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RESEARCH ARTICLE

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Nanoceria and bulk cerium oxide effects on the germination of *Asplenium adiantum-nigrum* spores

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Abstract

Aim of the study: The effect of cerium oxide engineered nanoparticles on the spore germination of the fern *Asplenium adiantum-nigrum*.

Area of study: France, Britanny Region, Finistére Department, Plougonvelin, in rocks near the sea.

Material and methods: Asplenium spores were cultured *in vitro* on agar medium with Nano-CeO_2 (less than 25 nm particle size) and bulk-CeO_2. The addition of each nano- and bulk particles ranged from 0 to 3000 mg L^{-1}. Observations on rhizoidal and prothallial cells during first stages of gametophyte development were made. The No-Observed-Adverse-Effect concentration (NOAEC) and Lowest-Observed-Adverse-Effect-Concentration (LOEC) values for spore germination rate data were analyzed.

Main results: Germination was speeded up by 100 to 2000 mg L^{-1} nanoceria, while bulk cerium oxide had the same effect for 500 to 2000 mg L^{-1} concentrations. Present results showed cellular damage in the protonema while rhizoid cells seemed not to be affected, as growth and membrane integrity remained.

Research highlights: Both nanosized and bulk cerium oxide are toxic for the fern *Asplenium adiantum-nigrum*, although diverse toxicity patterns were shown for both materials. Diverse toxic effects have been observed: chloroplast membrane damage and lysis, cell wall and membrane disruption which leads to cell lysis; and alterations in morphology and development.

Keywords: Nanoparticles; rhizoid; prothallus; chloroplast; fern.

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Introduction

Industrial manufactures have produced a range of engineered nanoparticles (ENPs) which have resulted in a higher level of nanosized particles that had always been present in nature. This increase is raising serious concerns over their potential impact on the environment and potential adverse effects on ecosystems, as well as on human health. Dissimilar to bulk material, nanoparticles have individual physical and chemical properties derived from their morphology and composition. Size, shape, purity and catalytic activity of nanoparticles determine their interaction with the environment and living organisms (Darlington *et al.*, 2009). There are very few reports on the effect of nanoceria on terrestrial plants (i.e. Lopez-Moreno *et al.*, 2010a; Lopez-Moreno *et al.*, 2010b). Furthermore, interactions of nanoparticles with other organisms that share similarities with higher plant cells, such as algae, have been poorly studied, remaining unclear the general consequences of nanoparticle exposure for plant cells (Zhang *et al.*, 2012). These studies are mainly based on seed germination tests, seedling growth, nanoparticle uptake and alterations in chlorophyll content or photosynthetic process (Remedios *et al.*, 2012).

Cerium oxide nanoparticles (nanoceria) is a nanosized material which includes particles of 100 nm or less. Nanoceria has a wide range of applications being its main use the formulation of slurries for the chemomechanical planarization of silicon wafers in the production of integrated circuits. Diverse results have been achieved; positive effects on plant growth were reached only at low concentrations (Diatloff *et al.*, 2008) while toxicity was shown at higher concentrations (Lopez-Moreno *et al.*, 2010b). Furthermore, several studies demonstrated that nanoparticles can also have no significant or even positive effects on plants (Remedios *et al.*, 2012). However, all these studies analyze the effects of nanoceria in seed plants and fewer have focused on algae (Rodea-Palomares *et al.*, 2011; Rodea-Palomares *et al.*, 2012).

Ferns, with more than 9,000 species, are the second most successful lineage of vascular plants, after angiosperms. They have a very long evolutionary history related to so important events as the origin of land plants and emergence of the seed. Also, ferns are relevant ecological elements in many ecosystems, especially in the tropics, where they can be dominant (Prada, 2004). Germination is a biological process of capital relevance for seed and spore plants and fungi. It is defined as the set of mechanisms occurring in the dormant germ (seed or spore) that culminates with the growth of the embryo or cell to form a seedling or sporeling able to establish in the substrate (Gabriel y Galán, 2010).

Fern spores are unicellular haploid structures of specific variable size, produced via meiosis, with the faculty to create a gametophyte. Despite of a certain variation in the developmental pattern, the process is as follows: a) the spore germinates, with appearance of the first rhizoidal and prothallial cells; b) a filamentous prothallus is developed, first as uniseriate, then biseriate and planar; c) a meristem is organized, which produces an adult, pre-sexual gametophyte (Gabriel y Galán, 2010). The rhizoid is a single, elongated, nonphotosynthetic cell that is thought to function in anchoring and absorption of nutrients. The protonemal initial eventually gives rise to the photosynthetic prothallus of the fern gametophyte (Banks, 1999). For many ferns, the whole process is very quick, lasting from some days to several weeks. Many physiological and ecological aspects of ferns germination have been studied (Weinberg, 1969; Lloyd, 1970; Raghavan, 1989; Sheffield, 1996; Gabriel y Galán, 2010).

Asplenium adiantum-nigrum is a frequent understory species of Mediterranean evergreen oak forests (Rodà *et al*. 1999) The haploid phase of the fern *Asplenium adiantum-nigrum* L. has been previously studied (Prada *et al.*, 1995). Germination and development processes fit the typical leptosporangiate fern model. Ferns, terrestrial and aquatic, have the capacity to take up large amounts of trace elements (Ozaki *et al.*, 2000) and light rare elements, such as cerium (Shan *et al.*, 2003), through their shoots. This ability makes them ideal environmental indicators of contamination, especially those ferns which can tolerate a wide range of environmental extremes (Chang *et al.*, 2009). In this sense, the species *Asplenium adiantum-nigrum* exhibits its resistance to metals and potential to clean up toxic metals growing on mine refuse (Prasad, 2003).

The rationale of this study is based on two facts: first, there is no previous study on the influence of CeO_2 on ferns; second, due to is relative developmental simplicity and speed, fern spore germination and early developmental stages and the tolerance of ferns to some pollutants make ferns an interesting model to study possible toxic effects of bulk-CeO_2 and nanoceria.

Material and methods

Chemicals

Nano-CeO_2 (less than 25 nm particle size) and bulk-CeO_2 were purchased from Sigma-Aldrich Chemical Co. and used as received. The less than 25 nm particle size nanoceria was chosen due to its demonstrated high toxicity (Rodea-Palomares *et al.*, 2011).

Chemicals preparation and addition to culture medium

Dispersion of chemicals (nanosized and bulk materials) was achieved by adding a suitable amount to ultrapure water, and the dispersions were sufficiently shaken and sonicated to break up agglomerates. Each treatment concentration was prepared separately, without dilution, by weighting them and dispersing them in agar (8.5 g/L) medium solution. The addition of each nano- and bulk particles ranged from 0 to 3000 mg L^{-1}. Treatments were 0, 100, 500, 1000, 2000 and 3000 mg L^{-1} of both bulk and nanosized CeO_2.

All the nanosized and bulk-CeO_2 solutions were prepared fresh in a final volume of 10 ml and sonicated for 30 s before addition to the tissue culture medium. After autoclave sterilization (121ºC and 1 atm, 20 min), test units (plastic Petri dishes; 9 cm diameter) were immediately hardened in a freezer to avoid the possible precipitation of chemicals (Lee *et al.*, 2008).

Spore germination and exposure

As biological material, we used spores from the leptosporangiate fern *Asplenium adiantum-nigrum*, from the following location: France, Britanny Region, Finistére Department, Plougonvelin, in rocks near the sea; Gabriel y Galán s/n, oct 2011. Voucher specimen is deposited in the herbarium MACB (Biology, Universidad Complutense de Madrid).

Spore samples were taken from dry material maintained at room temperature (approximately 20ºC) until sowing. Multispore cultures on mineral agar medium (Dyer, 1979) were established by shaking fertile pinnae onto weight paper and placing the spores in plastic Petri dishes. The density of the cultures was approximately 20 spores cm^{-2}. Three dishes were sown for each treatment. The test units were placed in an incubator at a controlled temperature of 22 ± 1ºC under cool white fluorescent lamps (irradiance of 50 µmol m^{-2} s^{-1}) in a 16-h light/ 8-h dark cycle.

A spore was considered germinated when the rhizoid was evident, emerging from the opened spore wall. The germination percentage was recorded every day until there was no further increase. Indices of No-Observed-Adverse-Effect concentration (NOAEC) values (mg/L) and Lowest-Observed-Adverse-Effect-Concentration (LOEC) values (mg/L) (OECD, 2014) were obtained from the evaluation of spore germination rate of *Asplenium adiantum-nigrum* exposed to nanoceria and bulk-CeO_2.

Observations on rhizoidal and prothallial cells during first stages of gametophyte development were also made. The lengths of the rhizoids were scored at the end of the experiment. Bright-field micrographs were taken with a Nikon microscope equipped with a Coolpix digital camera.

Statistical data treatment

Each concentration point was conducted in triplicate. A total of 300 spores were analyzed in each treatment (100 in each replicate). Under a compound light microscope, germinated spores were counted from a pool of 100 spores randomly selected in each Petri dish, excluding those abortive or irregularly formed. Data were reported as mean ± standard error (SE). Data were analyzed using a one-way analysis of variance and the Duncan's test.

Analysis of variance (ANOVA) was used to determine the NOAEC and LOEC values for spore germination rate data. The Dunnett's test was used to calculate the minimum difference between the control and the treatment means detected as being statistically significant. Significant difference was defined as that with a p value < 0.05 in all statistical analyses. All the statistical analyses were implemented using the statistical package Statistica v. 9 (StatSoft, 2009).

Results

Spores of *Asplenium adiantum-nigrum* examined in the present study began to germinate between seven and nine days after sowing, reaching maximum percentages that ranged between 18 and 100%, depending on the treatment. In the control, seven days after sowing, a 67% spores germinated, and by day nine, germination was complete (100%) (Fig. 1). Seven days after sowing (Fig. 1A), a significant effect of nanoceria and also of most of the bulk-CeO_2 concentrations tested was observed on spore germination as compared to the control. The germination rate of spores treated with nanoceria 100 to 2000 mg L^{-1} was significantly higher, showing a stimulating, speeding effect of this product. On the other hand, the germination at 3000 mg L^{-1} was significantly lower, with a 14.3% rate of germinated spores. Bulk-CeO_2 also showed a speeding up effect on germination at 500 to 2000 mg L^{-1}, with the maximum percentage of spore germination (90.7%) observed at 500 mg L^{-1} bulk-CeO_2, while no significant effects were observed at other concentrations (Fig. 1A). The lowest-observed-adverse-effect-concentration (LOEC) concentration for spore germination rate (Table 1) was determined as the lowest tested nanoceria treatment (100 mg L^{-1}), while for bulk-CeO_2 LOEC was 500 mg L^{-1} and the no-observed-adverse-effect concentration (NOAEC) was ≤100 mg L^{-1}.

Fig. 1B shows the results on spore germination eight days after sowing. At this point, only the highest concentration tested (3000 mg L^{-1}) showed a toxic effect for both nanoceria and bulk materials (Table 1).

The end of the experiment was established nine days after sowing, when spore germination was complete (100%) in the control. In most treatments, germination also reached a 100%. Fig. 1C shows the final results. There were no differences between the unexposed plants and most nanoceria treatments. Only for the 3000 mg L^{-1} nanoceria treatment an important toxic effect was observed. Diverse results were obtained for bulk material. A lower number of germinated spores was observed above 1000 mg L^{-1} concentration and this effect was significant at higher concentrations (Table 1). Thus, the observed effect on spore germination was dependent on CeO_2 concentration and particle size (nano- or bulk material).

The rhizoid growth was estimated as relative length value in comparison to the control (Fig. 2). Significant decreases in rhizoid growth were observed for 2000 and 3000 mg L^{-1} nano-CeO_2 and for 3000 mg L^{-1} bulk-CeO_2 while lower concentrations had no significant effect.

In order to further explore the cellular mechanisms of the observed toxicity, we took bright-field micro-

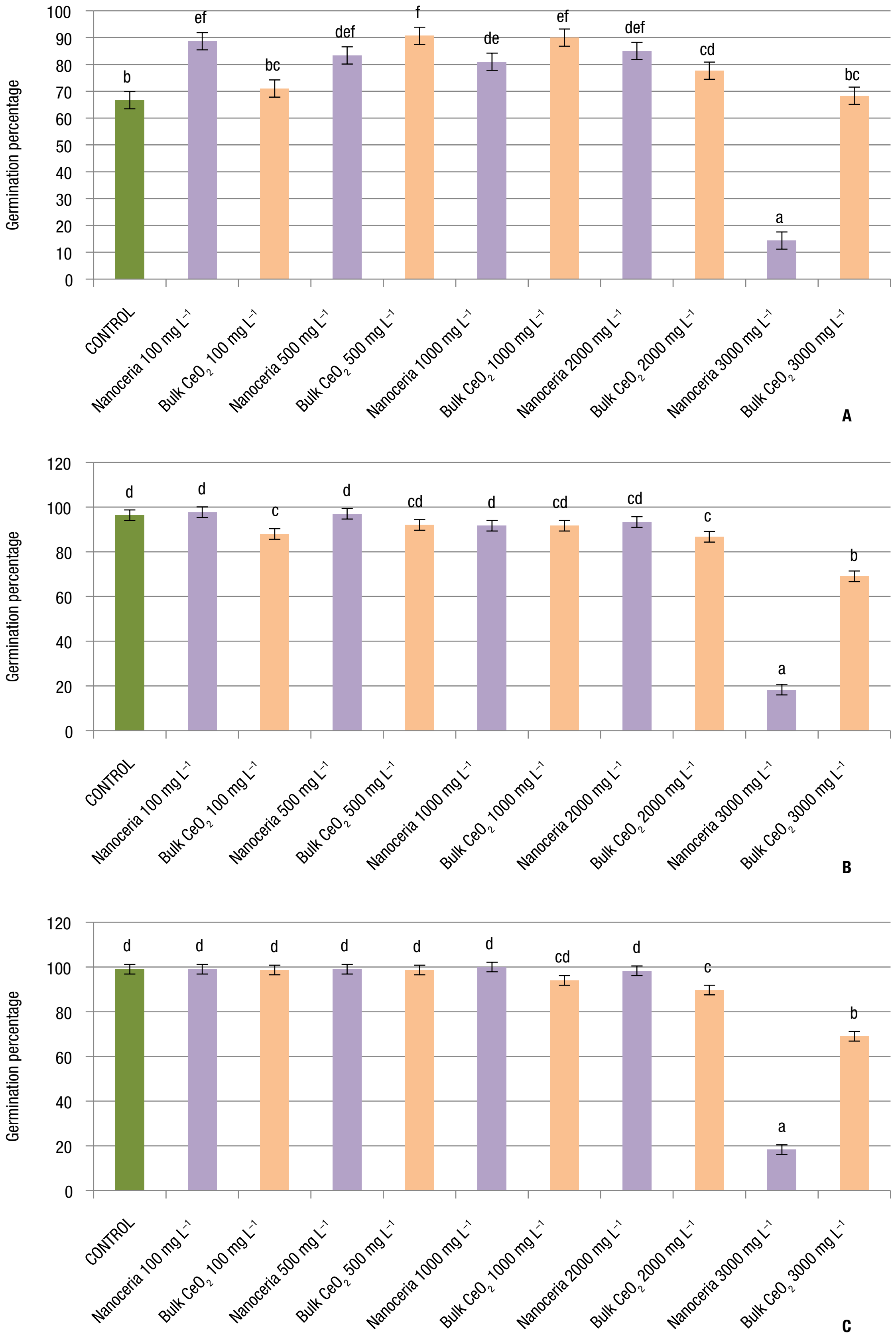

Figure 1. Effects of diverse nanoceria and bulk-CeO_2 treatments on the germination of *Asplenium adiantum-nigrum* recorded at: (A) seven days after sowing, (B) eight days after sowing, and (C) nine days (end-point) after sowing. The values are given as mean ± SE (standard error) of three replicates. Data with different letters are significantly different at $P < 0.05$ (One-way ANOVA; Duncan's test).

Table 1. Estimation of No-Observed-Adverse-Effect concentration (NOAEC) values (mg/L) and Lowest-Observed-Adverse-Effect-Concentration (LOEC) values (mg/L) obtained from the evaluation of spore germination rate of *Asplenium adiantum-nigrum* exposed to nanoceria and bulk-CeO_2

Treatment	Seven Days After Sowing		Eight Days After Sowing		Nine Days After Sowing	
	NOAEC (%)[a]	LOEC (%)[a]	NOAEC (%)[a]	LOEC (%)[a]	NOAEC (%)[a]	LOEC (%)[a]
Nanoceria	<100 mg L^{-1}	100 mg L^{-1}	≤2000 mg L^{-1}	3000 mg L^{-1}	≤2000 mg L^{-1}	3000 mg L^{-1}
Bulk-CeO_2	≤100 mg L^{-1}	500 mg L^{-1}	≤2000 mg L^{-1}	3000 mg L^{-1}	≤1000 mg L^{-1}	2000 mg L^{-1}

[a] These values were derived from ANOVA using Dunnett's procedure with $p < 0.05$.

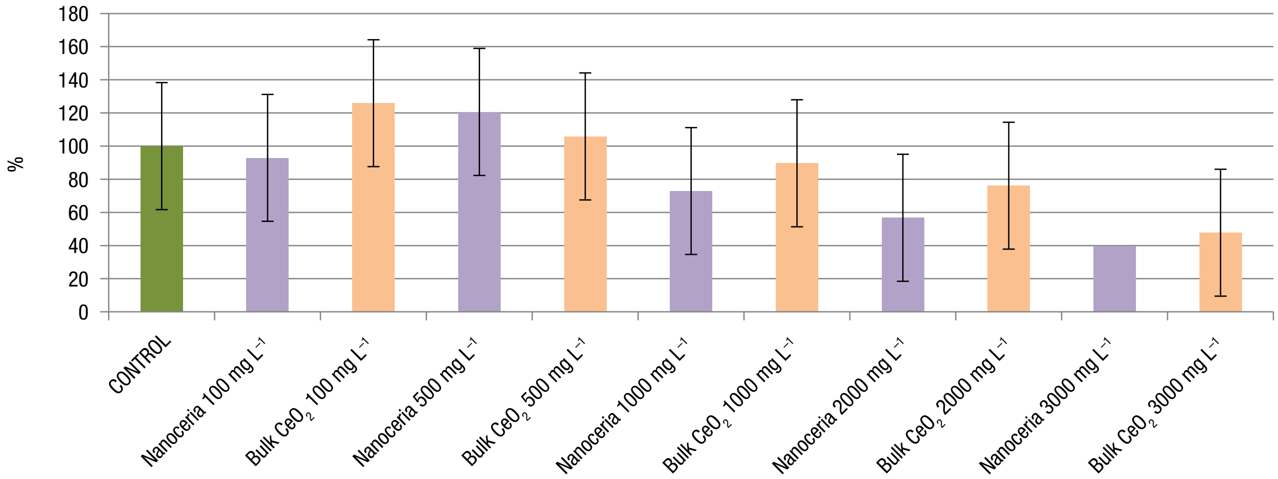

Figure 2. Effects of nanoceria and bulk-CeO_2 treatments on rhizoid growth of *Asplenium adiantum-nigrum*. Values are related to rhizoid growth in control treatment (0 mg L^{-1}). The values are given as mean ± C.I. (95% confidence interval) of three replicates.

graphs of *Asplenium adiantum-nigrum* germinated spores exposed to the treatments. In the control (Fig. 3A), as well as in 100 (Fig. 3B) and 500 mg L^{-1} nanoceria (Fig. 3C), well formed protonema cells with bright green chloroplasts were clearly visible. In contrast, nanoceria at concentration above 1000 mg L^{-1} and bulk material above100 mg L^{-1} generally resulted in germinated spores, many of which manifested abnormalities, such as damages to their internal structure, collapsed 2-3 celled filaments (Fig. 3E); filaments of 2-3 cells aborted and collapsed (Fig. 3I), chloroplast damaged cells (Fig. 3D-K) and multiple rhizoid formation or aborted protonema (Fig. 4), that sharply contrast with the healthy controls.

Discussion

The germination of the spores of *Asplenium adiantum-nigrum* is affected by the addition of CeO_2 to the culture medium. Materials surface chemistry is vital in biological interaction (Karakoti *et al.*, 2006). In general, for a fixed mass of particles, surface area increases as particle size becomes smaller. Thus, a dose-dependence on particle surface area may explain the greater toxicity of nanoparticles compared to an equal mass of fine particles of the same material (Donaldson *et al.*, 2002; Monteiller *et al.*, 2007; Sager, 2009). However, other reports (Warheit *et al.*, 2006; Warheit *et al.*, 2007) have questioned this hypothesis and, in agreement with other results (Rodea-Palomares *et al.*, 2011), our data do not support the view that surface area might work better than mass concentration as a dose variable when photosynthetic organisms are tested.

Differences in the germination rate have been observed between spores subjected either to nanoceria or bulk-CeO_2 even for the lowest concentration tested (100 mg L^{-1}, Fig. 1 A and B). At this concentration, germination was speeded up by nanoceria, while spore germination in bulk-CeO_2 was retarded (Fig. 1 B). On the other hand, only 500 to 2000 mg L^{-1} concentrations of bulk-CeO_2 showed a similar pattern. In fact, bulk material responds to a dose/response curve (Fig. 1 B). Differences between the control and the treatments decreased for the eighth and ninth day after sowing, when only the highest concentration (3000 mg L^{-1}) treatment showed toxicity for both chemicals, although it was stronger for nanoceria (Fig. 1 B and C). The parameters in the spore germination test, including NOAEC and LOEC, were lower for bulk-CeO_2 end-point (nine days) than for nanoceria (Table 1). This supports the requirement of the use of both, nano and bulk material for toxicity tests.

Taking into account the differences among angiosperms and ferns, some authors (Lopez-Moreno *et al.*, 2010b) showed, at concentrations of nanoceria from 500 to 2000 mg L^{-1}, relatively low to moderate toxicity on seed germination of alfalfa (*Medicago sativa*), cucumber (*Cucumis sativa*), tomato (*Solanum lycopersicum*) and corn (*Zea mays*). Nanoceria did not affect germination in lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), cabbage (*Brassica oleracea*), soybean (*Glycine max*), carrot (*Daucus carota*), perennial ryegrass (*Lolium perenne*), corn (*Zea mays*), cucumber (*Cucumis sativus*), oat (*Avena sativa*), and onion (*Allium cepa*) at concentrations between 250 and 1000 mg L^{-1} (Andersen *et al.*, 2016). The toxic effect at the highest concentration tested (3000 mg L^{-1}) was more marked for nanoceria than for bulk-CeO_2. At this highest nanoparticle concentration, cell toxicity could be related to concentration and to the presence of nanoparticle aggregates (Rodea-Palomares *et al.*, 2011).

No significant differences were observed for rhizoid growth among the control treatments (Fig. 2). The rhizoid is the cell responsible for the absorption of nutrients and, keeping again the differences, acts as the root. It has been reported previously (Lopez-Moreno *et al.*, 2010b) that root growth was reduced in alfalfa and tomato but was significantly promoted in cucumber and corn. Andersen *et al.* (2016) found that nanoceria alter average root length, and hence root growth was decreased in cabbage and corn, but was promoted in cucumber and onion. Ma *et al.* (2010) only detected a reduction on the root elongation in lettuce but no effect was detected for a suspension of 2000 mg L^{-1} nanoceria for rape, radish, wheat, cabbage, tomato, and cucumber. All these results suggest that the effects produced on early plant growth of nanoceria is species dependent. Nevertheless, rhizoid is a unicellular structure and, in this sense, differences with an organ (root) are evident. Furthermore, alterations in normal development of gametophyte were observed. For example, as shown in Fig. 4, more than one rhizoid was emitted by single spores. However, we did not observe cell damage in rhizoid structures.

Two explanations have been proposed for CeO_2 toxicity effects: mechanical damage to the cell membranes due to the numerous edges, corners, and reactive sites present in the crystal structure of the nanoparticles (Rogers *et al.*, 2010); or the generation of ROS (reactive oxygen species), thus inducing oxidative stress and cell toxicity leading to lipid/protein oxidation (Thill *et al.*, 2006; Park *et al.*, 2008; Zeyons *et al.*, 2009). Although we did not examine the presence of particles anchored to the cell surface, we did observe membrane rupture, cytoplasm leakage, and intracellular damage including chloroplast lysis for most treatments (Fig. 3 D-K). Similar results were obtained in the green alga *Pseudokirchneriella subcapitata* (Rodea-Palomares *et al.*, 2011). These authors indicate that nanoceria cytotoxicity could be mediated by the effect of nanoceria

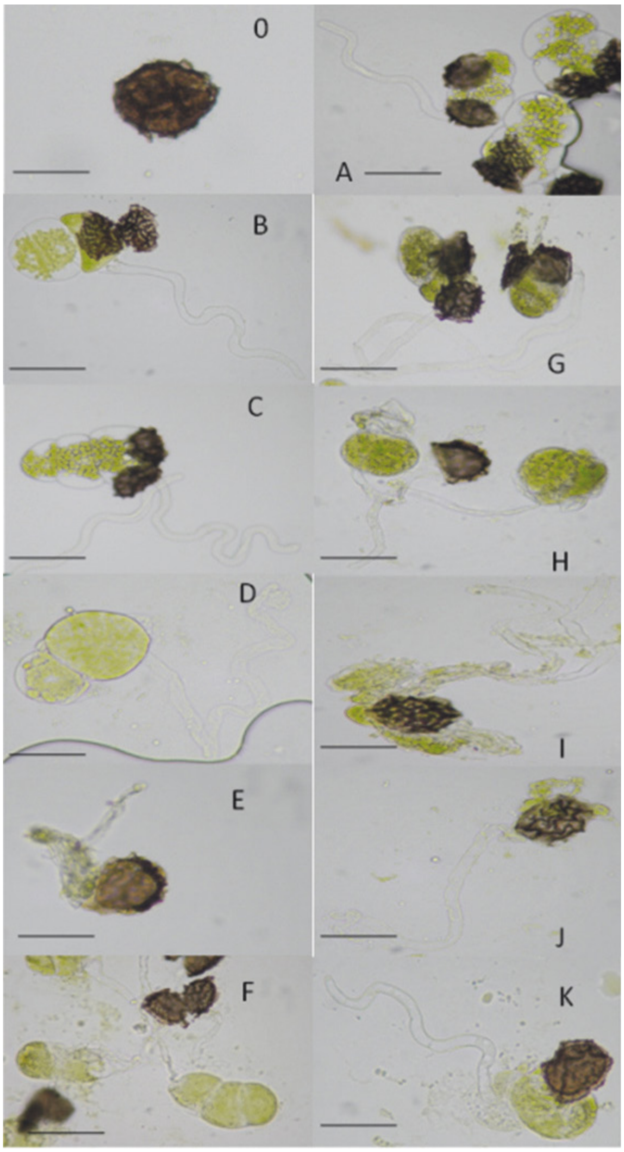

Figure 3. Bright-field micrographs of *Asplenium adiantum-nigrum* exposed to diverse nanoceria and bulk cerium oxide treatments. (0) Non-germinated spore. (A) Control plate, with early 2-3 celled filaments, showing rhizoids of normal length and well-developed chloroplasts. (B) Spores exposed to 100 mg L^{-1} nanoceria. (C) Spores exposed to 500 mg L^{-1} nanoceria. (D) Spores exposed to 1000 mg L^{-1} nanoceria. (E) Spores exposed to 2000 mg L^{-1} nanoceria. (F) Spores exposed to 3000 mg L^{-1} nanoceria. (G) Spores exposed to 100 mg L^{-1} bulk cerium oxide. (H) Spores exposed to 500 mg L^{-1} bulk cerium oxide. (I) Spores exposed to 1000 mg L^{-1} bulk cerium oxide. (J) Spores exposed to 2000 mg L^{-1} bulk cerium oxide. (K) Spores exposed to 3000 mg L^{-1} bulk cerium oxide. Bar = 100 µm (0); 57 µm (A); 40 µm (B-K).

Figure 4. Bright-field micrograph of *Asplenium adiantum-nigrum* exposed to 2000 mg L^{-1} nanoceria. Three rhizoids and an aborted protonema were emitted by a single spore. Bar = 40 µm.

on photosynthesis, which could be involved in the generation of ROS when photosynthetic reactions are not well balanced (Rico *et al*., 2015). Nanoceria can increase the production of hydrogen peroxide through an oxidative reaction (Rodea-Palomares *et al.*, 2012), which can cause lipid peroxidation compromising membrane integrity. We have observed critical chloroplast damage, which consequently resulted in cell and gametophyte death for all the bulk-CeO_2 treatments (Fig. 3G, H, I, J, and K), and for the high concentrations of nanoceria (1000, 2000 and 3000 mg L^{-1}, Fig. 3D, E, and F), while in the lower concentrations of nanoceria (100 and 500 mg L^{-1}, Fig. 3B and C) well defined chloroplasts were observed.

Present results showed cellular damage in the protonema, while rhizoid cells seemed not to be affected (growth and membrane integrity remained) either by bulk-CeO_2 or nanoceria treatments. As chloroplasts are present in the protonema and absent in the rhizoid, the question remains open to a possible role of CeO_2 toxicity through oxidative stress. In such hypothesis, photosynthetic cells would be more affected by the reduction of cerium (IV) to cerium (III) (Rodea-Palomares *et al.*, 2012). In agreement with this, the main driver of toxicity was found to be the percentage of surface content of Ce^{3+} sites (Pulido-Reyes *et al*., 2015).

Conclusions

Cerium oxide is toxic for *Asplenium adiantum-nigrum*. The sensibility of this species to CeO_2 makes it especially interesting as indicator of its presence. Diverse toxic effects have been observed: i) chloroplast membrane damage and lysis, ii) cell wall and membrane disruption which leads to cell lysis; and iii) alterations in morphology and development. Nevertheless, this toxicity is not apparent when low/moderate concentrations of nanoceria are added to the culture medium.

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