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Short Communication

Destructive Effect of Copper Oxide Nanoparticles on Ultrastructure of Chloroplast, Plastoglobules and Starch Grains in Spring Barley (*Hordeum sativum*)

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Abstract

This study focused on determining the phytotoxic mechanism of copper oxide nanoparticles (CuO-NPs); destroying chloroplasts, plastoglobules and starch granules in spring barley (*Hordeum sativum* distichum) leaves. Recently, plastoglobule emerged as an important role player in chloroplast lipid metabolism operating as a thylakoid microdomain for metabolite synthesis, repair, and disposal. In the past few decades, application of NPs in agriculture has increased drastically which imposed the threat on crops by damaging xylem vessels and photosynthetic apparatus. The study was performed on spring barley grown in a hydroponic condition, supplemented with a high concentration of CuO-NPs to observe the toxicity on the ultrastructure of chloroplasts, plastoglobules and starch granules. The ultrathin cross sections of spring barley leaves revealed that the NPs decreased the counts of plastoglobule and starch granule on chloroplast. However, the size of plastoglobule and chloroplast increased in comparison to control. In addition, swollen starch granules and scattered thylakoids were also found. This is the first detailed study of NPs toxicity on the crop plants. The results indicated that the chloroplast could be as one of the important characteristics in the evaluation of NPs toxicity on plants. © 2019 Friends Science Publishers

Keywords: Anatomy; Chlorophyll; CuO; Nanoparticle; Spring barley; Ultrastructural changes

Introduction

Environmental contamination by nanoparticles (NPs) is increasing enormously in recent years due to their wide applications in different sectors. Soil is the main terrestrial sink for these anthropogenic pollutants. The actual figure of the global production of NPs to the knowledge of the author is unavailable. However, BBC (2015) reported the global nanotechnology market in the environmental applications is expected to reach approximately $41.8 billion by 2020. In comparison, the global market for nanomaterials should reach $5.3 billion by 2021, up from $1.6 billion in 2016 (BBC, 2017). There is no data available on the concentration of copper oxide (CuO) NPs in the soil; however, Cu could range from 2–100 mg kg^{-1} in unpolluted soils (Nagajyoti *et al*., 2010). Estimation of NP concentrations in the environment is based on predictive calculations, but it might not be entirely accurate.

Different plants exhibit specific behaviours towards excess metal ions present in the growth medium, and also cultivar may respond differently (Gul *et al*., 2018). The exact mechanism of plant defence towards NP toxicity is not fully understood. Nanoparticles can enter into the plant via root and translocate to the above-ground tissues, and affect plant growth performance by damaging photosynthetic machinery with other physiological changes and anatomical modifications either by releasing ions from particles or by direct interactions with plant tissues (Rico *et al*., 2011; Rajput *et al*., 2018a, b).

Photosynthesis is a key bioenergetic process for the conversion of light energy into chemical energy, which is performed by chloroplast, and components of the photosynthetic machinery embedded in a highly dynamic matrix such as thylakoid membranes (Rottet *et al*., 2015). Thylakoids play an important role in the photosynthesis as the light reaction occurs at the thylakoids membrane (Järvi *et al*., 2013). Thylakoid consist lipid bilayer enclosing lumen is known as "sacs". Majority of thylakoids are stacked (granal thylakoids) and interconnected by non-stacked lamellae (stromal thylakoids) (Daum and Kühlbrandt, 2011). At the curved regions of the thylakoid membrane, globular lipid droplets are embedded, named plastoglobules. Plastoglobules are lipoprotein particles surrounded by a lipid monolayer, structurally and functionally associated with the thylakoid membranes via the outer lipid leaflet in chloroplast (Kessler and Vidi,

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2007). Plastoglobule can be an oval or tubular shape. Austin *et al*. (2006) demonstrated that plastoglobules attach to thylakoids through a half-lipid bilayer, and play a crucial role in lipid biosynthesis. Chloroplasts are the most vulnerable organelles in plant.

Copper is widely distributed in plant tissues and is an essential micronutrient for plant growth (Burachevskaya *et al*., 2018). As an element, Cu may become toxic above a certain plant-specific threshold (An, 2006). Copper may catalyse the production of free radicals (Hänsch and Mendel, 2009; Ivask *et al*., 2010), induce oxidative stress, and act in a genotoxic manner (Ahamed *et al*., 2010). CuO-NPs are highly toxic to plants as well as human cells (Assadian *et al*., 2018). According to a recent review, Cu and CuO-NPs affected germination rate, photosynthesis, transpiration rate and yield quality, damaged and/or corroded roots and decreased root and shoot length and biomass in the following species: *Lactuca sativa*, *Medicago sativa*, *Triticum estivum*, *Vigna radiata*, *Phaseolus vulgaris*, *Zea mays*, *Cucumis sativus*, *Coriandrum sativum*, *Oryza sativa*, *Spinacia oleracea*, *Allium cepa*, *Brassica juncea*, *Solanum lycopersicum*, *Glycine max*, *Daucus carota*, *Ipomoea batatas*, *Cicer arietinum*, *Raphanus sativus* and *Cucurbita pepo* (Rajput *et al.*, 2018b). These studies are limited to morphological parameters and did not examine ultrastructural modifications. However, there is a need to examine the effects of CuO NPs at the cell ultrastructural level.

The Russian Federation is currently, and has historically been the world's leading barley-producing country (FAO/UN, 2012). The territory of Rostov region in southern Russia is the largest producer of agricultural crops. Therefore, there is a demand to assess the risks associated with CuO-NPs on spring barley (*Hordeum sativum* distichum). Our study examined spring barley that was grown in hydroponic system determine the effects of CuO-NPs on the ultrastructure of chloroplasts, plastoglobules and starch granules by measuring their areas and counts. This is the first detailed study on NPs toxicity on chloroplast ultrastructure. Data from our study could as basis to understand the phytotoxicity, and its input into the food chain.

Materials and Methods

Commercial grade copper (II) oxide (CuO, size 30–50 nm, APS powder) NP bought from Alfa Aesar, USA, were used in the experiment. CuO-NPs were poured in double distilled water to prepare the required concentration (10 g L^{-1}). This concentration (10 g L^{-1}) was selected with consideration for the existing levels of Cu contamination in soils adjacent to industrial enterprises of the Rostov-on-Don region (Arenas-Lago *et al*., 2014). These industries, especially Novocherkassk power plant, contributed more than 50% of the atmospheric pollutant emission in the region (Minkina *et al*., 2016). Several other studies also indicated a high concentration of Cu in Kola Peninsula soils of the Russian territory (Paton *et al*., 2006; Boyd *et al*., 2009). The NPs suspension was stirred using a magnetic stirrer for 30 min. The widely cultivated spring barley (*Hordeum sativum* distichum cv. Travnik) seeds in the Rostov region, Russia were used. Seeds were obtained from the Botanical Garden, Southern Federal University, Russia. According to the international standard (ISO 11269-1, 2012) it is recommended to use barley vulgaris (*Hordeum vulgare*) for biotesting. Cleaned Petri dishes (95 mm diameter) were taken, and 25 seeds were placed at an equal distance over a round piece of filter paper; this procedure was performed in triplicate. Seeds were surface sterilized and washed 5-6 times with distilled water. Five mL of the CuO-NP suspension was applied to the petri dish, and it was maintained in a growth chamber at 28^{o}C. After successful germination, seedlings were transferred to plastic vessels. In each vessel, 10 germinated seeds were placed (in triplicate) in 50 mL a water with or without 10 g L^{-1}. The vessels were placed in the growth chamber at 25±2^{o}C, with a16 h light and 8 dark cycle. After 4 weeks of barley growth, newly formed leaves were collected and prepared for transmission electron microscopy (TEM). One mm samples were collected and fixed using 2.5% glutaraldehyde, prepared in 0.1 M phosphate buffered saline (PBS), at room temperature for 2 h. After washing with PBS, samples were incubated for 1 h in a 1% OsO_4, 0.2 M PBS solution, and dehydrated with increasing concentrations of ethanol and acetone. Ultrathin cross sections were prepared using a microtome (Leica EM UC6, Germany) and examined using a TEM (Tecnai G2 Spirit Bio TWIN, Netherlands). Ten images from different places of each sample were obtained, and processed by Image_J software to measure the areas of chloroplasts, plastoglobules, and starch granules. SPSS-19 software was used for statistical analysis and data presented in form of mean values with standard errors (SE).

Results

The number of plastoglobules on chloroplast decreased more than 4 times (4.9±0.48 counts/plastid) as compared to control (20.8±2.41 counts/plastid) (Fig. 1b and 2a, b), and areas of plastoglobule increased from 0.0203±0.0021 µm^2 to 0.1104±0.0168 µm^2 (Fig. 1c and 2c, d). Swollen chloroplasts with their shear area being significantly larger than control (2.7703±0.2028 to 4.4495±0.7367 µm^2) (Fig. 1a and 2b, d, f) were measured. Starch granules were either absent or present as single bigger size (0.2408±0.0648 µm^2) in CuO-NPs treated barley leaves with relation to control (0.0595±0.0096 µm^2) (Fig. 1d and 2e, f). Except this, damage to cell membranes of chloroplasts, swollen and scattered thylakoids were also observed (Fig. 2b and d, f). Electron dense material caused by exposure of CuO-NPs was visible as in the form of dark areas mainly near the cell wall (Fig. 2h).

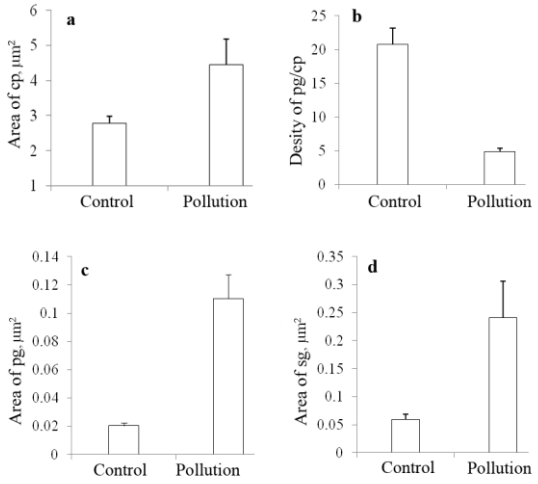

Fig. 1: Effects of CuO-NPs on chloroplast (cp) (a), plastoglobule (pg) (b, c) and starch grain (sg) (d) in spring barley (*Hordeum sativum*) leaves

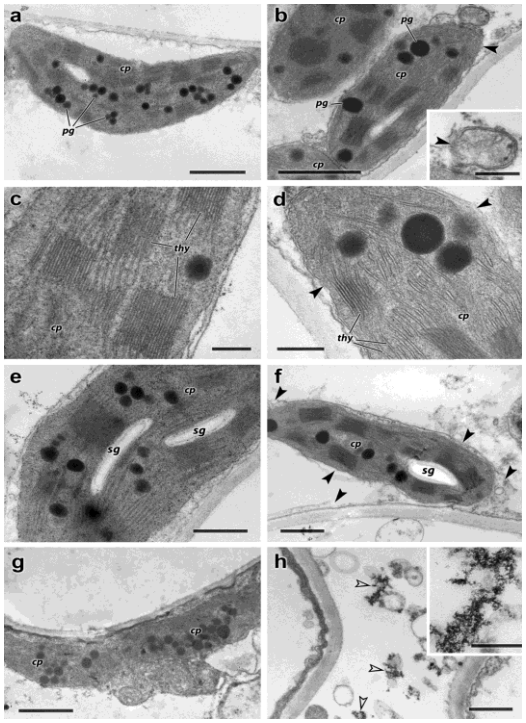

Fig. 2: Effects of CuO-NPs on chloroplast (cp), plastoglobule (pg), starch granule (sg), thylakoid (thy) in control (a, c, e, g), in pollution (b, d, f, h) in spring barley (*Hordeum sativum* distichum) leaves. Arrows indicate membrane damages and electron dense materials. Scale bar: (a) 1 µm, (b) 1 µm, (insert) 0.25 µm(c) 0.2 µm, (d) 0.25 µm, (e) 0.5 µm, (f) 0.25 µm, (g) 1 µm, (h) 0.5 µm, (insert) 0.2 µm

Discussion

Transmission electron microscopy images of spring barley leaves revealed that the CuO-NPs were absorbed into the roots and subsequently transported to the stems and leaves via xylem sap. The excess amount of Cu-NPs in leaves affected thylakoids; became disorganized, swollen, starch grains either absent in stroma or present single big, and widen interthylakoidal space. Furthermore, the perforation of the outer and inner membrane surrounding the chloroplast was observed. The chloroplasts in control were in proper shape, smooth and intact external double membranes, organized thylakoids and electron opaque stroma, which is a typical structure of a healthy and functional chloroplast. The areas of chloroplasts, plastoglobules, and starch granules increased, and the count of plastoglobules and starch granules decreased significantly in CuO-NPs treated leaves. The study conducted by Abreu de Freitas *et al.* (2015) showed Cu toxicity increased the count of plastoglobules but starch granules disappeared in *Inga subnuda* subs. *luschnathiana* leaves. Another study on Cu toxicity confirms the disappearance of starch granules and an increased count of plastoglobules in *Thlaspi ochroleucum* and *Origanum vulgare* (Ouzounidou *et al*., 1992; Panou-Filotheou *et al*., 2001). In contrast to these results, we did not notice increases in plastoglobule counts. Zhang *et al.* (2010) observed results similar to our study and suggested an increase in the size of chloroplasts in *Arabidopsis thaliana* leaves under high temperature. However, the author also suggests an increase in plastoglobules density. Previous studies performed on abiotic and biotic stresses, also suggest increase in counts of plastoglobules in different plant species (Hernández *et al*., 1995; Locy *et al*., 1996; Eymery and Rey, 1999). Long-term Cu toxicity decreased photosynthetic activity and disturbed the architecture of chloroplasts, particularly the thylakoid membranes on *Quercus robur* L. seedlings (Olchowik *et al*., 2017). However, CuO-NP reacts differently on hydroponically grown barley leave's chloroplast, especially for plastoglobules. There is limited literature available on NPs toxicity on chloroplast ultrastructure and rarely on plastoglobules and starch granules. Nhan *et al*. (2015) found swollen and ruptured external surface of chloroplast in Bt-transgenic cotton leaves by CeO_2 NPs and Cvjetko *et al*. (2017) also observed changes in the size of chloroplast in AgNP-treated and $AgNO_3$-treated tobacco leaves. Silver NPs affected chloroplast ultrastructure by disorganizing the chloroplast, grana, and thylakoids of barley leaves grown in a model experiment (Fayez *et al*., 2017). Changes in chloroplast size would affect the light-path inside leaf cells, and consequently photosynthesis (Zhang *et al*., 2010).

Conclusion

The results showed swollen chloroplasts, increased size of plastoglobules, starch grains and decreased counts of plastoglobule in CuO-NPs treated spring barley leaves. This indicates that the structural changes in chloroplasts, plastoglobules, starch granules, and thylakoids are the significant results of NPs toxicity. It may be explained as a protective mechanism against damage to the photosynthetic

apparatus and could be one of the important parameters for evaluating NPs toxicity on crops. Present results could help to increase the scientific understanding of the phytotoxicity of NPs of spring barley.

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