Evaluation of stress effects of copper oxide nanoparticles in *Brassica napus* L. seedlings

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Abstract Rapid growth of nanotechnology has enabled the production and use of engineered nanoparticles (ENPs) in several industries as well as in agriculture areas. This has raised ecotoxicological concerns due to the release of ENPs to the environment. In the present study, we investigated the effects of interactions of copper oxide nanoparticles (CuO NPs) on physiological, biochemical, and molecular indices in seedlings of an important oil seed crop *Brassica napus* L. The seedlings were treated with 0, 20, 50, 100, 200, 400, and 500 mg/L of CuO NPs for 14 days in half-strength semi-solid Murashige and Skoog medium. The CuO NPs treatment significantly reduced shoot and root growth as well as plant biomass. Shortening and thickening of primary and lateral roots and inhibition of lateral root growth was observed at higher concentrations. An increase in reactive oxygen species generation, and malondialdehyde accumulation was observed. Histochemical staining of roots with propidium iodide and aniline blue indicated cell death and callose formation in roots. Transcriptional modulation of genes related to oxidative stress viz. CuZn superoxide dismutase, catalase, and ascorbate peroxidase was observed. Element content analysis showed an increase in Cu content and decrease in Fe, Mn, and Zn contents. Overall, exposure to CuO NPs caused oxidative

injury, cell death, callose formation, and decreased the micro nutrient contents in *B. napus* seedlings.

Keywords *Brassica napus* · Copper oxide nanoparticles · Root system · Oxidative stress · Callose formation · Microelements

Introduction

The rapid growth of nanotechnology enhanced the use of engineered nanoparticles (ENPs) in various products. This has raised concerns about the release of ENPs from these products, thereby causing concerns due to the possibility of contamination of natural as well as agricultural ecosystems (Navarro et al. 2008). Previous studies have shown that the environmental pollution of ENPs could occur as a result of their release from different sources such as industries, waste treatment plants, and also by their direct use for agricultural purposes. For instance, the direct application of sewage sludge in agricultural soils for nutrient supply may also be contaminated with ENPs (Gardea-Torresdey et al. 2014). This will result in the accumulation of ENPs in agricultural soils as well as their entry into plants. The ENPs can also enter into food chains as a result of their widespread use for delivery of fertilizers and nanopesticides (Das et al. 2009; Wang et al. 2012).

Copper (Cu) is a microelement required for the proper growth, development, and survival of plants. In agricultural fields, excess accumulation of Cu can occur due to mining operations, sewage sludge disposal, and the use of poor quality water for irrigation purposes (Yruela 2009). Exposure to excess Cu leads to growth retardation as well as oxidative stress injuries in plants (Schützendübel and

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Polle 2002; Yruela 2005). Due to their physico-chemical properties, copper oxide nanoparticles (CuO NPs) were used in bioactive coatings, antimicrobial and antifouling paints, air and liquid filtration units, lubricant oils, textiles, and superconducting materials (<http://www.nanotechproject.org>). However, studies have shown the release of CuO NPs to the environment from these products could cause ecotoxicological threats owing to their toxic effects on plants (Wang et al. 2012).

Earlier investigations have proved that the toxic responses of metals varies between different types of plants and even the different cultivars of the same crop also showed differential sensitivity to metals (Ali et al. 2014). For instance, differential sensitivity to chromium (Cr) among different genotypes of the same species has been reported (Shahandeh and Hossner 2002). Gill et al. (2014) also reported genotypic variation in response to Cr toxicity in four oilseed rape cultivars. According to a study by Ali et al. (2014), the stress responses of two cultivars of *B. napus* exposed to same concentrations of Cd showed different responses. Feigl et al. (2013) reported that when exposed to Cu excess the morphological adaptation strategy, especially, the root system development in *B. juncea* and *B. napus* showed differences. In a previous study, we observed toxic responses in *B. juncea* seedlings exposed to CuO NPs (Nair and Chung 2015). In the present study, to check the species specific effects, using the same concentrations of CuO NPs and growth conditions as described in the previous study, the physiological, biochemical, and molecular level effects of CuO NPs were investigated in *B. napus* seedlings.

Materials and methods

Plant culture and exposure

The commercially available CuO NPs purchased from the Sigma-Aldrich (St. Louis, MO, USA) was used in the present study. The size distribution and morphological analysis of CuO NPs was carried out using Dynamic Light Scattering spectrometer (DLS-7000, Otsuka Electronics Co., Inc., Osaka, Japan) and transmission electron microscopic (TEM; Carl Zeiss, Oberkochen and Baden-Württemberg, Germany) analysis. The TEM analysis was conducted by drop coating of CuO NPs suspension on a 400-mesh carbon-coated copper grid (Tedpella Inc., Reading, USA). The grids were dried overnight and imaged using TEM.

Different concentrations of CuO NPs (0, 20, 50, 100, 200, 400, and 500 mg/L) were mixed by vortexing with autoclaved semi-solid half-strength Murashige and Skoog medium (100 mL; Duchefa Biochemie, Haarlem, The

Netherlands). One hundred milliliter of medium was poured into each of the culture vessels (120 × 80 mm; Phytohealth, SPL Life Sciences, Gyeonggi-do, South Korea) and solidified by keeping at 4 °C for 5 min for avoiding precipitation of CuO NPs.

The seeds were disinfected with 10% (v/v) sodium hypochlorite solution for 20 min and thereafter washed five times with autoclaved deionized water. The seeds were germinated by keeping under dark conditions on wet Whatman #1 paper for 48 h. In each vessel, 10 germinated seeds were placed. Four replications were maintained for each of the treatments and the study was repeated thrice. The seedlings were incubated in a growth chamber (Hanbaek Scientific Co., Gyeonggi-do, South Korea) under 16:8 h of light:dark period at 26 ± 2 °C for 14 days.

Morphological measurements

For growth measurements, the seedlings were removed from the growth medium, washed several times with autoclaved deionized water, and blotted dry on Whatman #1 paper. The shoot and root lengths were determined using a scale and the fresh weights (FW) were determined with the help of an electronic balance (Mettler Toledo, Switzerland). Morphological analysis of the roots was conducted using a stereomicroscope (Nikon, Japan) and photographed with a digital camera (Canon, Japan).

Analysis of microelement content

Samples collected from the control as well as seedlings grown in the presence of low (50 mg/L), medium (200 mg/L), and high (400 mg/L) concentrations of CuO NPs were dried at 70 °C for 72 h in a dry air oven. The dried samples (20 mg) were digested with concentrated HNO₃ at 115 °C for 12 h using a dry bath (JBI-01; JMC R&D Inc., Daejeon, South Korea). The samples were made up to 25 mL with autoclaved deionized water after digestion. The samples were filtered through 0.2 µm nylon filters (Chromedisc[®] syringe filter, Seoul, South Korea). The microelement content viz. Cu, Fe, Zn, and Mn analysis was done at NICEM (National Instrumentation Center for Environmental Management, Seoul National University, Seoul, Korea) using inductively coupled plasma emission spectrometer (ICP-730ES; Varian, USA).

Analysis of hydrogen peroxide content and lipid peroxidation

The hydrogen peroxide (H₂O₂) content in shoots and roots was analyzed using the protocol of Brennan and Frenkel (1977). The samples (100 mg, Fresh weight; FW) were homogenized in 4 mL cold acetone and filtered through

Whatman filter paper #1. To the filtrate (2 mL), 1 mL of 20% titanium chloride in concentrated HCl (v/v) was added. One microliter of concentrated ammonia solution (6 N) was added to the mixture and centrifuged at 5000g for 30 min. The precipitate was collected and re-dissolved in 1 mol/L H₂SO₄, washed, and dissolved in water. The H₂O₂ content was measured at 415 nm using UV–VIS spectrophotometer (Optizen, South Korea) and was quantified using a standard curve generated using known concentrations of H₂O₂.

The lipid peroxidation level was determined as per Heath and Packer's (1968) procedure. After homogenizing the samples (100 mg each; FW) in 5 mL of 0.1% (w/v) trichloroacetic acid solution, they were centrifuged at 7000g for 10 min at room temperature. Two milliliter of 0.67% (w/v) thiobarbituric acid was mixed with 2 mL of supernatant and boiled at 95 °C for 30 min. The samples were cooled immediately and centrifuged at 6000g for 5 min and absorbance was measured at 532 and 600 nm using a UV–VIS spectrophotometer (Optizen UV/VIS spectrophotometer, South Korea). The malondialdehyde (MDA) concentration was calculated using extinction coefficient 155 mmol⁻¹ cm⁻¹.

Microscopic analysis

ROS generation in shoots was determined by treating with 3,3'-diaminobenzidine (DAB) (Thordal-Christensen et al. 1997). The fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (HPF) (Invitrogen, USA) was used to check ROS generation in roots as described by Kwasniewski et al. (2013). Callose formation in roots was determined by treating with aniline blue for 1 h under dark conditions (Horst et al. 1997). The treated roots were rinsed with 0.1 M K₃PO₄ (pH 8) before imaging. Root cell death was determined using propidium iodide (PI) treatment as described by Kwon et al. (2013). The root tips were stained with 5 mg/L PI (after dissolving in water) in a watch glass. The DAB stained stem samples were photographed with Canon Power Shot (Canon, Japan) camera and the HPF, aniline blue, and PI-treated roots were imaged and analyzed with fluorescent microscope and associated software (BX61-32 FDIC, Olympus, Japan).

Gene expression analysis

One microgram from the total RNA isolated using the RNA extraction and clean up kit (Qiagen, Valencia, CA, USA) was used for the synthesis of cDNA. The cDNA was synthesized using QuantiTect[®] reverse transcription kit (Qiagen). The list of primers for the *CuZnSOD*, *CAT*, *APX*, and *ACT* (internal control) genes is given in Supplementary Table 1. Gene expression analysis was carried out using

the Chromo-4TM thermocycler (Bio-Rad, USA). The reaction mix included 1 µL of cDNA, 0.2 µM forward and reverse primers and 10 µL of 2X IQ SYBR Green Super Mix (Bio-Rad) and made up to 20 µL using deionized water. The reaction was conducted with an initial denaturation at 95 °C for 7 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. After that a melting curve analysis was conducted. The relative expression level of different genes as compared to the internal control was calculated using the Bio-Rad gene expression analysis software.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA) programme. Treatment differences as compared to the controls were examined with Dunnett's post hoc test and significant differences were determined at $p < 0.05$ probability level.

Results

Characterization of CuO NPs

TEM analysis revealed different morphologies viz. near spherical, rectangular, and rod shaped. Size distribution analysis using dynamic light scattering spectrometer showed that the CuO NPs were 10–50 nm in size with majority of them being 30 nm (Supplementary Fig. 1A, B).

Morphological observations

The growth of the seedling showed reduction after exposure to increasing concentrations of CuO NPs (Fig. 1a). Exposure to CuO NPs changed the root architecture as evidenced by thickening and reduction of primary and lateral roots. Inhibition of lateral root formation was observed in seedlings treated with above 20 mg/L of CuO NPs (Fig. 1b). Significant reduction in length and fresh weights of shoots and roots occurred with increasing concentrations of CuO NPs exposure (Fig. 1c, d).

Analysis of microelements

The Cu content was significantly increased in shoots and roots of *B. napus* seedlings. In comparison with shoots, higher amount of Cu accumulation was detected in roots (Fig. 2a). However, significant decrease in Zn, Fe, and Mn contents occurred in CuO NPs exposed seedlings as compared to the controls (Fig. 2b–d).

Fig. 1 **a** *Brassica napus* seedlings exposed to different concentrations of CuO NPs for 14 days. **b** Root morphology of *B. napus* seedlings treated with different concentrations of CuO NPs for 14 days. (From left to right: control and seedlings grown in the presence of 20, 50, 100, 200, 400, and 500 mg/L of CuO NPs,

respectively). Arrow mark indicates lateral root. Scale bar indicate 1 cm. **c** Average shoot and root length and **d** fresh weight of *B. napus* seedlings. Data are mean $\pm$ SE of three replications and asterisks indicate significant difference at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ level

Hydrogen peroxide and lipid peroxidation

The H_2O_2 content did not change significantly in shoots after exposure to 20 and 50 mg/L of CuO NPs, but it was significantly increased in shoots upon exposure to 100, 200, 400, and 500 mg/L of CuO NPs (Fig. 3a). In roots, treatment with 20 mg/L of CuO NPs did not result in any significant change in H_2O_2 generation. However, the H_2O_2 content significantly increased when treated with 50, 100, 200, 400, and 500 mg/L of CuO NPs (Fig. 3a). Except for 20 and 50 mg/L of CuO NPs exposure, the lipid peroxidation levels showed significant increase in shoots after exposure to all other concentrations (Fig. 3b). In roots, except for 20 mg/L, the lipid peroxidation level significantly increased after treatment with all other concentrations of CuO NPs (Fig. 3b).

Microscopic analysis

A gradual increase in H_2O_2 generation was observed in stems as indicated an increase in brown coloration after treatment with DAB stain (Fig. 4a). In roots, HPF

treatment indicated an increase in ROS generation as indicated by an increase in fluorescence levels as a result of exposure to increasing concentrations of CuO NPs. The HPF fluorescence level was high at the root tips (Fig. 4b). Exposure to various concentrations of CuO NPs resulted in a concentration dependant increase in callose formation in roots (Fig. 4c). Examination of root cell viability using PI stain indicated cell death, in the root tips of seedlings exposed to 100–500 mg/L of CuO NPs (Fig. 4d).

Gene expression

The relative expression of *CuZnSOD* did not show significant change in shoots of *B. napus* seedlings grown in the presence of 20, 50, and 100 mg/L of CuO NPs (Fig. 5a). However, the *CuZnSOD* gene expression significantly increased as a result of treatment with 200, 400, and 500 mg/L of CuO NPs (Fig. 5a). In roots, the expression of *CuZnSOD* gene was significantly increased when grown in the presence of 200, 400, and 500 mg/L of CuO NPs (Fig. 5a). In shoots of *B. napus* seedlings, the

Fig. 2 Effects of 50, 200, and 400 mg/L of copper oxide nanoparticles on microelement **a** Cu, **b** Zn, **c** Fe, and **d** Mn content in *B. napus* L. seedlings. Data are mean $\pm$ SE of three replications and significant

differences were indicated with asterisks at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ level

Fig. 3 **a** Hydrogen peroxide formation and **b** lipid peroxidation in shoots and roots of *B. napus* seedlings exposed to different concentrations of CuO NPs for 14 days. Data are mean $\pm$ SE of

three replications and asterisks indicate significant difference at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ level

expression of *CAT* gene did not change significantly after exposure to various concentrations of CuO NPs (Fig. 5b). The *CAT* gene showed no significant alteration in its expression levels in roots of seedlings exposed to 20, 50, and 100 mg/L of CuO NPs (Fig. 5b). However, significant

up-regulation of *CAT* gene occurred in roots upon exposure to 200, 400, and 500 mg/L of CuO NPs (Fig. 5b). The *APX* gene showed no significant change in its expression in shoots and roots at any of the treatments (Fig. 5c).

Fig. 4 **a** Histochemical detection of hydrogen peroxide formation in shoots. **b** Hydrogen peroxide formation in roots detected with 3'-(p-hydroxyphenyl) fluorescein treatment. **c** Callose deposition in roots detected using aniline blue and **d** cytotoxicity assessment of roots using propidium iodide treatment. (In each panel from left to right; control and seedlings treated with 20, 50, 100, 200, 400, and 500 mg/L of CuONPs, respectively)

Discussion

The rise in the production and usage of ENPs and their subsequent release and accumulation in the environment will have serious impacts on plant growth and development (Zhu et al. 2008). In our previous study, under the same growth conditions and exposure concentrations, alteration of root growth such as reduction in primary and lateral root growth was observed in *B. juncea* seedlings (Nair and Chung 2015). Exposure to CuO NPs led to changes in the root system architecture such as reduction in primary root growth as well as inhibition of lateral root growth in *B. napus* seedlings. As compared to *B. juncea* seedlings (Nair and Chung 2015), the toxic effects of CuO NPs on various parameters tested were more pronounced in *B. napus* seedlings, though the increased shoots and roots Cu contents of two species, as a result of CuO NPs exposure, were

not very different (Nair and Chung 2015). Lateral root growth was observed even at the highest exposure concentration of CuO NPs in *B. juncea* seedlings (Nair and Chung 2015) as compared to complete inhibition in *B. napus* seedlings.

Lipid peroxidation serves as an indicator of the extent of oxidative damage under oxidative stress. It has been known that exposure to excess Cu could enhance the ROS generation which in turn cause peroxidative damage to membrane lipids (Dietz et al. 1999). In this study, the lipid peroxidation level showed significant increase in *B. napus* seedlings treated with CuO NPs. The presence of higher concentrations of Cu, as revealed by the nutrient analysis, might have led to the increased lipid peroxidation levels in *B. napus* seedlings. Treatment with PI indicated cytotoxicity, in the root tips of *B. napus* seedlings treated with CuO NPs. As reported earlier as in the case of excess Cu (Feigl et al. 2013), the enhanced ROS generation as a result of the increased Cu levels under CuO NPs treatment caused the cytotoxicity in roots of *B. napus* seedlings.

In this study, inhibition of lateral root growth was observed after treatment with CuO NPs in *B. napus* seedlings. It was reported that loss of cell viability in root tips (in the meristem) could lead to the inhibition of root elongation in *Brassica* spp. (Feigl et al. 2013). Previous studies have shown that elongations of roots in plants are affected by the redox status in their apical region (Córdoba-Pedregosa et al. 2005). Changes in the redox status contribute to cell wall secondary thickening resulting in the reduction of cell growth and are negatively correlated with root elongation (Feigl et al. 2013). In this study, for the first time, we observed callose deposition as a result of CuO NPs exposure in roots of *B. napus* seedlings. It has been known from previous studies that Cu is an efficient inducer of callose formation in *B. napus* roots (Feigl et al. 2013). The callose deposition could inhibit cell wall loosening and thereby negatively affect root growth by inhibiting the passage of signal molecules or supply of carbon required for root growth in plants (Jones et al. 2006). Moreover, previous studies have shown that the disturbances in the auxin and cytokinin levels could also alter the primary and lateral root growth in plants (Aloni et al. 2006; Laplaze et al. 2007; Nibau et al. 2008). It has been reported that exposure to water soluble fullerenes caused abnormalities in hormone distribution resulting in the shortening of root growth in *A. thaliana* (Liu et al. 2010).

It was observed that exposure to CuO NPs significantly increased the ROS generation in *B. napus* seedlings. The presence of excess Cu content, as observed in micronutrient analysis, might have led to the excess ROS generation. The presence of excess Cu has been reported to cause dysfunction of the electron transport chain and thereby enhancing the ROS generation in plants. Plant cells have

Fig. 5 Effect of different concentrations of CuO NPs on the expression of **a** CuZn superoxide dismutase (*CuZnSOD*), **b** catalase (*CAT*), and **c** ascorbate peroxidase (*APX*) genes in shoots and roots of *B. napus* seedlings after 14 days of exposure. Data are mean $\pm$ SE of

three replications and *asterisks* indicate significant difference as compared to the control (control = 1) (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

evolved various antioxidant systems to cope with excess ROS generation (Sandmann and Boger 1980). It has been shown that increased ROS levels lead to the activation of genes coding for antioxidative enzymes such as CuZnSOD and CAT (Ezaki et al. 2000). The SODs accelerate the conversion of superoxides to H_2O_2 and CATs are involved in H_2O_2 breakdown (Bowler et al. 1992). APX has stronger substrate affinity than CAT and the induction of APX will be necessary for the efficient removal of H_2O_2 (Tripathi et al. 2006). In *B. napus*, we observed an increase in the expression levels of *CuZnSOD* and *CAT* genes. However, no significant change in the expression of *APX* gene was observed. Therefore, the gene expression results indicate that there was an enhanced production and inadequate removal of ROS in *B. napus* seedlings treated with CuO NPs.

In the present study, for the first time, we observed that exposure to CuO NPs has resulted in significant reduction in microelement content in *B. napus* seedlings. The possible reason for the observed reduction in microelements viz. Zn, Fe, and Mn in both shoots and roots of *B. napus* seedlings might be due to the competition in the uptake of

elements in the presence of excess Cu (Luo and Rimmer 1995; Lequeux et al. 2010). For example, in the case of Zn and Fe, the Cu–Zn antagonism or the competition of transport molecules might have resulted in the reduction in Zn level (Luo and Rimmer 1995; Feigl et al. 2013). The Mn deficiency might be due to the reducing effect of Cu on Mn leading to the reduced uptake of Mn (Feigl et al. 2013). Another reason for the observed changes might be due to the inhibition of water and nutrient uptake as a result of CuO NPs exposure. A previous study by Asli and Neumann (2009) showed that titanium dioxide nanoparticles interfered with the ability of maize by forming aggregates along the root cell walls.

Conclusion

In conclusion, it is observed that CuO NPs caused multi-level toxic effects on the growth and development of *B. napus* seedlings. Modification of root system architecture was observed due to CuO NPs exposure. Exposure to CuO NPs enriched the Cu content in the seedlings and decreased

Fe, Zn, and Mn microelements. Moreover, excess ROS accumulation, especially in the root tips, was observed leading to lipid peroxidation and cell death. Callose formation was also observed in roots of *B. napus* seedlings exposed to CuO NPs. Further studies are required to understand the molecular mechanisms and the role of hormonal changes in root growth modification in *B. napus* as a result of CuO NPs exposure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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