and Halliwell (1976). In the case of CAT, APX, and GR, 1 unit of activity was defined as the amount of enzyme that can oxidize 1 μmole of substrate per minute. Further experimental details are reported elsewhere (Morelli and Scarano 2004). All enzymatic activities were calculated per mg of protein. The protein content in the cell extract was determined according to Bradford (1976) using bovine serum albumin as standard. Enzymatic assays were carried out at 25°C using a double-beam Jasco UV/Visible Spectrometer (Model V-550, Lecco, Italy).

2.5. Statistical analysis

All the experiments were performed using at least four replicates, and the means and standard deviations were calculated. The statistical differences between control and treated samples were analyzed by two-way analysis of variance (ANOVA) followed by Student t-test using Statistica 8.0 (StatSoft, 2007). Significant differences were expressed as $p < 0.05$ and $p < 0.01$.

3. Results and discussion

3.1. Main soil characteristics

The topsoil used in this study is characterized by a sandy loam texture with sub-alkaline pH, typical of Tuscany soils (Bretzel and Calderisi 2006). Its OM content is within typical values of Italian soils (FAO 2005) and the CEC is classified as normal to high, according to Italian Society of Soil Science (SISS 2010), which indicates a soil with good capacity to bind cations and to provide the essential nutrients for the growth of plants. The original soil was spiked with B in order to increase its availability to plants. The availability of B and most of the macronutrients, together with the main soil characteristics are shown in Table 1.

3.2. Impact of CeO_2 NPs on sunflower plants

3.2.1. Effect of CeO_2 NPs in biomass production

The root, stem, and leaf biomass of plants grown in original soil (unspiked soil) was not significantly affected by the presence of nano-CeO₂, at all tested concentrations (Fig.1a-c). Moreover, none of the NP treatments produced visible symptoms of toxicity in leaves or roots. Fig. 2a, for example, shows leaves and roots exposed to nano-CeO₂ at 400 mg kg⁻¹. Previous studies in soil systems have also shown no visible signs of toxicity or biomass reduction under nano-CeO₂ exposure. Ma et al. (2016) observed no-effects on shoot height and total biomass at different growth stages (vegetative, floral, and seed) of *Brassica rapa* grown in soil irrigated with suspensions of nano-CeO₂ at 10 and 100 mg kg⁻¹. Recently, a field lysimeter study showed that nano-CeO₂ at 100 and 400 mg kg⁻¹ did not affect the biomass and grain yield of wheat (*Triticum aestivum*) (Du et al. 2015). However, different impacts on lettuce biomass were observed by Gui et al. (2015), who reported no effect on biomass at low level of CeO₂ NPs (50 mg kg⁻¹) and shoot and root biomass reduction at a very high concentration (1000 mg kg⁻¹). Moreover, Priester et al. (2012) reported a reduced growth of roots and shoots of soybean (*Glycine max*) cultivated in farm soil amended with 100 mg kg⁻¹ nano-CeO₂. The different responses have been attributed to plant species, soil properties, and different levels of Ce³⁺ on the surface of NPs (Morales et al. 2013, Zhao et al. 2012).

In our study, increasing concentrations of nano-CeO₂ in B-spiked soil did not produce a significant effect on plant biomass (Fig.1a-c), compared to plants from untreated soil (0 NPs). However, in B-spiked soil, typical B phytotoxicity symptoms were evident in older leaves (significant chlorosis and necrosis), regardless the presence or absence of nano-CeO₂ (Fig. 2b,c). Roots from B-spiked soil did not present any visible sign of toxicity (Fig. 2b,c). However, significant biomass reduction, respect to control (unspiked, untreated soil) was found at all NP concentrations (Fig. 1a), which could be attributed to B phytotoxicity. Interestingly, the 400 mg kg⁻¹ nano-CeO₂ treatment showed no reduction of root biomass. Independently of the NP concentration in soil, the biomass from stems and leaves (Fig. 1b,c) showed no significant difference, respect to the control, although a trend in biomass reduction was observable in both tissues. It is possible that statistically significant differences will be observed in a longer study, which is supported by the early symptoms observed in older leaves (Fig 2).

3.2.2. Ce and B content in plants tissues

Cerium concentration in roots increased at increasing levels of nano-CeO₂ (Fig.3a), where a high positive and significant correlation (coefficient of determination R²=0.8957, *p* = 0.01) among Ce in roots and nano-CeO₂ concentration in soil was found, suggesting that the uptake occurred *via* simple diffusion. Although there was a huge amount of Ce in roots, very little was translocated to stems (around 0.1-0.3 mg kg⁻¹ Ce, Fig 3b) or leaves (below detection limit of ICP-OES, DL=0.1 mg

L⁻¹). In addition, there were no visible effects on roots and leaves (Fig. 2a) nor a significant influence on growth (Fig. 1a). Similar results were found by Majumdar et al. (2014) who observed a linear increase of Ce accumulation in roots of hydroponic bean (*Phaseolus vulgaris*) plants with low translocation to the leaves. The spectroscopic analyses suggested the entry of nano-CeO₂ through the gaps in the Casparian strip at the emergence of secondary roots. Other crops or edible plants such as soybean (Lopez-Moreno et al. 2010), cucumber (*Cucumis sativus*) (Ma et al. 2015), rice (Rico et al. 2013a,b), and maize (*Zea mays*) (Birbaïum et al. 2010), among others, showed higher accumulation of nano-CeO₂ in the roots in a dose dependent manner. Apparently, the presence of Ce in sunflower plants did not produce a severe impact on plant development, in terms of biomass of either roots or shoots (Fig. 1 and Fig. 2). This suggests that sunflower could have an efficient tolerance mechanism. Possibly, immobilization of the nano-CeO₂ is occurring in the roots, and at the same time preventing the translocation to the shoots (Hossain et al. 2012), in analogy to the most common form of heavy metal tolerance mechanism (retention at root level). The immobilization of a large fraction of heavy metals in the apoplastic space of roots or complexation at the cell wall-plasma membrane is an adaptive strategy for tolerance in plants and could be associated also to NPs or its dissociated ions. In fact, Zhao et al. (2012) observed the presence of nano-CeO₂ mostly in cell walls of cortex tissues of corn plants, while Zhang et al. (2012) observed needle-like clusters of CePO₄ in both the root epidermis and root intercellular spaces of hydroponic cucumber, exposed to nano-CeO₂ at 2000 mg L⁻¹.

Cerium content in sunflower tissues from B-spiked soil showed similar trend as in the original soil. In roots, a highly positive and significant correlation with NPs in the soil (Fig. 3a, empty bars, coefficient of determination R²=0.9992 at p=0.01) suggested the uptake via simple diffusion. However, a lower amount of Ce in roots was observed, respect to plants grown in unspiked soil (Fig. 3a). This reduction was more pronounced at 100-400 mg kg⁻¹ (around 60%) and around 40% at 800 mg kg⁻¹ nano-CeO₂ treatments. These results could indicate a reduced NPs availability for plant uptake when high levels of B were present in this specific soil. However, additional studies are needed to better understand the interaction of NPs with the B available fraction and soil constituents. Studies on soils with different properties showed that, particularly the dissolved organic matter, could increase the NPs' leaching to deeper soil and their availability for plant uptake (Lv et al. 2014, Tassi et al. 2012, Zhao et al. 2012). Conversely, a reduced mobility, and perhaps reduced availability for plant uptake have been reported, due to increasing interaction among the NPs and the active surfaces in soil (clays, oxides and/or high molecular weight organic matter) and/or due to NPs coagulation in the small pore soil (De Castro et al. 2012, Li et al. 2011, Rodrigues et al. 2016). The mobility of NPs in soils could be affected by the high ionic strength of

soil pore-water, which facilitates NPs' aggregation and precipitation in soil pores. The values of OM and EC of our soil system are considered within normal values for Italian soils, and no relevant soil property could be associated with plant uptake and availability of nano-CeO₂.

Regarding B accumulation in plants, as expected, it was mainly transferred to the leaves; however, significant differences of B concentration were measured at different levels of NPs in soil. Roots showed a similar B concentration of about 40 mg kg⁻¹ (Fig. 4a, roots) regardless of the applied amount of nano-CeO₂. Stems generally had a B concentration around 20 mg kg⁻¹; however, at 800 mg kg⁻¹ nano-CeO₂, B concentration was reduced by about 30% (Fig. 4a, stems). In leaves, the presence of 100 mg kg⁻¹ nano-CeO₂ in soil did not significantly affect B accumulation, compared with control; however, at 400 and 800 mg nano-CeO₂ kg⁻¹, leaves showed a B reduction of about 37% (Fig. 4a, leaves). Moreover, a tendency of root biomass reduction, although not significantly different from the control, was observed (Fig. 1a), endorsing the roots sensibility to B reduction (Asab et al. 2003). Our results suggested that high levels of NPs in soil could interfere with B translocation from the roots to the upper parts, which give rise to a reduction of nutritional status of plants, since B is an essential micronutrient. Several studies demonstrated that B deficiency could interfere with the development and maintenance of cell wall and membrane function, nitrogen assimilation, stress response, photosynthesis and plant growth (Camacho-Cristobal et al. 2011, Cong et al. 2015, Dell and Huang 1997). Boron deficiency in plants is also a subject of interest since it is a constraint for crop production. For example, deformed seed and low oil content on sunflower with low B levels were found by Chatterjee and Nautiyal (2000) and Eggert and von Wirén (2015). On the other hand, several metal oxide NPs impact the physiology of plants: Fe₂O₃ NPs reduced root hydraulic conductivity of sunflowers grown in hydroponics (Martinez-Fernandez et al. 2016); TiO₂ NPs inhibited leaf growth and transpiration of hydroponic maize via physical effects on roots transport (Asli and Newman 2009); nano-CeO₂ affected nutrient and water uptake, reducing the nutrient status of rice grains (Rico et al. 2013c). It is still not known how metallic NPs influence the transport of nutrients and water through the roots; however, physical interactions between plant root tissues and NPs have been considered as a possible cause. Although further studies are needed to clarify the mechanism involved in the interference of nano-CeO₂ with B nutritional status of sunflower plants, our results suggest an interaction of NPs with the available B at root level, thus reducing the B transport to the upper parts, and/or NPs' interference on the water transport system. Boric acid [B(OH)₃] is the form of B mainly present in soil solution at soil pH near neutrality, and is the neutral-molecule-form of B mostly taken up by plants. Recently, the identification of B transporters in plants suggests that the process occurs not only by a passive diffusion but also by an active transport to xylem and facilitated diffusion through channels

1 belonging to intrinsic proteins, in response to environmental B conditions (deficiency or toxicity) to
2 maintain B homeostasis (Miwa and Fujiwara 2010). On the other hand, it was demonstrated that
3 nano-CeO₂ are able to carry out redox reactions with inorganic ions. Liu et al. (2015) observed that
4 nano-CeO₂ perform redox reaction with ferrous ions, improving the colloidal stability by enhancing
5 the zeta potential and hydrophilicity of the NPs with a potential impact on their transport. Our
6 results suggest a kind of interaction between nano-CeO₂ and B(OH)₃ at the root level, influencing
7 the nutritional status of sunflower at higher levels of NPs in soil. To our knowledge, this is the first
8 study showing the implication of nano-CeO₂ on the B-nutritional status of sunflower plants

14 As expected, a higher B concentration was found in all sunflower tissues from B-spiked soil,
15 compared with unspiked soil (Fig.4a,b). In particular, B in leaves increased about 10 times (660-710
16 mg kg⁻¹) compared to leaves from unspiked soil (Fig. 4a,b) and chlorosis on the tip of old leaves
17 was clearly visible (Fig. 2b,c). Moreover, a small but significant reduction of B content was
18 observed in roots and leaves due to the presence of nano-CeO₂ (Fig. 4b). Boron values in roots were
19 significantly reduced by about 15% at 100, 200 and 800 mg kg⁻¹ NP treatments and about 10% in
20 leaves of plants treated with the higher concentrations of NP (400 and 800 mg kg⁻¹). These results
21 could suggest a tendency to reduce B toxicity in nano-CeO₂ treated soils, although the visible
22 necrosis in older leaves from all NP treatments indicated a non-complete 'alleviation' of B toxicity
23 in plants. This result should be further explored to provide indications of some possible beneficial
24 effect of CeO₂ NPs.

3.2.3. *Stress enzymes in leaves*

38 In order to have information on the status of plant antioxidant defense system when subject
39 to increasing amount of NPs, sunflower leaves were evaluated for the following antioxidant
40 enzymes: SOD, CAT, APX and GR (Fig. 5 a-d). The activity of SOD, CAT, APX, and GR in leaves
41 from plants grown under the influence of NPs did not significantly differ from the control (Fig. 5a-
42 d). However, a slight increase (not significant) in CAT activity was observed with nano-CeO₂
43 concentrations above 200 mg kg⁻¹, suggesting an activation of the enzyme to some extent. This
44 result suggests that the reduction of B concentration in tissues at high NP levels, did not fall below
45 the B critical threshold; thus, sunflower leaves did not show any appreciable oxidative stress. In
46 fact, other studies with B-deficient sunflower observed two- and fourfold higher peroxidase activity
47 in leaves and roots, respectively in comparison to B-sufficient plants (El-Shintinawy 1999) and a
48 decrease of GR activities, concomitant with an increase of APX (Cakmak and Ronheld 1997).

58 The presence of huge levels of B in sunflower leaves from B-spiked soil without NP
59 treatments brought to a significant increase of SOD and a drastic reduction of GR, compared with
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leaves from control plants (Fig.5 a, c). On the other hand, no alteration of CAT and APX activities was observed in comparison with control plants (Fig 5 b,d). These results are in agreement with data on the involvement of stress enzymes, indicating an oxidative damage in sunflower under toxic B levels. The activation or inactivation of stress enzymes depends on several factors including plant species (Giansoldati et al. 2012), tolerant or sensible cultivars (Karabal et al. 2003), and the type of stressor, among others. At increasing amount of NPs in B-spiked soil, the SOD activity tends to decrease and restore the control value at the highest NP treatment (800 mg kg⁻¹) (Fig. 5a), behaviour that could be hypothetically attributed to a sort of SOD-mimicking action by nano-CeO₂ in plants. The activity of the other enzymes did not show influence of NPs (Fig 5b-d).

It is generally accepted that the toxicity of CeO₂ NPs may be caused by the excessive production of ROS with a pro-oxidative effect. Several studies on food plants grown in soil treated with nano-CeO₂ showed an overproduction of H₂O₂ and increase in antioxidant enzymatic activity (Du et al. 2015, Gui et al. 2015, Rico et al. 2013a). On the contrary, the anti-oxidative or ROS scavenging activity of nano-CeO₂ mimicking the action of common stress enzymes, such as SOD and CAT, has been observed in animal cells (Andreescu et al. 2014) and also in plant cells and chloroplasts (Boghossian 2015, Corral-Diaz et al. 2014, Gomez-Garay et al. 2014, Ma et al. 2016, Rico et al. 2013b,c). SOD- and CAT-mimicking action was attributed to the mixed valence (Ce³⁺/Ce⁴⁺) on the surface of CeO₂ NPs and the oxidation-reduction cycle between these two valence states (Xue et al. 2011). Taken together, our results suggest that the oxidative stress in plants due to high levels of B was not influenced by the presence of nano-CeO₂. More studies are needed in order to fully understand the changes (or no-alteration) of enzymatic activity caused by a stressor (excess of B), when a potential antioxidant factor (CeO₂ NPs) is present.

4. Conclusion

The results demonstrated that Ce was accumulated in sunflower roots in a dose dependent manner, with very low translocation to the upper plant parts. In addition, no significant effects on biomass production or visible traits were observed. Although an apparent mechanism of tolerance to nano-CeO₂ was demonstrated in sunflower plants, higher levels of NPs in the soil resulted in a detrimental effect on B nutritional status (reduction of about 30%) of plants grown in original soil. However, there was only a marginal reduction in B concentration (about 10%) in plants grown with excess of B, which could indicate a sort of counteraction by nano-CeO₂. In addition, plants from the original soil with reduced B status showed no observable and sensible oxidative stress in leaves, while plants with high levels of B showed a modulation of the antioxidant defense system.

1 However, further studies are needed to better understand the effects and interactions between nano-
2 CeO₂ and B in the soil-plant system.
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5 **Author contribution**

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7 All authors participated in data analysis and drafting manuscript, read and approved the final
8 version. Barbafieri M., Tassi E., Peralta-Videa J.R. and Gardea-Torredey J.L. participated in
9 conceiving the study; Tassi E. participated in designing the experiment and phytotoxicity studies;
10 Morelli E. participated in stress enzymatic studies; Giorgetti L. participated in phytotoxicity studies.
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Tables

Table 1. Main characteristics of the soil used.

Parameter	value
pH (H ₂ O)	7.42
OM (%)	1.76
CEC (cmol ⁽⁺⁾ kg ⁻¹)	36.1
EC (μS cm ⁻¹)	96.8
Sand (%)	46.1
Silt (%)	23.4
Clay (%)	27.5
N _{total} (mg kg ⁻¹)	1240
P _{assimilable} (mg kg ⁻¹)	180
K _{available} (mg kg ⁻¹)	1815
Ca _{available} (mg kg ⁻¹)	5000
Mg _{available} (mg kg ⁻¹)	5002
B (mg kg ⁻¹)	14.1 ± 2.52 (total)
	1.4 ± 0.03 (available)
B-spiked soil	60.7 ± 7.4 (total)
	25.6 ± 3.31 (available)

Figure Captions

Figure 1. Dry biomass of a) roots, b) stems, and c) leaves of sunflower grown in CeO₂ NPs treated soils: unspiked soil and B-spiked soil. Bars are mean values with standard deviation (n=4). Asterisks above bars denote significant differences at level $p \leq 0.05$ with respect to the control (unspiked, untreated).

Figure 2. Leaves and roots of sunflower plants from different soils: a) unspiked and 400 mg kg⁻¹ CeO₂ NPs treatment; b) B-spiked and no CeO₂ NPs treatment; c) B-spiked and 400 mg kg⁻¹ CeO₂ NPs treatment. Arrows indicate phytotoxic effect in older leaves.

Figure 3. Ce concentration in a) roots, b) stems of sunflower grown in unspiked soil and B-spiked soils treated with CeO₂ NPs. Bars are mean values with standard deviation (n=4).

Figure 4. Boron concentration in the different sunflower tissues from plants grown in CeO₂ NPs treated soils: a) unspiked soil, b) B-spiked soil. Bars are mean values with standard deviation (n=4). Asterisks above bars * and ** denote significant differences at level $p \leq 0.05$ and $p \leq 0.01$, respectively, with respect to untreated soil.

Figure 5. Activity of stress enzymes in sunflower leaves from unspiked soil and B-spiked soil: a) SOD, b) CAT, c) GR, d) APX. Values are means $\pm$ SD (n=4). Different letters are significantly different ($p \leq 0.05$) with respect to control soil (unspiked, untreated).

Figure 1.

Figure 2.

Figure 3.

Figure 4

Figure 5