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TOXICOLOGICAL EFFECT OF CUO NANOPARTICLES TO *BRASSICA NIGRA* L. SEEDLINGS: A COMPARATIVE *IN VIVO* AND *IN VITRO* RESPONSE

HIRA ZAFAR[1], IHSAN-UL-HAQ[2], SYEDA NASREEN[3] AND MUHAMMAD ZIA[1*]

[1]*Department of Biotechnology, Quaid-i-Azam University Islamabad Pakistan*
[2]*Department of Pharmacy, Quaid-i-Azam University Islamabad, Pakistan 45320*
[3]*Oilseed Program, National Agricultural Research Centre Islamabad, Pakistan*
[*]*Corresponding author's email: ziachaudhary@gmail.com*

Abstract

Metallic nanoparticles including CuO are considered toxic when released in the environment. However, the toxicity is concentration dependent. In present investigation, we report toxicological consequences of CuO nanoparticles under *In vivo* and *In vitro* conditions to *Brassica nigra* L (a model legume plant). To investigate LD50 (lethal dose), the seeds were germinated in presence of CuO NPs (1-1000 mg/L for *In vitro* and 10-200 mg/kg soil for *In vivo* condition). In both, *In vitro* and *In vivo* cases no or positive effect was observed on plant length, fresh and dry weight up to threshold concentration. Above that negative effect was observed on all physiological parameters. Under *In vitro* conditions Total phenolics and flavonoids (TPC and TFC), total antioxidative potential (TAC), total reducing power (TRP) and free radical scavenging activity fluctuated in *Brassica* plantlets by change in NPs concentration. While under *In vivo* application in *Brassica* plantlets TPC, TAC and TFC increased up to some concentration and then decreased while increasing the NPs concentration. Minor significant change was observed in TRP and DPPH based free radical scavenging activity continuously increased by increasing NPs concentration. HPLC analysis was performed for determination of rutin, gallic acid and caeffic acid in plants. All the three antioxidative molecules were found at varying concentration when plants were grown in presence of CuO NPs. The results conclude that under *In vivo* and *In vitro* condition NPs have different toxicity to plants which results in differential plant response to scavenge the free radicals and oxidative stress.

Key words: Antioxidative activities, *Brassica nigra*, Nanoparticles, CuO, toxicity, HPLC

Introduction

Importance of metal oxide nanoparticles (NPs) is increasing progressively with their abundant applications in materials science and nanotechnology industries, being employed in sensors, catalysis, and environmental remediation, and for their incorporation into commercial products (Franklin *et al.,* 2007). It is apparent that the increasing development in nanotechnology is also paving way for the higher risk exposure to humans and environment, however the data on the potential risks are scarce particularly the data explaining toxicity to the higher plants (Song *et al*., 2016). The mechanism of nanomaterials effect on biological systems is dependent on their shape, size, surface properties and composition (Perreault *et al.,* 2010). Factors responsible for the toxicity of metallic NPs include both the nanoparticulate form and the toxic metal ions being released from the NPs, hence it is very challenging to evaluate their toxicological properties(Javed *et al*., 2017; Franklin *et al.,* 2007).

A common judgment from phytonanotoxicity studies is that exposure of NPs (CuO NPs, CeO2 NPs, Ag NPs, etc) to terrestrial plant species such as onion (*Allium cepa* L.),corn (*Zea mays*), rice (*Oryza sativa* L.) and wheat (T*riticum aestivum* L.) produce excess amounts of reactive oxygen species (ROS) (Dimpka *et al.*, 2012, Zhao *et al.*, 2012). During normal physiological conditions, ROS continuously produce and are eliminated by antioxidant defense mechanisms thus maintaining a balance. Under pathological conditions, overproduction of ROS causes the imbalance leading to oxidative modification of cellular membranes or intracellular molecules (Gulcin, 2006). The toxicity by metals include direct oxidative injury by enduring Fenton and Haber-Weiss reactions leading to production of oxygen free radicals or ROS in plants (Emamverdian *et al.,* 2015). ROS consequently then disrupt the cell homeostasis, cause DNA strand breakage, proteins or cell membranes defragmentation and damage to photosynthetic pigments, thus initiating cell death (Schützendübel & Polle, 2002) (Hatata & Abdel-Aal, 2008). Plants antioxidant defense mechanism includes a lot of antioxidants that act against ROS. Example includes phenolic compounds that directly trap the free radicals or scavenge them with antioxidant enzymes through a series of coupled reactions (Rao *et al.,* 1996). In *Brassica nigra*, several bioactive natural compounds have been proposed to exhibit the radical-scavenging activity (Rajamurugan *et al.*, 2012). It is necessary to determine the relationship between metal-based NPs and ROS induced, and to gain information on the roles of defensive antioxidants on ROS scavenging (Zafar *et al*., 2016a).

Copper oxide (CuO) has been recognized as an industrially important material i.e. catalysis, batteries, magnetic storage media, antibacterial composites, solar energy conversion, and gas sensing and field emission devices. There are limited reports on phytotoxicity of CuO NPs on plants. CuO NPs have been stated to inhibit the growth of Lemna minor with more significant effects on roots (Song *et al.,* 2016). No significant effect of CuO NPs have been shown on maize (*Zea mays* L.) germination, however seedlings growth was found to be inhibited. The transport and redistribution of NPs in the plant was examined (Wang *et al*., 2012). *Phaseolus radiates* (mung bean) and *Triticum aestivum* (wheat) were also reported to give positive results on phytotoxicity tests of Cu NPs. *Phaseolus radiates* showed more reduced growth than *Triticum aestivum* (Lee *et al,.* 2008). Radish

(*Raphanus sativus*), annual ryegrass (*Lolium rigidum*) and perennial ryegrass were investigated for strong plant growth retardation as well as accumulation of mutagenic DNA lesions in effect of CuO NPs (Atta *et al.,* 2012).

The aims of this study are to characterize the effect of CuO NPs on *In vivo* and *In vitro* growth of *Brassica nigra* (a model plant) as well as the antioxidant potential of *Brassica nigra* altered in response to toxic levels of NPs. Furthermore, the differential response towards NPs by the *In vivo* and *in vitro* mode of growth was also investigated.

Materials and Methods

Materials: All the chemicals and reagents were obtained from Sigma Aldrich and Merck. MS media was attained from Phytotech. Coprecipitation method was employed to synthesize CuO nanoparticles (NPs) of approximately 53nm size determined through scanning electron microscopy (SEM). *Brassica nigra* seeds were obtained from NARC Islamabad, Pakistan presenting near 98% germination.

Preparation of NP supplemented media: NPs were suspended at 0 (control), 1, 10, 20, 30, 40, 50, 100, 200, 400, 600, 800 and 1000mg/l to prepare CuO supplemented media. Sucrose (3%) was added as carbon source. Sonication for 30min was employed to avoid aggregation. The pH was maintained as 5.7. For solidification, 0.44% agar was used and was dissolved by heating for 1-2min. Media was dispensed as 30ml/100ml conical flask while thoroughly shaking to avoid settlement of NPs at the bottom. The media was sterilized in an autoclave at 121ºC, 15psi pressure for 45min. The autoclaved media was then cooled upto 45ºC and to prevent agglomeration, the flasks were shaken well and kept at -4ºC to speed up solidification.

Preparation of NP supplemented soil pots: *In vivo* experiment was carried out in pots of capacity approx300g. Soil contained clay, sand and manure in a ratio of 2:1:1. NPs were augmented at the concentration of 0 (control), 10, 25, 50, 75, 100, 200 mg/kg and was thoroughly mixed manually in plastic bags to attain the uniform presence of NPs throughout. The *In vivo* concentrations were kept differential from *In vitro* because of the effect of soil medium.

Germination experiments

In vitro: Under sterile conditions, seeds of *Brassica nigra* were treated with 0.1% mercuric chloride (w/v) for 1-2 min. Afterwards they were thoroughly rinsed with sterilized distilled water. Three flasks for each concentration were prepared by inoculating five seeds in each flask. Flasks were placed in growth room at 25C under dark for seed germination. The germination efficiency was observed at 5th day of seed inoculation. After the appearance of plumule, the flasks were transferred to 16/8 light and dark conditions for further growth. Allowing the seedlings to grow for 12-14 days to fully develop their shoots and roots, their lengths were measured. Fresh weights of whole plants were also recorded and were processed for drying at 45C, 0.06Pa for 4 days under vacuum.

In vivo: The prepared pots were sprinkled with *Brassica nigra* seeds, approx. 20 seeds per pot. Water was provided by carefully spraying the top layer of soil to allow seed germination. The pots were kept in growth room to provide optimum environment. The pots were regularly watered to maintain the moisture required by seeds to germinate. After the seeds showed up seedlings after 6 days, their presence was ensured to 16/8 light and dark conditions for further growth. To allow the seedlings to fully develop shoots and roots, they were monitored daily and watered properly. After 20-25 days they were checked for whole plant length and fresh weight, and were processed for vacuum drying at 45ºC, 0.06Pa for 4 days.

Sample preparation for antioxidant assays: After both the *In vitro* and *In vivo* grown seedlings were finely dried under vacuum, their dry weight was recorded. DMSO was used as a solvent to prepare the samples of 100mg/ml concentration. Later sonicating for 20 mins, centrifugation gave the supernatant that was further used for the analysis of antioxidant activities using standard protocols.

Determination of total flavonoid content: Standard protocol described by Haq *et al.,* (2010) was used to determine the total flavonoid content. In short, the DMSO dissolved samples (20µl) were taken in 96 well plate. Then was added 10µl of each potassium acetate (1M) and aluminium chloride (10%w/v) solutions in each well. Distilled water (160µl) was added to make up total volume 200µl and kept for incubation for 30min. Absorbance readings were taken at 415nm on microplate reader (Bioteck, USA). The calibration curve ($y=0.0269x+0.00765$, $R^2=0.998$) was drawn by using quercitin as standard at 0 to 40µg/ml and the flavonoid content was established in µg quesrcitin equivalent per mg dry weight (µg QE/mg DW).

Determination of total phenolic content: The total phenolic contents were determined using standard protocol (Fatima *et al*., 2015). Briefly, 20µl of each extract was transferred in microplate wells and Folin Ciocalteu reagent (90µl) was added. After incubation for 5min, 90µl of sodium carbonate (7.5% w/v in H_2O) was added to each well. The reaction mixtures were incubated for 1 hr and absorbance was measured at 650nm by using microplate reader (Bioteck, USA). Gallic acid in DMSO was used as positive control in this assay. A calibration curve ($y=0.0135x+0.0846$, $R^2=0.986$) was obtained in parallel under the same operation conditions using gallic acid (6.25-50 µg/ml). The resultant TPC is determined as µg gallic acid equivalent per mg dry weight (µg GAE/mg DW).

DPPH free radical scavenging assay: 2,2-diphenyl-1-picryl hydrazyl (DPPH) reagent was used for the determination of free radical scavenging activity. Briefly, 10µl of samples were taken in microplate wells, added the DPPH (0.004% w/v in methanol) reagent in 190µl quantity into each. After incubation in dark for 1 hour at 37ºC, the optical density was measured at 515nm.

Ascorbic acid (1mg/ml DMSO) was used as a positive control. Percent inhibition was calculated by the following formula:

$$\text{Percent inhibition of the test sample} = \% \text{ Scavenging activity} = (1 - Ab_s / Ab_c) \times 100$$

where Ab_s is the absorbance of DPPH solution with sample, whereas Ab_cindicates the absorbance of negative control (containing the reagent and solvent only).

Determination of total antioxidant capacity: Jafri *et al*., (2014) (phosphomolybdenum) method with slight modification was employed for the evaluation of total antioxidant capacity. Samples were taken as 100µl in appendorf tubes and added 900µl of reagent into it. These were then kept in waterbath for incubation at 95°C for 90min. After cooling to room temperature, the reaction mixture (200µl) of each appendorfs was transferred to 96 well plate and the reading at 630nm was noted using microplate reader. Ascorbic acid (1mg/ml) was used as positive control.

Determination of total reducing power: Using ascorbic acid as positive control, samples (100µl) were taken in eppendorf tubes and 0.2M phosphate buffer was added. Potassium ferricyanide (1%) was added in amount 250µl and kept for incubation at 50°C for 20min. Added 200µl of trichloroacetic acid solution (10%) to the mixture and centrifuged at 3000rpm for 10min. The upper layer (150µl) of centrifuged mixture was isolated into the microplate wells and 50µl of ferric chloride (0.1%) was added into each isolate. Microplate was read at 630nm.

HPLC DAD analysis: Caffeic acid, gallic acid and rutin were used as reference standards. Their stock solutions were prepared and diluted further by using methanol solvent to obtain a 50µg/ml as a final concentration. The solutions were prepared fresh just before the analysis. Sartolon polyamide membrane filter of 0.2µm was used for filtration.

For HPLC analysis, samples were dissolved in methanol at 10mg/ml concentration. The vials were sonicated for a while, filtered the solution through a 0.2µm sartolon polyamide membrane filter. Samples were assured to be prepared fresh and were stored at 4°C if not tested for more than one hour.Adopted a previously reported protocol by Jafri *et al*., (2014) for the determination and quantification of polyphenols in *Brassica nigra* grown *In vitro* and *In vivo* using HPLC-DAD analysis. The system had a C8 analytical column in conjunction with a diode array DAD detector. Two mobile phases named A and B of different composition were prepared for polyphenols analysis. Mobile phase A had acetonitile: methanol: water: acetic acid in a ratio of 5:10:85:1, while mobile phase B contained acetonitrile: methanol: acetic acid in a ratio of 40:60:1 and their flow rate being maintained at 1ml/min. Sample volume of 20 µl was injected through an injection port into the column. Next analysis was preceded by a 5 min column reconditioning phase. The gradient volume of mobile phase B in the first 0-20 min was 0-50%, from 20-25 min it was 50-100% and from 25-30 min it was 100%. Absorbance was measured at four different wavelengths for respective compounds i.e. 257 nm and 325 nm (rutin and gallic acid: 257 nm, caffeic acid: 325 nm).

Results and Discussion

Brassica nigra propagated in the occurrence of CuO nanoparticles showed perceptible change in the germination potential, plant morphology as well as in biochemical characteristics.

***In vitro* & *In vivo* effect of NPs on seed germination and plant morphology:** The toxicology of NPs on plants studied till now has given both positive and negative effects. The major factors that are considered for the variability are nature of NPs, their minute size as well as the concentration (Zafar *et al*., 2016b).

CuO NPs exhibited inhibitory effect on seedlings length at all concentrations even the smallest quantity (1mg/l) showed a decrease of 35.7% in length. The inhibition was increased slightly with the increase in concentration reaching its maximum at 1000mg/l showing 82.69% decrease.The drastic effect of CuO NPs was more evident on plantlet roots at all concentrations. Shoot length was also poorly influenced as well as the leaf area (Fig. 1).

The *In vivo* effect of CuO NPs is the first ever study on *Brassica nigra* being reported (Fig. 2). Seeds were allowed to grow in soil (2:1:1 ratio of clay, sand and manure) at different concentrations of NPs. Seed germination is the physiological process involving water imbibition that leads to development of root primordia (Adhikari *et al.,* 2012). The toxic effect of CuO NPs was more pronounced in the roots and thus whole plant lengths were recorded. This observation is related to the reports by Yang & Watts (2005) and Adhikari *et al*., (2012) who found that nanoparticles retard root elongation more predominantly. Whole plant length was measured for determining *In vivo* effect of NPs on *Brassica nigra*. Descending order of inhibition was observed as the concentration in the pots was increased gradually. Maximum retardation of length calculated was 21.43% and was presented by the least quantity of CuO NPs (10mg/kg). Apparently, plant growth remained more or less the same from 50mg/kg and onwards.

Fig. 1. *In vitro* growth of *Brassica nigra* on MS media in the presence of CuO NPs.

Fig. 2. *In vivo* growth of *Brassica nigra*on soil containing nanoparticles.

Table 1. *In vitro* effect of CuO NPs on *Brassica nigra* seed germination, plantlet length, fresh and dry weight.

Concentration (mg/l)	Length (cm)	FW (mg)	DW (mg)
Control	14.50 ± 2.03^{a}	73.28 ± 2.69^{a}	1.74 ± 0.46^{b}
1	9.33 ± 1.85b^{b}	53.11 ± 2.07^{b}	1.38 ± 0.89^{cd}
10	7.26 ± 1.26^{bc}	52.84 ± 1.98^{b}	1.35 ± 0.67^{cd}
20	8.81 ± 1.52^{b}	53.63 ± 2.47^{b}	1.62 ± 0.82^{bc}
30	8.96 ± 1.84^{b}	51.21 ± 2.01^{b}	1.55 ± 0.43^{c}
40	8.42 ± 1.31^{b}	48.97 ± 1.39^{c}	1.79 ± 0.41^{b}
50	8.14 ± 1.17^{bc}	48.06 ± 1.83^{c}	2.35 ± 0.95^{a}
100	5.09 ± 1.06^{c}	30.00 ± 1.52^{d}	1.79 ± 0.32^{b}
200	4.26 ± 0.67^{d}	26.32 ± 1.72^{e}	1.34 ± 0.36^{cd}
400	2.72 ± 0.90^{e}	21.06 ± 1.09^{f}	1.51 ± 0.01^{c}
600	2.64 ± 0.79^{e}	16.80 ± 1.39^{g}	1.68 ± 0.05^{bc}
800	2.53 ± 0.72^{e}	16.81 ± 1.21^{g}	1.34 ± 0.21^{cd}
1000	2.51 ± 0.69^{e}	18.50 ± 1.01^{fg}	1.32 ± 0.13^{cd}

Table 2. *In vivo* effect of CuO NPs on *Brassica nigra* seed germination, plantlet length, and fresh and dry weight.

Concentration (mg/l)	Length (cm)	FW (mg)	DW (mg)
10	4.73 ± 0.38^{b}	24.76 ± 0.09^{e}	2.17 ± 0.17^{a}
25	4.98 ± 0.53^{b}	31.56 ± 0.10^{d}	1.78 ± 0.04^{b}
50	5.54 ± 0.72^{ab}	37.37 ± 0.52^{b}	1.24 ± 0.50^{d}
75	5.30 ± 0.43^{ab}	38.23 ± 0.13^{b}	1.14 ± 0.02^{e}
100	5.60 ± 1.62^{ab}	33.23 ± 0.52^{c}	1.41 ± 0.08^{c}
200	4.74 ± 0.63^{b}	34.13 ± 1.29^{c}	1.10 ± 0.12^{e}
Control	6.02 ± 0.85^{a}	41.52 ± 1.56^{a}	2.29 ± 0.05^{a}

Phytotoxicity of CuO NPs was evident from the experimental results. Seed coats are selectively permeable and play a crucial role in protecting the embryo against harmful substances. Radicles, however when get penetrated from the seed coat come in direct contact with the nanoparticles. Consequently, root elongation of delicate species would have a dose-dependent response. Whole plant lengths recorded included the root and shoot lengths combined. Since roots are the primary tissues that are threatened by the excess concentrations of pollutants, the lethal symptoms seem to be predominant in roots than in shoots. In contradicton to *In vitro* results, the *In vivo* plantlets showed the opposite trend, the inhibitory effect appeared to decrease with increase in concentration.

In vitro fresh weights were also seen to be prominently reduced by elevating the NPs level. Maximum reduction (74.75%) was observed in flasks having1000mg/l conc of NPs. Dry weight however presented a differential pattern with control plant exhibiting 1.74mg (Table 1). The maximum decrease (24.14%) in DW was presented by plantlets at 1000mg/l. There was perceived an increase of 0.61mg in DW at CuO-50mg/l. Comparatively, the *In vivo* results considered for the respective concentrations showed relatively similar pattern as by their lengths (Table 2). Lessened FW at 10mg/l concentration of CuO NPs was about 10.76mg. The loss in weight became insignificant as the concentrations were elevated. However, the weight of dried *In vivo* whole plants when calculated displayed a considerable increase at the inhibitory concentrations. Dry weight for 10mg/l and 25mg/l concentrations was increased by 68.22% and 37.98% respectively (Table 2).

Fresh and dry biomass production were found to be in accordance with the wholeplant length for corresponding nano-CuO treatments *In vivo* and *In vitro*, except for some concentrations. In *In vitro* experiment, 35% increase of DW at CuO-50mg/l depicted that CuO at nano level might dissipate Cu ions in MS culture media that altered the biochemical processes thus elevating the major biomass of the plant associated with the increased biomolecules (Table 1). Furthermore, the ions might be reducing the water content accumulation at this concentration thus may explain the lower fresh weight than control. The same concentration when considered for *In vivo* elevates the fresh weight depicting an increased capability of water retaining potential (Table 2).

***In vitro* and *In vivo* effect of CuO NPs on plant antioxidative response:** The antioxidative response was resoluted through five assays; DPPH free radical scavenging activity, total antioxidant capacity, total reducing power, total flavonoid and phenolic contents. The *In vitro* results were inconsistent, though an increase in DPPH capacity was detected compared to control 79.9% (maximum 9.7% at CuO-10mg/l) (Fig. 3a).

The reducing power was highly increased at initial concentrations (1-30mg/l) with 100% increase at CuO-20mg/l and around 50% at others. Onwards the power was reduced to 38% at 200mg/l and then gradually increased with concentration.

There was no significant difference in total flavonoid contents (Fig. 4A). The plantlets showed comparatively the same trend at all concentrations. For TPC, the ascending trend followed was altering initially at 20mg/l to 200mg/l. CuO NPs at 400mg/l gave the somewhat maximum quantity of phenolics, followed by 1000mg/l as displayed in the Fig. 4A. Total antioxidant capacity was also observed to be elevated as the concentration of NPs was increased. At CuO NPs 100mg/l and onwards, the capacity was seen to be doubled reaching more than 3 folds and 6 folds capacity at 800mg/l and 1000mg/l concentrations respectively (Fig. 5A).

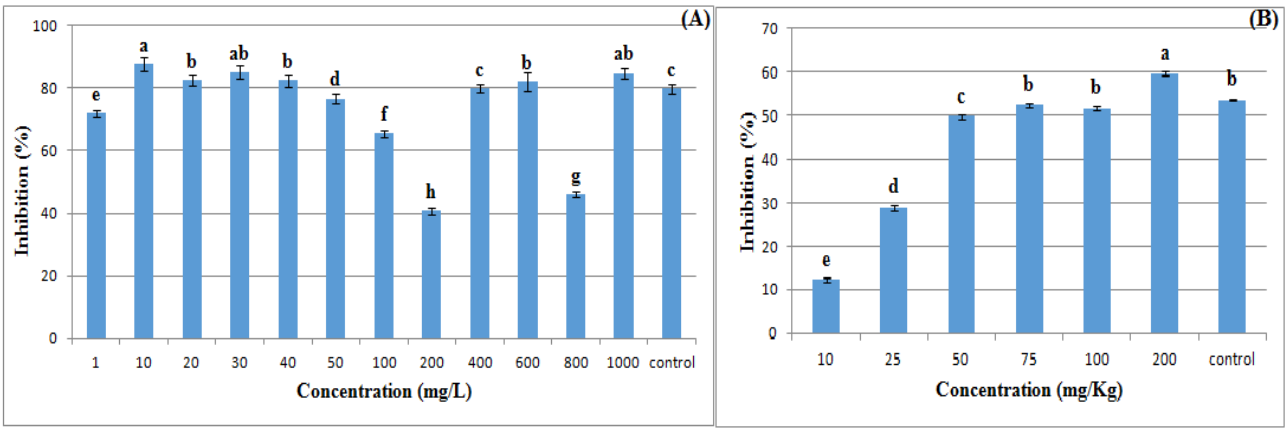

Fig. 3. DPPH scavenging assay (A) *In vitro*, (B) *In vivo*.

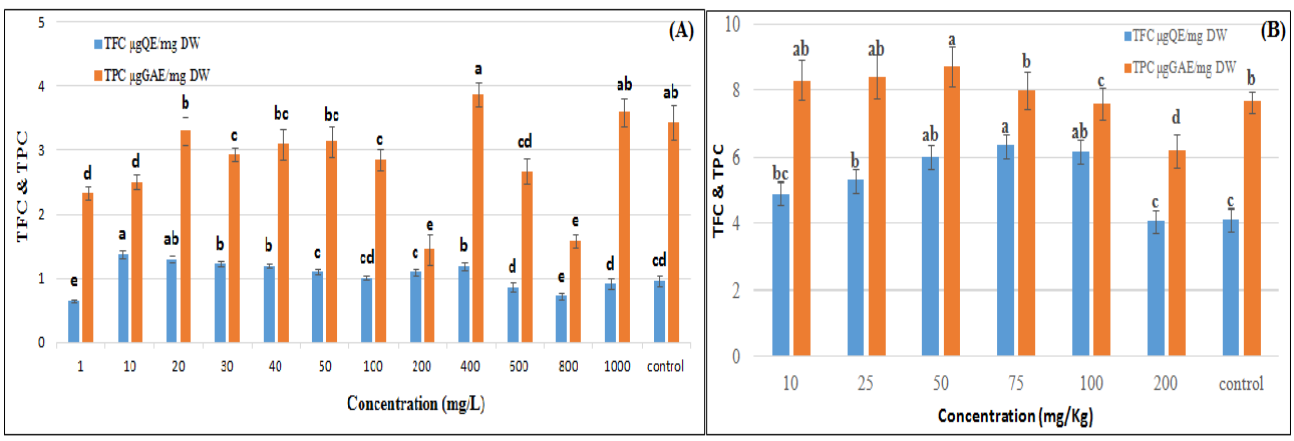

Fig. 4. Total flavonoid content, total phenolic content (A) *In vitro,* (B) *In vivo*.

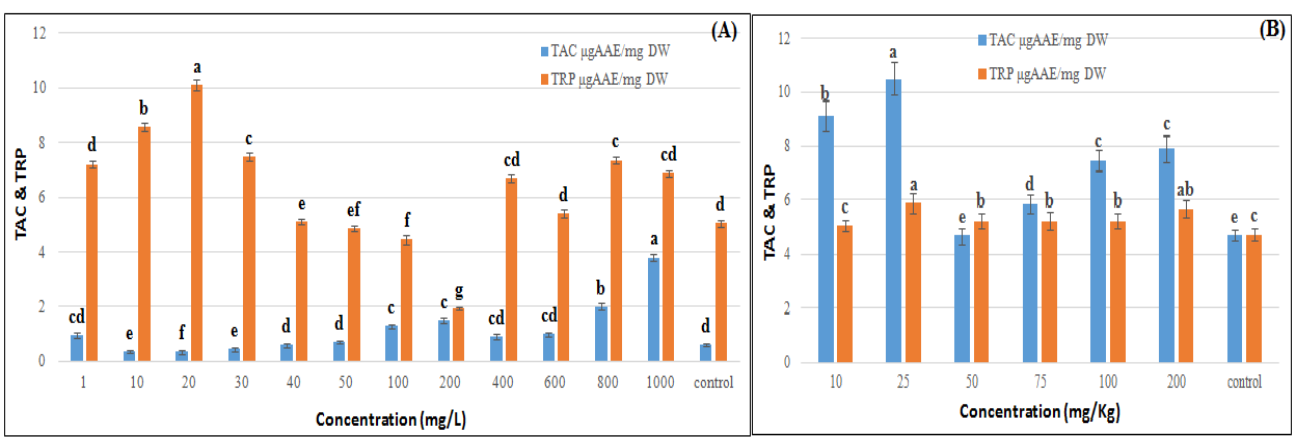

Fig. 5. Total antioxidant capacity, reducing power (A) *In vitro,* (B) *In vivo*.

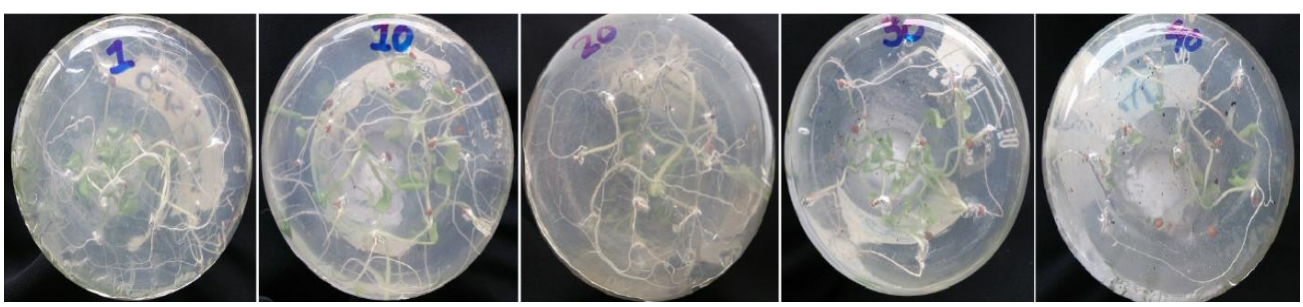

Fig. 6. *In vitro* flasks showing retardation in rooting system.

Table 3. Quantification of rutin, caffeic acid and gallic acid in *In vitro* grown *Brassica nigra*.

In vitro concentration	Rutin	Gallic acid	Caffeic acid
mg/l	ug/100 mgDW	ug/100 mgDW	ug/100 mgDW
1	5.88 ± 0.03^{j}	8.46 ± 0.14^{f}	9.64 ± 0.32^{d}
10	9.3 ± 0.12^{i}	8.4 ± 0.20^{f}	17.42 ± 0.21^{b}
20	9.58 ± 0.02^{h}	11.98 ± 0.19^{de}	19.48 ± 0.09^{a}
30	10.36 ± 0.19^{g}	11.24 ± 0.18^{de}	17.06 ± 0.16^{b}
40	11.88 ± 0.23^{f}	10.82 ± 0.15^{e}	10.06 ± 0.29^{cd}
50	13.54 ± 0.21^{e}	12.14 ± 0.21^{d}	9.06 ± 0.23^{d}
100	14.86 ± 0.27^{d}	12.38 ± 0.18^{d}	11.94 ± 0.18^{c}
200	17.28 ± 0.34^{c}	12.26 ± 0.24^{d}	10.38 ± 0.53^{cd}
400	23.12 ± 0.32^{b}	14.02 ± 0.35^{c}	11.24 ± 0.41^{c}
600	27.06 ± 0.64^{a}	16.54 ± 0.31^{a}	8.56 ± 0.32^{de}
800	24.2 ± 0.26^{b}	13.36 ± 0.56^{cd}	8.32 ± 0.25^{de}
1000	25.36 ± 0.51^{b}	15.66 ± 0.42^{b}	7.74 ± 0.31^{e}
Control	2.38 ± 0.09^{k}	15.48 ± 0.37^{b}	7.3 ± 0.30^{f}

When grown in soil, the antioxidant potential of *Brassica nigra* presented somehow variable trend. Total flavonoid and phenolic contents of plantlets grown *In vivo* were gradually elevated as the concentration was increased, maximum increase was in flavonoids (54%) and phenolics (13.7%) at 75mg/kg and 50mg/kg respectively (Fig. 4B). In case of TAC, all concentrations were detected to increase the capacity to a considerable level except 50mg/kg, which depicted the antioxidant capacity was same as control. The capacity was found to be 20% more than doubled at 25mg/kg of NPs *In vivo* (Fig. 5B). Total reducing power showed insignificant increase at all concentrations (Fig. 5B) while DPPH % inhibition at lowest concentration was minimum that is 12.31 (Fig. 3B), that increased with the increase in concentration reaching upto 59.72 (relative to control–53.72). The increase of flavonoids and phenolics at the concentrations 75mg/kg and 50 mg/kg respectively can be analogous with their corresponding fresh weights (Fig. 4B).

The toxicological studies of NPs are in an early stage. There are few reports on applications of NPs on plants, nevertheless the precise mechanisms of toxicity remain unclear. The key factors defining the toxicity may be dependent on the interaction between NPs and biomolecules. The toxicity mainly encompasses unfolding of proteins, fibrillation, thiol-cross linking and loss of plants enzymatic activity (Chang *et al.,* 2012). NPs can diffuse across the plasma membrane because of their small size and the positive ions present on their surfaces. Cu2+ ions entry into the cells can occur by transport and ion/voltage-gated channels. The local concentration of ions thus increase and disrupt cellular homeostasis causing in cell toxicity (Chang *et al.,* 2012; Inelova *et al*., 2018). CuO NPs are intrinsically oxidative (reducing agents) and can thus remove electrons from numerous biological entities inside plant cells and subsequently transfer them to other biomolecules (Atha *et al.,* 2012). The unusual response depicted by the *In vitro* CuO concentration 50mg/l was the reduced DPPH % inhibition. This can be explained in one way by the corresponding higher dry biomass. Since Cu is an essential nutrient for plant growth, plays a vital role in photosynthetic reactions, enzymes activation, RNA synthesis and improves the photosystems (Adhikari *et al.,* 2012). It can be explained by higher stem elongtation than roots and increased surface area, that 50mg/l concentration in flasks might render Cu ions a compatible amount for plant growth thus increased dry weight and reduced DPPH. *Brassica nigra* showed inhibition in growth even at very small concentrations, however reaching 50mg/l the NPs brings about more of the morphological impact than physiological variation. Resultantly, the production of ROS was also decreased as evident by the DPPH scavenging activity. The same concentration of CuONPs has been found to cause serious damage to Lemna minor cells and thus accumulated more ROS (Song *et al.,* 2016). Ions from NPs can induce production of ROS by a variety of chemical reactions. ROS can break DNA strands and disturb gene expression. Furthermore, Cu2+ ions have the ability to form chelates with biomolecules or dislodge the metal ions in specific metalloproteins, which may result in functional protein inactivation (Chang *et al.,* 2012).

CuO NPs being highly dense and insoluble in water is? more likely to get aggregated that does not allow the plants to be exposed to the actually proposed concentrations. This might explain the variations in the antioxidant potential thus considered. Furthermore, the high temperature during sterilization may cause the MS media to get denser not allowing the bigger aggregated clumps of NPs to diffuse through it and enter the roots. Initial inhibitory effect on roots causes the root cells to halt the cell division and reduced growth. This is another aspect that reflects variable response due to the differential exposure to NPs because of short primary roots, retarded secondary roots and absence of root hairs as apparent in Fig. 6. The roots since grown very weak and fragile might cut and some portion might remain inside the media causing minute changes in results, in other words called handling error.

With metal stress in soil, more the antioxidant compounds are produced, more likely the water retaining capability as they are increased. But as the concentration of NPs in the soil increased, so would have increased their interaction with the soil constituents making them unavailable for diffusion into the seedlings. Furthermore, the regular watering of seedlings may cause the NPs to be drawn with water and get reduced in concentration within the diffusion zone of the seedlings. The unusual trend shown by seedlings was their scavenging capability i.e. DPPH for the minimum concentrations was lowest, that increased gradually with the concentration. This most likely would be due to the changes in DNA brought out by NPs. This change might have resulted in molecules or compounds that act CuO NPs are capable of imposing

damage to plant DNA directly by redox interactions. These can also operate indirectly by promoting the oxidation of other biomolecules (such as membrane lipids) within the plant that can produce •OH which is then capable of attacking DNA at diffusion controlled rates (Atha *et al.*, 2012).

***In vitro* and *In vivo* analysis of rutin, gallic acid &caffeic acid by HPLC:** The HPLC analysis of *Brassica nigra* illustrates the production of antioxidants in response to metal stress *In vivo* and *In vitro*. Quantification of rutin, kaempferol and quercetin in *Brassica oleracea* was done by (Ahmed & Rao, 2014). In present study, the standards used for quantification were gallic acid, caffeic acid and rutin.

The *In vitro* growth showed an ascending quantity of rutin with increasing concentration of nanoparticles (Table 3). Even at the lowest concentration (1mg/l of CuO NPs), the quantity of rutin was increased by more than 2 folds. Maximum elevation (almost 11 times) was seen at 600mg/l and 1000mg/l concentrations. Gallic acid showed a decreased quantity at lower concentrations that elevated to levels comparable to control as the concentration was increased gradually. Caffeic acid also showed an increase at lower doses of CuO NPs i.e an increase of approx. 2.5 times was observed at 10, 20 and 30mg/l. Further rise in concentration gradually decreases the caffeic acid to control level.

The *In vivo* trial also elevated the quantity of rutin even at lowest concentration (10mg/kg). This rise in rutin quantity reached maximum to about three times at 75mg/kg of CuO NPs (Table 4) and then decreased onwards. Gallic acid was also found to be maximum at 75mg/kg with approx. double the value. Caffeic acid however showed comparatively an insignificant increase in quantity.

Hence, it can be rightly said that the main phenolic compound which increased drastically was rutin. Gallic acid and caffeic acid did rise too but rutin pathway showed to be activated as a major response both *In vivo* and *In vitro*. In conditions *In vitro*, Cu ions penetrating the plant causes the production of reactive oxygen species activating the antioxidant defense system of the plant. At lower concentrations, the caffeic acid rises to act against the free radicals but as the stress increases gradually, so increases the production of rutin inside the cells. The maximum stress might be at the concentration 600mg/l as depicted by the quantity of rutin.

In vivo stress showed somewhat reduced response compared to *In vitro*, due to the difference in growth medium and bioavailability of nanoparticles. Nevertheless, the increase of rutin was still much higher than control even at small doses of nanoparticles. Rutin reaching three times and gallic acid 2 times the control quantities at 75mg/kg illustrates the maximum stress triggered *In vivo* at this concentration.

Table 4. Quantification of rutin, caffeic acid and gallic acid in *In vivo* grown *Brassica nigra*.

Concentration	Rutin	Gallic acid	Caffeic acid
mg/kg	ug/100 mgDW	ug/100 mgDW	ug/100 mgDW
10	9.48 ± 0.18^{c}	6.22 ± 0.13^{bc}	10.22 ± 0.28^{b}
25	10.04 ± 0.42^{b}	5.32 ± 0.20^{c}	8.48 ± 0.18^{c}
50	10.42 ± 0.19^{b}	6.68 ± 0.31^{b}	9.84 ± 0.21^{bc}
75	11.54 ± 0.42^{a}	8.02 ± 0.27^{a}	10.36 ± 0.32^{b}
100	4.96 ± 0.13^{e}	7.52 ± 0.26^{ab}	11.34 ± 0.42^{a}
200	5.32 ± 0.17^{d}	6.62 ± 0.17^{b}	11.12 ± 0.36^{a}
Control	4.04 ± 0.11^{f}	4.84 ± 0.10^{d}	10.04 ± 0.29^{b}

Conclusion

In vitro and *In vivo* experiments carried out depicted an overall inhibition phenomenon at the applied concentration range. CuO NPs affect the growth of *Brassica nigra* seedlings at different concentrations. The external environmental factors and growth medium account for the variable response exhibited by the seedlings. Despite these factors, the more comparable of the growth was found at 50mg/l for *In vitro* where a slight stem elongation was also observed. Beyond this concentration, the retardation in growth was considerably increased.CuO NPs was observed to damage roots pronouncedly compared to shoots. The inhibition of secondary roots and root hairs was predominant.The antioxidant response exhibited by *Brassica nigra* in NPs presence showed variations with respect to control. *In vitro* application of CuO NPs resulted in significantly increased yet variable reducing power. TAC was dramatically increased under Cu stress. TFC and TPC were also variably altered. Same was the case with *In vivo* antioxidant response. This was confirmed by higher production of rutin even at small NPs concentrations.The *In vitro* stress seemed to be maximum at 600mg/l while *In vivo* stress at 75mg/kg where gallic acid was also raised.

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