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Phytotoxic Mechanism of Nanoparticles: Destruction of Chloroplasts and Vascular Bundles and Alteration of Nutrient Absorption

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This study focused on determining the phytotoxic mechanism of CeO₂ nanoparticles (NPs): destroying chloroplasts and vascular bundles and altering absorption of nutrients on conventional and Bt-transgenic cottons. Experiments were designed with three concentrations of CeO₂ NPs including: 0, 100 and 500 mg·L⁻¹, and each treatment was three replications. Results indicate that absorbed CeO₂ nanoparticles significantly reduced the Zn, Mg, Fe, and P levels in xylem sap compared with the control group and decreased indole-3-acetic acid (IAA) and abscisic acid (ABA) concentrations in the roots of conventional cotton. Transmission electron microscopy (TEM) images revealed that CeO₂ NPs were absorbed into the roots and subsequently transported to the stems and leaves of both conventional and Bt-transgenic cotton plants via xylem sap. In addition, the majority of aggregated CeO₂ NPs were attached to the external surface of chloroplasts, which were swollen and ruptured, especially in Bt-transgenic cotton. The vascular bundles were destroyed by CeO₂ nanoparticles, and more damage was observed in transgenic cotton than conventional cotton.

Nanomaterials have been applied in many aspects of life processes and products, including wastewater treatment, food processing, catalytic materials, and biomedical products¹⁻⁴. They are used in agriculture to enhance seed germination and plant growth and to protect crops from biotic stresses, such as insects, fungi, and bacteria⁵⁻⁸. However, given the unique properties of nanoparticles (NPs), living organisms in the ecosystem could suffer from oxidative stress induced by NPs⁹⁻¹¹. A common conclusion from recent studies was that the nanotoxicity might depend on either metal speciation or the plant species. Ma *et al.*¹² reported that exposure to 2000 mg·L⁻¹ NPs from the lanthanide series, such as lanthanum oxide (La₂O₃), gadolinium oxide (Gd₂O₃), and ytterbium oxide (Yb₂O₃), greatly inhibits root growth in radish, tomato, rape, lettuce, wheat, cabbage, and cucumber plants. According to Lin and Xing¹³, a concentration of 200 mg·L⁻¹ of nano-sized Zn (zinc, 35 nm) and ZnO (zinc oxide, 20 nm) inhibited germination in ryegrass and corn, respectively. The root growth of radish, rape canola, ryegrass, lettuce, corn, and cucumber species was also inhibited upon exposure to 2000 mg·L⁻¹ nano-sized Zn and ZnO¹³. Silicon oxide NPs (SiO₂) notably inhibit plant growth and the shoot and root biomasses of *Bacillus thuringiensis* (Bt)-transgenic cotton¹⁴. Le *et al.*¹⁴ reported that the height, shoot biomass, and root biomass of Bt-transgenic cotton were 65.39%, 68.55%, and 57.69%, respectively, of those of the control group when

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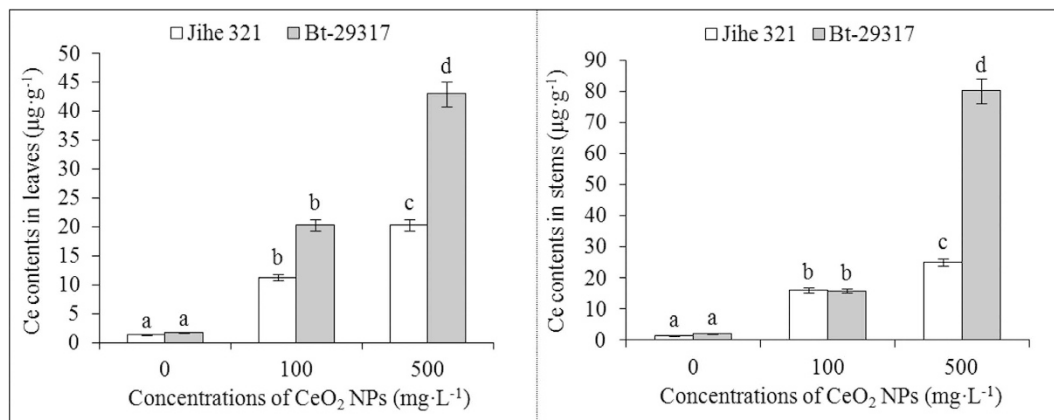


Figure 1. Ce contents in leaves and stems of Bt-transgenic and conventional cottons. The means are averaged from three replicates and error bars correspond to standard derivations of mean. Different small letters in the same plant indicate significant difference at $p < 0.05$ level between control and CeO₂ NPs treatments, and different small letters in the same CeO₂ NPs concentration show significant difference at $p < 0.05$ level between conventional and Bt-transgenic cottons.

exposed to 2000 mg·L⁻¹ of SiO₂ NPs. Furthermore, Li *et al.* found that cerium oxide (CeO₂) NPs disrupt the uptake of nutrient elements in Bt-transgenic cotton compared with its parental conventional cotton plant¹⁵. Thus, the phytotoxic mechanism of nanomaterials must be thoroughly understood before application in fields.

Transgenic plants offer numerous advantages to producers, consumers, and the environment. Indeed, engineered plants are highly tolerant to pesticides and herbicides, demonstrate resistance towards pathogens caused by microorganisms, and exhibit increased production yields and enhanced biofuel content, which are considered novel and sustainable energy sources¹⁶. Bt-transgenic cotton contains one foreign gene derived from the bacterium *Bacillus thuringiensis* that encodes the Cry toxin, which effectively kills bollworms and therefore controls yield losses^{17–19}. In China, Bt-transgenic cotton was approved for commercial use in the Yellow River and Changjiang River regions in 1997 and 2000, respectively²⁰. Today, because of the widespread uses of Bt-transgenic cotton, China has become the largest cotton producing country in the world²¹. However, studies on the effects of NPs on transgenic plants and crops are minimally documented.

In this study, we investigated the effect of CeO₂ NPs on both conventional and Bt-transgenic cotton. Upon exposure to 100 or 500 mg·L⁻¹ CeO₂ NPs, the nutrient levels, enzyme activities, and hormone concentrations in both types of cotton indicated that NPs have adverse effects regardless of the plant type. The results provide a preliminary basis for the assessment of the safety of NPs in the sustainable development of transgenic agriculture.

Results and discussion

Ce distribution in the stem and leaves. The Ce content in the leaves and stems of old Bt-transgenic and conventional cotton plants after treatment with CeO₂ NPs is presented in Fig. 1. The Ce content in the leaves increased with increasing doses. In the presence of 100 mg·L⁻¹ CeO₂ NPs, the Ce content in Bt-transgenic leaves was 1.8-fold higher than in conventional cotton and 12.5 fold higher than in Bt-transgenic cotton in the control group. Upon treatment with 500 mg·L⁻¹ CeO₂ NPs, the Ce content was significantly higher in the leaves of Bt-transgenic cotton than in the leaves of conventional cotton. Additionally, the Ce content in Bt-transgenic cotton was 2.1- and 26.4-fold higher than conventional cotton exposed to 500 mg·L⁻¹ CeO₂ NPs and the Bt-transgenic cotton control group, respectively. Similarly, the Ce content in the stems of both Bt-transgenic and conventional cotton increased with increasing CeO₂ NP concentrations. When treated with 500 mg·L⁻¹ CeO₂ NPs, the Ce content in the stems of Bt-transgenic cotton was greatly elevated compared with conventional cotton. The Ce content in 500 mg·L⁻¹ CeO₂ NP-treated Bt-transgenic stems was 80.15 µg·g⁻¹, which was 3.2 times the Ce content in similarly treated conventional cotton. These results suggested that CeO₂ NPs were taken up by the cotton plants and transported to the stems and leaves from the root system. This result is consistent with previous studies stating that CeO₂ NPs are transported to shoots from the cotton root system¹⁵ and that CuO NPs enter maize through the root system²². Lin and Xing²³ reported that ZnO nanoparticles taken up by ryegrass roots but that few ZnO nanoparticles were transported to the shoots.

Effect of CeO₂ NPs on nutrients in xylem sap. Figure 2 presents nutrient concentrations in xylem sap from Bt-transgenic and conventional cotton plants exposed to CeO₂ NPs. Zn, Mg, Fe, and P levels in the xylem sap after exposure to CeO₂ NPs significantly decreased ($p < 0.05$), whereas the Mn

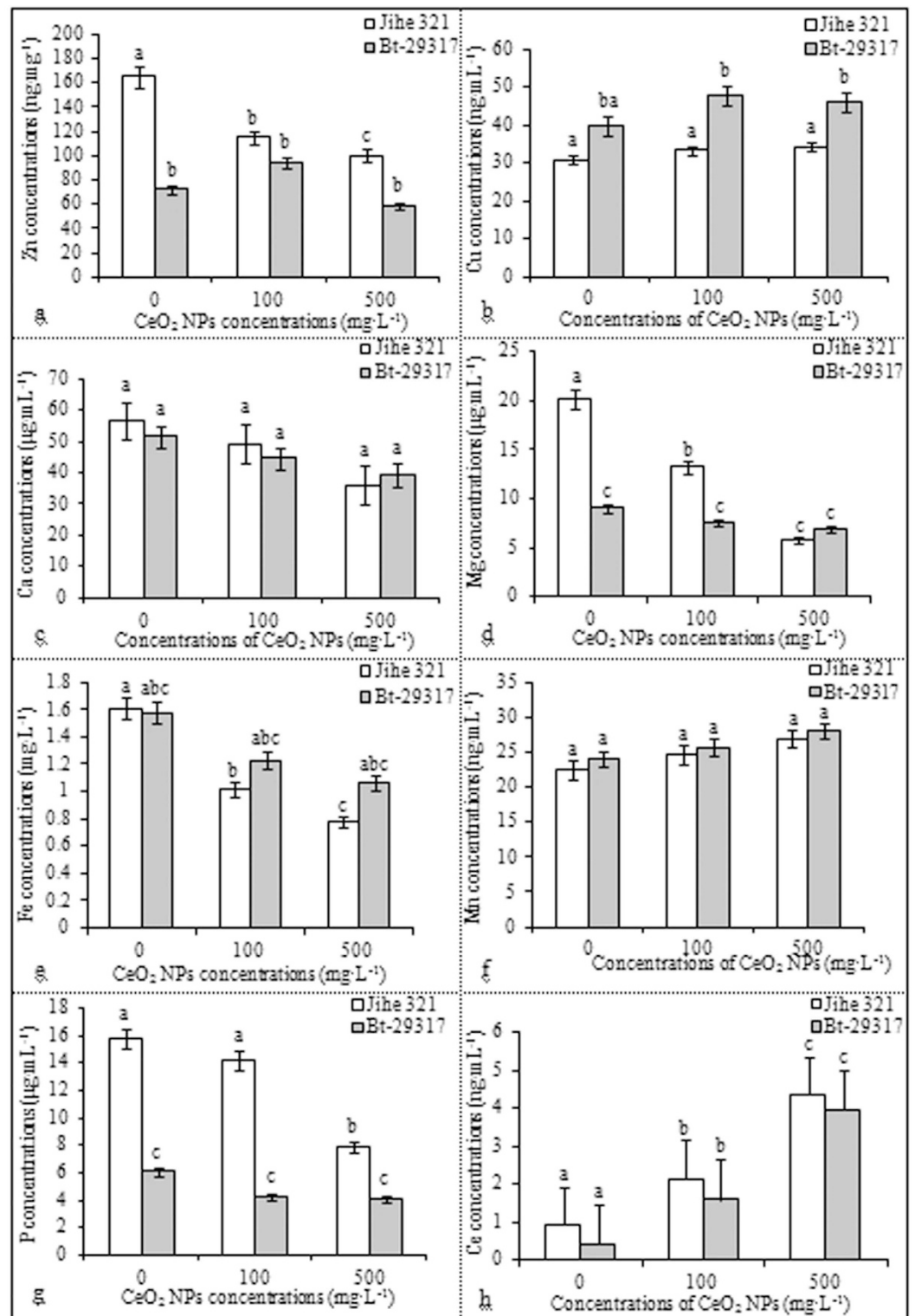


Figure 2. Effects of CeO₂ NPs on the nutrient concentrations in the xylem sap. The means are averaged from three replicates and error bars corresponded to standard derivations of three values. Different small letters in the same plant indicate significant difference at $p < 0.05$ level between control and CeO₂ NPs treatments, and different small letters in the same CeO₂ NPs concentration show significant difference at $p < 0.05$ level between conventional and Bt-transgenic cottons.

concentration increased in CeO₂ NP-treated cotton plants. The concentration of Zn in the xylem sap of conventional cotton plants decreased upon treatment with CeO₂ NPs. As the exposure dose increased, more effects on the Zn uptake were observed. However, no significant differences in Zn content were

noted between CeO₂ NP-treated Bt-transgenic cotton and the control group. Zn levels in Bt-transgenic cotton were significantly lower ($p < 0.05$) than in the control group and conventional cotton, especially upon exposure to 500 mg·L⁻¹ of CeO₂ NPs. Similarly, Mg, Fe, and P concentrations in the xylem sap of conventional cotton significantly decreased ($p < 0.05$) after treatment with CeO₂ NPs. Mg plays a critical role in photosynthesis; Mg is involved in chlorophyll synthesis and in the ultrastructure of chloroplasts²⁴. The Mg concentration in conventional cotton treated with CeO₂ NPs was significantly lower ($p < 0.05$) than in the control group (Fig. 2d). By contrast, Mg levels were significantly lower ($p < 0.05$) in transgenic cotton than in conventional cotton treated with 100 mg·L⁻¹ CeO₂ NPs and the control group. No significant differences were noted between the control and CeO₂ NP-treated groups in transgenic cotton. Similarly, the Fe content in conventional cotton significantly differed ($p < 0.05$) between the control and the CeO₂ NP-treated cotton samples, but no effect of CeO₂ NPs was observed in transgenic cotton and between cultivars. The P level in the xylem sap of both transgenic and conventional cotton decreased after exposure to CeO₂ NPs. The high CeO₂ NP dose significantly affected ($p < 0.05$) the P content in the xylem sap of conventional cotton, but no significant differences in P levels were noted between the CeO₂ NP-treated Bt-transgenic cotton and the control group. However, the P levels significantly differed ($p < 0.05$) between conventional and Bt-transgenic cotton in both the control and CeO₂ NP-treated samples. These results suggested that CeO₂ NPs did not affect P levels in the xylem sap of Bt-transgenic cotton (Fig. 2g).

No significant differences in Cu, Ca, and Mn levels in the xylem sap of both conventional and Bt-transgenic cotton were noted between the CeO₂ NP-treated and the control samples (Fig. 2b,c,f). The effects of CeO₂ NPs on the concentration of Cu in the xylem sap of transgenic cotton were different from those noted in conventional cotton. The Cu concentration in the xylem sap of transgenic cotton was elevated in both CeO₂ NP-treated samples, but no effects were observed in conventional cotton. Moreover, the Cu level in Bt-transgenic cotton was significantly higher ($p < 0.05$) than in conventional cotton exposed to CeO₂ NPs. These results suggest that the Cu uptake in transgenic cotton was more sensitive to CeO₂ NPs than that in conventional cotton.

The Ce concentration in the xylem sap of both conventional and Bt-transgenic cotton increased significantly with increasing CeO₂ NP concentrations. Minimal Ce was noted in the xylem sap of conventional and transgenic cotton plants not exposed to CeO₂ NPs (Fig. 2h). In the CeO₂ NP-treated samples, the concentration of Ce in the xylem sap of both conventional and transgenic cotton increased significantly, indicating that CeO₂ NPs can be absorbed by the root and transported to the shoot via xylem sap. This result is supported by previous studies by Zhang *et al.*²⁵ and Li *et al.*¹⁵ that indicate that CeO₂ NPs entered the roots and were subsequently transported to the shoots of cucumber and cotton, respectively. The presence of Ce in plant tissues is further demonstrated using transmission electron microscopy (TEM).

CeO₂ NPs observed in xylem sap. We observed CeO₂ NPs in the xylem sap of both transgenic and conventional cotton plants (Fig. 3c,f), suggesting that the CeO₂ NPs were transported from the roots to the shoots via xylem sap. Many studies report that NPs are transferred to the shoots from the root system²⁵. These results suggested that CeO₂ NPs were taken up by cotton plants. Similarly, Li *et al.*¹⁵ reported that a higher concentration of Ce was observed in CeO₂ NP-treated shoots than in the control group, but our results demonstrate that CeO₂ NPs were transported and aggregated to form clusters.

Effect of CeO₂ NPs on enzyme activities. No significant differences in root peroxidase (POD) activities, leaf superoxide dismutase (SOD) activities, and protein concentration were noted in either Bt-transgenic or conventional cotton between the CeO₂ NP-treated and control samples as well as between cultivars exposed to similar CeO₂ NP concentrations (Fig. 4). POD activity was significantly higher in the leaves ($p < 0.05$) of Bt-transgenic cotton than in conventional cotton upon exposure to 500 mg·L⁻¹ CeO₂ NPs. Fig. 4 (B1, B2) indicates no significant difference in SOD activity in the leaves and roots of Bt-transgenic cotton and the leaves of conventional cotton for the CeO₂ NP-treated and control groups. Upon exposure to 500 mg·L⁻¹ CeO₂ NPs, SOD activity in the roots of conventional cotton significantly differed ($p < 0.05$) from that of the control group. In addition, SOD activity in Bt-transgenic cotton roots was significantly lower ($p < 0.05$) than in conventional cotton without CeO₂ NP treatment. However, no significant difference in root SOD activity was noted between conventional and Bt-transgenic cotton plants upon CeO₂ exposure. The effects of CeO₂ NPs on catalase (CAT) activity in the leaves and roots of Bt-transgenic and conventional cotton are presented in Fig. 4 (C1, C2). Upon CeO₂ exposure, CAT activity in the leaves of conventional cotton plants significantly differed ($p < 0.05$) from that of the control group. However, a significant difference ($p < 0.05$) in the CAT activity in the leaves and roots of Bt-transgenic cotton was noted between samples treated with 100 mg·L⁻¹ CeO₂ NPs and the control group. By contrast, the CAT activity in the leaves of conventional cotton plants was lower than that of Bt-transgenic cotton in the control group, but the levels were obviously higher ($p < 0.05$) than in Bt-transgenic cotton treated with 100 mg·L⁻¹ CeO₂ NPs. Similar results were observed regarding root CAT activity upon exposure to 100 mg·L⁻¹ CeO₂ NPs. These results are consistent with the previous

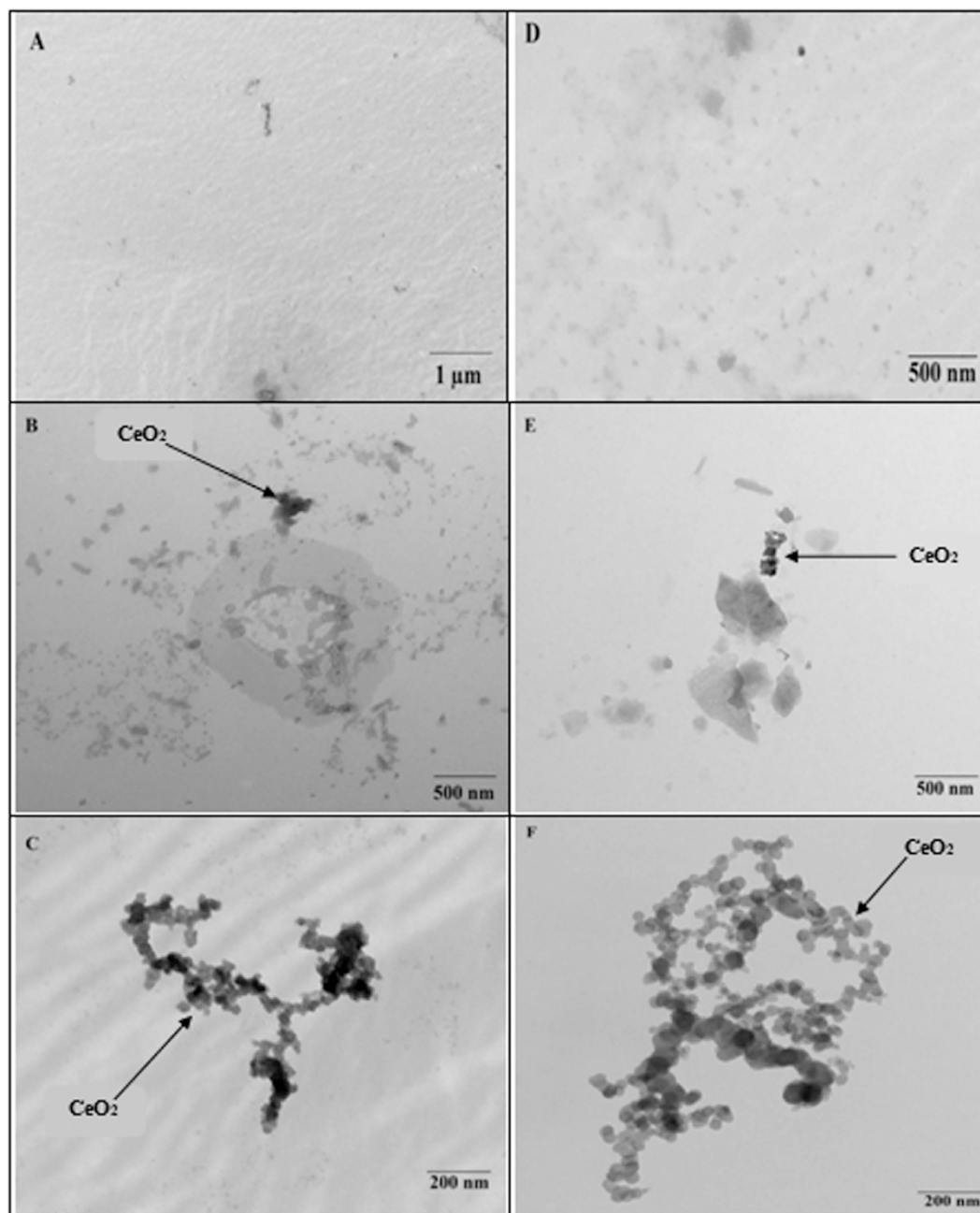


Figure 3. TEM images of conventional and transgenic cotton xylem sap treated with different concentration of CeO_2 NPs. A-B-C and D-E-F are TEM images of non-transgenic and Bt-transgenic cotton under 0, 100 and $500 \text{ mg}\cdot\text{L}^{-1}$ CeO_2 NPs treatments, respectively.

study by Ali²⁶, who investigated the effects of nanoparticles on *Triticum aestivum*. According to Zhao *et al.*²⁷, reduced CAT activity was noted in corn plants grown in organic soil treated with $400 \text{ mg}\cdot\text{kg}^{-1}$ ZnO NPs. Arnab Mukherjee²⁸ reported decreased CAT activity in green peas (*Pisum sativum L*) treated with ZnO NPs.

The protein concentration in the leaves and roots of both conventional and Bt-transgenic cotton was not affected upon exposure to CeO_2 NPs compared with the control group (Fig. 4 D1, D2). A significant difference ($p < 0.05$) in the root protein concentrations were noted between conventional and Bt-transgenic cotton samples treated with $100 \text{ mg}\cdot\text{L}^{-1}$ CeO_2 NPs and control groups. Root protein concentrations was higher in conventional cotton than in Bt-transgenic cotton for both control groups and samples exposed to CeO_2 NPs (Fig. 4 D2). These results indicated that CeO_2 NPs did not affect protein concentrations in the leaves and roots of both conventional and Bt-transgenic cotton plants. This finding differs from previous studies by Salama²⁹, who reported the effects of silver nanoparticles on the protein

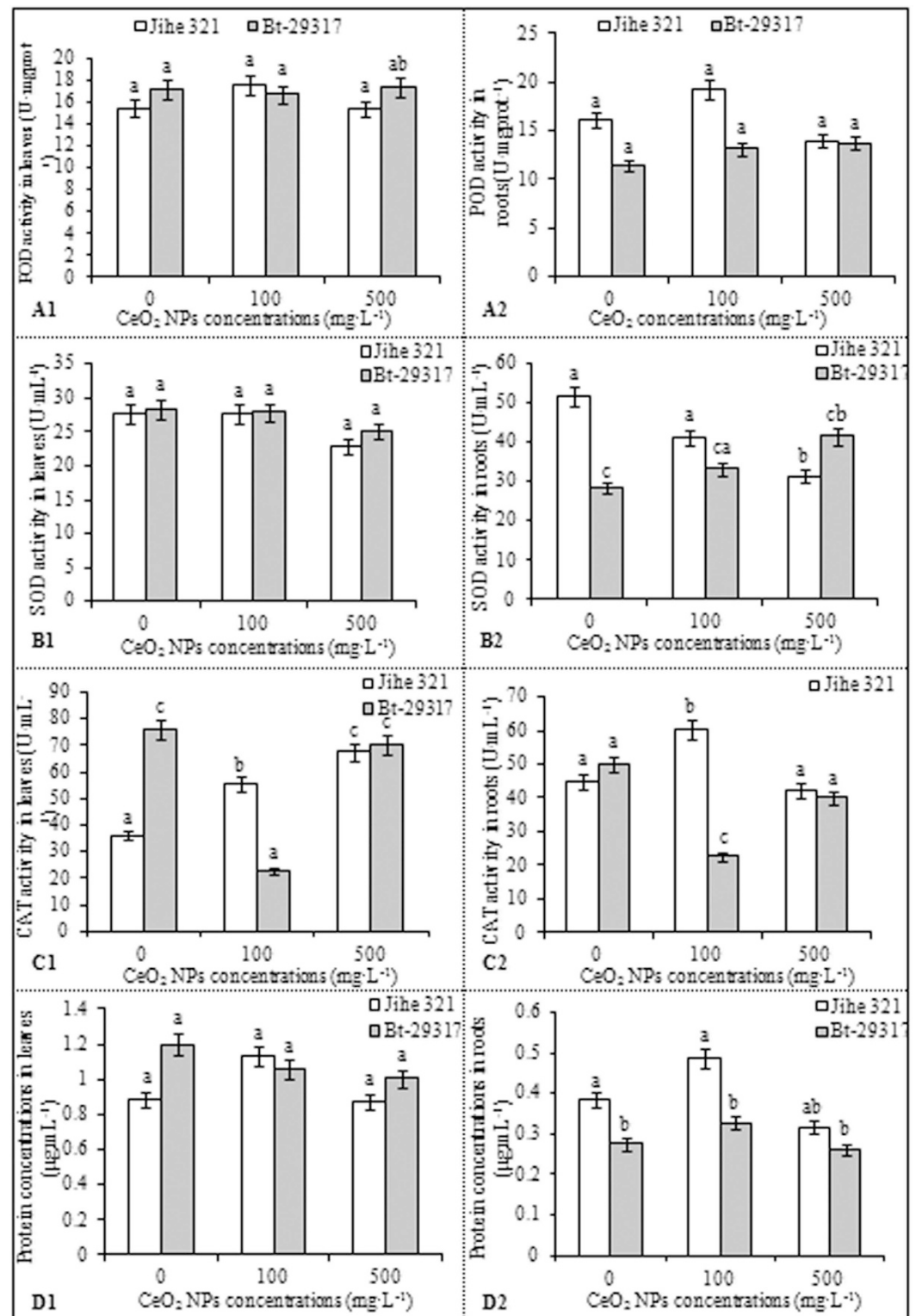


Figure 4. Effects of CeO_2 NPs concentrations on the enzymes activities of conventional and transgenic cottons. A1, B1, C1, D1 and A2, B2, C2, D2 were POD, SOD, CAT activities and protein concentration in the leaves and roots of non-transgenic and Bt-transgenic cottons, respectively. The mean is averaged from three replicates and error bars correspond to standard derivation of mean. Different small letters in the same plant indicate significant difference at $p < 0.05$ level between control and CeO_2 NPs treatments, and different small letters in the same CeO_2 NPs concentration indicate significant difference at $p < 0.05$ level between conventional and Bt-transgenic cottons.

content of common bean (*Phaseolus vulgaris*) and corn (*Zea mays*). This finding suggests that various nanoparticles have different effects on the protein content of different plants.

Effects of CeO₂ NPs on hormone concentration. Figure 4 (A1, A2) indicates that the IAA content in the leaves and roots of Bt-transgenic cotton did not significantly differ between the CeO₂ NP-treated samples and the control groups. However, IAA levels in the leaves of conventional cotton treated with 500 mg·L⁻¹ CeO₂ NPs were 1.29-fold higher ($p < 0.05$) than in the control group. Additionally, root IAA levels of Bt-transgenic cotton exposed to 100 and 500 mg·L⁻¹ CeO₂ NPs were 1.25- and 1.19-fold higher ($p < 0.05$), respectively, than those of conventional cotton, whereas CeO₂ NPs were not observed to affect IAA in the leaves of Bt-transgenic and conventional cotton. Similarly, no significant difference was noted in the ABA content of the leaves of conventional or Bt-transgenic cotton treated with CeO₂ NPs compared with the control group. Upon exposure to 100 mg·L⁻¹ CeO₂ NPs, ABA levels in the roots of Bt-transgenic cotton were 81.98% lower ($p < 0.05$) than that of the control group; values were not reported for treatment at 500 mg·L⁻¹ (Fig. 5 B1, B2). By contrast, trans-zeatinriboside (t-ZR) content in the leaves and roots of Bt-transgenic cotton was not affected by CeO₂ NPs compared with the control group. The t-ZR content in the leaves and roots of conventional cotton exposed to 500 mg·L⁻¹ CeO₂ NPs was 76.57% and 91.26% lower ($p < 0.05$), respectively, than that in the leaves and roots of the control group. In addition, the t-ZR content in conventional cotton leaves and roots was 97.31% and 86.43% ($p < 0.05$), respectively, than in the leaves and roots of Bt-transgenic cotton exposed to 500 mg·L⁻¹ CeO₂ NPs (Fig. 5 C1, C2). This finding suggests that high concentrations of CeO₂ NPs affect t-ZR content in conventional cotton but not Bt-transgenic cotton. No significant difference was observed regarding gibberellic acid (GA) content in the leaves and roots of conventional and Bt-transgenic cotton plants from the CeO₂ NP-treated samples and the control groups (Fig. 5 D1, D2). However, the leaf GA content in conventional cotton was considerably higher ($p < 0.05$) than in Bt-transgenic cotton upon CeO₂ NP treatment. Furthermore, IAA and ABA concentrations in the leaves were 2-fold higher than in the roots of both Bt-transgenic and conventional cotton plants, whereas the t-ZR concentrations were 1.5-fold higher in the leaves than in the roots. Leaf GA content was similar to that observed in the roots. These results indicate that hormone concentrations were higher in leaves than in roots under similar CeO₂ NP concentrations. This result suggests that CeO₂ NPs exert different effects on hormones and affect different plant regions in Bt-transgenic and conventional cotton plants.

TEM images of CeO₂ NP distribution in the leaves, stems, and roots. CeO₂ NPs were observed in the leaf cells of both conventional and transgenic cotton and more CeO₂ NPs were noted in the leaves of transgenic cotton than in those of conventional cotton (Fig. 6). At 100 mg·L⁻¹ CeO₂ NPs, most CeO₂ NPs were distributed around the chloroplast and adsorbed on the outer membrane of the chloroplast; however, fewer CeO₂ NPs were observed on the outer membrane of chloroplasts in the leaves of conventional cotton. As the CeO₂ NP concentration increased to 500 mg·L⁻¹, more CeO₂ NPs were adsorbed onto the outer membrane of the chloroplast in the leaves of transgenic cotton. In addition, many chloroplasts began to swell, rupture, and exhibit deformities. In contrast, chloroplasts in the leaves of conventional cotton exhibited no obvious change. These observations are consistent with the conclusion by Li *et al.*¹⁵ that Bt-transgenic cotton is more sensitive to CeO₂ NPs than is conventional cotton. All of these data indicate that chloroplasts are the most vulnerable organelle in the leaves and that the chloroplasts of transgenic cotton are more vulnerable than those of conventional cotton.

Numerous vascular bundle fragments were noted in both conventional and transgenic cotton exposed to 100 mg·L⁻¹ CeO₂ NPs. Compared with the CeO₂ NP distribution on the inner wall of the vascular bundles of conventional cotton, fewer NPs were observed in transgenic cotton (Fig. 7 C2, D2). With 500 mg·L⁻¹ CeO₂ NPs, the vascular bundles in conventional cotton were deformed and damaged (Fig. 7 C3, D3). These data imply that most CeO₂ NPs were detained in vascular bundles in conventional cotton, whereas most CeO₂ NPs were transported into the leaves of transgenic cotton.

Figure 8 presents TEM images of conventional and Bt-transgenic cotton roots under various treatments. The adsorption of CeO₂ NPs into the root cells and CeO₂ NP aggregation on the root surface of both conventional and Bt-transgenic cotton were evident, and CeO₂ NP aggregation increased as the CeO₂ NPs exposure dose reached 500 mg·L⁻¹ (Fig. 8 E2, E3, F2, F3). Li *et al.*¹⁵ reported that CeO₂ NPs were observed in the roots of Bt-transgenic cotton and its parental non-transgenic cotton based on TEM. In studies by Jose *et al.*³⁰, synchrotron microfocused X-ray fluorescence (μ -XRF) and micro X-ray absorption near-edge structure (μ -XANES) analyses were performed to determine the presence of CeO₂ NPs in soybean (*Glycine max*) tissues. X-ray absorption spectroscopy (XAS) was used to demonstrate the uptake of CeO₂ NPs into alfalfa (*Medicago sativa*), corn (*Zea mays*), cucumber (*Cucumis sativus*), and tomato (*Lycopersicon esculentum*)³¹.

Conclusions

CeO₂ NPs were absorbed, transported by the xylem sap, and distributed to the roots, stems, and leaves of both conventional cotton and transgenic cotton. CeO₂ NPs significantly reduced the absorption of Zn, Mg, Fe, and P in the xylem sap of conventional cotton but increased Mn absorption. SOD activity in the roots and CAT activity in the leaves and roots of Bt-transgenic cotton plants were affected by low CeO₂ NP concentrations. In addition, the root IAA content was obviously higher ($p < 0.05$) in

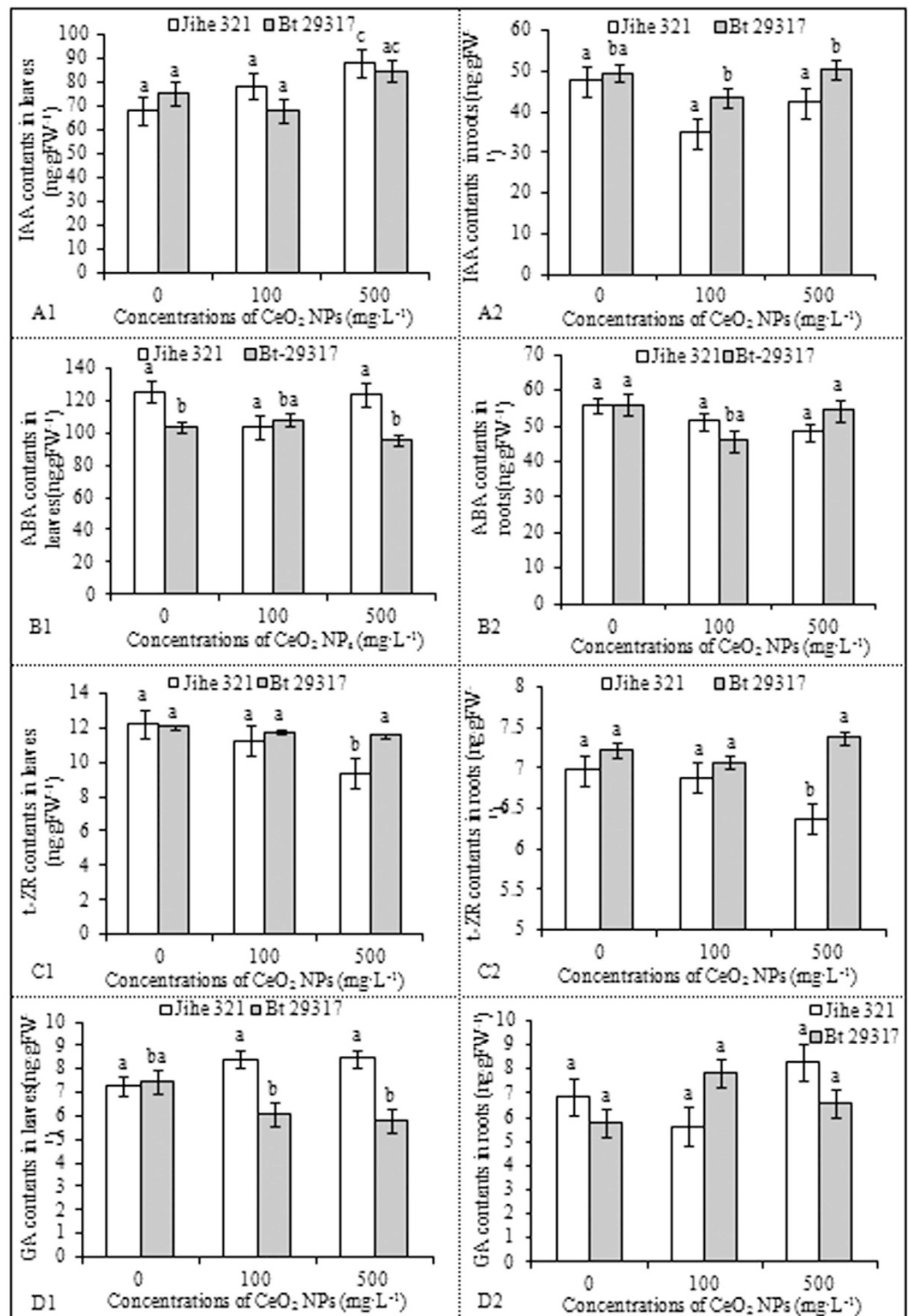


Figure 5. Effects of CeO₂ NPs concentrations on contents of IAA, ABA, t-ZR and GA in the leaves and roots of Bt-transgenic and non-transgenic cotton. A1, B1, C1, D1 and A2, B2, C2, D2 were IAA, ABA, t-ZR and GA contents in the leaves and roots, respectively. The mean is averaged from three replicates and error bars correspond to standard deviation of mean. Different small letters in the same plant indicate significant difference at $p < 0.05$ level between control and CeO₂ NPs treatments, and different small letters in the same CeO₂ NPs concentration indicate significant difference at $p < 0.05$ level between conventional and Bt-transgenic cottons.

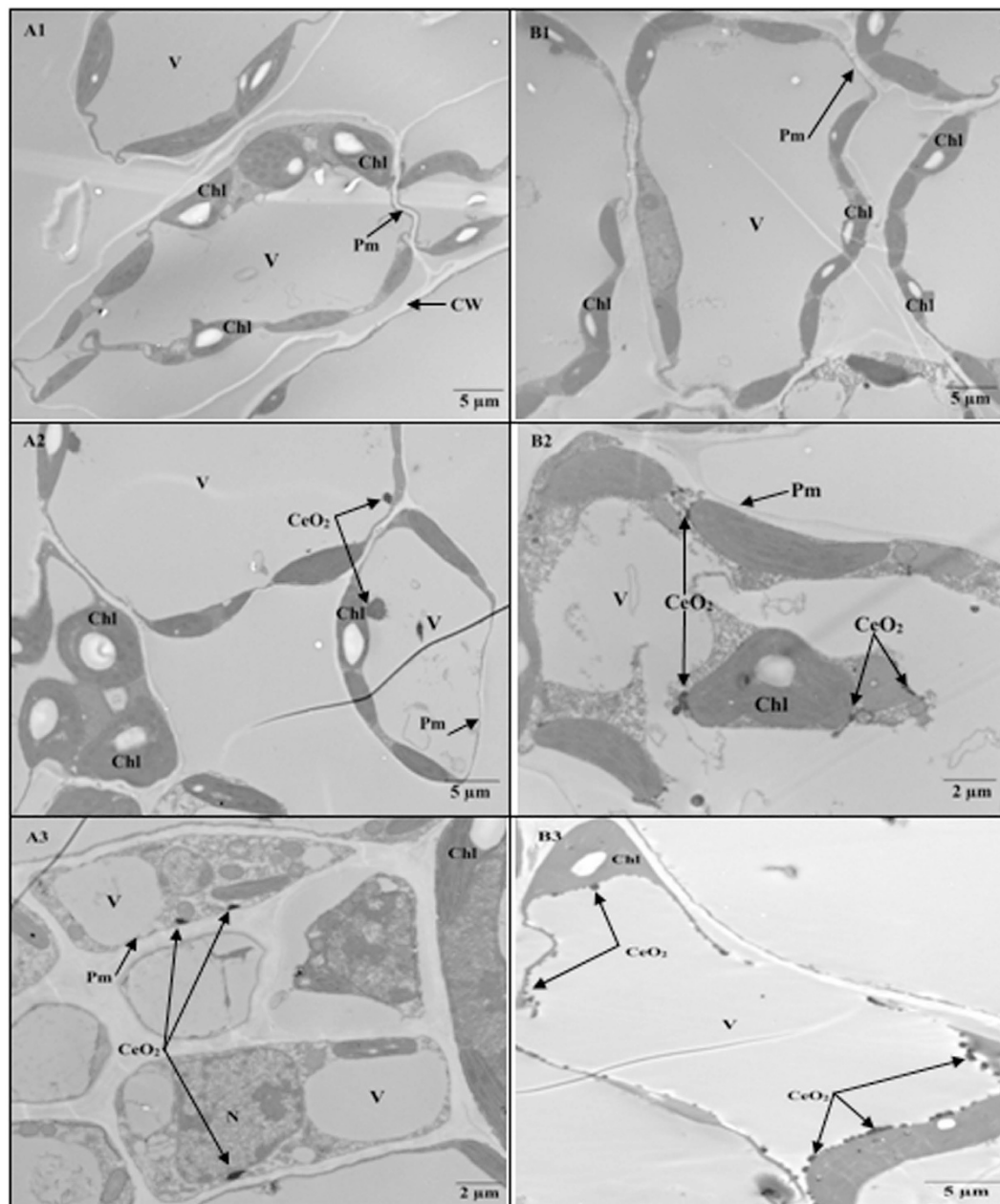


Figure 6. TEM images of conventional and Bt-transgenic cotton leaves. A1-A2-A3 and B1-B2-B3 are TEM images of conventional and Bt-transgenic cottons under 0, 100 and 500 mg·L⁻¹ CeO₂ NPs treatments, respectively. Chloroplast (Chl), Vacuole (V), Plasma membrane (Pm), Nucleus (N) and Cell wall (CW).

Bt-transgenic cotton than in conventional cotton upon CeO₂ NP treatment; the ABA content in the roots of Bt-transgenic cotton treated with 100 mg·L⁻¹ CeO₂ NPs was lower than that of the control group, but CeO₂ NPs had no effect on GA and t-ZR content in the leaves and roots of Bt-transgenic cotton.

Most CeO₂ NPs aggregated on the external surface of chloroplasts, which were swollen and ruptured, especially in Bt-transgenic cotton. This phenomenon could result in decreased Zn, Mg, Fe, and P levels in xylem sap because ruptured chloroplasts can release these elements, which are components of chloroplasts or chlorophyll. In addition, numerous fragments emerged in vascular bundles as their structure was destroyed. This destruction was more evident in transgenic cotton than in conventional cotton. The endocytosis of CeO₂ NPs was observed by TEM on the surface of the root of transgenic cotton; this observation may be indicative of the mechanism by which CeO₂ NPs entered the plant cells. The presence of CeO₂ NPs on the surface and in the internal cells of the root is dependent on the CeO₂ NP concentration.

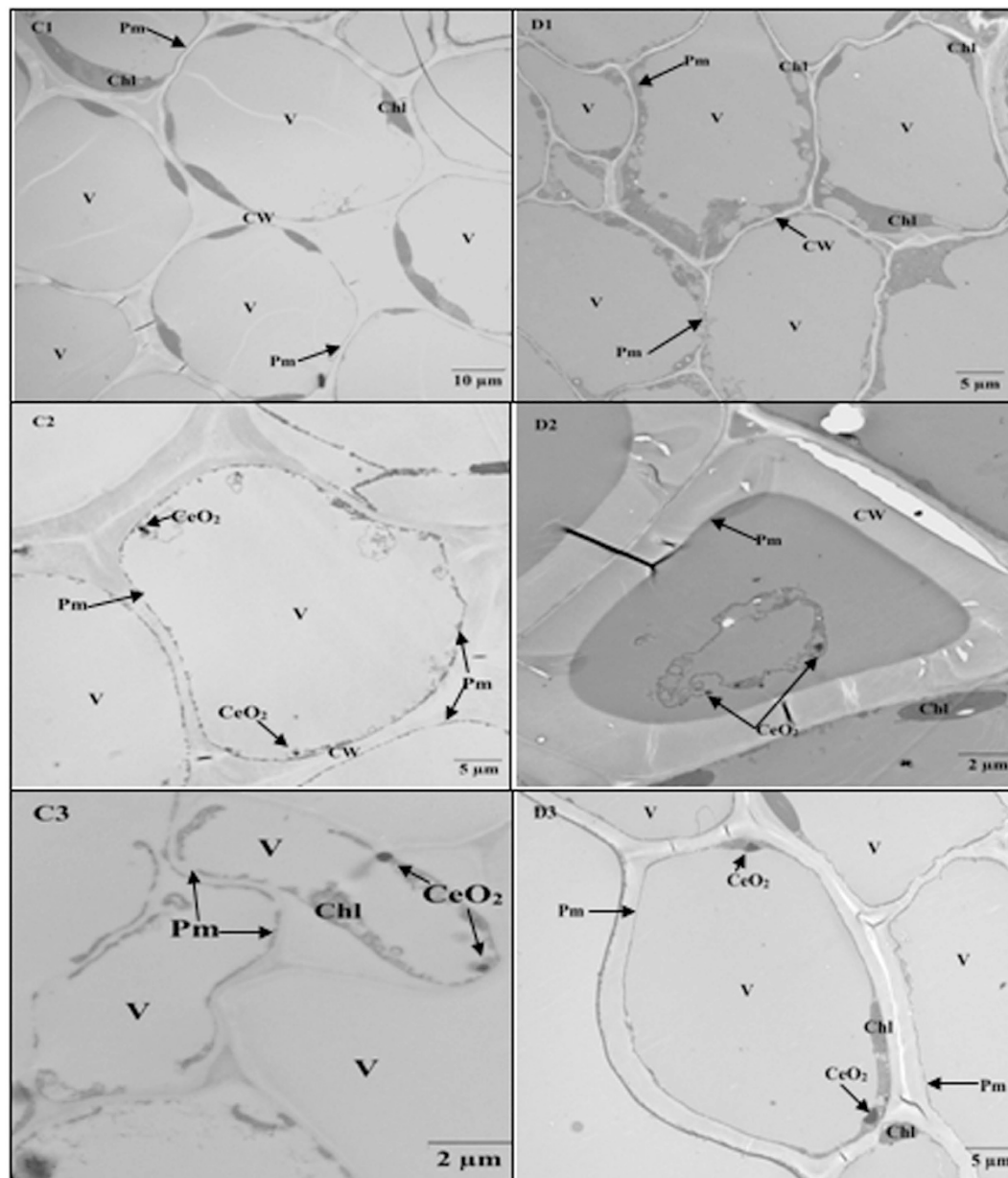


Figure 7. TEM images of conventional and Bt-transgenic cotton stems. C1-C2-C3 and D1-D2-D3 are TEM images of conventional and Bt-transgenic cotton under 0, 100 and 500 mg·L⁻¹ CeO₂ NPs treatments, respectively. Chloroplast (Chl), Vacuole (V), Plasma membrane (Pm), Nucleus (N), Cell wall (CW).

Methods

Sample preparation and experiments. CeO₂ NPs and other chemicals were purchased from Shanghai Hufeng Bioscience Technology Company (Shanghai, China) and Beijing Chemical Plant (Beijing, China). Experiments and samples were performed according to previous studies by Li *et al.*¹⁵, who investigated the effects of CeO₂ NPs on Bt-transgenic cotton at different concentrations (0, 10, 100, 500, and 2000 mg·L⁻¹). The results indicate that root biomass and nutrient uptake in Bt-transgenic cotton were affected by low concentrations of CeO₂ NPs (100 and 500 mg·L⁻¹) in contrast to conventional cotton. In addition, based on a preliminary experiment involving various concentrations of CeO₂ NPs (0, 10, 100, 500, 1000, and 2000 mg·L⁻¹) (please see the supplementary information), three concentrations of CeO₂ were selected (0, 100, and 500 mg·L⁻¹ CeO₂ NPs). Bt-transgenic cotton (Bt-29317) and conventional cotton (Jihe 321) were germinated in sterilised, moist sand, and two seedlings were then transplanted to 4.0-L pots containing 3.0 L of nutrient solution. After one week, the nutrient solution was amended with 0, 100, and 500 mg·L⁻¹ CeO₂ NPs. The average diameter of the CeO₂ NPs was 10 ± 3.2 nm, as measured by TEM before use in the experiments (please see the supplementary information). TEM also indicated that the CeO₂ NPs exhibited an octahedral morphology and easily agglomerated (Fig. 9).

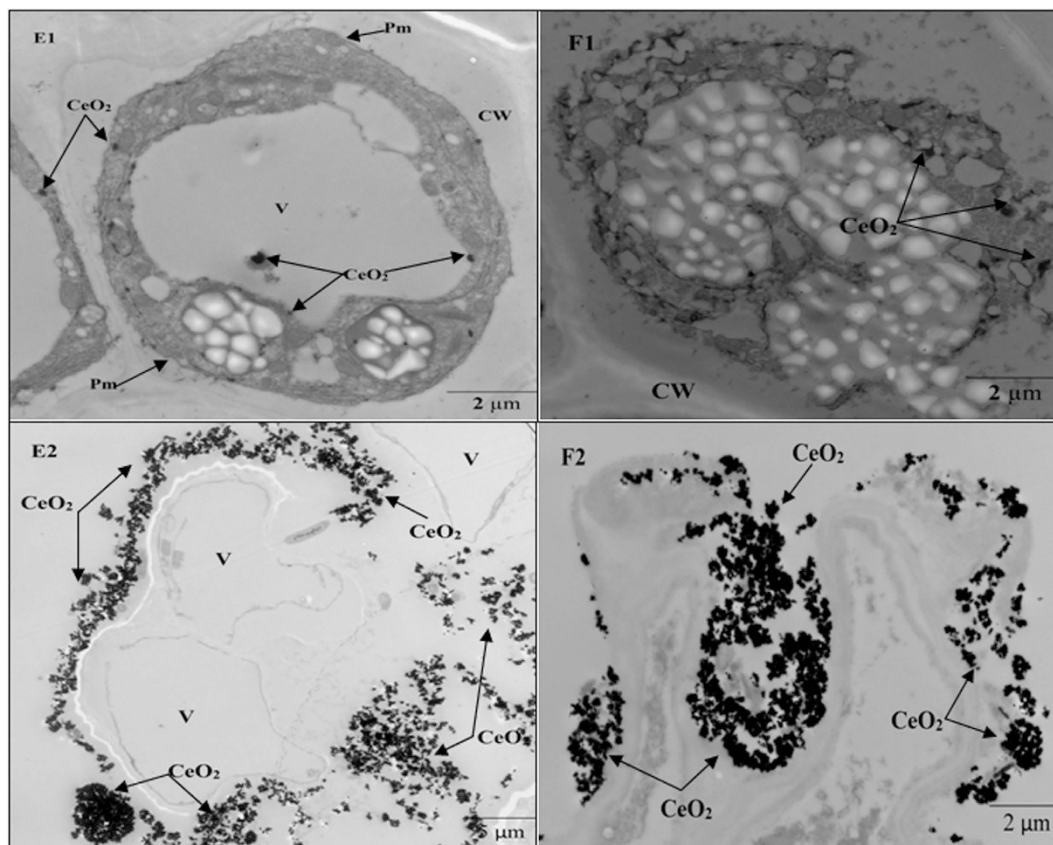


Figure 8. TEM images of conventional and Bt-transgenic cotton roots. E1-E2 and F1-F2 are TEM images of conventional and Bt-transgenic cotton under 100 and 500 mg·L⁻¹ CeO₂ NPs treatments, respectively. Chloroplast (Chl), Vacuole (V), Plasma membrane (Pm) and Cell wall (CW).

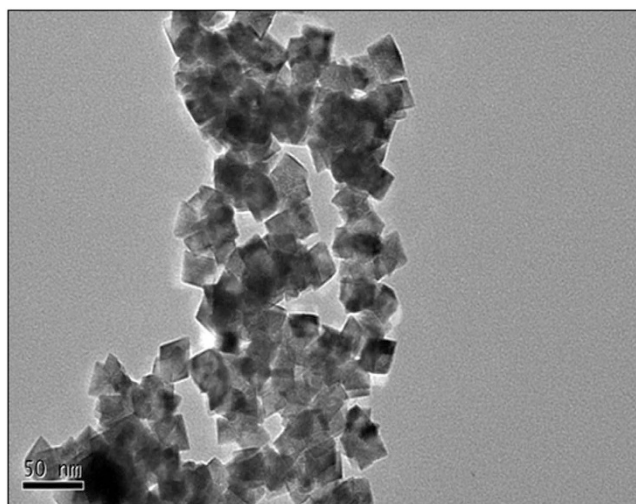


Figure 9. TEM image of CeO₂ nanoparticles.

The cotton plants were grown in the greenhouse of the China Agricultural University from May to June of 2014, and three replicates were performed for each treatment.

Determination of nutrients and Ce content in xylem sap. Xylem sap was collected 2 cm below the cotyledon with a surgical blade when the plants developed 8 to 9 leaves. The harvested samples were rinsed with deionised water to remove any disrupted and residual cell. The cut stem was connected to a centrifugal tube by a flexible silicon tube (15 mm length, 2 mm internal diameter). Xylem sap was

collected for 15 min by a pressure chamber (0.2–0.3 MPa)²³. The Ce, Cu, and Zn content in xylem sap was determined using inductively coupled plasma mass spectrometry (ICP-MS) (DRC-II). The Ca, Mg, Fe, Mg, and P content was determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES) (iCap 6000).

Measurement of enzyme activities and protein content. After washing with tap and deionised water, fresh leaves and roots were separately harvested, cut, and mixed. Approximately 0.2 g of fresh sample was packed with aluminium paper and soaked into liquid nitrogen to protect the enzyme activities before storing at 4 °C. Each 0.2 g sample was homogenised in 1.8 mL of normal saline solution to generate a 10% sample compound solution. Then, the tissues were finely ground and centrifuged at 3500 RPM for 10 min. The extract was used to measure CAT, SOD, and POD activities as previously described by Xu *et al.*³² and Cho *et al.*³³. CAT activity was measured by monitoring the degradation of H₂O₂ at 240 nm³² and SOD activity was determined by measuring inhibition of the photochemical reduction of nitroblue-tetrazolium at 560 nm³⁴. POD activity in the extract was measured by following the formation of guaiacol dehydrogenation products, as determined by an increase in absorbance at 470 nm³⁵. Protein concentrations were determined using bovine serum albumin (BSA) as the standard in the Bradford assay³⁶.

Hormones. The extraction and purification of ABA, IAA, t-ZR, and GA were performed according to the previous studies of He *et al.*³⁷. Approximately 0.2 g of fresh leaves and root samples was separately homogenised in 2 mL of 80% methanol (containing 40 mg·L⁻¹ butylated hydroxy toluene) and stored at -20 °C for 48 h. The solution was then centrifuged at 3500 RPM for 15 min. The precipitates were re-suspended in 1 mL of 80% methanol at -20 °C for 16 h. C₁₈ Sep-Pak cartridges (Waters, Milford, USA) were applied for the purification of the combined extracts. The samples were then evaporated under vacuum to remove the organic solvent and were dissolved in 2.0 mL of TBS buffer (Tris-buffered saline; 50 mM Tris, pH 7.8, 1 mM MgCl₂, 10 mM NaCl, 0.1% Tween, 0.1% gelatin). ABA, IAA, t-ZR, and GA levels were determined by enzyme-linked immunosorbent assay (ELISA) kits using monoclonal antibodies (Phytodetek, Agdia, Elkhart, IN, USA). Absorbance was recorded at 450 nm.

Transmission Electron Microscopy. The plant tissues for transmission electron microscopy (TEM) observation were collected from Bt-transgenic and conventional cotton after two weeks of CeO₂ NP exposure. The roots, stems, and leaves were thoroughly washed with deionised water, prefixed in 2.5% glutaraldehyde, dehydrated in a graded acetone series, and embedded in Spurr's resin. The samples were sectioned for TEM analysis using a microtome JEM-1230 (JEOL, Ltd., Japan) with a diamond knife^{25,38,39}.

Data analysis. Each treatment was performed in triplicate, and the results presented in the figures are the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test and an independent samples t-test using SPSS 22.0 software. A confidence interval of 95% (p < 0.05) was considered significant in all cases.

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Author Contributions

Y.R. conceived the experiments. L.V.N. and Y.R. designed the research. L.V.N., C.M., Y.R., S.T., X.L., B.X. and L.L. performed the experiments and analysed the data. L.V.N. and Y.R. wrote the paper. All authors have read and approved the final manuscript.

Additional Information

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