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Article

Effects of Nano-Cerium Oxide on Seed Germination and Seedling Growth of *Vitex negundo*

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Abstract: Cerium oxide nanoparticles (CeO_2NPs) are likely to have dramatic impacts on plant performances, yet the effects of CeO_2NPs on seed germination and seedling growth have not been fully explored. In this study, the seed germination and seedling growth of subshrub species *Vitex negundo* under different concentrations of CeO_2NPs (low-1 mg/L, medium-100 mg/L, high-500 mg/L) have been discussed. Results showed that: (1) The seed germination rate reduces by 11.25% and 2.5% under the low and medium concentrations of CeO_2NPs, respectively, but increased by 7.08% under the high concentration; (2) CeO_2NPs had significant effects on the growth traits (root length, shoot height and biomass) of seedlings, being the highest under the medium concentration and the lowest under the highest concentration; (3) The superoxide dismutase activity was the maximum (355.91 U/g), but the protein concentration was the minimum (3.85 ug/mL) under the high concentration of CeO_2NPs. Our results indicated that the effects of CeO_2NPs on seed germination and seedling growth are concentration-dependency, i.e., low and medium concentrations inhibited while high concentration promoted seed germination, however, seedling growth showed opposite responses. Therefore, appropriate CeO_2NPs concentrations are beneficial to the seed germination and seedling growth of *Vitex negundo* and improve the physiological performance of seedlings and enhance their adaptability to environmental adversity.

Keywords: CeO_2NPs; malonaldehyde; seed germination; physiological responses; *Vitex negundo*

1 Introduction

The rapidly increasing application of nanoparticles (with size < 100 nm) in various areas (e.g., textiles, electronics, pharmaceutics, cosmetics and environmental remediation) has drawn an increasing attention [1]. Depending on the type, nanoparticles may be released to the atmosphere as well as to the soil and surface water in the form of aerosols. For example, nanoparticles released to the atmosphere may be deposited in the soil, and affect not only soil microbial communities but also plants [2]. Nanotechnology also have been used in agricultural researches, such as propagation technique, waste disposal and transformation, crop disease prevention, and some treatments on plants also used various

nanocides [3–5]. However, the interaction mechanisms between nanoparticles and biological systems at the molecular level is still unknown [6,7].

Many evidences have confirmed that rare earth nanomaterials to some extent could significantly promote plant growth. The physical and chemical properties of nanoparticles are important factors for their accessibility to organisms. In addition, it is observed the bioaccumulation of nanoparticles when the concentration was increased [2]. Lu et al. [8] found that a combination of nanosized SiO_2 and TiO_2 increased the nitrate reductase in tissues, increased the absorbing and utilization efficiency of water and nutrients, promoted the antioxidant system, and consequently facilitated the seed germination and plant growth of *Glycine max*. Similarly, TiO_2 nanoparticles promoted the seed germination and plant growth of *Spinacia oleracea* [9]. However, the effects of nanomaterials on plant performances are not always to be positive, which strongly depend on nanomaterials' concentrations and plant species. For example, artificial ZnO nanomaterials reduce the biomass of ryegrass, narrow its root tips, and even disintegrate its epidermal and cortical cells [10,11]; the seed germination rate of ryegrass and scallion were significantly reduced under high concentration (100 mg/L) of ZnONPs. Therefore, the seed germination was inhibited at high concentration of ZnONPs [12,13], although Zn is an essential element for plants. Previous studies also pointed out that although metal oxide nanomaterials have no effects on the seed germination of corn and rice, $nA1O_3$, nZnO and nCuO significantly inhibit their root growth. Copper oxide nanomaterials could interrupt the balance of metabolism in rice, damage the integrity of cell membrane structure and reduce the chlorophyll content, and eventually restrict rice growth [14,15]. For the phytotoxicity and bioavailability of copper nanoparticles, Lee et al. [16] found that the growth rate of *Phaseolus radiatus* and *Triticum aestivum* were constrained and the seedling lengths were negatively related to the exposure concentration of nanoparticles. However, whether the effects of nanoparticles on plants is concentration dependency has not been fully tested.

Cerium (Ce) is an abundant rare earth element (about 68 ppm) in the crust. Some of its compounds can enhance the resistance to environmental stress, the activities of peroxidase, catalase and nitrate reductase [17,18], improve some essential element and chlorophyll contents in plants [19–21]. The interaction between plants and their corresponding atmospheric and edaphic environments strongly depends on the concentration of Ce compounds [22–24]. For instance, Gui et al. [25] found that the growth rate of lettuce was greatly promoted at the concentration of 100 mg/kg CeO_2NPs . The toxicity of nanomaterials may be caused by the excessive production of reactive oxygen species (ROS). Nanomaterials induce cells to produce ROS by interfering with the electron transfer of the mitochondrial respiratory chain, which attacks membrane lipids, damages cell structures and causes oxidative stress [26]. Navarro et al. [27] found that nanoparticles could sequester nutrients on their surfaces and thus serve as a nutrient stock to organisms, particularly those nanoparticles having high specific surface area. At present, many researches had been carried out on accessing the biological effects of nanomaterials on crop species, but the effects of nanomaterials on plant performances in other natural and wild species has been rarely explored.

Vitex negundo is a typical wild deciduous subshrub in northern China and plays an important role in soil and water conservation [28]. This species performs a high tolerance to drought and cold conditions and has a dense root system. Since the reproduction and population recruitment of this species mainly depends on seeds, and the stages of seed germination and seedling growth are susceptible to abiotic stresses, the present study aims to explore the effects of nanocomposite materials (CeO_2NPs) on the seed germination and seedling growth of *Vitex negundo*, and determine the appropriate concentration of CeO_2NPs for this species. The results will provide scientific evidences for the safe application of CeO_2NPs and potential ecological risk assessment.

2 Materials and Methods

2.1 Seed Soaking and Treatment with CeO_2NPs

The seeds used in this study were conventional varieties, provided by Henan Agricultural University, and the seeds were all in the same batch. The neat, consistent, and full seeds were selected and sterilized with 30% hydrogen peroxide for 10 min, and then soaked in ultrapure water for 24 h, so that the germ could break through the seed coat. Then, the seeds were selected and placed in a petri dish with a diameter of 10 cm and a height of 11.5 cm and supported with filter paper. Twenty seeds were evenly distributed in each petri dish. The purchased CeO_2NPs solution (29500 mg/L) was considered as the original solution, and the ultrapure water was used to prepare the treatment solution with three concentrations (low-1.0 mg/L, medium-100 mg/L, high-500 mg/L). Then the solutions were oscillated for 15 min using an ultrasonic instrument to effectively open the soft agglomeration of nanoparticles. The powder was dispersed in the medium with many uniform small agglomerates due to strong impact, shearing and grinding, and the nanometer-CeO_2 was fully dispersed in water to get a more uniform solution. The 7 mL CeO_2NPs treatment solution was added to each petri dish, one group was treated with ultrapure water only as a control group, and each treatment was repeated 4 times. The total number of seeds were 960 (20 seeds × 12 test groups × 4 concentrations of treatment solution). The petri dish was placed in a bag of breathable and waterproof film (PM 996, Parafilm M®, US) and cultured in a greenhouse incubator for 15 days. It was cultured for 16 h at 25–30°C in the daytime and 15–20°C for 8 h during night. The germination of seeds was observed every three or four days.

2.2 Seed Germination Rate

The germination rate of seeds was determined on the 10th day after the treatment with CeO_2NPs solution. The germination rate was calculated by Eq. (1).

$$GR = \frac{n}{N} \times 100\% \tag{1}$$

where *GR* is the germination rate of *Vitex negundo* seeds, *n* is the number of germinated seeds in 10 days, *N* is the number of seeds tested.

2.3 Seedling Growth Indexes

After 15 days of seeds cultured, 10 seedlings were randomly selected from each group, and the morphological indexes such as root length, bud length and shoot biomass of seedlings were measured. The root length, bud length and shoot biomass were used to represent the growth characteristics of seedlings, and indicate the toxicity or stress of CeO_2NPs treatments on seedling growth.

2.4 Physiological Characteristics

2.4.1 Malondialdehyde (MDA)

MDA was measured by the thiobarbituric acid (TBA) method [10], the specific test steps were as follows:

(1) Solution configuration for MDA detection: 0.6 g TBA, dissolved with a small amount of 1M NaOH and stabilized with 10% TCA (trichloroacetic acid) to 100 mL.

(2) MDA extraction: About 0.07 g tissue was weighed, and then 1 mL of extraction liquid was extracted with an adjustable pipette and add to plant tissue for grinding (homogenize on ice). Then the table centrifuge was adjusted to 8000 g under 4°C for 10 min. After centrifugation, the supernatant was taken and placed on the ice for preparation. The digital display thermostatic water-bath pot was adjusted to 100°C, after temperature was stable, kept the mixed liquid in the water bath for 30 min (to prevent water loss, the cap of the mixed liquid bottle needs to be tightly closed). After 30 min in a water bath, the mixed solution was placed on an ice bath to cool, and then adjusted to 10000 g with a desktop centrifuge, centrifuged at

room temperature for 10 min. The absorbance of each sample at 450 nm, 532 nm and 600 nm were determined by absorbing 200 μl supernatant into a 96-well plate. MDA content was calculated by Eq. (2).

$$\text{MDA content} = \frac{(12.9 \times (A532 - A600) - 1.12 \times A450)}{\left(W \times \frac{V_{sample}}{V_{sample\ total}}\right)} \times V_{total} \quad (2)$$

where V_{total} is reaction system total volume (mL), V_{sample} is add sample volume (ml), W is the sample quality (g), $V_{\text{sample total}}$ is add extraction liquid volume (ml).

2.4.2 Catalase (CAT)

The measurement was finished by ultraviolet spectrophotometry [29]. Taken five 10 ml test tubes, one of which was a blank control (distilled water) and four were sample measuring tubes. Add 50 uL of supernatant, 25 mg/L PBS (pH 7.0, containing 0.1 mg/L EDTA, 1% PVP) 3.4 mL, then add 100 mg/L H_2O_2 to the samples at 25°C reaction. Then the CAT was immediately measured after each addition of a measuring tube and quickly poured into a quartz cuvette. The measurement was conducted every 1 min, the total measuring time was 4 min. A change of 0.01 per $\Delta A240$ min was used as an enzyme activity unit. The CAT activity was calculated by Eq. (3).

$$CAT\ activity = \frac{\Delta A240 \times V_T}{0.1 \times V_1 \times t \times FW} \quad (3)$$

where *ΔA240*-absorbance of reaction solution, V_T-the total volume of enzyme extract (mL), V_1-the measured volume of enzyme solution (mL), *FW*-sample fresh weight (g), 0.1-each drop in A240 is 0.1 per unit of enzyme activity (μ), *t*-add H_2O_2 last reading time (min).

2.4.3 Superoxide Dismutase (SOD)

The activity of SOD was detected by the micro method [30]. First, according to the treatment group, taken 0.1 g of seedling tissue from each petri dish, and added 1 mL of extraction solution, put in a pre-chilled mortar on ice and grind until uniform and repeated three times for each group. After that, the sample solution was transferred to a centrifuge tube in a water bath at 100°C for 30 min and then placed in a frozen centrifuge at 8000 g under 4°C for 10 min. The supernatant was taken and placed on the ice for measurement. Meanwhile, the reagents of 1, 2 and 4 were bathed in water at 15°C for more than 5 min before the experiment. Then, each group of individual samples was added the reagent with pipette in accordance with Tab. 1, and put them into a spectrophotometer to detect the light absorption value A. The SOD activity was calculated by Eq. (4).

Table 1: SOD assay reagent addition sequence

Reagent name (μL)	Measuring tube	Control tube	Blank tube 1	Blank tube 2
Reagent 1	45	45	45	45
Reagent 2	100	100	100	100
Reagent 4	35	35	35	35
Sample	18	18	–	–
Double distilled water	–	2	18	20
Reagent 3	2	–	2	–

$$SOD\ activity = \frac{\frac{\text{inhibition percentage}}{1 - \text{inhibition percentage}} \times V_{total}}{\frac{V_{sample}}{V_{sample\ total}} \times W} \times \text{sample dilution times} \quad (4)$$

where Inhibition percentage-$\frac{\Delta A_{blank} - \Delta A_{\text{determination}}}{\Delta A_{blank}} \times 100\%$, V_{total}-reaction system total volume, mL, V_{sample}-add sample volume (mL), W-sample fresh weight (g), $V_{\text{sample total}}$-add extraction liquid product (mL).

2.4.4 Protein Concentration

The protein concentration of seedlings was determined by BCA (bicinchonininc acid) protein quantification method [31], and the BCA protein concentration determination kit was selected. The BCA protein quantification method is a fast, sensitive, stable, and reliable protein quantification method for determination of protein in the range of 10–2000 g/ml. The advantage of the BCA method is that the protein concentration will not be affected by most chemicals in the samples, and it had the maximum absorption value at 562 nm, and the concentration of protein to be measured can be calculated by comparing with the standard curve. The basic principle is that BCA and other reagents composed of Cu^{2+}, $CuSO_4$ and other reagents are mixed and displayed as apple green, which is BCA working reagent in alkaline conditions. When BCA is combined with protein, the protein reduces Cu^{2+} to Cu^{+}, and one Cu^{+} chelate two BCA molecules. The working reagent is formed from the original apple green to form a purple complex. The maximum light absorption intensity is proportional to the protein concentration. Specific operation steps were as follows:

1. BCA solution preparation: Taken 20 mL reagent A (alkaline BCA solution) and 0.4 mL reagent B (copper sulfate solution) and mixed them thoroughly.
2. Added protein standards to the protein standard wells of 96-well plates at 0, 1, 2, 4, 6, 8, and 10 μL, added sterile double distilled water to make up to 10 μL, take 10 μL of the test sample and added to 96-well plates.
3. Added 200 μL of BCA solution (the volume ratio of sample to working solution was 1:20) to the sample well and protein standard well to mix.
4. Bathed at 37°C for 30 min and cooled to room temperature.
5. The absorbance was measured at 562 nm with a microplate reader.
6. A standard curve was prepared, and the sample concentration was obtained from the standard curve.

2.5 Statistical Analysis

The data were represented by mean ± standard deviation. After data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test), One-way ANOVA was used to compare the seed germination rate and growth indexes of seedling among different treatments, and $p < 0.05$ as the difference was statistically significant. SPSS 22.0 was used for statistical analysis and Origin 9.1 was used for drawing.

3 Results

3.1 The Effect of CeO_2NPs on the Seed Germination of Vitex negundo

CeO_2NPs had significant effects on the germination rate of seeds, especially under the concentration of 1 mg/L and 500 mg/L (Fig. 1). The seed germination rate was reduced by 11.25% under the low concentration (1 mg/L) of CeO_2NPs solution, but increased by 7.08% under the high concentration (500 mg/L). The seed germination rate under the 500 mg/L concentration was significantly higher than that under the 1 mg/L concentration ($p < 0.05$) (Fig. 1).

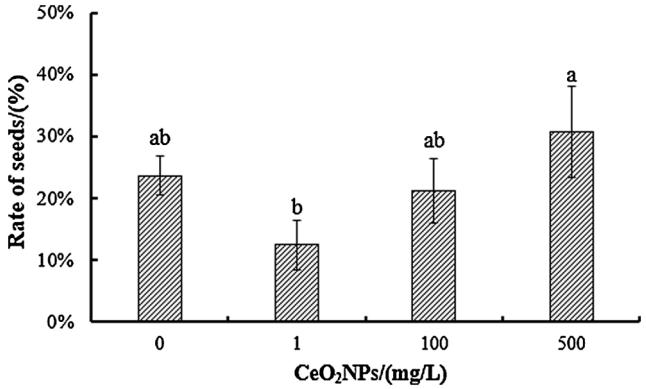

Figure 1: Germination rate of *Vitex negundo* seeds under different concentrations of CeO_2NPs. Different letters indicate the significant differences in seed germination rates under different concentrations of CeO_2NPs, and the significant level is $p < 0.05$

3.2 The Effect of CeO_2NPs on the Seedling Growth of Vitex negundo

Different concentrations of CeO_2NPs had significantly effects on the root length, shoot height and biomass of *Vitex negundo* seedlings ($p < 0.05$). Generally, three growth indexes of seedlings first increased and then decreased with the increasing concentration of CeO_2NPs. Specifically, the seedling treated with 100 mg/L CeO_2NPs had the highest growth indexes, while those treated with 500 mg/L had the lowest growth indexes. The root length, shoot height and biomass of the seedlings treated with 100 mg/L CeO_2NPs were significantly higher compared with the control ($p < 0.05$) (Fig. 2). The root length, shoot height and biomass of seedlings were the highest under 100 mg/L concentration, which was 22.63% higher than the control. Shoot height increased significantly by 13.10%, and shoot biomass increased significantly by 13.97%. However, high concentration of CeO_2NPs inhibited the growth of seedlings to different degrees. Compared with the control, the biomass of seedlings treated with 500 mg/L CeO_2NPs was significantly reduced ($p < 0.05$). The root length, shoot height and biomass of the seedlings treated with 500 mg/L decreased by 13.87%, 6.90% and 14.53%, respectively (Fig. 2).

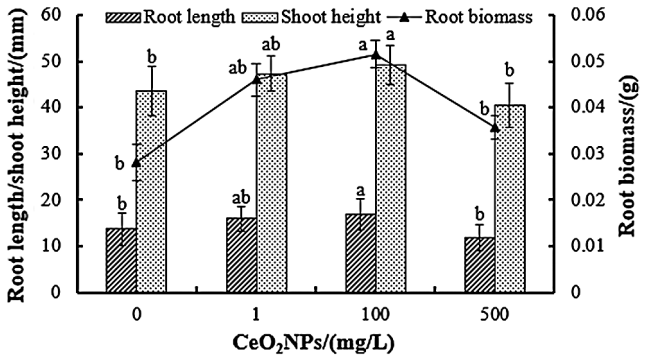

Figure 2: The effect of CeO_2NPs concentration on the growth index of *Vitex negundo* seedlings. Different letters indicate the significant differences in the growth index under different concentrations of CeO_2NPs, and the significant level is $p < 0.05$

3.3 The Effect of CeO_2NPs on the Physiological Characteristics of Vitex negundo Seedings

CeO_2NPs had significant effects on the CAT activity of seedlings under the low concentration CeO_2NPs solution (1 mg/L), and the MDA content under CeO_2NPs solution of 100 mg/L was significantly higher than

the control ($p < 0.05$). The SOD activity was significantly higher but the CAT activity and protein concentration were lower than the control. The CeO_2NPs solution with the high concentration (500 mg/L) had an inhibitory effect on the CAT activity and protein concentration of seedlings while promoted the activity of SOD (Fig. 3).

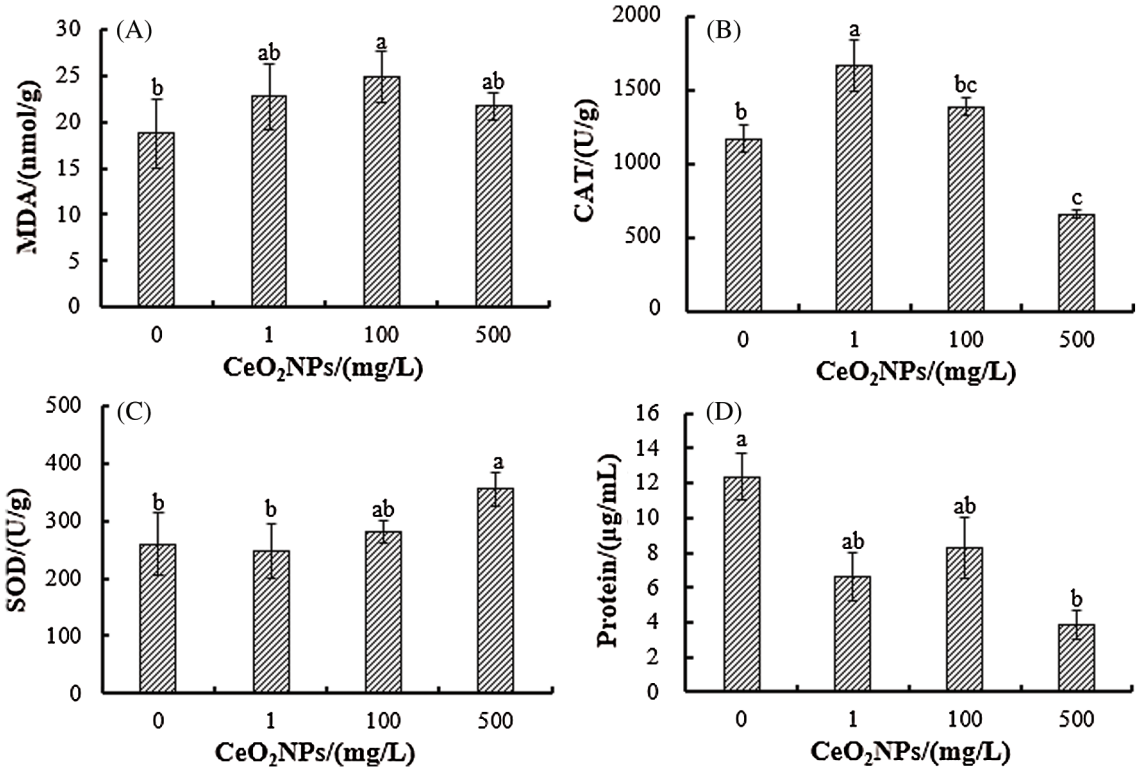

Figure 3: Effects of different concentrations of CeO_2NPs on the physiological characteristics of *Vitex negundo* seedlings. Different letters indicate the significant differences in the physiological characteristics under different concentrations of CeO_2NPs, and the significant level is $p < 0.05$. A–MDA content, B–CAT activity, C–SOD activity, D–protein concentration

4 Discussion

4.1 Effects of CeO_2NPs on Seed Germination Rate

Seed germination is a key period of plant life cycle and one of the most sensitive periods to environmental factors [23]. Our results showed that the effects of CeO_2NPs on the seed germination of *Vitex negundo* strongly depended on its concentration—The low concentration had an inhibitory effect, but the high concentration had a positive effect on seed germination. Previous studies also found that other nanoparticles have similar effects on the seed germination of other species. For example, at the lower concentration of ZnONPs, the seed germination rate of mung bean was inhibited, and at the higher concentration of ZnONPs, the seed germination was facilitated [32].

4.2 Effects on Seedling Growth

The growth characteristics of seedlings reflect the phytotoxicity of CeO_2NPs on the seedling growth [33]. The selected growth indexes of seedlings all increased first and then decreased with the increasing concentration of CeO_2NPs. Therefore, it can be inferred that lower concentrations of CeO_2NPs has a

certain promoting effect on the growth of *Vitex negundo* seedlings, while higher concentrations showed an inhibitory effect, which was consistent with the findings on *Arabidopsis thaliana* [34], onions [33] and ryegrass [35]. At the high concentration (500 mg/L) of CeO_2NPs, the decrease of each growth index reflects its toxic effect on the seedlings of *Vitex negundo*, which also indicated that the root and shoot lost seed coat protection after seed germination, and nanoparticles could directly effect on young roots and shoots and then affect their growth.

4.3 Changes in Physiological Characteristics

MDA is one of the most important products of membrane lipid peroxidation when plant organs are aging or damaged. Its production will aggravate membrane damage, and its content can reflect the extent to which plants are subjected to stresses [36]. The treatments with different concentrations of CeO_2NPs resulted in an increase in MDA content in the seedlings. The 100 mg/L concentration of CeO_2NPs had the most significant influence on MDA content of seedlings. It indicated that under the stress of CeO_2NPs, plant cells increased the antioxidant enzyme activity for self-protection, but not enough to resist the toxic effects of CeO_2NPs. Instead of reducing membrane lipid peroxidation, the active oxygen accumulation has occurred, leading to excessive MDA in plants and resulting in oxidative stress [37]. When the plant under adversity or aging, H_2O_2 accumulates due to the increased active oxygen metabolism in plant tissues, and cell membrane is damaged, which accelerates cell senescence and disintegration. CAT can remove H_2O_2, which is one of the important enzymatic defense systems in plants [38]. CAT activity increased most at the low concentration of 1 mg/L CeO_2NPs, and with the increase of CeO_2NPs concentration, the overall activity of seedlings decreased, indicating that the CeO_2NPs can promote the CAT activity in seedlings at low concentration, but the high concentration showed more obvious inhibitory effect.

The activity of SOD is an important respiratory enzyme in plants. From the perspective of environmental biology, peroxidase is critical to determine the adaptability of plants to environmental factors. Higher SOD activity can improve the resistance ability of plants to environmental stress such as water stress [14]. The SOD enzyme activity of seedlings treated with different concentrations of CeO_2NPs was higher than that of the control group except for the concentration of 1 mg/L. Among them, the SOD enzyme activity of seedlings treated with the concentration of 500 mg/L was the strongest. The toxicity of CeO_2NPs at a lower concentration was relatively low, and the oxidative damage of the seedlings was not obvious. However, the antioxidant enzyme system of seedlings was not enough to resist the damage of tissues and cells induced by high concentration of CeO_2NPs, so high concentration of CeO_2NPs had strong toxicity.

The protein concentration of *Vitex negundo* seedlings first increase and then decrease with the increasing concentrations of CeO_2NPs. Specifically, the protein concentration of seedlings treated with the concentration of 500 mg/L was the lowest. The protein concentration of the seedlings treated with 1 mg/L and 100 mg/L concentration of CeO_2NPs was also lower than that of the control. Because seeds contain enzymes and membrane-bound proteins, they will accumulate protein as the main storage reserve [29]. Proteins were involved in oxidative stress to a certain extent. In addition, the seedlings treated with the concentration of 500 mg/L faced strongest oxidative stress. It was found that different concentrations of CeO_2NPs had different toxic effects on the physiological characteristics of seedlings.

5 Conclusions

Our study demonstrated that the effects of CeO_2NPs on the seed germination and seedling growth of *Vitex negundo* are concentration-dependency, i.e., low and medium concentrations inhibited while high concentration promoted seed germination, however, seedling growth showed opposite responses. Meanwhile, the CeO_2NPs treatment of high concentration (500 mg/L) had oxidative stress and significantly inhibited the growth of *Vitex negundo* seedlings. Future research should focus on the

existing morphology of nanoparticles in plant tissues treated with different nano-materials, and provide sufficient basis for a clear elaboration of the toxicogenic mechanism of nano-materials on plants.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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