

Available online on 15.03.2019 at <http://jddtonline.info>

Journal of Drug Delivery and Therapeutics

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Research Article

Nano-cuprous oxide enhances seed germination and seedling growth in *Lycopersicon esculentum* plants

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ABSTRACT

This study was carried out to determine the influence of cuprous oxide nanoparticles (Cu₂O NPs) biosynthesised from leaf extracts of *Flacourtia montana* on the tomato *Lycopersicon esculentum* seed germination, seedling growth and vigour index. Here we examined the promotory and phytotoxic effect of Cu₂O NPs (0-160ppm) on tomato seeds resulted in dosage dependent response. The highest germination percentage (95%) was observed at 20ppm Cu₂O NPs, however, above 20ppm Cu₂O NPs, there is a reduction in the seed germination. The tomato seedlings showed increased root and shoot elongation up to 20ppm Cu₂O NPs concentration, further increase in NPs concentration caused the negative effect on plants growth and development. The leaf pigments showed increasing trend in tomato plants after treatment with Cu₂O NPs up to 20ppm as compared to control. Phytotoxicity of Cu₂O NPs in tomato seedlings demonstrated by lower contents of chlorophyll a, b and carotenoid pigments. The study of effect on antioxidant enzymes showed increases in activity with increase in Cu₂O NPs concentration for two enzymes, Super oxide dismutase (SOD) and Glutathione Peroxidase (GPX) out of five enzymes treated. High antioxidant activity of enzymes is followed by the increased lipid peroxidation and decrease in free radical scavenging activity by the DPPH. The activity of Catalase, Phenyl Alanine Aminolyase and Poly Phenol Oxidase enzymes were found to increase up to 20ppm as compared to control and above this, all three enzymes showed decrease in activity. Uptake of Cu₂O NPs nanoparticle by tomato seedlings was confirmed by atomic absorption spectroscopy.

Keywords: Nano-Cuprous Oxide, *Flacourtia montana*, Tomato, antioxidant enzymes, lipid peroxidation

Article Info: Received 06 Feb 2019; Review Completed 09 March 2019; Accepted 12 March 2019; Available online 15 March 2019



Cite this article as:

Ananda S, Shobha G, Shashidhara KS, Vishwaprakash M, Nano-cuprous oxide enhances seed germination and seedling growth in *Lycopersicon esculentum* plants, Journal of Drug Delivery and Therapeutics. 2019; 9(2):296-302
<http://dx.doi.org/10.22270/jddt.v9i2.2554>

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INTRODUCTION

Nanomaterials have many applications in agriculture in terms of plant protection and nutrition due to their size-dependent qualities, high surface-to-volume ratio and unique optical properties¹. Research and development in this field growing rapidly. Hence it pave way for wide advances in agricultural research, transfer of agricultural and food wastes to energy and other useful by-products through enzymatic nanobio processing, disease prevention, and treatment in plants using various nanocides².

Nanoparticles in agriculture can act as potential candidates for modulating the redox status thereby changing the development of the plants. It is reported that ionic silver (Ag⁺) released from AgNPs inhibits respiratory enzymes and induces oxidative stress by generation of reactive oxygen species (ROS)^{3, 4,5,6,7}. The interaction mechanisms at the

molecular level between nanoparticles and biological systems are largely unknown⁸.

Choosing an appropriate technique to increase seed tolerance to adverse environmental conditions will enhance the seed germination percentage. Nanoparticles of many metal oxides by absorbing water, oxygen and nutrients and having the antimicrobial properties can affect the seed germination %, improve growth and plant metabolism⁹.

NPs closely interact with their surrounding environment and plants are essential base component of all ecosystem. There is considerable concern about the potentially harmful effects of these NPs and they can have significant negative effects on many organisms¹⁰, especially plants. NPs will inevitably interact with plant and these interactions such as uptake and accumulation in plant biomass will greatly affect their fate and transport in the environment¹¹.

Recently many studies have showed the physiological responses of plant seedlings to nanoparticles during germination, but the influence on the seed germination and root growth varied significantly among the plants and nanoparticle types. For example, TiO₂ on fennel seed germination¹², ZnO on cucumber¹³, Lahiani and co-workers¹⁴ proved that MWCNTs on corn, barley, and soybean. In contrast, CuO NPs decreased the germination seeds, shoot length and shoot weight of rice¹⁵. Ag NPs increased the germination percent of *Pennisetum glaucum*¹⁶. Graphene had no obvious effects on wheat seed germination, but decreased the number of wheat roots¹⁷. CuO nanoparticles have no effects on maize seeds germination, while inhibited the root elongation¹⁸.

Copper as a micro element required for the normal growth and development of the plants. A nano metal copper functions as that of copper by slow release of ions. In present study green synthesised cuprous oxide nanoparticle from *Flacourtia montana*¹⁹ and characterized was used to check their role in enhancing the germination of tomato seeds and seedling stage growth and also the phytotoxicity effects on plants.

MATERIALS AND METHODS

Collection of Tomato (*Lycopersicum esculentum*) seeds

Tomato seeds of PKM-1 variety procured from local market and surface sterilized in 5% Sodium Hypochlorite solution for 10min followed by with multiple washing in distilled water.

Seed germination test

The disinfected tomato seeds were soaked with sonicated Cu₂O NPs arranged in the proportion of 1:10 (w/v) and control in distilled water for 12h with gentle shaking in an orbital shaker at 150rpm to ensure every one of the seeds were evenly in contact with the solutions²⁰.

To contemplate the impact of NPs on seed germination, the 12h Cu₂O NPs treated seeds in various nanoparticle concentrations were exchanged onto wet cotton two fold layer filter paper in transparent box having size 15* 6.5 cm for 14 days until two cotyledon leaves arise and in each treatment, fifty seeds were taken, all kept 1cm apart from each other. 10 mL of appropriate concentrations of NPs arrangement was included and distilled water was used as control. After a time of 14 days, the tomato seedlings were randomly taken out to quantify the seed germination parameters, chlorophyll content, antioxidant properties and nanoparticle uptake by the plant.

Seed germination percentage

The seed germination was recorded from 2-6 days interval. Seeds were considered as germinated when their radicle showed at least 2mm length. The germination percentage was calculated according to formula²¹.

Germination percentage (GP %) = (Gf/n) × 100

Where, 'Gf' is the total number of germinated seeds at the end of the experiment, 'n' is the total number of seeds used in the test.

Root and shoot length

The root and shoot length was measured at 14 days after treatment using a meter scale and expressed in centimetre.

Seedling vigour index

The seedling vigour index (VI) was calculated by using the method suggested by ²².

Vigour Index =

$$(\text{Mean root length} + \text{Mean shoot length}) \times \text{Germination (\%)}$$

Biomass study

The randomly selected 10 plants after 14 days were washed with distilled water, blotter dried and fresh weight was determined. Dry weight was determined after drying the plant material in oven at 70°C for 24h and recorded²³.

Estimation of leaf Chlorophyll pigment

Chlorophyll estimation was carried out as describe by Lichtenthaler²⁴ and expressed in mg/g fresh weight. Briefly seedlings were ground in 5ml of 80 % of acetone and supernatant was measured at 470, 645 and 663 nm. Leaf pigments were analysed by using the formula

$$\text{Chla (mg/g FW)} = 12.21 \times \text{A663} - 2.81 \times \text{A645}$$

$$\text{Chlb (mg /g FW)} = 20.13 \times \text{A645} - 3.27 \times \text{A663}$$

$$\text{Carotenoids (mg/ g FW)} =$$

$$[1000 \times \text{A470} - 3.27 \times (\text{Chl a}) - 104(\text{Chl b})]/198$$

Were, ΔA = Absorbance at respective wavelength, V = Volume of extract (ml), W = Fresh weight of the sample (mg)

Antioxidant enzyme activities

Preparation of crude enzyme extract

Crude enzyme extract was prepared by grinding tomato plant seedlings (500mg) of 14 days old in 10ml of 50mM sodium phosphate buffer (pH 7) containing 5mM EDTA. All the steps in the extraction process were carried out at 0 to 4 °C. The activity of enzyme assay was expressed as μM/ml/min.mg of protein (Activity (U)/mg of protein).

Superoxide dismutase (SOD: EC 1.15.1.1) Assay

SOD activity was carried out by measuring inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described Mirsa and Fridovich²⁵ with some modification. Briefly, the reaction mixture contained 0.1 ml of enzyme extract, 0.5 ml of sodium phosphate buffer, 1ml of Na₂CO₃, 0.4ml of NBT and 0.2 ml of EDTA and 0.4ml of Hydroxylamine hydrochloride. After 15min incubation the absorbance was recorded at 560nm against the blank without extract. The activity was calculated using molar extinction coefficient of 4020 M⁻¹ cm⁻¹.

Glutathione Peroxidase (GPX: EC1.6.4.2) Assay

It was measured according to Mohandas et al. ²⁶ with some modification. Briefly, reaction mixture contained sodium phosphate buffer, EDTA, sodium azide, 0.1 ml of extract, glutathione, NADPH, hydrogen peroxide, and 0.1 ml of PMS. The activity was calculated as μmol NADPH oxidized/min/mg of protein using molar extinction coefficient of 6220 M⁻¹ cm⁻¹ at 560nm.

Catalase (CAT: EC 1.11.1.6) Assay

Catalase activity was carried out as described by Aebi²⁷ at 240nm using a molar absorption of 43.6M⁻¹cm⁻¹ by monitoring the decrease in absorbance resulting from the decomposition of H₂O₂. The reaction mixture contained 1.5ml of sodium phosphate buffer, 0.5 ml of H₂O₂ and 0.1 ml of enzyme extract. The activity expressed using coefficient of absorbance at 40 M⁻¹ cm⁻¹.

Phenyl Alanine Aminolyase (PAL) (PAL: EC 1.10.3.1) Assay

PAL assay was carried out as given by Goldson and co-workers²⁸ by using L-phenylalanine as a substrate. The total

volume 3ml of reaction mixture contained 0.1 ml enzyme extract with 50 mM potassium phosphate buffer (pH 7) and 200 mL of substrate and incubated at 37° C for 15min. The change in the activity was measured for 1min at 290nm and activity was expressed using molar absorptivity of 19.73 $\text{mM}^{-1} \text{cm}^{-1}$.

PolyPhenol Oxidase (PPO) Assay

Polyphenol oxidase activity was determined as per the procedure given by Mahadevan and Sridhar²⁹. The reaction mixture consists of 3.0 ml of 0.5 mM sodium phosphate buffer (pH 6.5) and 0.1 ml of the enzyme extract. To start the reaction 1.0 ml of 0.01 M catechol was added and the rate of increase in absorbance was measured at 420 nm for one min. The activity was expressed as change in absorbance/min/mg protein using a molar absorptivity of 1010 $\text{M}^{-1} \text{cm}^{-1}$.

Antioxidant properties

Determination of Malondialdehyde (MDA) content: Lipid peroxidation was determined by measuring MDA formation using the thiobarbituric acid (TBA) reaction³⁰. Briefly the reaction mixture contained 2mL trichloro acetic acid leaf extract, 2mL 0.6%TBA and the mixture was incubated in boiling water for 30min and the absorbance was recorded at 532nm and 450nm and 600nm against TCA blank. After subtracting the non-specific absorbance at 600nm, the MDA content was determined by its extinction coefficient of 155 mM/cm and is expressed as nano moles per ml of fresh weight.

Determination of free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method

Protocol of Lee et al.³¹ was followed with some modifications. The reaction mixture containing 0.5ml of methanolic extracts of sample and 1.5ml of 0.2 mM DPPH methanol solution was incubated at dark for 30min at room temperature and absorbance was recorded at 515nm. The scavenging activity of the extract was calculated using the formula and expressed as percentage of DPPH discoloration using the equation.

$$\% \text{ DPPH scavenging} = (\text{AC} - \text{AS} / \text{AC}) \times 100$$

Where, 'AC' is the absorbance of the control and 'AS' is the absorbance of the sample.

Determination of Total Antioxidant Capacity (TAC)

The reaction mixture contained 0.5ml of extract with 0.45ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The extract was kept in water bath at 95°C for 90min and then cooled. The absorbance was measured at 695nm against methanol and expressed as the number of milligram equivalent of ascorbic acid (GAE) per gram of fresh weight³².

Nanoparticle uptake in plant tissues by Atomic Absorption Spectrometry

Tomato seedlings 0.1–0.4g were pre-treated with 1% HNO_3 solution for 24h heated to 80°C for 2h in sand bath. Then, 10mL of 70% per chloric acid was added and the solution was heated to 200°C until it became clear and filtered through 0.45 μm Whatman filter paper. The filtrate was analysed for Cu concentrations using inductively coupled plasma atomic absorption spectroscopy (Agilent technologies, 200 Series).

Estimation of Total Soluble Protein: By using BSA as a standard curve, protein concentration of leaf extract was determined by the method of Lowery et al.³³.

Statistical Analysis

Each treatment in the experiment was conducted with three replications and the results were presented as Mean \pm SE (Standard error). One-way ANOVA was conducted to identify significant differences between treatments, the differences were considered significant when $P < 0.05$.

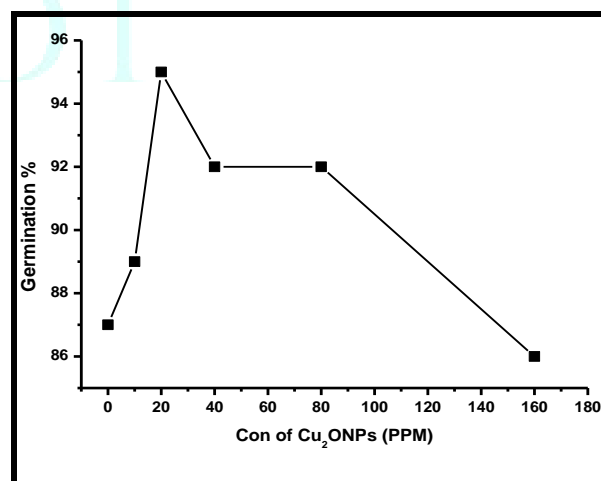
RESULTS AND DISCUSSION

Nanomaterials have many applications in agriculture in terms of plant protection and nutrition due to their size-dependent qualities, high surface-to-volume ratio and unique optical properties. Nanoparticles in the field of agriculture can act as potential candidates for modulating the redox status thereby changing the growth and development of the plants at various stages. The accumulation of NPs of metals at high levels in the plant not only impact their growth, but can cause phytotoxicity effect on plants and also pose a route for contamination of the food chain. Here we studied the effect of Cu_2O NPs on tomato (*Lycopersicon esculentum*) seed germination and seedling vigour.

Effect on Seed Germination

There is no negative effect of Cu_2O NPs on the tomato seed germination (Figure 1). All the treatments were prompted more than 85% of germination. The highest germination percentage 95% was observed at 20ppm Cu_2O NPs, however, above 20ppm NPs concentration there is a reduction in the seed germination as compared to control.

Seed germination gives an appropriate establishment for further plant development, improvement and good economic yield. During germination, the NPs were presumed to diffuse through nano gaps on seed coats, bringing about enhanced germination, followed by slow and minimal release of NPs and also probably due to the seed coat, which can act as protector for the embryo. In the meantime higher concentration on Cu_2O NPs acted as toxic for the development of plants which suggests that the seeds were likely stressed by the presence of Cu_2O NPs at higher concentrations. Similar results were reported by Singh and his co-workers³⁵.



Figures 1: Effect of Cuprous oxide nanoparticle on seed germination

The phytotoxicity of NPs was assessed according to the root and shoot length and biomass study. In the present study, the tomato seedlings showed increased root and shoot elongation up to 20ppm NPs concentration, further increase in NPs concentration caused the negative effect on plants growth and development. The root and shoot length (Figure 2) observed to be maximum of 12.4 ± 0.31 cm and $5.55 \pm$

0.24cm at 20ppm NPs concentration. The biomass study at 20ppm NPs concentration has demonstrated an increase in fresh weight to 0.28 ± 0.006 g per plant and dry weight to 0.13 ± 0.003 g per plant (Figure 3). The seedling vigour index which is calculated based on the biomass was also found to be highest (1605.10 ± 47.9) at 20ppm NPs concentration (Figure 4).

The impact of Cu NPs and its oxide forms has been studied on various plants which had given conflicting outcomes. De la Rosa and co workers¹³ reported the 20% decrease in seed germination and 50% decrease in root elongation with 800 and 1600 mg/l concentration of NPs and an increase in biomass weight at 250 mg/l of when tomato seeds treated with 8nm size ZnO NPs. Song et al.³⁵ reported the inhibition of root length, biomass and chlorophyll pigments at lower concentration of 40nm CuO on *L. minor*. Singh co-workers³⁴ have reported the plant exposure to 10mg/l NPs has showed increased in anti oxidant activity and above this concentration there is a decrease in the antioxidant activity in the Petriplate assay.

The major reason for the decrease in the root length might be due to aggregation of nano particles and stopping up of root pores along these lines which lessen the uptake of water by the seeds³⁶ and increment in the root length might be because of the antimicrobial activities that enhances the plant opposition against stress³⁷. Biomass production was in accordance with the root and shoot length of tomato seedlings. There is decrease in biomass under high concentration of cuprous oxide NPs treatment suggesting the toxic effect of NPs at higher concentrations. These results were similar to the results reported by Fatma et al.³⁸.

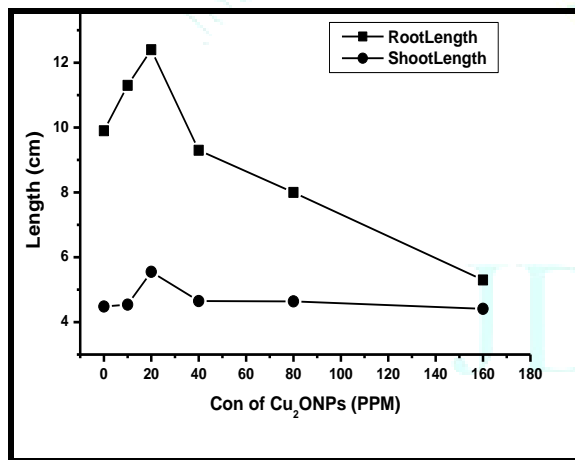


Figure 2: Effect of Cuprous oxide nanoparticle on Root and Shoot Elongation

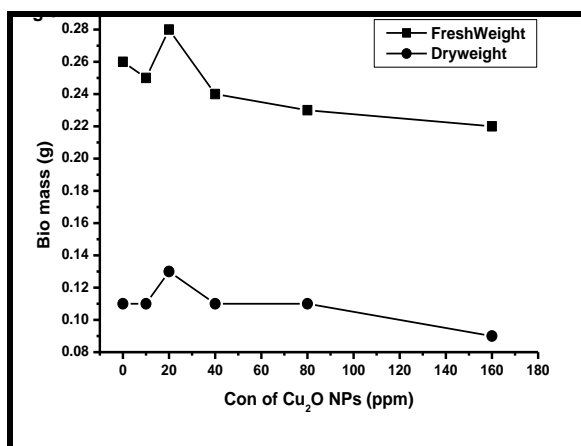


Figure 3: Effect of Cuprous oxide nanoparticle on Tomato Biomass gain

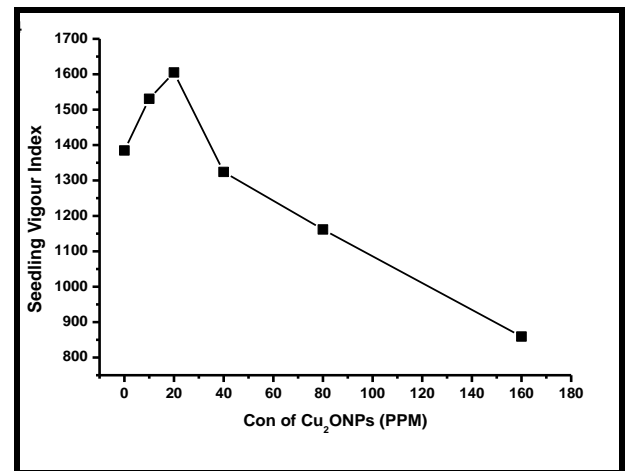


Figure 4: Effect of Cuprous oxide nanoparticle on Tomato Seedling Vigour

Effect on Photosynthetic Pigments (Chl-a, b and Carotenoids)

The leaf pigments showed increasing trend in tomato plants after treatment with Cu₂O NPs up to 20ppm as compared to control and observed to be 16.94 mg/g fresh weight (FW), 14.11 mg/g FW and 21.24 mg/g FW (Figure 5) for Chl-a, Chl-b and Carotenoids respectively. The concentration of leaf pigments at 160ppm NPs was found to be lowest 8.47 ± 0.31 mg/g FW, 7.82 ± 0.49 mg/g FW and 10.70 ± 0.096 mg/g FW respectively for chl-a, chl-b and carotenoids. However the outcomes were statistically significant in comparison to control.

The increase in photosynthesis rate is affected by NPs might be because of correlative impact of Iron (Fe), Magnesium (Mg), and sulphur (S) on plants. The NPs can enhance the structure of chlorophyll, better capture of daylight and transfer of light energy to active electrons, increase light absorbance and facilitate formation of pigments and chemical activities³⁹. The decrease in the photosynthetic pigment may be due to formation reactive oxygen species (ROS) by NPs at higher concentration. The similar outcomes were reported by Fatma et al.³⁸ when tomato plant exposed to Ag NPs. Kareem et al.⁴⁰ reported decrease in the pigments concentration when *C. sativus* exposed to Cu NPs.

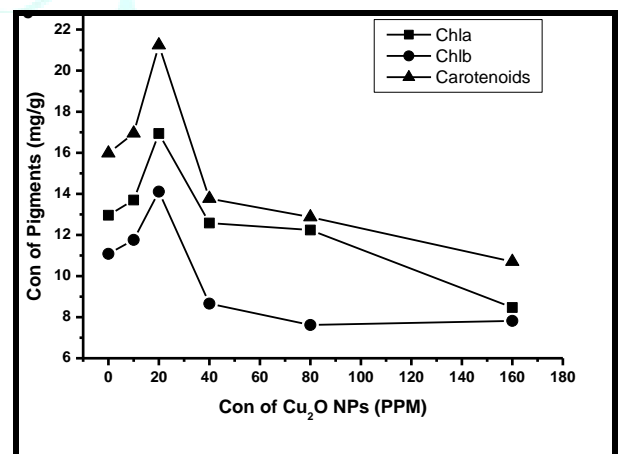


Figure 5: Effect of Cuprous oxide nanoparticles on Leaf Pigments of Tomato

Effect on Antioxidant Enzymes content

The study of effect of NPs on antioxidant enzymes showed increases in activity with increase in NPs concentration for two enzymes, Super oxide dismutase (SOD) and Glutathione Peroxidase (GPX) out of five enzymes treated. The activity of

SOD was found to be highest 13.47 ± 0.26 U/mg of protein and for GPX, 18.61 ± 0.17 U/mg of protein at 160ppm. The activity of Catalase (CAT), Phenyl Alanine Aminolyase (PAL) and Poly Phenol Oxidase (PPO) enzymes were found to increase up to the concentration of 20ppm as compared to control and above 20ppm NPs concentration, all three enzymes showed decrease in activity (Figure 6). Catalase enzyme showed lower activity than the control at 160ppm Cu_2O NPs treatment whereas the PAL and PPO showed lesser activity but more than the control even at highest NPs concentration.

During the stress condition the plants produce antioxidant enzymes which will scavenge the free radicals in the plant body. The SOD is a critical antioxidant enzyme having antioxidant impact against on superoxide anion, GPX scavenges peroxy, CAT acts on H_2O_2 thereby keep up the functional integration of the cell membrane. In present investigation the SOD and GPX level was increased as compared to control at higher levels of NPs and this may be due to scavenging activity of the enzymes on the free radicals present in the plant. Hence in the present perception there was no significant damage observed in tomato plant. Further increase in concentration of NPs, the activity of the enzymes also increased due to ROS creation in the plant. The CAT, PPO and PAL activity initially increased and later decreased this may be due to the plants have produced more free radicals under NPs stress and the activities of antioxidants were not stimulated to the optimum level, thus the damage caused by free radicals was not counteracted.

The simultaneous decreasing and increasing trend in the different enzymes were also observed by Hesham et al.⁴¹ where they have reported increased activity of SOD and GPX activities at 15 mg/l and 30 mg/l concentration of ZnO-NP in callus of tomato plant. Rao and Shekhawat⁴² reported increasing level of antioxidant enzyme APOX, CAT, SOD with different amounts of CuO NPs (200, 500, 1000, and 1500 mg/L).

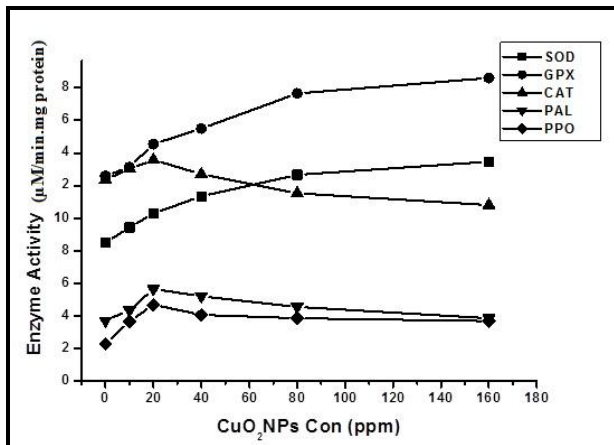


Figure 6: Effect of Cuprous oxide nanoparticles on activity of different Antioxidant enzymes (SOD: Super Oxide Dismutase, GPX: Glutathione Peroxidase, CAT: Catalase, PAL: Phenyl Alanine Aminolyase and PPO: PolyPhenol Oxidase)

Effect on antioxidant properties

Membrane damage was estimated as the contents of malondialdehyde (MDA). The MDA content was found to increase as the concentration of NPs increase and it was found to be maximum (0.84 ± 0.033 nmol/ml) at 160ppm treatment as compared to control (0.11 ± 0.006 nmol/ml) (Figure 7). The increased level of MDA in plant cells is due to stress created by NPs which was associated with an increase in protein content and defense enzyme activities. Song and

his co-worker³⁵ have reported increased MDA content in *L. minor* when exposed to Cu_2O Nps at 50 mg/l.

Figure 8 shows the dose-response curve of DPPH radical scavenging activity of the aqueous extracts of the tomato seedlings. It was observed that 20 and 40ppm treatments showed more scavenging activity (50%) than other concentrations of Cu_2O NPs treatments. As the concentration increases beyond 40ppm, the scavenging activity was reduced and this might be due to the fact that the plants produce more ROS which is induced by Cu_2O NPs.

The antioxidant activity of the extracts was determined by the phosphomolybdenum method. The total antioxidant activity (TAC) of the extracts increased with the increased concentration of the Cu_2O NPs (Figure 9). The TAC was found to be highest (4.66 mg GAE/g FW) at higher concentration of NPs (160ppm). The results were supported by Zaka et al.⁴³ study where they have reported that the TAC was found to be $7 \mu\text{g}$ Ascorbic acid/mg FW and DPPH 12% on *Eurica sativa* when exposed to Cu_2O NPs.

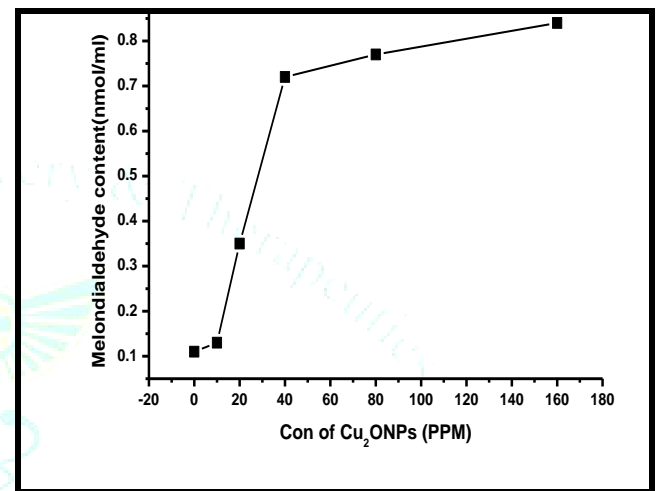


Figure 7: Effect of Cuprous oxide nanoparticle on Malondialdehyde content in tomato

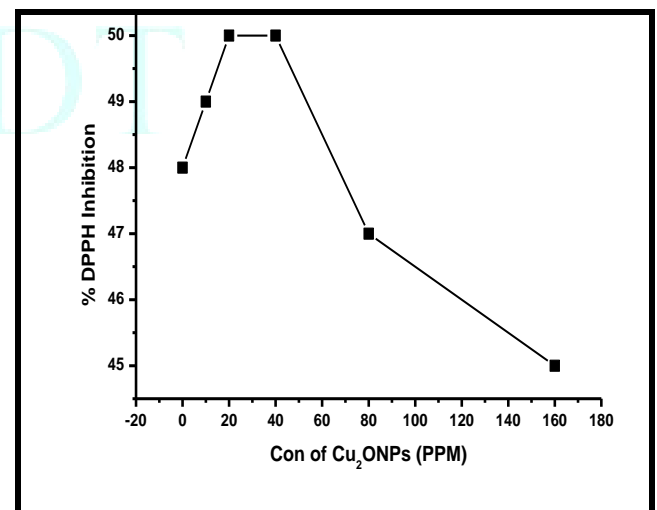


Figure 8: Effect of Cuprous oxide nanoparticle on DPPH activity.

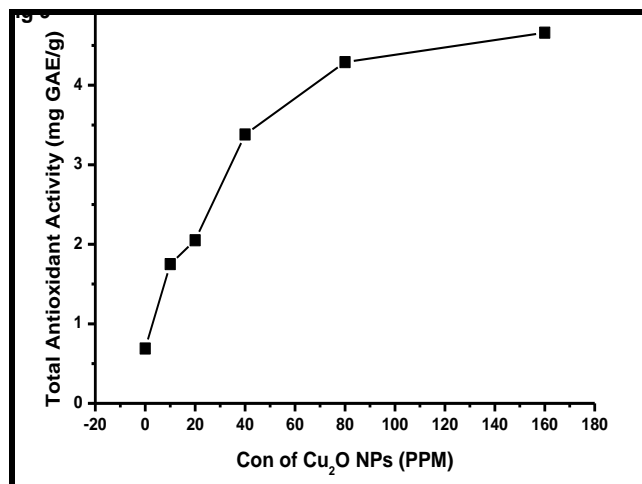


Figure 9: Effect of Cuprous oxide nanoparticle on Total Antioxidant activity

Nanoparticle uptake by tomato seedlings

The uptake of Cu₂O NPs by tomato plant seedlings showed increasing trend with increased concentration of NPs up to 160ppm, but the rate of increase in uptake of Cu₂O NPs was exponential only up to 20ppm (Figure. 10). At highest level of treatment (160ppm) of Cu₂O NPs, the uptake was found to be 0.25ppm as compared to control 0.005ppm. The uptake of different NPs including copper oxide nanoparticles by plants has been reported by many workers^{44, 45, 46}.

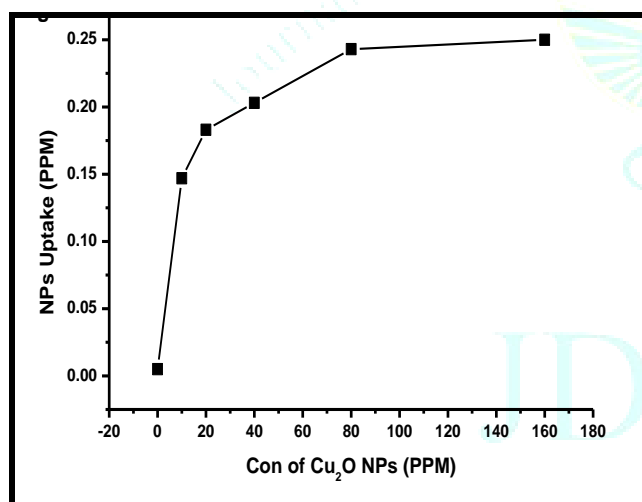


Figure 10: Uptake of Cu₂O NPs (PPM) by tomato seedlings estimated by Atomic absorption spectrometry

CONCLUSION

The cuprous oxide nanoparticles have enhanced the germination at lower concentration up to 20ppm. The exposure of tomato seedlings to cuprous oxide nanoparticles showed dose dependent response and maximum promotory activity of Cu₂O NPs was seen at 20ppm. At higher concentrations, there is a decline in the trend for all parameters under study except for SOD and GPX. Hence by judicious use of Cu₂O NPs at appropriate concentrations, the growth and development of the plant can be improved to greater extent in the plants. Further, there is more work to be done at green house and field conditions for determining the impact of NPs on plants for growth and development.

Conflict of Interest

There is no conflict of interest.

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