Ecotoxicology and Environmental Safety 155 (2018) 86–93

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Effects of manufactured nano-copper on copper uptake, bioaccumulation and enzyme activities in cowpea grown on soil substrate

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ARTICLE INFO

*Keywords:*
Translocation
Copper toxicity
Enzyme activity
Oxidative stress
Membrane damage
Nano particles

ABSTRACT

Increased use of nanoparticles-based products in agriculture portends important implications for agriculture. Therefore, the impact of nano-copper particles (< 25 nm and 60–80 nm) on Cu uptake, bioaccumulation (roots, leaves and seeds), activity of ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and lipid peroxidation in leaves and roots of *Vigna unguiculata* (cowpea) was studied. Plants were exposed to four levels (0, 125, 500 and 1000 mg/kg) of 25 nm or 60–80 nm nano-Cu for 65 days. Results indicated significant ($P < .05$) uptake of Cu at all nano-Cu levels compared to control, and bioaccumulation increased in seeds by at least 250%. Response of antioxidant enzymes to both nano-Cu types was concentration-dependent. Activity of APX and GR was enhanced in leaves and roots in response to both nano-Cu treatments in similar patterns compared to control. Both nano-Cu increased CAT activity in roots while SOD activity reduced in both leaves and roots. This shows that response of antioxidant enzymes to nano-Cu toxicity was organ-specific in cowpea. Malondialdehyde, a measure of lipid peroxidation, increased at 500 −1000 mg/kg of 25 nm nano-Cu in leaves by average of 8.4%, and 60–80 nm nano-Cu in root by 52.8%, showing particle-size and organ-dependent toxicity of nano-Cu. In conclusion, exposure of cowpea to nano-Cu treatments increased both the uptake and bioaccumulation of Cu, and also promoted the activity of APX and GR in root and leaf tissues of cowpea. Therefore, APX- and GR-activity level could be a useful predictive biomarker of nano-Cu toxicity in cowpea.

## 1. Introduction

Nanoparticles have received a tremendous global attention, especially in agriculture. In particular, nano-based copper materials are being actively developed specifically for applications in agriculture and food preservation based on the antifungal and antimicrobial properties of $Cu^{+2}$ (Montes et al., 2015; Majumder and Neogi, 2016), and Cu-based nanopesticides (Keller et al., 2017). The rate of production and application of nanoparticles have tremendously increased, and there is every possibility of plants' exposure to nanoparticles either through foliar or root uptake. The exact behavior of nanoparticles in plants is still not well-understood because different nanoparticles have differed pathway of behavior in plant system (Rao and Shekhawat, 2016). However, several studies have reported toxic effects of metal-based nanoparticles in the plant. For instance, phytotoxicity to *Lolium perenne* and *Raphanus sativus* by CuO and ZnO nanoparticles (Atha et al., 2012; Lin and Xing, 2007 respectively), *Pisum sativum* by ZnO nanoparticles (Mukherjee et al., 2013, 2016), *Arabidopsis thaliana* by $CeO_2$ nanoparticles (Ma et al., 2016), *Lactuca sativa and Medicago sativa* by CuO (Hong et al., 2015) and *Brassica juncea* by $TiO_2$ and CuO nanoparticles (Rao and Shekhawat, 2016) have been documented. The toxic response of plants to nanoparticles is induced by alterations in normal metabolism. This usually culminates into oxidative stress that always correlates with the generation of reactive oxygen species (ROS) in plant (Rao and Shekhawat, 2016). One of the antioxidant systems for safeguarding plants against oxidative stress by detoxifying excess ROS is the enzymatic antioxidant system. This comprises enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD),

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https://doi.org/10.1016/j.ecoenv.2018.02.070
Received 24 December 2017; Received in revised form 19 February 2018; Accepted 22 February 2018

glutathione reductase (GR) and guaiacol peroxidase (GPOx). The process of scavenging excess ROS has been reported to be achieved by the efficient activation of these antioxidant enzymes (Noctor and Foyer, 1998).

Investigations have shown that engineered carbon-based and metal-based nanoparticles are able to produce stress by generating excess reactive oxygen species (ROS) with the potential to affect metabolic activities in plants. For instance, the ROS-mediated oxidative stress with significant activation of antioxidant defense system has been reported in several species that were exposed to nanoparticles such as graphene oxide nano-sheet (Anjum et al., 2013, 2014), $CeO_2$ (Rico et al., 2013), ZnO (Mukherjee et al., 2013), CuO and $TiO_2$ (Rao and Shekhawat, 2016).

However, to the best of our knowledge, so far no study has been done to investigate the activity of antioxidant enzymes in response to nano-copper exposures plants such as cowpea. Hence, this study aims to give an insight into the effects of engineered nano-copper of two particle sizes (< 25 nm and 60–80 nm) on Cu uptake by roots, bioaccumulation in leaves and seeds and the activity of stress enzyme in leaf and root tissues of *Vigna unguiculata* L. (Warp).

## 2. Materials and methods

### 2.1. Source and properties of nano copper particles

Nano-copper particles of two different particle sizes (25 nm and 60–80 nm) were purchased from Ionic Liquids Technologies GmbH, as powders with characteristics presented in Table S1. The morphology of the nanoparticles was further assessed by field emission scanning electron microscopy (FESEM-Carl Zeiss, SUPRA® 55 with GEMINI® Technology) coupled with energy dispersive X-ray spectroscopy (OXFORD-X Act), and images taken at 20 KeV. X-ray diffraction analysis (XRD) of the nanoparticles was carried out using a Schimadzu X-ray diffractometer-XRD 6000 (under 40 kV/44 mA -X-Ray, 2θ/θ-continuous scanning mode, fixed monochromator). Data was taken from the 2θ range of 5–80 degrees with a step of 0.02°. The test soil used for the study was collected from the Botanical garden of the University of Ilorin, Nigeria, air-dried and sieved through a-2 mm mesh. The soil (n = 5) was characterized for selected physico-chemical properties and has the following values ($\bar{x}\pm SD$): $pH_{water}$ = 6.29 ± 0.08, organic carbon = 1.35 ± 0.06%, organic matter content = 2.34% ± 0.11, EC = 1.53 dS/m, CEC = 2.84 ± 0.56 cmol/kg, Ca = 331.0 ± 9.90 mg/kg, Mg = 77.2 ± 9.62 mg/kg, Na = 112.4 ± 16.69 mg/kg, K = 118.8 ± 13.65 mg/kg and Cu = 30.6 ± 4.42 mg/kg. Briefly for soil cation determination, 1 g of air-dried soil was digested using aqua regia method and analyzed for Ca, Mg, Na, K and Cu by Flame atomic absorption spectrophotometer (FAAS)- Bulk Scientific- ACCUSYS 211.

### 2.2. Experimental set-up

The concentrations employed in the study were 125, 500, and 1000 mg/kg, respectively for the two nano-Cu (25 nm and 60–80 nm) and the soil mass per container was 1.75 kg. Treatment with 1000 mg/kg of nanoparticles was the maximum as any concentration above this level renders the study to be environmental irrelevant for any nanomaterial (Bouguerra et al., 2016). In addition, the environmentally-relevant exposure setting of copper nanoparticles is very important in ecotoxicological studies to obtain a clear insight into the action of Cu nanoparticles, and its associated safety concerns in the soil-plant system (Anjum et al., 2015). Furthermore, 1000 mg/kg is the maximum concentration recommended for ecotoxicological test of chemical substances according ISO guideline 11269–2 (ISO, 2012). Soil was spiked with various concentrations in triplicates by mixing nano-Cu directly with test soil with 1% of the total mass of the air-dried soil used as the carrier (Hund-Rinke and Klawonn, 2013) and homogenized properly using a stainless spatula. Amended soils and control (n = 3) were afterward irrigated with deionized water (DW) to saturation and allowed to age for 7 days in a screen house under ambient environmental conditions. Seeds of cowpea (Accession number- A1 8462) for the study was supplied by the International Institute for Tropical Agriculture (IITA, Nigeria). The seed were planted in the amended soils and irrigated with DW according to water requirement of the plants throughout the period of the experiment. After germination, seedlings were thinned to maximum of 3 stands per container and allowed to grow for the exposure period of 65 days without fertigation before plant samples were collected for biochemical analysis. For the analysis, the whole root system were harvested and for leaves, only matured leaves without sign of senescence were collected.

### 2.3. Determination of Cu content and selected mineral elements

Concentration of copper content and mineral nutrients (Ca, Mg, Mn and Zn) in the roots, leaves and seeds of cowpea were determined after exposure, following the digestion procedure of EPA 3050. Briefly, 0.2 g of dried powdered sample was first subjected to digestion in 5 ml $HNO_3$ for 10 min and later heated with 1 ml $H_2O_2$ and 2.5 ml HCl for 15 min before digest was filtered and made up to 25 ml with deionized water. Concentration of Cu, Ca, Mg, Mn and Zn were determined by FAAS (Bulk Scientific- ACCUSYS 211). Detection limit for Cu, Ca, Mg, Mn, Zn, Na, and K are 0.005 mg/l, 0.05 mg/l. 0.005 mg/l, 0.03 mg/l. 0.005 mg/l, 0.005 mg/l and 0.01 mg/l respectively. Quality control and quality assurance was ensured by the use of internal control, replicate digestion and standard reference material -IAEA 359-cabbage (percentage recovery ranged from 92% −105% for studied elements).

### 2.4. Determination of biochemical parameters

#### 2.4.1. Determination of antioxidant enzyme activity

i. Crude extract for ascorbate peroxidase (E.C.1.1.11.1) was prepared according to Xu and Chen (2011). Approximately 0.5 g of fresh root (whole roots) or leaf (mature and non-senescence) sample was homogenized at 1:10 (w:v) ratio in 4 ml of 4 °C 100 mM phosphate buffer (pH 6.0) containing 2 mM EDTA, 4 mM dithiothreitol, and 2% (w/v) polyvinylpyrrolidone (PVP). The resultant homogenate was filtered, centrifuged at 10,000*g* for 25 min at 4 °C, and the collected supernatant was stored as above for determination of antioxidant enzyme activities.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981). The crude extract was reacted with mixture of 4 µl 0.5 mM ascorbate, 10 µl 0.2 mM $H_2O_2$, 886 µl 50 mM potassium phosphate buffer (pH 7.4), and the absorbance was read at 290 nm for 2 min (extinction coefficient 2.8 mM $cm^{-1}$).

ii. Crude extract for catalase (E.C.1.11.1.6), superoxide dismutase (E.C.1.15.1.1) and glutathione reductase (E.C.1.6.4.2) was prepared according to Lee and Lee (2000). 1 g of fresh root (whole roots) or leaf (mature and non-senescence) sample was homogenized in at 1:10 (w/v) ratio in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVP) and 0.5% (v/) Triton X-100 at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000*g* for 20 min at 4 °C. The supernatant was collected and store at −80 °C for determination of antioxidant enzyme activities.

Catalase (CAT) activity of the plant sample was determined according to the method of Aebi (1974). 20 µl crude extract was mixed with 980 µl 10 mM $H_2O_2$ prepared in 25 mM phosphate buffer and the absorbance was read at 240 nm for 3 min (extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$).

Superoxide dismutase (SOD) activity was determined according to Beyer and Fridovich (1987). 3 ml of reaction mixture of 450 µl 500 µM nitroblue tetrazolium (NBT), 500 µl 78 mM/l methionine, 200 µl 1.5 mM EDTA, 300 µl 0.02 mM riboflavin, 1500 µl 100 mM potassium phosphate buffer (pH 7.8) and 50 µl crude extract were prepared. The