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BRIEF COMMUNICATION

The responses of germinating seedlings of green peas to copper oxide nanoparticles

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Abstract

The effects of copper oxide nanoparticles (CuONPs) on germinating seedlings of green pea (*Pisum sativum* L.) were studied. The seedlings were grown in a half-strength Murashige and Skoog semisolid medium containing 0, 50, 100, 200, 400, and 500 mg dm^{-3} CuONPs for 14 d under controlled growth chamber conditions. Exposures to 100, 200, 400, and 500 mg dm^{-3} CuONPs significantly reduced plant growth (shoot and root lengths) and increased reactive oxygen species (ROS) generation and lipid peroxidation. Gene expression study using real-time polymerase chain reaction showed no significant change in the expression of genes coding CuZn-superoxide dismutase (*CuZnSOD*), catalase (*CAT*), and ascorbate peroxidase (*APX*) in shoots. However in roots, a significant increase in the expression of the *CuZnSOD* gene was observed under the exposures to 100, 200, 400, and 500 mg dm^{-3} CuONPs, in the expression of the *CAT* gene under 100 and 200 mg dm^{-3} CuONPs, and in the expression of *APX* under 200 and 400 mg dm^{-3} CuONPs.

Additional key words: chlorophyll, gene expression, lipid peroxidation, oxidative stress, *Pisum sativum.*

Copper oxide nanoparticles (CuONPs) are widely used in several products, such as antimicrobial and antifouling paints, bioactive coatings, air and liquid filtration, textiles, and lubricant oils and super conducting materials (www.nanotechproject.org). The phytotoxic effects of CuONPs, *e.g.*, an inhibition of seed germination, reduction in plant growth and biomass, and induction of oxidative stress, have been reported in various plants (Lin and Xing 2007, Stampoulis *et al*. 2009). However, only limited studies has been undertaken on the toxic effect of CuONPs in food crops (Shaw and Hossain 2013). In the present investigation, the toxic effects of CuONPs were studied in a green pea (*Pisum sativum* L.). The effects of CuONPs on seedling development (shoot and root lengths), total chlorophyll content, ROS generation, and lipid peroxidation were studied. Further, the modulation of expression of genes involved in antioxidative responses, such as those coding CuZn-superoxide dismutase (*CuZnSOD*), catalase (*CAT*), and ascorbate peroxidase (*APX*), were evaluated. Since Cu ions released from CuONPs to the exposure medium may cause toxic responses, the toxic effect of CuONPs was compared with the effect of Cu^{2+} ions, and a medium devoid of CuONPs was used as negative control.

A semi solid half-strength Murashige and Skoog (MS) medium (pH 5.7) with 20 g dm^{-3} sucrose and 5 g dm^{-3} agar (*Duchefa Biochemie*, Haarlem, The Netherlands) was used. CuONPs were purchased from *Sigma-Aldrich* (St. Louis, MO, USA). The characterization of CuONPs and the amount of Cu^{2+} ions released to deionized water from the highest concentration of CuONPs tested (*i.e.*, 500 mg dm^{-3}) was measured based on our earlier study (Nair and Chung 2014). As described by Lee *et al.* (2008), $CuCl_2 . 2 H_2O$ (*Sigma-Aldrich*) was used for the preparation of a Cu^{2+} solution. After autoclaving, different concentrations of CuONPs and $CuCl_2$ were vortexed with 100 cm^3 of a growth medium to reach final concentrations of 0, 50, 100, 200, 400, and 500 mg dm^{-3}

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; CuONPs - copper oxide nanoparticles; CuZnSOD - CuZn-superoxide dismutase; HPF - 3'-hydroxyphenyl flourescein; ICP-AES - inductively coupled plasma-atomic emission spectrophotometry; MS - Murashige and Skoog; NBT - nitroblue tetrazolium; PCR - polymerase chain reaction; ROS - reactive oxygen species.

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CuONPs and 0.2 mg dm^{-3} Cu^{2+}, and distributed to plant growth vessels (120 × 80 mm; *Phytohealth*, *SPL Life Sciences*, Gyeonggi-do, South Korea). The medium was solidified immediately by keeping in a refrigerator at 4 °C for preventing precipitation of CuONPs.

Seeds were surface sterilized with a sodium hypochlorite solution (10 %, m/v) for 15 min and rinsed with sterilized deionized water five times. The seeds were germinated in the dark on wet *Whatman #1* filter paper in disposable plastic Petri dishes (*SPL Life Sciences*). Ten germinated seeds with 1 mm root length were transferred to each culture vessel and incubated in a growth chamber (*Hanbaek Scientific Co.*, Gyeonggi-do, South Korea) under a 16-h photoperiod, an irradiance of 80 μmol m^{-2} s^{-1}, and a temperature of 26 ± 1 °C for 14 d. Four replications were maintained for the control and all treatments and the experiment was repeated twice.

For analysis of Cu content in plants, root and shoot tissues from the control and CuONPs exposed plants were thoroughly washed with deionized water and dried at 75 °C for 24 h in a dry air oven. The dried plant samples (100 mg) were digested with concentrated HNO_3 at 115 °C for 1 h, diluted to 25 cm^3 with deionized water, and filtered through 0.2 μm nylon filters (*Chromedisc*® syringe filter, Seoul, South Korea). The Cu content was determined by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES; *Ultrace*, Jobin Yvon, France) using samples collected from three biological replications.

Shoot and root lengths were measured using a ruler, and fresh mass (FM) of shoots and roots were determined by weighing them on a digital balance (*Mettler Toledo*, Greifensee, Switzerland). Total chlorophyll content was determined according to Lichtenthaler (1987). Hydrogen peroxide content in shoots and roots was measured using the method described by Rao *et al*. (1997), and lipid peroxidation determined as per the method of Heath and Packer (1968). *In vivo* detection of superoxide and hydrogen peroxide formation in roots was done by treating them with nitroblue tetrazolium (NBT) (Fryer *et al.* 2002) and 3'-hydroxyphenyl flourescein (HPF; *Invitrogen*, Carlsbad, USA) (Kwasniewski *et al*. 2013). The NBT and HPF treated root samples were mounted on 26 × 76 mm microscope slides (*Citoglas*®, Jiangsu, China) and photographed using a digital camera (*Canon*, Tokyo, Japan) and a fluorescence microscope equipped with digital camera (*BX61-32 FDIC*, *Olympus*, Tokyo, Japan), respectively.

Total RNA was isolated from roots and shoots using an RNA isolation kit and cleaned up using an RNA-clean up kit (*Qiagen*, Valencia, CA, USA). The quality and quantity of the RNA preparation was verified by absorbance spectrophotometry (A_{260}/A_{280} > 1.8) (a UV/Vis spectrophotometer, *Optizen*, Daejeon, South Korea) and agarose gel elecrophoresis. One microgram of total RNA was used for cDNA synthesis using a *QuantiTect*® reverse transcription kit (*Qiagen*) integrated with a genomic DNA contamination removal step as per the manufacturer's instructions. Primers for the *CuZnSOD* [forward (F): 5'-TTCAGTCAGGAGGGAAATGG-3'; reverse (R): 5'-TCCAAGATCACCAGCATGTC-3'], *CAT* (F: 5'-CTAGGGGAGCAAGTGCAAAG-3'; 5'-GACGGGAAAGTTGTTTCCAA-'3'), *APX* (F: 5'-GGAAGGCTATGGGGCTTAG-3'; R: 5'-TCAACA AGAGGGCGGAATAC-3'), and *ACT* (F: 5'-GTT TGGATCTTGCTGGTCGT-3'; R: 5'-GAACCTCTC AGCTCCGATTG-3') genes were designed with publically available sequences in *NCBI* Genbank (http://www.ncbi.nlm.nih.gov) using the online *Primer3* program (http://frodo.wi.mit.edu/primer3/). The *P. sativum ACT* gene was used as internal control to normalize the gene expressions.

The mRNA expression analysis was done using a *Chromo-4 Opticon* real-time PCR system (*Bio-Rad*, Hercules, USA) according to the manufacturer's instructions. A reaction mixture included 1 mm^3 of cDNA, 0.2 μM corresponding forward and reverse primers, and 10 mm^3 of 2× *IQ SYBR Green Super Mix* (*Bio-Rad*) and made up to to 20 mm^3 using sterilized deionized water. Reactions were run with an initial template denaturing step 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, and a melting curve analysis was done. The relative expression of different genes under different exposure conditions were compared with the internal control using the gene expression analysis software (*Bio-Rad*). Data obtained from different experiments were analyzed using the one-way analysis of variance (*ANOVA*) with the *SPSS 12.0 KO* (*SPSS Inc.*, Chicago, IL, USA) programme. The Dunnett's post-doc test was done to determine the effect of different exposures on various parameters. Differences at a probability of $P < 0.05$ were considered as signifi cant and at probabilities of $P < 0.01$ and $P < 0.001$ as highly significant.

The morphological analysis of CuONPs in the exposure medium using transmission electron microscopy shows that particles remained as single or in an agglomerated form (Fig. 1 Suppl.). The analysis of dissolution of CuONPs using ICP-AES in deionized water after 24 h showed the presence of 0.025 ± 0.002 μg dm^{-3} Cu ions.

No significant changes in the shoot and root lengths and FM were observed after the exposure to 0.2 mg dm^{-3} Cu^{2+} ions and 50 mg dm^{-3} CuONPs as compared to the controls (Fig. 1*A*,*B*). However, significant reductions in the shoot and root lengths and FM were observed upon the exposures to 100, 200, 400, and 500 mg dm^{-3} CuONPs (Fig. 1*A*,*B*). Except in the case of 500 mg dm^{-3} CuONPs, no significant change in the total chlorophyll content was observed (Fig. 1*C*). The analysis of Cu content showed an average of 3.2 ± 1.0 mg(Cu) g^{-1}(DM) in the control seedlings. However, increases in the Cu content to 5.1 ± 0.8, 7.3 ± 0.9, and 17.6 ± 1.1 mg g^{-1}(DM) were observed upon the exposures to 50, 200, and 500 mg dm^{-3} CuONPs, respectively.

The exposures to 0.2 mg dm^{-3} Cu^{2+} and to different concentrations of CuONPs did not change the H_2O_2 content and lipid peroxidation in shoots as compared to

the controls (Fig. 1*D*,*E*). Similarly, no significant changes in the H_2O_2 content and lipid peroxidation were observed in roots upon the exposures to 0.2 mg dm^{-3} Cu^{2+} and 50 mg dm^{-3} CuONPs (Fig. 1*D*,*E*). However, the exposures to 100, 200, 400, and 500 mg dm^{-3} CuONPs significantly increased the H_2O_2 content and lipid peroxidation in roots (Fig. 1*D*,*E*). The *in vivo* detection of superoxide generation using NBT staining showed no change in roots of the control and Cu^{2+} exposed plants (Fig. 2*A* Suppl.). However, a gradual increase in superoxide generation was observed in roots after the treatment with different concentrations of CuONPs (Fig. 2*A* Suppl.). The detection of H_2O_2 generation using the HPF treatment showed no increase in ROS generation

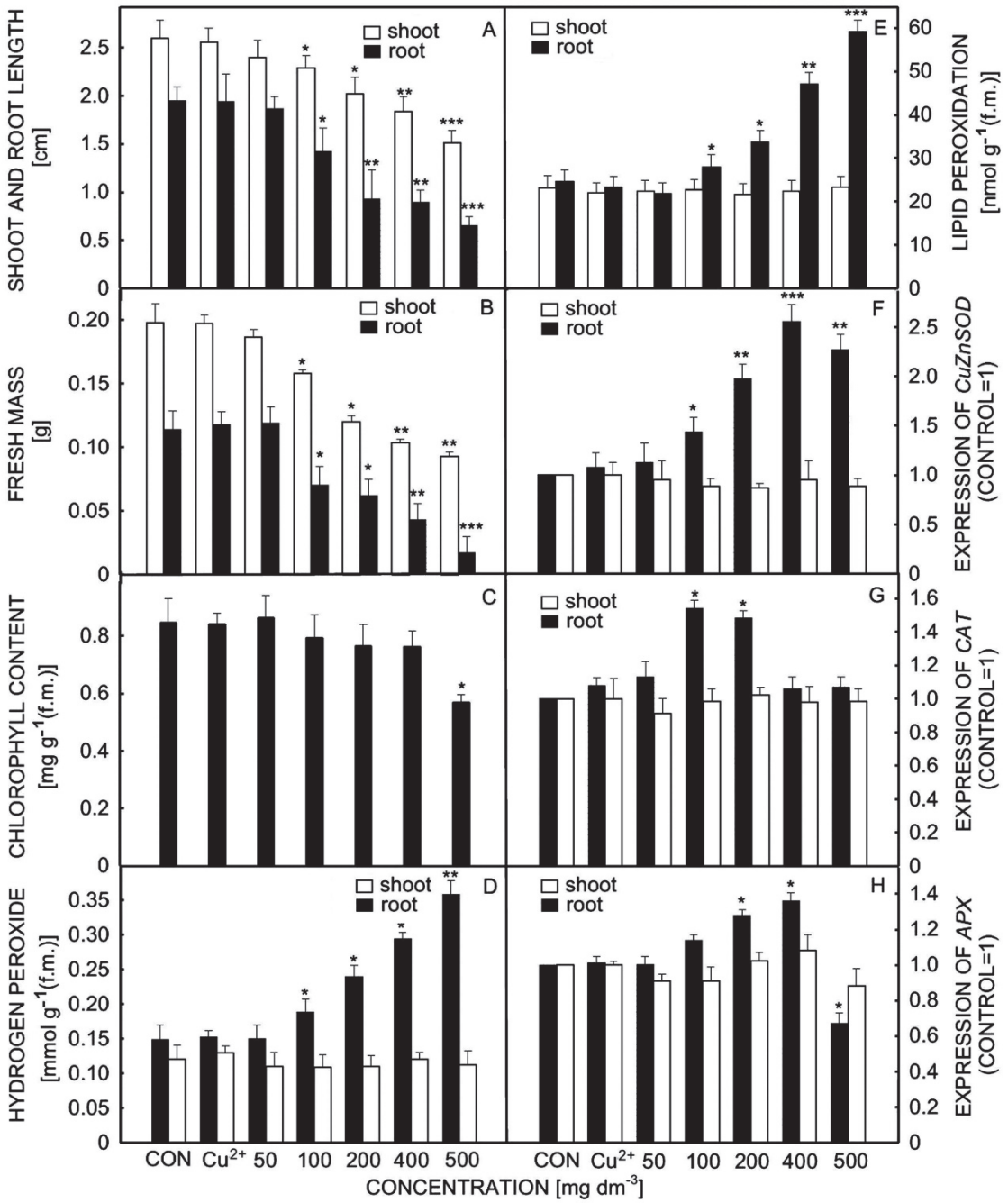

Fig. 1. Average shoot and root lengths (*A*), shoot and root fresh mass (*B*), total chlorophyll content (*C*), hydrogen peroxide content (*D*), lipid peroxidation **(***E*), *CuZnSOD* expression (*F*), *CAT* expression (*G*), and *APX* expression (*H*) in green pea seedlings exposed to 0.2 mg dm^{-3} Cu^{2+} and 50, 100, 200, 400, and 500 mg dm^{-3} CuONPs. Data are means ± SE of four replicates. The *asterisks* indicate significant differences at $P < 0.05$*, $P < 0.01$**, and $P < 0.001$***.

in roots exposed to Cu^{2+} as compared to the control. However, in roots, the ROS generation increased upon the exposures to increasing concentrations of CuONPs (Fig. 2*B* Suppl.).

The expression of *CuZnSOD* did not change significantly in shoots after the treatment with Cu^{2+} and different concentrations of CuONPs (Fig. 1*F*). No significant changes in the expression of the *CuZnSOD* gene were also observed in roots upon the exposures to Cu^{2+} and 50 mg dm^{-3} CuONPs (Fig. 1*F*). However, a significant increase in the expression of *CuZnSOD* was observed in roots after the exposures to 100 - 500 mg dm^{-3} CuONPs (Fig. 1*F*). The expression of the *CAT* gene did not change significantly in shoots under any of the exposures as compared to the controls. In roots, the expression of *CAT* showed no significant changes after the exposures to Cu^{2+} ions and 50, 400, and 500 mg dm^{-3} CuONPs. However, a significant increase in the *CAT* expression was observed in roots upon the exposures to 100 and 200 mg dm^{-3} CuONPs (Fig. 1*G*). The analysis of the expression of the *APX* gene did not show any significant changes in shoots upon the exposures to Cu^{2+} and different concentrations of CuONPs. In roots, no changes in the *APX* gene expression were observed after the exposures to Cu^{2+} and 50 and 100 mg dm^{-3} CuONPs. However, the *APX* expression in roots significantly increased upon the exposures to 200 and 400 mg dm^{-3} CuONPs but decreased upon the exposure to 500 mg dm^{-3} CuONPs (Fig. 1*H*).

The phytotoxic dose of CuONPs varies between different plant species. For example, according to Wu *et al.* (2012), the phytotoxic doses of CuONPs in lettuce, radish, and cucumber are 12.9, 397.6, and 175.4 mg dm^{-3}, respectively. Since there is not specific regulations for studying the effect of engineered nanoparticles in plants, the guidelines established by the US Environmental Protection Agency (USEPA 1996) is generally followed. Therefore, in this study, the selection of the phytotoxic doses of CuONPs was done based on their effect on inhibition of root growth. It was observed that more than a 50 % reduction in root growth was observed after the exposure to 200 mg dm^{-3} CuONPs. Moreover, the exposure to CuONPs strongly inhibited seedling development, and the inhibition was positively correlated with the concentration of CuONPs. In accordance with our results, Shaw and Hossain (2013) has also reported that shoot and root growth of rice seedlings is significantly reduced under CuONPs stress. However, in this study, it was observed that the exposure to 0.2 mg dm^{-3} Cu^{2+} ions has not affected shoot as well as root development of the green pea seedlings. It is well known that trace amount of Cu is required for normal plant growth and development since it is an essential micronutrient. Therefore, low concentrations of Cu might support seedling development as observed in this study and elsewhere (Lee *et al.* 2008). In a recent study, Shi *et al.* (2014) also reported that phytotoxicity in a copper tolerant plant *Elshotzia spendens* is due to CuONPs exposure and not as result of exposure to soluble Cu ions or bulk CuONPs. In this study, it was observed that the exposure to the highest concentration of CuONPs significantly reduced the total chlorophyll content in the green pea seedlings, and this might be due to the presence of excess Cu as result of the CuONPs exposure. It has been known from earlier studies that the presence of excess Cu can interfere with enzymes associated with chlorophyll biosynthesis (Maksymiec *et al.* 1994) or can lead to Fe deficiency as result of antagonism between Cu and Fe uptake (Pätsikkä *et al.* 2002). In accordance with our hypothesis, Hong *et al.* (2015) reported that except for $CuCl_2$, all Cu treatments including CuONPs significantly reduce Fe content in shoots and roots of alfalfa and lettuce.

In this study, both qualitative and quantitative measurements indicate that the exposure to CuONPs increased ROS generation and lipid peroxidation in roots of the green pea seedlings. Based on the Cu content analysis, it can be assumed that the increase in the Cu content in the CuONPs exposed plants might have enhanced the production of ROS in the green pea seedlings. Since Cu is redox active element, the presence of excess Cu could lead to an increased ROS generation in plants either through Fenton or Haber-Weiss reactions (Takeshi *et al.* 1997), or through interaction with the photosynthetic electron transport system (Sandmann and Boger 1980). It has been known from previous studies that exposure to CuONPs can result in their uptake to the plants (Wang *et al.* 2012) and also lead to their dissolution as result of a reduced pH and interaction with organic acids and proteins inside the plant tissues (Shi *et al.* 2014). Moreover, the up-regulation of the *SOD* gene in roots indicates an excess ROS generation and the conversion of superoxide to H_2O_2 in roots under CuONPs stress (Bowler *et al.* 1992). It is known that cells are protected from the excess accumulation of H_2O_2 either by the action of *CAT* or through the ascorbate-glutathione cycle by the action of *APX* which reduces H_2O_2 to H_2O. However, though the expression of the *CAT* and *APX* genes were up-regulated, incomplete scavenging H_2O_2 might lead to an increased ROS generation and a subsequent increase in lipid peroxidation levels in roots. In recent studies, Shaw and Hossain (2013) and Shaw *et al.* (2014) also reported that though the activity of *APX* increases, the H_2O_2 content in leaves of rice and barley seedlings does not decrease when they are subjected to CuONPs. Enhanced ROS generation as well as lipid peroxidation has also been reported from different biological models and plants under CuONPs stress (Wang *et al.* 2012, Melegari *et al.* 2013, Shaw and Hossain 2013, Nair and Chung 2014, Shaw *et al.* 2014).

References

Bowler, C., Van Montagu, M., Inzé, D.: Superoxide dismutase and stress tolerance. - Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 83-116, 1992.

Fryer, M.J., Oxborough, K., Mullineaux, P.M., Baker, N.R.: Imaging of photooxidative stress responses in leaves. - J. exp. Bot. **53**: 1249-1254, 2002.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplasts. I. Kinetics and stochiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hong, J., Rico, C.M., Zhao, L., Adeleye, A.S., Keller, A.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L.: Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). - Environ. Sci. Processes Impacts **23**:177-185, 2015.

Kwasniewski, M., Chwialkowski, K., Kwasniewska, J., Kusak, J., Siwinski, K., Szarejko, I.: Accumulation of peroxidase-related reactive oxygen species in trichoblasts correlates with root hair initiation in barley. - J. Plant Physiol. **170**: 185-195, 2013.

Lee, W.M., An, Y.J., Yoon, H., Kweon, H.S.: Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water-insoluble nanoparticles. - Environ. Toxicol. Chem. **27**: 1915-1921, 2008.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Methods Enzymol. **148**: 350-382, 1987.

Lin, D., Xing, B.: Phytotoxicity of nanoparticles: inhibition of seed germination and root growth.- Environ. Pollut. **150**: 243-250, 2007.

Maksymiec, W., Russa, R., Urbanik-Sypniewska, T., Baszynski, T.: Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages. - Physiol. Plant. **91**: 715-721, 1994.

Melegari, S.P., Perreault, F., Popovic, R.H.R.C., Radovan, Matias, W.G.: Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. - Aquat. Toxicol. **142-143**: 431-440, 2013.

Nair, P.M.G., Chung, I.M.: Impact of copper oxide nano-particles exposure on *Arabidopsis thaliana* growth, root system development, root lignification and molecular level changes. - Environ. Sci. Pollut. Res. **21**: 12709-12722, 2014.

Pätsikkä, E., Kairavuo, M., Šeršen, F., Aro, E.M., Tyystjärvi, E.: Excess copper predisposes photosystem II to photo-inhibition *in vivo* by outcompeting iron and causing decrease in leaf chlorophyll. - Plant Physiol. **129**: 1359-1367, 2002.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C.B.: Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes. Salicylic acid-mediated oxidative damage requires H_2O_2. - Plant Physiol. **115**: 137-149, 1997.

Sandmann, G., Boger, P.: Copper-mediated lipid peroxidation processes in photosynthetic membranes. - Plant Physiol. **66**: 797-800, 1980.

Shaw, A.K., Ghosh, S., Kalaji, H.M., Bosa, K., Brestic, M., Zivcak, M., Hossain, Z.: Nano-CuO stress induced modulation of antioxidative defense and photosynthetic performance of syrian barley (*Hordeum vulgare* L.). - Environ. Exp. Bot. **102**: 37-47, 2014.

Shaw, A.K., Hossain, Z.: Impact of nano-CuO stress on rice (*Oryza sativa* L.) seedlings. - Chemosphere **93**: 906-915, 2013.

Shi, J., Peng, C., Yang, Y., Yang, J., Zhang, H., Yuan, X., Chen, Y., Hu, T.: Phytotoxicity and accumulation of copper oxide nanoparticles to the Cu-tolerant plant *Elshotzia spendens*. - Nanotoxicology **8**: 179-188, 2014.

Stampoulis, D., Sinha, S.K., White, J.C.: Assay-dependent phytotoxicity of nanoparticles to plants. - Environ. Sci. Technol. **43**: 9473-9479, 2009.

Takeshi, M., Sook, M.L., Noriyuki, S., Takakiro, I.: Identification of S100b protein as copper-binding protein and its suppression of copper-induced cell damage. - J. biol. Chem. **272**: 23037-23041, 1997.

Wang, Z., Xie, X., Zhao, J., Liu, X., Feng, W., White, J.C., Xing, B.: Xylem and phloem based transport of CuO nanoparticles in maize (*Zea mays* L.). - Environ. Sci. Technol. **46**: 4434-4441, 2012.

Wu, S.G., Huang, L., Head, J., Chen, D.R., Kong, I.C., Tang, Y.J.: Phytotoxicity of metal oxide nanoparticles is related to both dissolved metals ions and adsorption of particles on seed surfaces. - J. Petroleum Environ. Biotechnol. **3**: 126, 2012.