Tropical Plant Research
An International Journal
© Society for Tropical Plant Research

ISSN (E): 2349 – 1183
ISSN (P): 2349 – 9265
4(2): 246–253, 2017
DOI: 10.22271/tpr.2017.v4.i2.034

Research article

Synthesis and characterization of copper oxide nanoparticles and its impact on germination of *Vigna radiata* (L.) R. Wilczek

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Abstract: Nanoparticles (NPs) and its disposal via anthropogenic activities are a new concern nowadays. The impact of copper on plant metabolism has not yet been studied in detail. In this study, Copper oxide nanoparticles (CuO NPs) were synthesized by wet precipitation method using copper acetate monohydrate precursor. Powder X-ray diffraction, particle size analyzer, scanning electron and transmission electron microscope analysis revealed that synthesized CuO NPs are in nano size range. The impact of synthesized NPs on germination and seedling growth of *Vigna radiata* were tested. Germination and seedling growth of the plants were almost unaffected at lower concentrations while significant inhibition was recorded at highest 1000 $mg.l^{-1}$ concentration of NPs as compared to control. Protein and sugar content were used as biochemical parameters to estimate the effects of different concentration of CuO NPs on the metabolism of seedlings. The amount of sugar and protein registered slight higher value at lower concentration and sharply decreased with increase in concentration. By analyzing this compiled information, it is evident that CuO NPs exhibits a toxic effect on growth and physiology of test plant while at very low concentration effect of NPs may be little favorable also.

Keywords: Anthropogenic activities - CuO NPs - Wet precipitation - Biochemical parameters.

[Cite as: Singh A, Singh NB, Hussain I, Singh H & Yadav V (2017) Synthesis and characterization of copper oxide nanoparticles and its impact on germination of *Vigna radiata* (L.) R. Wilczek. *Tropical Plant Research* 4(2): 246–253]

INTRODUCTION

Nanoparticles (NPs) are natural or anthropogenic materials having at least two dimensions between 1–100 nm. NPs possess numerous distinctive properties like high reactivity, unique optical properties and bio-compatibility in comparison to their bulk counterparts due to their very high surface area to volume ratio (Garima *et al.* 2011). In recent years, nanomaterials (NMs) have contributed in economy of many sectors including consumer products, energy, transportation, cosmetics, pharmaceuticals, antimicrobial agents and agriculture (Singh *et al.* 2015a). Nanotechnology has tremendous potential to boost agricultural production to fulfill food, fodder and fiber requirements of humans. Use of NPs as fertilizer has a great potential to augment crop yield and diminish environmental threats (Singh *et al.* 2013, Roohizadeh *et al.* 2015). On the other hand extensive release of NPs in the environment causing toxicity to the flora and fauna also (Franklin *et al.* 2007). The hazardous effects of metal oxide NPs can be due to the dissolved ionic metal, or there may be special hazards from NPs themselves due to improved bioactivity. Many authors have pointed out the importance of difference in the toxicity of NPs from heavy metal toxicity (Franklin *et al.* 2007, Griffitt *et al.* 2007, Karlsson *et al.* 2008).

Among the different types of metallic NPs, Copper oxide nanoparticles (CuO NPs) have been mainly used as preliminary material due to its natural abundance, low cost production, non-toxic nature with good electrical and optical properties. Copper (Cu) have position in block D and period four of the periodic table and it is a microelement required for the development of plant. Passam *et al.* (2007) reported that Cu in 10^{-14} to 10^{-16} M concentrations is required for normal development of plant, below which deficiency occurs however higher concentration than optimum exhibited toxicity. The Cu deficiency in plants is expressed as curled leaves;

Received: 29 October 2016 Published online: 30 June 2017
https://doi.org/10.22271/tpr.2017.v4.i2.034

petioles bent downwards and light chlorosis as well as permanent turgor loss in the young leaves while the higher concentration of Cu leads to toxicity, growth inhibition, photosynthesis interferences, photo respiration and increases oxidative stress (Yruela 2005, Passam *et al.* 2007, Manceau *et al.* 2008). The extensive use of CuO NPs has increased concerns over their potential toxic impacts on ecosystem and human health due to their discharge from different products to the environment (Chen *et al*. 2012). Previous reports have revealed that exposure to CuO NPs caused toxic effects on aquatic organisms such as protozoa, crustaceans, algae, and zebra fish (Nair & Chung 2014). The effect of Cu NPs on plant cells has not been studied sufficiently and the available results are uncertain. It has been reported that Cu is biologically available to mung bean and wheat germs (Lee *et al.* 2008). Cu exerts its toxic impact by penetrating the cell directly, apparently by causing oxidative damage to cell structures and molecules (Garnett 2007). Cu NPs show positive effects on germination (Shah & Belozerova 2009) but are phytotoxic at seedling growth (Lee *et al.* 2008).

Current progress in the field of nanotechnology has guide to the development a new perception of synthesizing nano sized particles of preferred size and shape (Kumar *et al.* 2015). Synthesis of metal NPs can be accomplished through various methods. Chemical approaches are the most popular methods for the production of NPs. There are several schemes for the synthesis of CuO NPs like precipitation methods, decomposition methods, plasma methods, pulsed wire explosion methods, sol gel methods, vapour deposition, electrochemical, radiolysis methods and so on (Hussain *et al.* 2016).

The purpose of this study was to synthesize CuO NPs via wet precipitation method and to analyze specific effect of CuO NPs at different concentration on the germination and growth of *Vigna radiata* (L.) R. Wilczek as it is one of the most important staple food crops in India.

MATERIALS AND METHODS

Synthesis of copper oxide nanoparticles

CuO NPs were synthesized through wet precipitation method (Zhu *et al.* 2011). Copper acetate monohydrate (99%), glacial acetic acid (99%) and sodium hydroxide were used without further purification as they are of analytical grade.

In a typical procedure, about 0.2 M of Copper acetate monohydrate ($Cu(CH_3COO)_2.H_2O$) was dissolved in 300 ml of water in round bottomed flask and 1 ml of glacial acetic acid (CH_3COOH) was added into that flask and heated to boiling under continuous stirring. After 30 minutes 15 ml of 8 M sodium hydroxide (NaOH) solution was gradually tipped as reducing agent with the help of a needle into the flask. The colour of the solution turned black from blue immediately after addition of NaOH. The reaction was carried out under stirring for 2 hrs, great quantity of black precipitate appeared. The mixture was cooled in room temperature and then centrifuged, obtained wet precipitate was washed twice with DDW to eliminate the impurity. The precipitate was then dried at room temperature for 48 hrs. Brownish black colored powder was obtained and this was carefully collected and stored for characterization.

Characterization of synthesized copper oxide nanoparticles

First round characterization of the CuO NPs was carried out by X-Ray diffraction (XRD). The XRD pattern of the powdered metal was recorded with the XRD equipment *i.e.* Rigakud'max 2200 diffracto meter with Cu-Kα radiation (k = 1.5406 Å). Particle size distribution of the CuO NPs was measured by computer controlled particle size analyzer (Nanotrac wave W3372) to find out the particle size distribution. SEM studies of the synthesized particles were done by JEOLJXA-8230. The synthesized CuO powder was mixed into DDW and sonicated for 30 min. A tiny drop of the sample was permitted to dry on glass slide to make a thin layer of NPs for the SEM investigation. TEM images were captured on a TECNAI 200 Kv TEM instrument. The nano powders were diluted with DDW and dispersed by ultrasonic bath. Then, one drop was placed on a carbon-coated copper grid and left to dry at room temperature.

Germination and seedling vigor index

Synthesized CuO NPs were suspended directly in DDW and discrete by ultra-sonic vibration for 30 min. The solutions for seed treatments were prepared in five different concentrations: 1 $mg.l^{-1}$, 10 $mg.l^{-1}$, 100 $mg.l^{-1}$, 500 $mg.l^{-1}$ and 1000 $mg.l^{-1}$ and labeled as Cu1, Cu2, Cu3, Cu4 and Cu5 respectively. Ten vigna seeds in triplicates were soaked in 20 mL of respective concentration for 3–4 hrs. The seeds imbibed in DDW only were taken as control and named as CuC. Treated vigna seeds were placed at equal distance in sterilized petriplates lined with Whatman No 1 filter papers. 2 ml of respective concentrations was added in each petriplate. Petriplates were covered and kept in a dark place for seven days. Seed germination and seedlings growth were recorded at regular interval of 24 hrs. Seedling vigor index (SVI) was calculated by the formula following Abdul-Baki & Anderson (1973).

$$\text{Seedling vigor index} = \text{Germination\%} \times (\text{root length} + \text{shoot length})$$

Protein and sugar content

Quantitative analysis of protein was done following Lowry *et al.* (1951). The quantity of protein was measured with reference to a typical curve of bovine serum albumin. The absorbance was calculated at 650 nm. Total soluble sugars quantification was done by following Hedge & Hofreiter (1962). The quantity of sugar was determined by the standard curve prepared from glucose. 0.05 mg fresh leaf tissue was homogenized in 5 ml of 95% ethanol. Following centrifugation, 1 ml of supernatant was thoroughly mixed with 4 ml anthrone reagent and heated for 10 min in boiling water bath. Following cooling, the absorbance was recorded at 620 nm.

Statistical Analysis

Treatments were arranged in a randomized block design with three replications. Data were statistically analyzed using ANOVA by using SPSS (Ver.10; SPSS Inc., Chicago, IL, USA). Fitting standard errors of the means (±SEM) were calculated for presentation of graphs. The treatment means were evaluated by Duncan's multiple range test (DMRT) at $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis of copper oxide nanoparticles

The conventional and well known method for synthesis of metallic NPs is wet-chemical procedure. This method involves growing of NPs in a liquid medium having different reactants, in particular reducing agent. The stabilizing agent is also added to the reaction mixture to avoid the agglomeration of NPs (Singh *et al.* 2016). Colour change of the reaction mixture was observed from deep blue to colour less and then to dark brown on vigorous stirring (Fig. 1). In this process, CuO produced due to reaction between Cu^{++} ions from copper acetate monohydrate and reducing agents *i.e.* NaOH. The synthesis process is based on the following chemical reactions:

$$Cu^{2+} + 2NaOH = Cu(OH)_2 + 2Na^+ \quad (1)$$
$$Cu(OH)_2 = CuO + H_2O \quad (2)$$

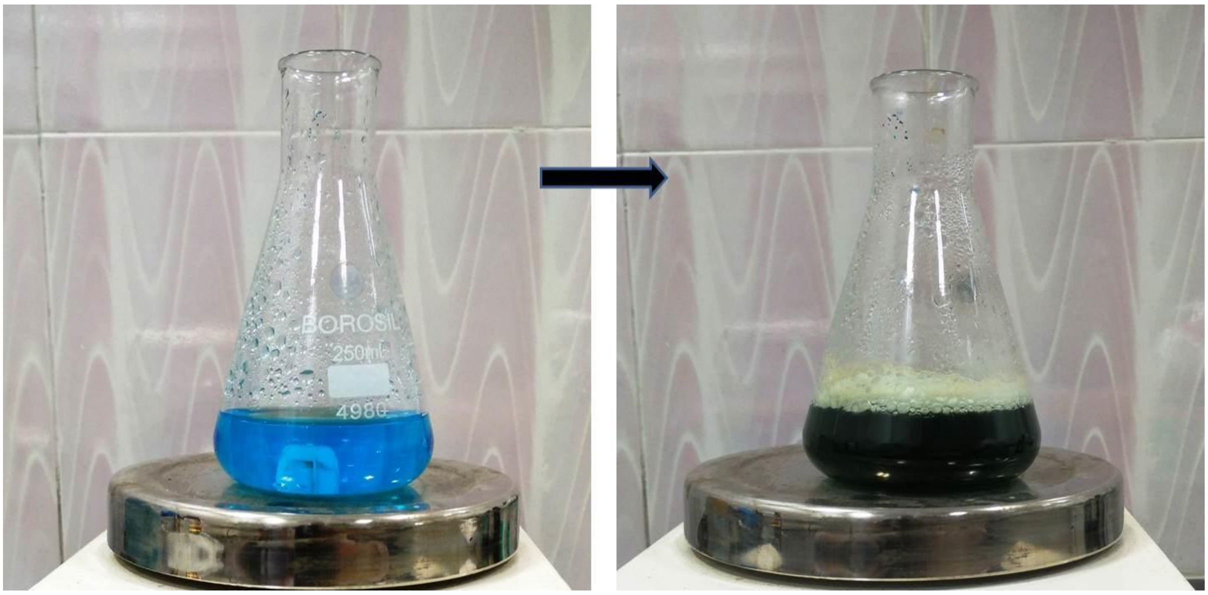

Figure 1. Picture of aqueous solution of $Cu(CH_3COO)_2H_2O$ before adding NaOH and after addition of NaOH.

Characterizations of copper oxide nanoparticles

X-ray diffraction (XRD) is recognized as an analytical method for detection and quantitative determination of various crystalline forms which are also known as 'phases' of compound there in powder and solid samples. The XRD technique was used to determine and confirm the crystal structure of the NPs. The XRD patterns of as synthesized CuO NPs are shown in figure 2(A). The characteristic peaks located at $2\theta = 30.5, 38.97°$ and 48.

74° are assigned to (111), (200) and (202) plane orientation of CuO (JCPDS 80-1268). XRD pattern showed broad diffraction peaks indicating crystalline and nanoscale dimensions of particles. All diffraction peaks can be indexed as typical monoclinic in structure and no other phases were observed.

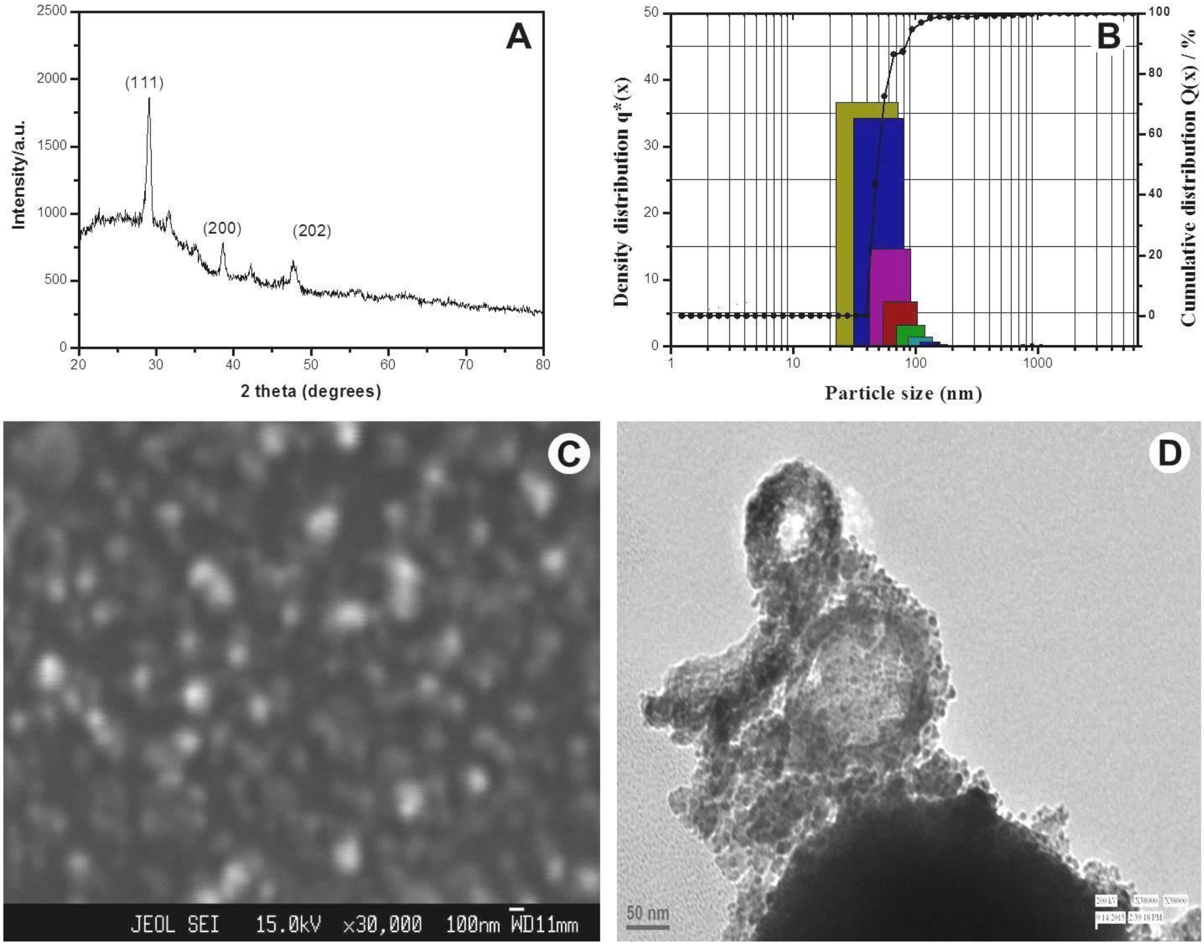

Figure 2. Characterization of synthesized CuO NPs (A) X-ray diffraction pattern of the synthesized CuO NPs (b) Particle size distribution image of the synthesized CuO NPs (C) SEM image of the synthesized CuO NPs (D) TEM image of the synthesized NPs.

Dynamic light scattering (DLS) is a method used to decide the size distribution profile of minute particles in suspension or polymers in solution. The size of the particles depends on the size of the size of surface structure, the particle core, concentration of particle and the type of ions in the medium. Particle size analyzer study exposes that the most of the particles in the solution are in range of 35 to 40 nm (Fig. 2B).

SEM image (Fig. 2C) of CuO NPs demonstrated the presence of some large particles, which can be credited to aggregation or overlapping of smaller particles with sizes around 100 nm. TEM image (Fig. 2D) showed a not well-dispersed and spherical shape for green synthesized CuO NPs with a size range of 20 to 40 nm. The larger particle size is due to the short clusters shown in the TEM image.

It is interesting to note that there is variation in the size of NPs measured with different characterization tools. This can be attributed with the findings of Singh *et al.* (2016), where authors stated that XRD measures the crystalline size which is smaller than the real particle size while diameter measured using DLS is hydrodynamic diameter of NPs, which is bigger than the actual particle size. In DLS measurement liquid layer moves with NPs during Brownian motion which results in the increased size of NPs than actual size.

Germination and seedling vigor index

Seed germination was recorded up to 6 days. CuO NPs did not show any toxicological effect on germination up to 500 $mg.l^{-1}$ concentration. Effect of different concentration of CuO NPs on seedling growth of *Vigna radiata* (L.) R. Wilczekis shown in figure 3. However slight enhancement in seed germination was recorded in lower concentrations of NPs *i.e.* 1 and 10 $mg.l^{-1}$. Highest concentration *i.e.* 1000 $mg.l^{-1}$ of NPs adversely affected the seed germination. Maximum 27% of inhibition is found in seed germination in seeds treated with highest concentration (Fig. 4A). Seed germination is the beginning of the physiological process that needs water imbibitions (Kathiravan *et al.* 2015). Enhanced seed germination in lower concentration of NPs may be attributed with the findings of Khodakovskaya *et al.* (2012) as stated that NPs have tendency to penetrate plant seed coats and enhance seed germination and growth. CuO NPs would have penetrated the cell wall and guides new pores formation as a result increase in water absorption which is favorable for seed germination. Germination of lettuce seeds was promoted by Cu NPs (14). In contrast to our result Adhikari *et al.* (2012) reported that the germination of soybean and chickpea was not checked up to 2,000 ppm concentration of CuO NPs. Seedling growth and seedling vigor index (SVI) also followed the same trend.

Figure 3. Image showing effect of CuO NPs on seed germination and seedling growth of *Vigna radiata* (L.) R. Wilczek seedling. CuC = Control; Cu1 (1 mg.l^{-1}); Cu2 (10 mgl^{-1}); Cu3 (100 mg.l^{-1}) Cu4 (500 mg.l^{-1}); Cu5 (1000 mg.l^{-1})

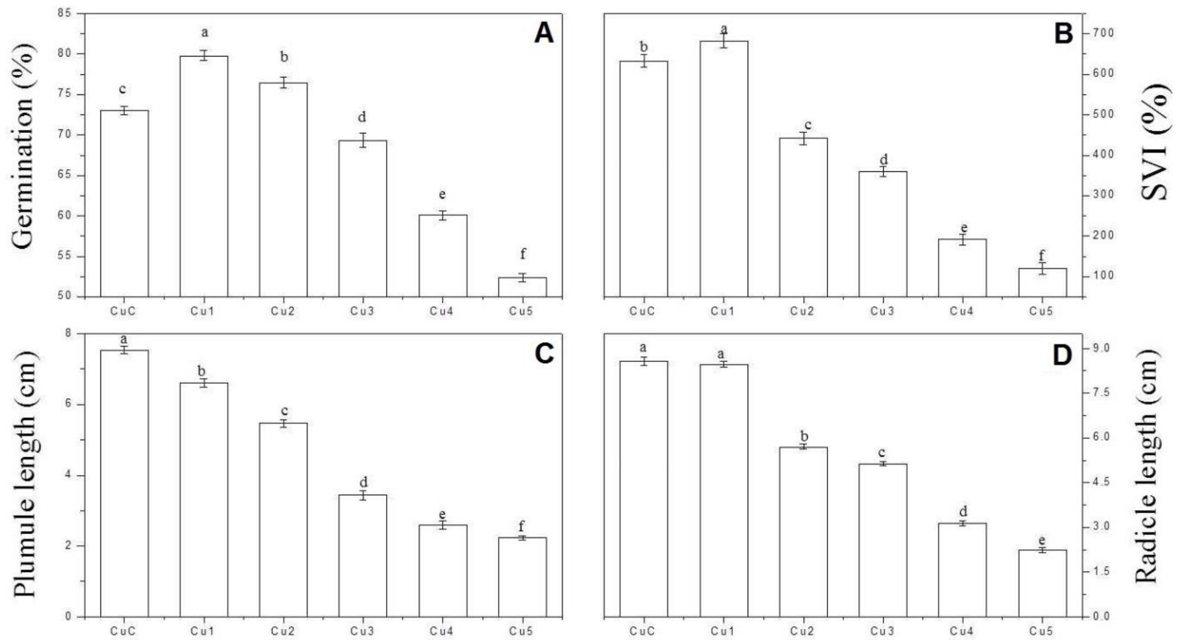

Figure 4. Effect of CuO NPs on (A) seed germination, (B) seedling vigor index (SVI), (C) plumule length and (D) radicle length of *Vigna radiata* (L.) R. Wilczek seedling. Data are means ± standard error of three independent experiments with three replicates in each experiment. Bars followed by different letters show significant differences at $p < 0.05$ significance level between treatments according to the Duncan's multiple range test. CuC = Control; Cu1 (1 mg.l^{-1}); Cu2 (10 mg.l^{-1}); Cu3 (100 mg.l^{-1}) Cu4 (500 mg.l^{-1}); Cu5 (1000 mg.l^{-1})

Radicle (RL) and plumule length (PL) also followed the germination result. However it is interesting to note that radicle length was found more susceptible to CuO NPs. There was no promoting effect found in RL at any concentration of NPs however decrease RL was concentration dependent. Declining trend in RL and PL at concentrations higher than 1000 mg.l^{-1} might be due to more absorption of NPs leading to phytotoxic effects. In this study seed germination was not significantly decreased by NPs because NPs could not pass through seed coats. NPs could contact radicals directly after its emergence through seed coat. Since roots are the first tissue to confront with high concentration of NPs therefore altered growth in radical has dose dependent response. NPs clog with root openings thus nutrient uptake inhibited resulted in decreased plant growth (Adhikari *et al.* 2012). It was observed that with increase in NPs concentration PL also was found to decline as PL is dependent on the growth and health of radicle. Effect of CuO NPs seems concentration dependent and higher concentration may be harmful to plants. Maximum inhibition of 74 and 70% was recorded in RL and PL respectively (Fig. 4). Toxic effects of Cu-NPs in various studies reporting that concentration were higher than 200 ppm (Doshi *et al.* 2008, Shah & Belozerova 2009). For mung bean seedling the best growth response for radicle and plumule was observed at lowest concentration. Pätsikkä *et al.* (2002) and Yruela (2005) stated that elevated concentration of Cu may injure thylakoid membranes, in that way distressing the functioning of photosystem II and the water-oxidizing complex of chloroplasts. There is 7% stimulation was recorded in the SVI of the seeds treated with 1 mg l^{-1} concentration.

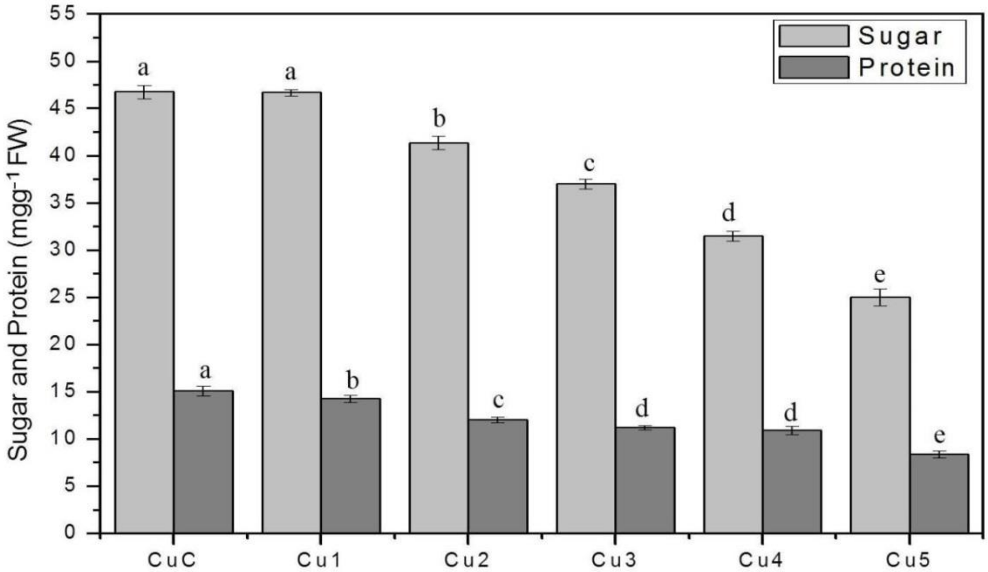

Figure 5. Effect of CuO NPs on sugar and protein content of *Vigna radiata* (L.) R. Wilczek seedling. Data are means ± standard error of three independent experiments with three replicates in each experiment. Bars followed by different letters show significant differences at $p < 0.05$ significance level between treatments according to the Duncan's multiple range test. CuC = Control; Cu1 (1 mg.l^{-1}); Cu2 (10 mg.l^{-1}); Cu3 (100 mg.l^{-1}) Cu4 (500 mg.l^{-1}); Cu5 (1000 mg.l^{-1})

The increase in CuO NPs concentrations on *vigna* seedlings appeared to be negative, primarily due to reduced sugar and protein content. A decrease in sugar and protein under unfavourable conditions allows the conservation of energy, thereby launching the appropriate defense response and also reducing the risk of damage. The protein and sugar content of seedling decreased significantly, reaching the minimum value in the plant treated with 1000 mg.l^{-1} of CuO NPs (Fig. 5). Maximum inhibition of 44 and 46% was recorded in protein and sugar content of the seeds treated with highest concentration respectively. Negative effects of Cu-NPs on root (Adhikari *et al.* 2012), seedling growth (Shah & Belozerova 2009) and shoot growth (Stampoulis *et al.* 2009) on different plants have been reported. The significant reduction in protein and sugar content might be due to the reduced biomass upon exposure to higher concentrations of CuO NPs or due to the membrane damage under oxidative stress (Halliwell & Gutteridge 1989). It has been reported in various studies that metal oxide NPs at their high concentration alters the physiological processes of the plants (Singh *et al.* 2015b, Hussain *et al.* 2015).

CONCLUSION

Heavy use and release of NPs in the environment causing toxic impact on crop plants. Wet precipitation method was found to be very efficient in producing small sized CuO NPs. The influence of synthesized NPs on germination and seedling growth as well as protein and sugar content of *Vigna radiata* (L.) R. Wilczek was investigated. The results showed that exposure to higher concentrations of CuO NPs has resulted in significant reduction in the seed germination and caused retardation of seedling growth in test plant. Moreover, exposure to CuO NPs has resulted in inhibition of protein and sugar content of seedlings which affected plant metabolism. However, it is interesting to note that lower doses of CuO NPs were found to be not even non-toxic while favoured seed germination, seedling growth and metabolism of the test plant to some extent. Cu being a micro element is essential for growth and metabolism of plants. Further study is required to explore the minimum and favourable concentrations of CuO NPs which may be beneficial for plant growth and metabolism to increase the productivity as well as safe release in the environment. Moreover these findings can guide for further investigation of the mechanism of NPs transport within the plant and the potential implications of NPs in the food chain.

ACKNOWLEDGMENTS

The authors are thankful to the University Grant Commission (UGC), New Delhi, India and University of Allahabad, India for providing financial assistance to Ajey Singh. Dr. Vikas Baranwal, Nanotechnology Application Centre, A.U, Dr. M. Dwivedi, Centre for Petrology and Mineralogy A.U, MNIT, Allahabad and SAIF, AIIMS (New Delhi) are acknowledged for providing XRD, SEM, DLS and TEM facilities respectively.

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