



Evaluation of Ecotoxicity of Nanoceria to Organisms of Different Trophic Levels

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ABSTRACT

The present study was aimed at evaluation of the toxicity potential of nanoceria on phosphobacteria (*Bacillus megaterium* ; soil ecosystem), azolla (*Anabaena azollae* and microalgae ; Aquatic ecosystem) and sorghum (*Sorghum bicolor* (L.) Moench) pollen grain and photosystem (PS) II quantum yield (terrestrial ecosystem). The study examined the differences in toxicity among a different concentration of nanoceria to each organism and differences in toxicity among the organisms. In each toxicity experiment, the concentration of nanoceria used are 0, 5, 10, 25, 50, 100, 200, 400, 500, and 1000 mg L⁻¹. The result indicated that nanoceria is not toxic to soil microbes, aquatic organisms and terrestrial plants at lower concentration (up to 25 mg L⁻¹). However, above 25 mg L⁻¹ concentration, differential responses between nanoceria and organisms were observed. Higher concentration (500 and 1000 mg L⁻¹) inhibited the growth of phosphobacteria, microalgae, and pollen germination and PS II quantum yield. The adverse effect caused by nanoceria could be associated with the concentration of nanoceria, differences in interactions with the cell with nanoceria, and oxidative damage caused by nanoceria. Among the assays, pollen germination was found to be more sensitive to the nanoceria in the medium, followed by photosystem II quantum yield.

Received : 05th February, 2021

Revised : 10th February, 2021

Accepted : 22nd February, 2021

Key words: *Azolla*; *Ecotoxicity*; *Microalgae*; *Nanoceria*; *Phosphobacteria*; *Pollen germination*

INTRODUCTION

The nanoparticles have potential applications in several sectors like medical science, electronics and biotechnology, including agriculture, where nanotechnology is used to synthesize nano fertilizers, carriers of agrochemicals and materials to mitigate abiotic stress (Prasad *et al.*, 2017). Research indicated that nanoparticles are involved in the alleviation of abiotic stress in sorghum [*Sorghum bicolor* (L.) Moench], wheat (*Triticum aestivum* L.), corn (*Zea mays* L.) (Djanaguiraman *et al.*, 2018a, b; Jaberzadeh *et al.*, 2013; Zaimenko *et al.*, 2014). In general, before adopting nanomaterials for agricultural use, nanomaterials have to be screened for ethical, societal, legal issues. In addition, a robust scientific assessment of nanomaterials' benefits and drawbacks, potential environmental, health, and safety risks of nanomaterials has to be studied. In general, toxicology depends on the dose, but in the case of nanoparticle, it depends on size, number, surface activity and aggregation (Paramo *et al.*, 2020). In metallic nanoparticles, the properties such as solubility and binding also determine the toxicity. Any functional or morphological changes in

the test organism due to exposure to nanomaterial is said to be their effects; if the effects are positive and enhance growth, then the nanomaterial has a positive influence. In contrast, if the material has adverse effects on morphology, function, and growth, it is negatively influenced. The significant aftermath of the nanoparticle toxicity in most of the organism is associated with increased production of reactive oxygen species leading to loss of membrane integrity and oxidation of biomolecules (Klaine *et al.*, 2008). When an engineered nanomaterial is released to the environment, either by natural or anthropogenic activities, the nanoparticle may be released as intact material, or it will be broken down into base compound, which is under the influence of environmental conditions and intrinsic properties of nanomaterials (Lowry *et al.*, 2012; Nowack *et al.*, 2012).

Studies indicated that the application of titanium oxide nanoparticle under drought stress in wheat had improved the yield, gluten and starch content (Jaberzadeh *et al.*, 2013). Likewise, the application of analcite, a member of the zeolite group, to wheat and maize has improved the antioxidants

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and antioxidant enzyme activity, resulting in better growth (Zaimenko *et al.*, 2014). In *Arabidopsis thaliana*, the zero-valent nano iron particle (nZVI) has decreased the effects of drought stress through altering stomatal movement by activating H⁺ ATPase activity (Kim *et al.*, 2015).

Cerium oxide nanoparticles (nanoceria) are extensively used in the electronics and automotive industry, and there is limited use in agriculture. It is not clear whether nanoceria act as a prooxidant or antioxidant molecule due to the conflicting reports on the toxicity of nanoceria. Cerium oxide nanoparticles have gained attention for exhibiting redox activities and free radical scavenging. The controlled environmental study has shown that cerium nanoparticle can alleviate the drought stress effects in sorghum through modulating antioxidant enzymes activity and oxidant production level (Djanaguiraman *et al.*, 2018b). Foliar application of silicon nanoparticle under salinity stress to cherry tomato has improved the photosynthesis rate, mesophyll conductance, and plant water use efficiency compared with unsprayed control (Haghighi *et al.*, 2013). Application of nTiO₂ to chickpea (*Cicer arietinum* L.) exposed to low-temperature stress has improved the membrane integrity in both tolerant and susceptible genotypes (Mohammadi *et al.*, 2013). Foliar application of copper nanoparticles against the *Fusarium* sp. was effective than the commercial ones (Brahmanwade *et al.*, 2015). This unprecedented growth in the production and utilization of nanoparticles creates concerns on the safety of the environment and organisms being exposed. Hence it is mandatory that whenever a nanoparticle is developed, it should be subjected to toxicity assays. Nanoceria has been considered as one type of emerging contaminants that pose great potential risks to the environment and human health. The effects of nanoceria on soil microbes, aquatic organisms and terrestrial organisms associated with agriculture has not been studied in detail.

Therefore, we conducted an experiment to understand the toxicity effects of nanoceria on azolla, microalgae, phosphobacteria, seed germination, photosystem (PS) II quantum yield, and pollen germination. We aim (i) to examine whether there are differences in toxicity among a different concentration of nanoceria to each organism and (ii) whether for the same nanoparticle, there are significant differences in toxicity among the organisms.

MATERIAL AND METHODS

Synthesis of Nanoceria

Nanoceria was synthesized as detailed by Ketzial and Nesaraj (2011) using cerium (III) nitrate

as a precursor. The cerium oxide nanoparticle synthesized was <25 nm in size and rod-shaped (Djanaguiraman *et al.*, 2018b).

Evaluation of nanoceria toxicity on various organisms

Phosphobacteria

Phosphobacteria (*Bacillus megaterium*) is a phosphate solubilizing organism and dwells in the rhizosphere of the plant. Phosphobacteria solubilizes immobile insoluble soil inorganic phosphates like Ca₃(PO₄)₂, FePO₄, and AlPO₄, through the production of organic acids, siderophores, and hydroxyl ions into mobile phosphate ions and helps in the uptake of phosphate by the plants. Phosphobacteria was subcultured in Luria-Bertani (LB) broth in Erlenmeyer flask under aseptic condition and incubated for 24 h in the shaker cum incubator at a temperature of 34 °C at 200 rpm. The LB broth consists of tryptone 10 g L⁻¹, yeast extract 5 g L⁻¹, sodium chloride 10 g L⁻¹ and 1.5 per cent agar for solidifying the medium. LB medium with different concentration of nanoceria (0, 5, 10, 25, 50, 100, 200, 400, 500 and 1000 mg L⁻¹) was prepared by adding the required quantity of nanoceria. After the addition of the nanoceria, the medium was sonicated for 10 min and autoclaved at 121°C for 20 min and 15 lbs sq inch⁻¹ pressure. The medium was then plated and kept undisturbed under aseptic condition. The subculture having an optical density of 0.1 was used for inoculation. The subculture was inoculated on the plates with a dilution factor of 10⁻⁴ concentration, and the inoculation was done by smearing on the plates with an L-rod. Then the plates were wrapped and kept in an incubator for a duration of 24 h. After incubation, the plates were counted for colony forming units (CFU) per plate to determine the effect of different concentrations of nanoceria on phosphobacteria.

Azolla

Azolla, a unique freshwater fern, symbiotically lives with the microscopic filamentous cyanobacteria called *Anabaena azollae*, which fixes the atmospheric nitrogen. In contrast, azolla provides the space for the survival of the bacteria. The azolla ferns were procured from the Department of Rice, Tamil Nadu Agricultural University, Coimbatore. In the present experiment azolla was grown in IRR1 2 medium. The medium consists of macronutrients such as CaCl₂.2H₂O 40 µM, MgSO₄.7H₂O 40 µM, K₂SO₄ 40 µM, NaH₂PO₄.H₂O 20 µM and micronutrients such as CuSO₄.5H₂O 0.01 µM, H₃BO₃ 0.2 µM, Na₂MoO₄.2H₂O 0.15 µM, ZnSO₄.7H₂O 0.01 µM, CoCl₂.6H₂O 0.01 µM, MnCl₂.4H₂O 0.5 µM, Fe-EDTA.Na₂ 0.5 µM (Periera *et al.*, 2009). The procured ferns were kept in the medium for overnight. Then, 500 mg of the fern was added to 50 mL of the medium amended with nanoceria at different concentrations (0, 5, 10, 25,

50, 100, 200, 400, 500 and 1000 mg L⁻¹). The light intensity of the growth room was 300 μmol m⁻²s⁻¹, and the temperature was 28 °C. The experiment was conducted for 10 d. At the end of the experiment, the ferns were collected and weighed to calculate the fresh weight. To understand the effect of nanoceria on *Anabaena* structure, 100 mg of fern was collected from each treatment. The fern was placed on the microscope slide and covered with a coverslip, then gently crushed using the forceps. The ferns were damaged resulting in the exposure of *Anabaena*, which was viewed in Olympus microscope at 400 times magnification.

Microalgae

Chlorella sorokiniana, microalgae, is a minute photoautotrophic organism that can be used as a biofuel or processed into proteins. The microalga was grown in the controlled condition in BG-11 medium, which contained sodium carbonate and sodium bicarbonate as the nitrogen source with macro and micronutrients and pH adjusted to 7.5. Medium with different concentration of nanoceria (0, 5, 10, 25, 50, 100, 200, 400, 500 and 1000 mg L⁻¹) was prepared in Erlenmeyer flask and sonicated, followed by autoclaving. The algal culture (50 μL per 100 mL of the medium) was added to the BG-11 medium under aseptic condition. After inoculation, the microalga was maintained for 14 d under a controlled environment. The light intensity of the growth room was 300 μmol m⁻²s⁻¹, and the temperature was 28 °C. After the expiry of the experiment, the algae were harvested by centrifugation at 3000 rpm and dried in an oven, maintained at 50 °C for arriving dry weight.

Sorghum seed germination

The sorghum seeds cv. Co (S) 30 was purchased from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore. The seeds were surface sterilized with 0.5% (v/v) sodium hypochlorite solution for 10 min, followed by water wash for three times. Then, the seeds were placed in a Petri dish lined with filter paper. For each Petri dish, 25 seeds were kept. Nanoceria at different concentrations (0, 5, 10, 25, 50, 100, 200, 400, 500 and 1000 mg L⁻¹) was prepared by adding the required quantity of nanoceria in water and Tween 80. The mixture was sonicated for 5 min, and 5 mL of the solution was added every day till the completion of the experiment. After 10 d, the number of seeds germinated, seedling shoot length, seedling root length and vigour index were observed. The emergence of the radicle was considered as criteria for germination. Vigour index is the product of germination percentage and seedling length.

Photosystem (PS) II quantum yield

A field experiment was conducted at Tamil Nadu

Agricultural University, Coimbatore, (11° N; 77° E; 426.7 m MSL) in a randomized block design with five replications. The sorghum genotype Co (S) 30 was sown during Kharif, and the crop was maintained as per the recommended package of practices. At the booting stage, ten plants were tagged for the foliar spray of different concentration of nanoceria (0, 5, 10, 25, 50, 100, 200, 400, 500, and 1000 mg L⁻¹). Nanoceria was sprayed in the evening, and the PS II quantum yield was recorded in the following day between 10 AM and 11 AM. The PS II quantum yield was recorded continuously for three days. Chlorophyll a fluorescence parameter was measured using a modulated fluorometer (OS5p+, OptiSciences, Hudson, NH) in tagged plants as explained by Djanaguiraman *et al.* (2013).

Pollen germination

In the field experiment, the primary panicle was tagged in 100 plants at the booting stage. These plants were not sprayed with any chemical. At anthesis, 5-10 florets were collected from tagged panicles, and the pollen grains were squeezed from the anthers by tweezers and placed on clean slides. *In-vitro* pollen germination was assessed by germinating the pollen in a medium containing 150 mg H₃BO₃, 500 mg Ca (NO₃)₂·4H₂O, 200 mg MgSO₄·7H₂O, 100 mg KNO₃ and 300 g sucrose, dissolved in 1 L of deionized water to which 15 g of agar per litre and different concentration of nanoceria (0, 5, 10, 25, 50, 100, 200, 400, 500, and 1000 mg L⁻¹) were added. The contents were slowly heated on a hotplate until the agar dissolves and forms a clear solution. Collection of pollen and dusting on the medium was done as detailed by Djanaguiraman *et al.* (2004).

STATISTICAL ANALYSES

The data were analyzed using SAS program. All the toxicity experiment of the nanoceria was conducted in a completely randomized design except the field experiment. Each experiment had at least four independent replications. Observations were analyzed using the PROC GLM procedure of SAS. The standard error was shown as an estimate of variability and means of various variables were separated for significance by the LSD test at a probability level of 0.05.

RESULT AND DISCUSSION

To increase food production to feed the ever-growing population, adopting new technologies like nanotechnology in agriculture is mandatory (Anjum *et al.*, 2013). However, the safety of the nanomaterial to the ecosystem is of high priority. In the present study, the toxicity of nanoceria was studied. The major findings from this study are (i) inhibition of sorghum photosystem II photochemistry

and pollen germination from 50 mg L⁻¹ of nanoceria, (ii) comparing azolla and microalgae growth, azolla growth was not inhibited by nanoceria; however, the growth of microalgae was inhibited from 100 mg L⁻¹ of nanoceria, (iii) there is no inhibition of seed germination due to nanoceria treatment; however, the seedling vigour was decreased from 50 mg L⁻¹ of nanoceria, and (iii) the growth of phosphobacteria was inhibited at higher concentration of nanoceria.

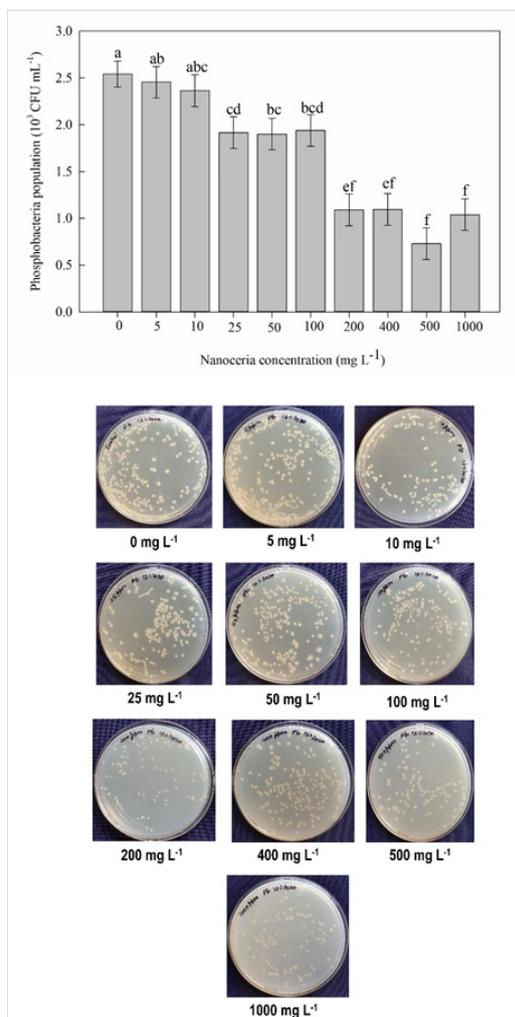


Figure 1. Phosphobacteria population dynamics as influenced by nanoceria at different concentration [10³ colony forming units (CFU) mL⁻¹]

Impact of nanoceria on phosphobacteria growth

The effect of nanoceria on phosphobacteria growth was assessed by counting colony-forming units (CFU), and there were significant differences among the treatments (Fig. 1). The result indicated that increasing the concentration of nanoceria on the growth medium decreased the colony-forming units and a severe decrease was observed from 200 mg L⁻¹ (Fig. 1). The decrease in the number of colonies may be associated with antimicrobial activity from 200 mg L⁻¹ concentration (Kuang *et al.*, 2011; Krishnan *et al.*, 2013).

Impact of nanoceria on dry biomass of azolla and microalgae

There were significant ($P < 0.01$) differences among the nanoceria concentration for dry biomass of azolla and microalgae (Fig. 2 and 3a). The azolla and microalgae dry biomass were significantly decreased from 200 mg L⁻¹ to 1000 mg L⁻¹ of nanoceria (Fig. 2 and 3a). Knowing the effects of size, surface charges and chemical reactivity of nanoceria, it is possible to predict that at higher concentrations, nanoceria may induce the production of reactive oxygen species (Auffan *et al.*, 2010).

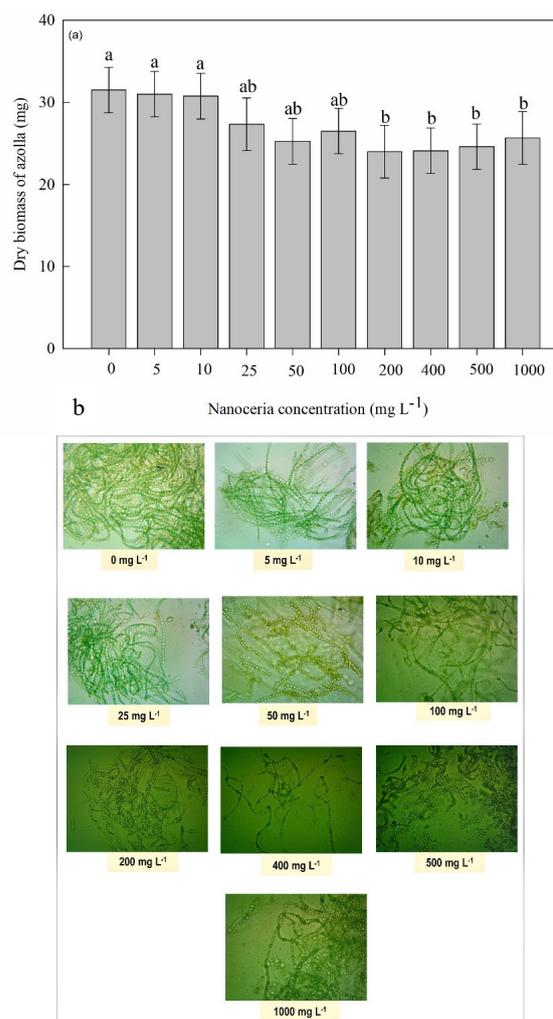


Figure 2. Effect of different concentration of nanoceria on (a) dry biomass of azolla (mg) and (b) structure of *Anabaena*

The structure of *Anabaena* was significantly altered due to exposure to nanoceria (Fig. 2b). *Anabaena*, a nitrogen-fixing blue green algae, forms symbiotic relationship with azolla. *Anabaena* forms heterocysts and vegetative cells, and the enzyme Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) is present in the vegetative tissue of the cyanobacteria. From (Fig. 6), it is evident that nanoceria from 100 mg L⁻¹ there was a

degeneration of vegetative tissue, which may affect the nitrogen fixation process in *Anabaena* (He *et al.*, 2021). Also, the heterocyst frequency reduced with 100 mg L⁻¹ nanoceria concentration (Fig. 2b) and subsequently may fix low levels of atmospheric nitrogen. Heterocysts differentiate from vegetative cells of some filamentous cyanobacteria to fix nitrogen for the entire filament under toxic growth conditions (Thiel *et al.*, 2001). Due to the electron sponge property of nanoceria under aqueous condition, the probability of adsorption of O₂⁻ on reduced (Ce³⁺) ceria will be higher than unreduced (Ce⁴⁺) ceria (Choi *et al.*, 2006), due to this property at higher concentration nanoceria can damage the vegetative cells and heterocyst through the production of reactive oxygen species (Heckert *et al.*, 2008; Rogers *et al.*, 2010).

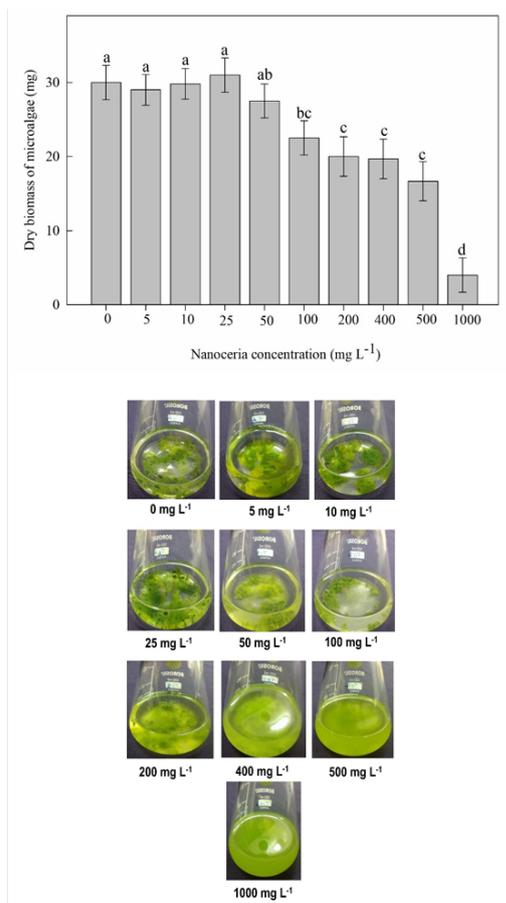


Figure.3. Effect of different concentration of nanoceria on (a) dry biomass of microalgae (mg) and (b) microalgae at different concentration of nanoceria

Impact of nanoceria on the maximum quantum efficiency of photosystem (PS) II photochemistry (Fv/Fm ratio) and *in-vitro* pollen germination of sorghum

The result indicated significant ($P < 0.01$) differences among the nanoceria concentrations for the maximum quantum efficiency of PS II photochemistry (Fv/Fm ratio) and *in-vitro* pollen

germination was observed (Fig. 4 and 5). Among the various nanoceria concentrations, Fv/Fm ratio started to decrease from 50 mg L⁻¹ to 1000 mg L⁻¹ (Fig. 4). Exposure of nanoceria to sorghum pollen germination medium decreased the *in-vitro* pollen germination from 50 mg L⁻¹ (Fig. 5). It is evident that increasing the concentration of nanoceria in the medium had significantly reduced the pollen germination percentage from 62% (0 mg L⁻¹) to 6.4 % (1000 mg L⁻¹) (Fig. 2).

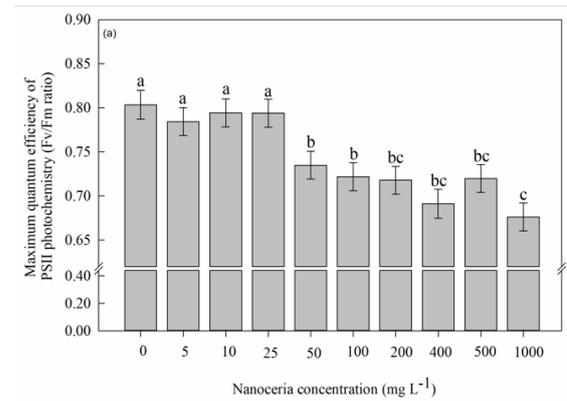


Figure 4. Effect of different concentration of nanoceria on maximum quantum efficiency of photosystem (PS) II photochemistry [Fv/Fm ratio] of sorghum

Comparing the effects of nanoceria on PS II photochemistry and pollen germination, the latter was most affected than the previous, which could be associated with the development phase of the plant (Tarafdar *et al.*, 2012). Also, studies indicated that the method of application also causes differential toxic effects (Corredor *et al.*, 2009). The toxicity of nanoceria at higher concentration may be associated with its morphology, size, number of nanoparticles and surface reactivity (Singh, 2016). The dual property of nanoceria i.e., no effect at low concentration and deleterious effect at higher concentration, may be related to the dual oxidation state of cerium at its surface, which can act as an oxidizer and reducer and surface adsorption property (Peng *et al.*, 2005; Sardesai *et al.*, 2013). Rogers *et al.*(2010) reported that nanoceria could produce hydroxyl radical from fatty acid. Furthermore, free Ce³⁺ can redox-cycling with hydrogen peroxide to generate damaging reactive oxygen species (Heckert *et al.*, 2008). This property of nanoceria might have affected the PSII quantum yield by inducing photoinhibition (Havaux, 1993).

Impact of nanoceria on seed germination and seedling vigour

The effects of nanoceria on seed germination and seedling vigour is presented in Fig. 7. Overall, the impact of nanoceria on sorghum seed germination was very low. However, the effects of nanoceria

on seedling vigour were high, as evidenced by a significant decrease in seedling vigour from 50 mg L⁻¹ of nanoceria. The decrease in seedling vigour is associated with decreased root length from 50 mg L⁻¹ to 1000 mg L⁻¹ (Fig. 8), and not with germination percentage and shoot length. Seed germination percentage, root elongation, seedling biomass and the number of leaves plant⁻¹ are considered as important physiological indices used to assess nanoparticle toxicity on plant species (Lee *et al.*, 2010). Comparable to the present study, substantial negative effects of nanoparticle on suppression of tissue elongation were reported (Dimkpa *et al.*, 2012; Ghosh *et al.*, 2015). Song *et al.* (2013) also observed a reduction in biomass and root length in tomato due to silver nanoparticle treatment.

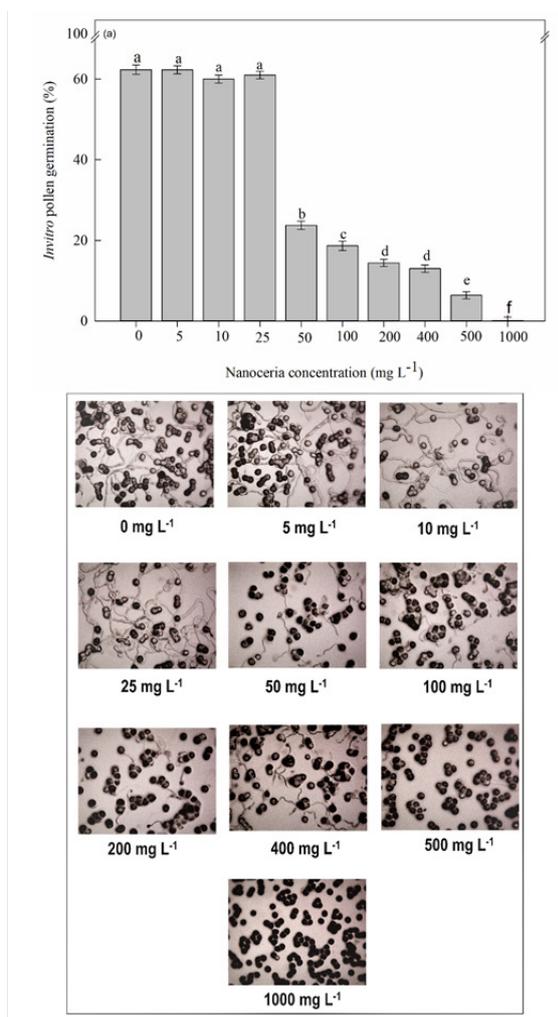


Figure 5. Effect of different concentration of nanoceria on pollen germination of sorghum

The overall mechanism associated with adverse effects of nanoceria on various processes

The present research indicated that the effects of nanoceria on different processes vary with concentration. In general, higher concentrations of nanoceria affects PS II quantum yield,

pollen germination, azolla growth, *Anabaena* structure, microalgae growth, seedling vigour, and phosphobacteria growth (Fig. 1 to 6). The metal and metal-based nanoparticle toxicity is associated with oxidative stress (Sharma *et al.*, 2012). The mechanism of deleterious effect may be associated with oxidative damage caused by nanoceria. Excess reactive oxygen species would cause oxidative stress, which emerges when the reactive oxygen species level exceeds the antioxidant defense mechanisms. The oxidative stress is caused by oxidants like superoxide radicals, hydrogen peroxide and lipoperoxides. During oxidative stress, the cells will undergo DNA damage, protein oxidation, electrolyte leakage, lipid peroxidation and membrane damage and eventually, cell death (Meriga *et al.*, 2004; Sharma *et al.*, 2012). Therefore, it is concluded that the deleterious effects of nanoceria at higher concentration may be associated with oxidative stress.

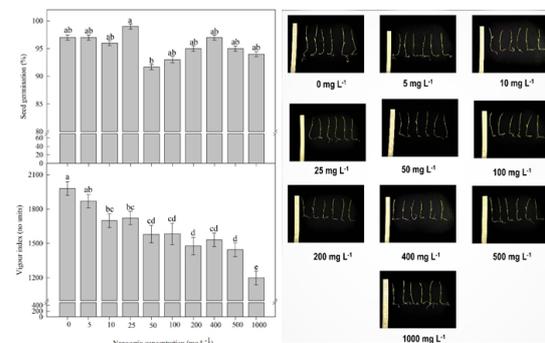


Figure 6. Effect of different concentration of nanoceria on sorghum seed germination

CONCLUSIONS

This study clearly showed that nanoceria is not toxic to terrestrial plants, aquatic organisms and soil microbes at a lower concentration, up to 25 mg L⁻¹. After that, differential responses were observed at different organisms and processes, which could be associated with the stage of spray or exposure methods of nanoceria to the organisms. Among the assays, pollen germination was found to be more sensitive to the nanoceria concentration in the medium, followed by photosystem II quantum yield. Therefore, this methodology can be adopted to understand the toxicity of nanomaterials on terrestrial species.

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