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Comparative physiological and biochemial effects of CuO NPs and bulk CuO phytotoxicity onto the maize (*Zea mays*) seedlings

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Abstract

Plant nanotoxicology is an emerging and less-explored area of research for the plant stress biologists. The present study assesses the toxicity of bulk-CuO and nano-CuO (size <50 nm and surface area = 29 m^2 g^{-1}) on maize (*Zea mays* cv. hybrids triple white Hi Tech.) seedlings. Five successive levels of stress (25, 50, 100, 150 and 200 mg L^{-1}) suspensions of CuO and CuONP were imposed and seedling growth performance was studied along control at 8 days of experiment. Modulation of enzymatic antioxidants (SOD, CAT, APX and POD) and non-enzymatic antioxidants (total phenolics and total flavonoids) activities under both-CuO stresses were investigated in detail to get an overview of their-stress response of maize. Seed germination was completely stopped under 150 and 200 mg L^{-1} CuONP. Generally, CuONP stress was most violent and 100 mg L^{-1} CuONP showed the same or more drastic effect in 150 and 200 Cu bulk. Photosynthetic pigments, shoot and root lengths reduced under both stresses by about 50% of control. Soluble proteins and total antioxidants increased gradually till it reached about 200% of control at 150 mg L^{-1} CuO and about 200-300% at 100 mg L^{-1} CuONP. In addition, increased reducing power activity coupled with both stresses which reached to 400% of control at 150 mg L^{-1} CuO and 100 mg L^{-1} CuONP. Moreover, superoxide dismutase increased by 800% of control at 100 mg L^{-1} CuONP.

Keywords: Nanotoxicology, CuO, oxide, reducing power, H_2O_2 scavenging%, metal chelating%, multivariate analysis.

1. Introduction

Advances in nanotechnology include the incorporation of many metallic nanoparticles (NPs) into diverse industrial, household and medical products (Lee *et al.,* 2008; Navarro *et al.,* 2008). Although, many of them are useful, some are toxic to micro and macro-flora, including plants and our food-crops.Improper handling and disposal of NP-containing wastes could result in environmental contamination. Uncontrolled release of metal oxide nanoparticles (NPs) into the environment due to human activities has become a serious threat to the ecological system.

The development of nanotechnology in physiology and biochemistry has expanded the application area of nano-materials in different fields due to their unique characters such as large surface areatovolume ratio, ability to engineer electron exchange and highly surface reactive capability (Scrinis and Lyons, 2007).

In recent years, many scientists have studied the effects of these materials on seed germination and plant growth with the aim to promote its use for agricultural applications. Most of these studies are focused on the potential phyto-toxicity of nanoparticles in higher plants and both positive and subsequently negative or inconsequential effects were represented. NPs have the potential for harmful effects on plants and their associated soil microflora (Dimkpa *et al.,* 2011; Lin and Xing, 2008; Nair *et al.,* 2010). At sub-lethal concentrations, NPs variably modify the production of secondary metabolites from bacterial that involved in plant growth and production (Dimkpa *et al.,* 2012) andmay pose a route for contamination of the food chain (Hatami and Ghorbanpour, 2014).

Some nano-materials are toxic to flora and fauna as they are used to inhibit their growth to prevent further multiplication (Siddiqi and Husen, 2016a). The toxicity response depends on the concentration, particle shape and size of the se nano-materials (Siddiqi and Husen, 2016b). Toxicity of NPs depends on many factors such as their conformation, surface characteristics, such as the presence of coatings, and their state of aggregation (Barrena *et al.,* 2009). The impact of nanoparticles on higher plants appears to depend on the species and age of the plants, the experimental conditions such as temperature, the duration and method of exposure. Bio-uptake and accumulation of nano-materials in plants may increase shoot height and decrease root length (Atha *et al.,* 2012), and they recorded to be reach to the shoots (Lee *et al.,* 2008). The pathway of water and nutrient-transport has one mechanism proposed to account for how the NPs cause their damage to the plant (Lin and

Xing, 2008). In addition, these metallic NPs may release soluble metals (Lee *et al.,* 2008; Lin and Xing, 2008) that are taken up by the plant.

Growth of food plants such as lettuce, cucumber, bean, rye, corn, and zucchini is impaired, depending on the concentration of Cu, Ag, CuO, Zn, TiO_2, and ZnO NPs (Atha *et al.,* 2012; Barrena *et al.,* 2009; Du *et al.,* 2011). It was shown that 40 and 60 mg L^{-1} NanoSilver-treated *Pelargomium zonale* encourage an efficient cellular electron exchange mechanism, which slows down electron leakage and consequently reduces the ROS production and malonaldehyde (MDA) content (Lu *et al.,* 2002). It was reported that activity of specific antioxidant enzymes was induced in *Brassica juncea* seedlings treated with silver nanoparticles (Priyadarshini *et al.,* 2012). For this instance, 10 mgL^{-1} AgNPs was found to inhibit seed germination in *Hordeum vulgare*and reduced shoot length in flax and barley (El-Temsah and Joner, 2012).

Exposure to ions such as Al, Cd, Fe, Mn, Cu, Ni, Zn, and U results in many symptoms including loss of chlorophyll pigmentation, increased lipid membrane peroxidation, altered ferric iron metabolism, modification in the levels of plant growth regulators and activities of stress-inducible enzymes (Dimkpa *et al.,* 2008).

Many studies found that physiological indexes were positively affected by nanoparticle treatments during thermal treatments (Mohammadi *et al.,* 2013). Moreover, it was shown that Titanium nanoparticles (TiO_2) not onlyreducedoxidative damage but also alleviated membrane damage indexes (electrolyte leakage) under cold stress treatment in chickpea genotypes. In spinach, TiO_2 NPs increase RUBISCO activase that enhance photosynthesis and plant growth (Gao *et al.,* 2008).

Keller and Lazareva (2013) have reported that about 5500-3000 tons of TiO2NPs are produced every year and more than 50% of which is used in personal care products followed by 550 tons/year for ZnONP and Ag NPs is about 55 tons (Piccinno *et al.,* 2012). Copper-based nanoparticles (NPs) are profusely used due to their optical, thermal, electrical, antibacterial, and catalytic applications (Peralta-Videa *et al.,* 2016). Its global production in 2010 was estimated in 200 tons, 36 of which ended up in soil and 11 in bodies of water (Keller *et al.,* 2013). Very recently, nanoparticulate forms of copper are starting to be used in agriculture for specific purposes. For instance, it has been used as alternative to bulk-Cu products to combat fungal diseases (Servin *et al.,* 2015). The production of Cu-based nano forms is predicted to reach to unanticipated levels. However, little is known about their physiological and biochemical effects in many agricultural crops (Du *et al.,* 2017). From previous publications, it is known that nano-CuO, at different concentrations, alters plant growth and development by increasing the reactive oxygen species (ROS) production and unbalancing homeostasis of essential elements (Du *et al.,* 2017).

Shaw *et al.* (2014) have shown that CuO nanoparticles reduced shoot and root growth of *Hordeum vulgare* seedlings. They have also reported that the CuO NPs induced the release of ROS, membrane damage and overall enzymatic activity not enough to cope with stress at 20-day exposure.It has been proposed that CuO nanoparticles would have been translocated via the vascular tissues and subsequently dissolved to produce Cu ions which resulted in deposition of lignin. Translocation of CuO nanoparticles is apparent but the production of Cu ions by dissolution is impossible because generation of Cu ions from copper nanoparticles is a redox process which requires a reducing agent such as hydrogen, phenol, protein or an acid (Siddiqi and Husen, 2017). The plants grown in presence of nanoparticles may absorb and translocate them in different tissues. It has been shown that CuO nanoparticles were reduced to Cu_2O and Cu_2S in maize plants (Wang *et al.,* 2012).

Duckweed exposed to CuO nanoparticles showed inhibition of photosynthetic activity due to the Cu^{2+} ions released from it (Perreault *et al.,* 2014). Carotenoids remained unchanged and chlorophyll reduction began at 100 mgL^{-1} CuO nanoparticles in mung beans (Nair *et al.,* 2014). Chlorophyll started decreasing at 100 mg L^{-1} (Nair and Chung, 2014). In another study, CuO nanoparticles reduced carotenoids and chlorophylls in mustard (Nair and Chung, 2015).

Copper oxide NPs have been shown to induce DNA damage in plants (Atha *et al.,* 2012). Growth inhibition in *Raphanus sativus*, *Lolium perenne* and *Lolium rigidum* under laboratory conditions has been reported. Germination of radish seeds in presence of CuO nanoparticles induces substantial accumulation of mutagenic DNA lesions. Radish and similar other plants produce oxygen-derived species (O_2^-, H_2O_2, OH·) during germination (Schopfer *et al.,* 2001). H_2O_2 enhances seed germination but in presence of peroxidase or transition metal ions such as copper produce an excess of OH· via the Fenton reaction (Halliwell and Gutteridge, 2007). It is therefore suggested that copper ions produced from CuO NPs may catalyze the formation of this free radicle. CuO NPs inhibited the radish root growth to the extent of 79% which is relatively much larger than that observed for Cu_2^+ ions alone. The stunted growth has been observed mainly in the root/shoot (Fernandes and Henriques, 1991).

Plants are generally protected against this oxidative stresses by a wide range of radical scavenging systems such as antioxidative enzymes like peroxidase, ascorbate peroxidase superoxide dismutase and catalase as well as nonenzymatic compounds include antioxidants such as ascorbate, glutathione, α-tocopherol and carotenoids (Azevedo Neto *et al.,* 2008; Zimmermann and Zentgraf, 2005). These components minimize the oxidative damage during exposure to metal oxide nanoparticles (Zhao *et al.,* 2012a). Therefore, controlling ROS levels would be clearly advantageous in improving the plant performance and longevity. *Zea mays* exposed to CeO_2 NPs (Zao *et al.,* 2012b) did not show lipid peroxidation and any physiological changes, although activity of catalase, ascorbate and upregulation of heat shock proteins was observed. However, no elevation of lipid peroxidation in

rice treated with this compound (0-500 mg L^{-1}) was recorded but ion leakage was observed at higher doses (Rico *et al.,* 2013).

The scientific information on potential harmful effects of NPs severely lags behind the development of nanotechnologies (Kahru and Ivask, 2013). The available nanotoxicity data are inconsistent because experimental approaches vary from article to article making it impossible to compare results (Schrurs and Lison, 2012). To overcome these problems, nanotoxicology community has recently started a discussion about the implementation of general guidelines for nano-toxicology research and establishment of common parameters that should be addressed in all nano-toxicological articles.

The present study was carried out to elucidate the potential effects of bulk (CuO) and nano-Copper (CuONP) particle application on photosynthetic pigments, seedling development, lipid peroxidation inhibition%, LOX, protein content, defense antioxidants (enzymatic and non-enzymatic) activity and metal chelating% in maize (*Zea mays*).

2. Materials and methods

2.1. CuO nanoparticle and bulk particle suspension preparation

CuO Nanoparticle (CuONP) tested for phytotoxicity was purchased from Sigma-Aldrich. Specific surface area, size, and purity of the compound were adopted (where available) from the manufacturer. CuO (size <50 nm, surface area = 29 $m^2.g^{-1}$). Bulk material tested is CuO (purity 99.99%) purchased also from Sigma-Aldrich. Tested CuO (Nano or Bulk) at a concentration of 200 mg. L^{-1} were sonicated for 30 minutes to ensure dispersion in the solution.This suspension was further diluted to obtain the remaining concentrations of 25, 50, 100, 150 and 200 mg. L^{-1}.

2.2. Phytotoxicity assay

Phytotoxicity of previously prepared suspensions of CuO (Nano or Bulk) was tested using Maize seeds (*Zea mays* cv. hybrids triple white Hi Tech.). Well selected seeds were immersed in 10% H_2O_2 solution for 10 min and rinsed thoroughly with deionized water to ensure surface sterility. The seeds were subsequently placed in Petri dishes (100 mm × 15 mm); there were 10 seeds per Petri dish. Seeds germinated on filter paper moistened with 10 mL of tested metal oxide (Nano or Bulk) suspensions in final concentrations 25, 50, 100, 150 and 200 mg. L^{-1}. All experiments were performed in six replications. The seeds were covered in the dark at 24°C. The length of roots and shoots were measured over the course of 8 days and compared to the untreated control. After measurement, whole seedlings were washed briefly with deionized water, in order to avoid washing out freshly acquired Cu^{+2}, blotted gently with filter paper. The seedlings were quickly weighted for fresh weight determination, then oven-dried at 70°C for 48 hours in order to determine dry weight. The Cu content in tissues from all treatments was measured by Atomic Absorption Spectroscopy (AAS) after HNO_3 digestion according to Sawhney and Frink (1991).

2.3. Preparation of the extract

Another fraction of fresh seedlings were immediately weighted and ground in a chilled mortar and pestle with 6 ml buffer solution containing Tris HCl 0.05 M, PH 7.0 consisting of 3 mM $MgCl_2$ and 1 mM EDTA. The extract was centrifuged at 4°C for 10 min at 5000 rpm and the supernatant obtained was used for the determination of enzymatic and non-enzymatic antioxidants and for the determination of antioxidant potential.

2.4. Determination of photosynthetic pigments

The photosynthetic pigments, *via*, chlorophyll a, chlorophyll b and carotenoids, were extracted from fresh plumules samples. The plumules tissues were suspended in 5 ml of 95% ethyl alcohol in a test tube at 60°C, until colorless. Then the total volume completed into 10 ml with 95% ethyl alcohol and absorbance readings were determined spectrophotometerically. Chlorophylls and carotenoids concentrations were calculated as mg g^{-1} FW at 663, 644 and 452 nm using equations as cited by Lichtenthaler (1987).

2.5. Determination of soluble proteins

Protein contents were determined in the plant extract by Folin reagent according to Lowry *et al.* (1951). A calibration curve was constructed using bovine serum albumin (BSA) and the data were expressed as mg BSA g^{-1} fresh matter.

2.6. Enzymatic antioxidants

2.6.1. Assay of superoxide dismutase

SOD activity Determination carried out according to of Beauchamp and Fedovich (1976) method. SOD Unit was expressed as the amount of enzyme causing the reduction of NBT by 50%. The expression of specific activity was in terms of units per mg of protein.

2.6.2. Catalase assay

CAT activity determination carried according to Aebi (1984). The decrease in H_2O_2 absorbance at A240 nm was used to calculate the activity.

2.6.3. Guaiacol peroxidase assay

GPX activity determination carried out following the method of Tatiana *et al.* (1999). The increase in absorbance at A470 nm due to the formation of tetraguaiacol was measured.

2.6.4. Assay of ascorbate peroxidase

APX activity was assayed following the method of Jiang and Zhang (2002). The decrease in A290 following the oxidation rate of ascorbic acid was measured.

2.6.5. Assay of lipoxygenase

The method of Minguez-Mosquera *et al.* (1993) was modified and used to assay lipoxygenase activity. The substrate was prepared by solubilizing 0.5 g linoleic acid with 0.5 g Tween 20 in deionized water and the final volume brought to 25 ml. Turbidity was cleared with a few drops of 2N NaOH. The plant extract was reacted with the substrate in a spectrophotometer cuvette containing 3 ml phosphate buffer 0.2 M, at pH 6.5 and the absorbance

measured at 234 nm at 20 s intervals for 1 min using a recording spectrophotometer. The rate of formation of conjugated diene reaction products, measured as an increase in A234 nm.

2.7. Determination of non-enzymatic antioxidants

2.7.1. Total phenolics determination

Total phenolic contents were assessed according to Singleton and Rossi (1965). Folin-Ciocalteau reagent method was used. The measurements carried out at A765 nm. Gallic acid equivalents were used to express the data as $\mu g\ g^{-1}$ FW using Molar Coefficient of 120 $\mu g^{-1}\ cm^{-1}\ ml^{-1}$.

2.7.2. Total flavonoids determination

Content of total flavonoid was measured according to Moreno *et al.* (2000). Quercetin equivalents were used to express the absorbance at A415 nm as mg g^{-1} FW.

2.8. Antioxidant ability assays

2.8.1. Total antioxidant activity

The total antioxidant contents were estimated following the method of Prieto *et al.* (1999). 0.1 ml of the plant extract was combined with 3 ml of the reagent solution (0.6 M H_2SO_4, 28 mMNa_3PO_4 and 4 mM ammonium molybdate); the mixture was incubated at 95 °C for 90 minutes and then cooled to room temperature. The absorbance was measured at 695 nm.

2.8.2. Reducing power assay

The reducing power of the samples was determined according to the procedure described by Oyaizu (1986) with modifications.0.1 ml of the plant extractwas mixed with 0.5 ml of 0.2 M phosphate buffer (pH = 6.6) and 0.5 ml of 0.1%$K_3[Fe(CN)_3]$; the mixture was incubated in a water bath for 20 minutes at 50 ºC. After adding 0.5 ml trichloroacetic acid, the mixture was centrifuged at 1000 rpm for 10 minutes. To the supernatant (1 ml), 1 ml distilled waterand 100 µl of 0.01% $FeCl_3$ were added. The mixture was then incubated at 37 ºC for 10 minutes; after which, the absorbance was measured at 700 nm. Ascorbic acid solution was used to construct a standard curve. The results were expressed as ascorbic acid equivalents as $\mu g\ g^{-1}$ fresh matter.

2.8.3. Hydroxyl radical (OH^-) scavenging assay

OH^- radical scavenging assay carried out according to Kunchandy and Rao (1990). Absorbance of plant extract was measured against a blank containing deoxyribose and buffer at A532 nm, and degradation inhibition of deoxyribose was used to calculate the inhibition in percent (I) was calculated by the formula I = (Abs control – Abs sample)/Abs control × 100.

2.8.4. Hydrogen peroxide (H_2O_2) scavenging

H_2O_2 radical scavenging assay carried out according to Long *et al.* (1999). Sodium pyruvate was used as the reference compound. The absorbance of the ferric-xylenol orange complex was measured at A560 nm.

2.8.5. Lipid peroxide formation inhibition

Lipid peroxidation inhibition% carried out according to Janero (1990). The absorbance of the upper organic layer was measured at A532 nm. The inhibition in percent (I) was calculated by the formula I = (Abs control – Abs sample)/Abs control × 100.

2.8.6. Metal chelating assay

Metal chelating ability carried out according to Decker and Barbara (1990). The absorbance of the solution was measured at A562 nm. EDTA was used as a positive control.

2.9. Statistical analysis

For all the experiments (complete randomized design) three samples were analyzed and all the assays were carried out in triplicate. All values described in results section were mean of three replications ± standard error. Analysis of variance (ANOVA) was carried out using SPSS v16.0, followed by Duncan's multiple range test between the means of treatments to determine the significant difference at the probability level < 0.05. All the assessed attributes subjected to cluster analysis using a Correlations similarity distance with the software PAST version 2.11 for Windows (Hammer *et al.,* 2001). The matrix was then analyzed with Principle Component Analysis (PCA) variance regression ordination, using the Sørensen coefficient as the distance measure, to check the magnitude of change in attributes along the CuO and CuONP gradients by the same software.

3. Results

Copper, as a heavy metal, stress caused a significant decrease in most of the investigated parameters and it unfortunately inter to the plant body in exponential manner by the increase of its concentration in the circumstance. In this respect, Cu reached to 260 $\mu g\ g^{-1}$ DW in the plants grown in 200 mg L^{-1} CuO and 82 $\mu g\ g^{-1}$ DW in the plants grown in 100 mg L^{-1} CuONP (Table 1).Generally, the maize growth was completely stopped under 150 and 200 mgL^{-1} CuONP. Maize seedling fresh and dry weight and shoot and root lengthsdecreased gradually in bulk-conditions till the minimum at 200 mgL^{-1} (Table 1).However, at Nano-conditions; the same effect was observed for all CuONP concentrations till 100 mgL^{-1} that induced the same responseofits twofold of bulk-conditions.Water content also decreased significantly by increase in CuO and CuONP concentrations (Table 1).

Plumule pigments showed somewhat different trends toward the different concentrations of both CuO and CuONP. Chlorophyll a and b content decreased in bulk Cuat 25, 50 and 100 mg L^{-1} and nano-Cu at 25 and 50 mg L^{-1} for about the half of control (no Copper) with no significant difference between them. Other significant decrease was recorded in 150 mg L^{-1} followed by slight increase in 200 mg L^{-1} under bulk-conditions and reached to the quadrate of the control at the last two bulk concentrations (150 and 200 mg L^{-1}). Differences in chlorophyll b between 25, 50 and 100 mg L^{-1} can be neglected with the unusual significant increase under 100 CuONP which statistically equal to the control.Furthermore, carotenoids follow the same steps with notable peak at 100 mg L^{-1} for both conditions (Table 1).

Table 1. Growth attributes and copper content of (*Zea mays* cv. hybrids triple white Hi Tech.) as influenced by the toxicity of bulk-CuO and nano-CuO (mean ± SE; n = 3)

Cu Conc. mg L^{-1}		Cu µg g^{-1} DW	FW mg	DW mg	WC%	Shoot Length (mm.)	Root Length (mm.)	Chlorophyll a mg g^{-1} FW	Chlorophyll b mg g^{-1} FW	Carotenoids mg g^{-1} FW
Control	**Zero**	10 ± 1.11^{a}	369 ± 4.5^{i}	33.20 ± 0.57^{i}	91.0 ± 2.5^{c}	54.67 ± 1.42^{d}	83.33 ± 3.02^{d}	109.43 ± 1.49^{d}	20.06 ± 0.07^{cd}	52.82 ± 0.94^{g}
Bulk (CuO)	**25**	14.2 ± 1.02^{b}	363 ± 2.9^{h}	32.17 ± 0.39^{h}	91.1 ± 2.9^{d}	35.33 ± 1.21^{c}	21.67 ± 1.34^{c}	44.21 ± 1.21^{c}	12.35 ± 0.30^{abc}	26.03 ± 0.53^{c}
	50	18.5 ± 1.41^{c}	249 ± 7.3^{e}	27.07 ± 0.45^{g}	89.1 ± 1.9^{b}	34.00 ± 1.21^{c}	11.67 ± 0.97^{abc}	40.13 ± 0.96^{c}	13.57 ± 0.03^{abcd}	30.20 ± 0.80^{d}
	100	49.8 ± 4.01^{f}	240 ± 3.5^{d}	20.93 ± 0.51^{e}	91.3 ± 3.5^{e}	20.00 ± 1.89^{ab}	6.67 ± 1.02^{ab}	42.65 ± 4.13^{c}	14.21 ± 0.94^{bcd}	37.31 ± 0.45^{f}
	150	70 ± 3.1^{g}	226 ± 2.1^{c}	18.33 ± 0.67^{c}	91.9 ± 2.8^{f}	16.67 ± 1.67^{ab}	3.00 ± 0.58^{a}	20.81 ± 0.75^{a}	6.05 ± 0.75^{a}	14.63 ± 0.35^{a}
	200	260 ± 5.21^{i}	206 ± 3.29^{b}	6.33 ± 0.44^{a}	96.9 ± 4.1^{i}	15.00 ± 1.70^{ab}	2.00 ± 0.00^{a}	28.53 ± 0.89^{b}	6.82 ± 0.35^{ab}	16.73 ± 0.58^{b}
Nano (CuONP)	**25**	20 ± 3^{d}	320 ± 3.08^{g}	21.53 ± 0.29^{f}	93.4 ± 3.4^{h}	23.33 ± 1.33^{b}	18.33 ± 1.33^{bc}	42.54 ± 4.02^{c}	12.69 ± 0.40^{abc}	31.64 ± 0.56^{e}
	50	27.33 ± 2.14^{e}	289 ± 1.33^{f}	20.17 ± 0.46^{d}	93.1 ± 2.8^{g}	16.67 ± 1.67^{ab}	10.00 ± 0.00^{abc}	43.49 ± 1.16^{c}	13.91 ± 0.74^{bcd}	29.41 ± 0.55^{d}
	100	82 ± 3.2^{h}	74 ± 3.41^{a}	12.50 ± 0.66^{b}	83.1 ± 2.9^{a}	9.67 ± 0.63^{a}	2.00 ± 0.00^{a}	41.88 ± 3.56^{c}	20.52 ± 0.50^{d}	36.03 ± 0.74^{f}

Values with same alphabets in superscript in a column do not differ significantly.

On the other hand, many pivotal primary (soluble proteins), secondary metabolites (total phenolics, total flavonoids) in addition to total antioxidants and reducing power showed counter response of the growth and pigments. All of them, in comparison to control, recorded significant increase under both CuO conditions. There was a general gradual increase in soluble protein contents under bulk-conditions till 150 mg L^{-1} CuO while CuONP caused drastic jump at 25 mg L^{-1} (double of the control). Comparing the two conditions show the ferocity of 25 mg L^{-1} CuONP that caused the same effect of 150 mg L^{-1} CuO on soluble proteins.

Table 2. Biochemical attributes of (*Zea mays* cv. hybrids triple white Hi Tech.) as influenced by the toxicity of bulk-CuO and nano-CuO (mean ± SE; n = 3)

Cu Conc. mg L^{-1}		Proteins mg g^{-1} FW	Phenolic µg g^{-1} FW	Flavonoids mg g^{-1} FW	Total Antioxidants Abs. (at 695 ml^{-1})	Lipid Peroxidation inhibition %.	Hydroxyl Radical Scavenging %	Reducing Power µg g^{-1} FW	H_2O_2 Radical Scavenging %	Metal chelating %
Control	**Zero**	46.86 ± 2.11^{a}	2.08 ± 0.10^{a}	0.86 ± 0.02^{a}	62.33 ± 1.57^{a}	91.16 ± 1.17^{e}	94.96 ± 1.47^{i}	0.23 ± 0.01^{a}	95.67 ± 1.49^{f}	93.20 ± 1.38^{i}
Bulk (CuO)	**25**	80.67 ± 1.10^{d}	3.05 ± 0.08^{c}	1.80 ± 0.05^{c}	101.54 ± 0.95^{ef}	89.88 ± 0.96^{e}	93.19 ± 0.96^{h}	0.30 ± 0.01^{b}	96.32 ± 1.19^{h}	92.01 ± 1.28^{h}
	50	90.77 ± 1.22^{e}	2.66 ± 0.06^{b}	2.05 ± 0.05^{d}	100.36 ± 3.04^{def}	83.36 ± 1.19^{d}	90.20 ± 1.96^{e}	0.38 ± 0.01^{c}	96.08 ± 0.29^{g}	86.27 ± 0.56^{f}
	100	72.95 ± 2.29^{c}	3.22 ± 0.04^{cd}	1.35 ± 0.04^{b}	108.17 ± 2.12^{f}	83.96 ± 0.95^{d}	89.17 ± 1.16^{d}	0.44 ± 0.02^{d}	92.05 ± 1.36^{c}	82.60 ± 1.81^{d}
	150	109.12 ± 2.4^{f}	3.71 ± 0.03^{e}	1.46 ± 0.05^{b}	95.88 ± 1.19^{de}	66.36 ± 2.44^{b}	78.26 ± 0.98^{a}	0.75 ± 0.02^{g}	88.77 ± 1.19^{a}	72.17 ± 1.25^{a}
	200	93.93 ± 1.32^{e}	2.77 ± 0.04^{b}	1.84 ± 0.05^{cd}	78.75 ± 0.94^{b}	74.10 ± 1.21^{c}	86.01 ± 1.37^{c}	0.56 ± 0.01^{e}	93.88 ± 1.47^{d}	79.11 ± 1.28^{c}
Nano (CuONP)	**25**	109.47 ± 0.82^{f}	3.38 ± 0.08^{d}	1.98 ± 0.08^{cd}	99.01 ± 2.56^{def}	83.53 ± 1.99^{d}	91.04 ± 0.85^{g}	0.46 ± 0.02^{d}	95.14 ± 1.00^{e}	85.40 ± 0.45^{e}
	50	65.40 ± 1.2^{b}	3.26 ± 0.06^{d}	1.91 ± 0.05^{cd}	85.40 ± 2.74^{bc}	85.80 ± 0.72^{d}	90.59 ± 1.18^{f}	0.40 ± 0.01^{c}	95.77 ± 0.93^{f}	89.63 ± 1.12^{g}
	100	65.27 ± 0.86^{b}	3.05 ± 0.03^{c}	2.35 ± 0.04^{e}	89.86 ± 1.28^{cd}	61.81 ± 1.41^{a}	81.58 ± 1.13^{b}	0.64 ± 0.01^{f}	89.76 ± 1.10^{b}	78.85 ± 0.97^{b}

Values with same alphabets in superscript in a column do not differ significantly.

Overall ability of the cell to detoxify ROS was measured by total antioxidants activity and reducing power. *Zea mays* seedlings raised their reducing power agents under bulk-conditions until they reachedthe maximum (about 3.5 folds of control) at 150 mg L^{-1} CuO while it turned to decrease at 200 mg L^{-1}. The same was occurred under 100 mg L^{-1} CuONP which recorded the maximum (0.64 µg. g^{-1} Fresh wt.). However, the reducing power activity was moderate and similar under 50 mg L^{-1} of both conditions.

Table 3. activities of lipoxygenase and some enzymatic antioxidants of (*Zea mays* cv. hybrids triple white Hi Tech.) as influenced by the toxicity of bulk-CuO and nano-CuO (mean ± SE; n = 3)

Cu Conc. mg L^{-1}		LOX min mg^{-1} proteins	SOD unit mg^{-1} proteins	POD min mg^{-1} proteins	CAT min mg^{-1} proteins	APX min mg^{-1} proteins
Control	**Zero**	1.89 ± 0.07^{b}	2.07 ± 0.14^{a}	0.03 ± 0.003^{b}	0.05 ± 0.003^{c}	0.04 ± 0.003^{c}
Bulk (CuO)	**25**	3.82 ± 0.10^{d}	2.28 ± 0.14^{a}	0.04 ± 0.001^{c}	0.04 ± 0.001^{a}	0.04 ± 0.002^{e}
	50	2.78 ± 0.14^{c}	2.16 ± 0.33^{a}	0.04 ± 0.001^{e}	0.06 ± 0.001^{f}	0.03 ± 0.002^{a}
	100	4.41 ± 0.05^{e}	4.45 ± 0.32^{bc}	0.07 ± 0.001^{h}	0.08 ± 0.001^{g}	0.07 ± 0.002^{g}
	150	5.56 ± 0.14^{f}	9.21 ± 0.17^{d}	0.06 ± 0.005^{f}	0.06 ± 0.002^{e}	0.05 ± 0.004^{f}
	200	1.34 ± 0.13^{a}	5.43 ± 0.32^{c}	0.04 ± 0.001^{d}	0.08 ± 0.001^{h}	0.08 ± 0.002^{h}
Nano (CuONP)	**25**	2.07 ± 0.09^{b}	2.32 ± 0.20^{a}	0.02 ± 0.003^{a}	0.04 ± 0.003^{b}	0.03 ± 0.002^{b}
	50	3.60 ± 0.07^{d}	3.42 ± 0.36^{ab}	0.07 ± 0.002^{g}	0.05 ± 0.003^{d}	0.04 ± 0.002^{d}
	100	3.81 ± 0.12^{d}	15.82 ± 0.50^{e}	0.07 ± 0.003^{i}	0.15 ± 0.003^{i}	0.15 ± 0.003^{i}

Values with same alphabets in superscript in a column do not differ significantly

Total antioxidants made an arch with the maximum at 100 mg L^{-1} CuO while this peak was recorded at 25 mg L^{-1} CuONP which statistically equal to the response under 100 mg L^{-1} CuO. Almost the same arch was representedin the response of total phenolic compounds while the significant increase continued for 150 mg L^{-1} CuO. These compounds showed approximately same response at nano-conditions (25 and 50 mg. L^{-1} CuONP). Total flavonoid contents increased due to 25 and 50 mg L^{-1}, under both conditions, to the double of control, while the substantial rise was at 100 mg L^{-1} CuONP.

Table 4. Correlation coefficient values (r2) among different parameters of Zea mays as affected by different CuO concentrations under bulk and nano-conditions

	FW	**DW**	**WC**	**SH**	**RL**	**Ch_a**	**Ch_b**	**Carot**	**Ph**	**RP**	**TA**	**Fl**	**Pro**	**LP**	**HR**	**H_2O_2**	**MC**	**LOX**	**SOD**	**POD**	**CAT**	**APX**
FW	1																					
DW	0.94***	1																				
WC	0.77*	0.68*	1																			
SH	0.87**	0.85**	0.62	1																		
RL	0.71*	0.59	0.56	0.89**	1																	
Ch_a	0.65	0.49	0.46	0.82**	0.96***	1																
Ch_b	0.37	0.17	0.01	0.36	0.51	0.70*	1															
Carot	0.54	0.36	0.27	0.61	0.75*	0.89**	0.90***	1														
Ph	-0.40	-0.36	-.15	0.72*	0.72*	0.77*	0.50	0.62	1													
RP	-.83**	0.73*	.75*	0.80**	0.69*	0.71*	0.45	0.63	0.65	1												
TA	-0.05	0.05	0.12	0.39	0.66	0.66	0.29	0.37	0.67	0.22	1											
Fl	-0.47	0.44	0.45	0.62	0.69*	0.62	0.03	0.40	0.34	0.35	0.40	1										

[*Continued*]

	FW	DW	WC	SH	RL	Ch_a	Ch_b	Carot	Ph	RP	TA	Fl	Pro	LP	HR	H_2O_2	MC	LOX	SOD	POD	CAT	APX
Pro	-0.38	-0.17	-0.15	-0.39	-0.56	-0.74*	-0.79*	-0.77*	0.62	0.53	0.52	0.32	1									
LP	0.80**	0.75**	0.89**	0.73*	0.59	0.56	0.19	0.45	-0.42	-0.94***	-0.08	-0.43	-0.31	1								
HR	0.76*	0.66	0.80**	0.73*	0.64	0.65	0.36	0.58	-0.57	-0.98***	-0.18	-0.30	-0.41	0.96***	1							
H_2O_2	0.61	0.59	0.74*	0.61	0.44	0.42	0.15	0.28	-.51	-0.87**	-0.18	-0.05	-0.20	0.87**	.911**	1						
MC	0.81**	0.68**	0.78*	0.73*	0.66	0.68*	0.50	0.61	-0.57	-0.97***	-0.25	-0.22	-0.56	0.89**	.951***	0.88**	1					
LOX	-0.03	0.01	-0.20	-0.36	-0.41	-0.41	-0.15	-0.28	0.68*	0.45	0.55	-0.02	0.12	-0.35	-0.53	-0.62	-0.42	1				
SOD	-0.63	-0.69*	-0.83**	-0.63	-0.43	-0.33	0.15	-0.17	0.31	0.77*	0.02	0.39	-0.03	-0.92***	-0.83**	-0.85**	-0.70*	0.42	1			
POD	-0.39	-0.41	-0.46	-0.61	-0.51	-0.36	0.08	-.13	0.42	0.44	0.24	0.16	-0.28	-0.45	-0.52	-0.58	-0.40	0.71*	0.59	1		
CAT	-0.61	-0.69*	-0.87**	-0.54	-0.37	-0.19	0.36	0.07	0.01	0.53	-0.01	0.42	-0.25	-0.75*	-0.59	-0.65	-0.51	0.16	0.88**	0.55	1	
APX	-0.61	-0.75*	-0.79*	-0.56	-0.35	-0.18	0.32	0.03	0.04	0.53	-0.06	0.40	-0.26	-0.74*	-0.58	-0.65	-0.49	0.14	0.89**	0.53	0.97**	1
cu	-0.77*	-0.73*	-0.52	-0.49	-0.40	-0.41	-0.49	-0.53	-0.02	0.50	-0.25	0.17	0.25	-0.50	-0.45	-0.29	-0.54	-0.31	0.32	0.06	0.34	0.45

**** = Correlation is significant at the 0.001 level*

*** = Correlation is significant at the 0.01 level*

** = Correlation is significant at the 0.05 level*

Protective reactions those occurred under toxicity stress to restrict the production of ROS such as hydroxyl radiclescavenging, H_2O_2 scavenging, metal chelating and lipid peroxidation inhibition, were represented in table 2. Notably, these four attributes trended similarly and significantly decreased gradually toward the increase in CuO till 150 mg L^{-1} and return to increase slightly at 200 mg L^{-1}.Also they statistically decreased under CuONP concentrations in comparison to bulk CuO and control specially at 100 mg L^{-1}, while there were no significant differences between 25 and 50 mg L^{-1} of this condition. Specifically, H_2O_2 radicle scavenging reactions were significantly higher than or equal to the control at 25 and 50 mg L^{-1} for both bulk and nano-conditions.

The enzymatic antioxidants (SOD, POD, CAT and APX)and lypoxygenase LOX, were almost showed the same direct relationbetween their responseand the increase in CuO toxicity under bulk (till 150 mg L^{-1}) and nano-conditions (till 100 mg L^{-1}), while the 200 under bulk-conditions recorded significant decrease even than the control

(Table 3). This concept can be applied completely on lipoxygenase activity (LOX), meanwhile superoxide dismutase seems to be unaffected at 25 and 50 mg L^{-1} for both conditions then jumped at 100 mg L^{-1} CuONP for 8 folds of the control and at 150 mg L^{-1} CuO for 4 folds of the control. Also peroxidase, recoded significant increase at 50 mg L^{-1} than the controlwith the maximum activity at 100 mg L^{-1} for both conditions. Ascorpate peroxidase and catalase activities at 25 and 50 mg L^{-1} were more or less equal to those of the control while it was different for 100 mg L^{-1}.

Table 4 show all possible positive and negative correlations among all assessed parameters. Significant positive correlations were found among plant biomass parameters (fresh weight, dry weight, shoot height and root length). Plant biomass production was also positively correlated with different antioxidant ability attributes such as hydroxyl radicle scavenging, inhibition of lipid peroxidation, metal chelating. However, there was negative correlation in plant biomass with the non-enzymatic antioxidants (free phenolics and flavonoids) and Copper contents. The reducing power components showed clear significant positive correlations with all assessed enzymatic antioxidants (SOD, CAT and APX), meanwhile these enzymes were also positively correlated to each other. However, there were significant negative correlations in the relation between these reducing power components on one hand and biomass, leaf pigments and the other antioxidant ability parameters on the other hand.

Subjection of the original data of all measured parameters to the Principle Component Analysis (PCA) interpreted in Figure1 which revealed the previously mentioned correlations on its first two axes. PCA axis 1 captures about 53.3% of the cumulative percentage followed by the second axis (19.2%). The distances between the attributes on axis 1 illustrate the degree of similarity; the closer the distance, the greater the resemblance and vice versa. Thus PCA biplot indicated great contrariness between the growth indicators and antioxidant activities (the right hand side of Figure 1) and Copper, enzymatic and non-enzymatic antioxidants contents (the left hand side).

Similarities among different studied attributes represented in the dendrogram (Figure 2) shows that studied attributes can be categorized in four major classes (A-D). Respectively as they shown in the dendrogram; class A included the growth attributes and most of the measured antioxidant ability components. Class B included the leaf pigments (Chlorophyll a, b and carotenoids). Meanwhile, class C included all of the measured enzymatic antioxidants, while D had the soluble proteins with total antioxidants, total phenolic compounds and total flavonoids.

4. Discussion

This study was undertaken to investigate the toxicity of CuO and CuONPs in *Zea mays* seedling development, growth and physiological responses. The toxic effect of engineered nanoparticles in plants used to be studied through various indicatorssuch as germination percentage, biomass production, shoot length, and root growth which were primarily based on studying the effect of heavy metals in plants (OECD, 2004). Based on previous studies, the phytotoxic dose of CuO nanoparticles varies according to the plant species. For example, Wu *et al.* (2012) reported that the phytotoxic dose of CuONPs was 397.6 mg L^{-1} for radish, 175.4 mg L^{-1} for cucumber and 12.9 mg L^{-1} for lettuce. In the present study, the phytotoxic concentrations of CuONPs those inhibited the seeds germinations at all were those equal or smaller than 150 mg L^{-1} while they can withstand till 100 mg L^{-1}.

Shi *et al.* (2014) in a study on *Elshotzia splendens* concluded that the phytotoxicity was due to CuONPs exposure and not from soluble Cu. In an earlier study, Lee *et al.* (2008) have also reported that exposure to very low concentrations of Cu^{2+} ions has not affected the shoot and root growth in *Phaseolus radiates* and *Triticum aestivum*. Also Prakash *et al.* (2014) recorded drastic roots-growth retardation and triggering their lignification in Soybeanunder 400-500 mg L^{-1} CuONPs.In accordance with the present study, the exposure to the highest concentration of CuONPs (i.e., 100 mg. L^{-1}) significantly reduced the shoot and root growth as well as the total chlorophyll and carotenoids contents in maize seedlings. Hence, Chlorophyll is the critical photosynthetic pigment and its levels can be a significant indicator of toxicity to plants (Ma *et al.,* 2015). However, exposure to CuO made the same effect at double of this dose (200 mg. L^{-1}). This might be due to the fact that lower concentration of Cu is essential for plant development since it is an essential micronutrient for the plant growth. However, higher exposure concentrations of CuO-nanoparticles might have resulted in the excess presence of Cu in maize roots leading to adverse effects on plant development (Prakash *et al.,* 2014).

A very high concentration of nanoparticles may severely affect the photosynthesis which may result in plant growth suppression or plant death. Several reports have observed significant decrease in plant growth and pigments as the result of nanoparticle exposure as in barely (Shaw *et al.,* 2014) and rice (Costa and Sharma, 2016). The significant reduction in chlorophyll attributes, as observed in this study, might be due to the reduction in biomass or as a result of the lipid peroxidation of chloroplast membranes due to the NP oxidative stress (Ma *et al.,* 2013). Similarly, significant reduction in chlorophyll attributes as a result of AgNPs exposure had also been reported by Ma *et al.* (2013) from *Arabidopsis thaliana*. While, unusual increase in Chlorophyll b and carotenoids at 100 mg L^{-1} CuONPwas reported with tomato under 250 mg TiO_2 NP Kg^{-1} (Raliya *et al.,* 2015) and 500 mg Ce_2ONP Kg^{-1} soil (Barrios *et al.,* 2016).

Previous studies reported that the surface of NPs could be covered by various macromolecules, and the chemical surface property and the ligand density of NPs could strongly influence the interaction between NP-bound ligands and cellular receptors (Rauch *et al.,*

2013). This could be due to the fact that macromolecules in root exudates altered physicochemical properties of the NPs surface and resulted in NPs accumulation in the root epidermis (Ghafariyan *et al.,* 2013). The plants grown in presence of nanoparticles may absorb and translocate them in different tissues. It has been shown that CuO nanoparticles were reduced to Cu_2O and Cu_2S in maize plants (Wang *et al.,* 2012). Several studies in *Zea mays* seedlings have proved that the uptake and translocation of CuONPs occurs through the roots to shoots via xylem and shoots to roots via phloem (Wang *et al.,* 2012). Moreover, Shi *et al.* (2014) reported that once inside the plant cells, the dissolution of CuONPs are promoted due to the reduced pH and by their interaction with organic acids and proteins inside the plant tissues. In accordance with earlier reports, Cu metal content analysis provided evidence for the presence of significantly high Cu content in roots of CuONP-exposed soybean seedlings (Prakash *et al.,* 2014) and our maize seedlings.

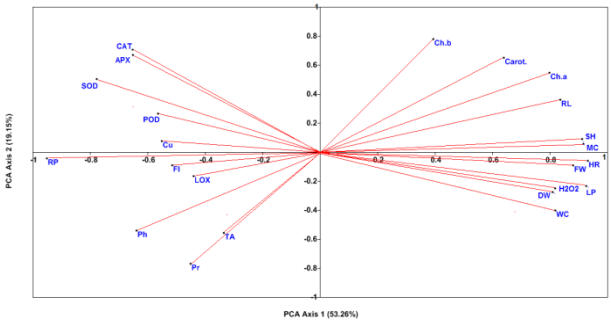

Figure 1. Loading plot of different studied attributes correlations to the first two Principle component analysis (PCA) axes. Horizontal and vertical arrows indicate the rise-direction of CuO and CuONP concentrations. **Abbreviations**: Cu = Cupper, Ph = Total Phenolics, TA = Total Antioxidants, Pr = Proteins, Fl = Total Flavonoids, RP = Reducing Power, LOX = Lipoxygenase, POD = Peroxidase, SOD = Superoxide Dismutase, CAT = Catalase, APX = Ascorbate peroxidase, Ch. A = Chlorophyll a, Ch. b = Chlorophyll b, Carot. = Carotenoids, LP = Lipid peroxidase, HR = Hydroxyl reductase, MC = Metal chelating agents, H_2O_2 = Hydrogen peroxide scavenging, WC = Water content, RL = Root length, SH = Shoot height, DW = Dry weight, FW = Fresh weight.

While redox biology implies a slight increment of the reactive oxygenated species level, meant to activate signaling pathways, oxidative stress involves elevated ROS amounts, resulting in the impairment of biomolecules such as nucleic acids, protein or lipids (Schieber and Chandel, 2014).

Oxidative stress was characterized by Sies (1991) as "a disturbance in the prooxidant to antioxidant balance in favor of the oxidant species, leading to potential damage". Oxidative stress has been understood as an excessive amount of ROS that is the outcome of an imbalance between the generation and depletion of ROS. Hence, oxidative stress is the repercussion of an enhanced free radical occurrence, but also of a reduced activity of the protective antioxidant defense system (Poljsak *et al.,* 2013).

From this point of view, the present study followed the incidence of oxidative stress resulted from Cu toxicity (CuO and CuONP) through detecting the activity of the protective antioxidant defense system. The general evaluation took place by detecting total antioxidant potentialandreducing power. While more specific evaluation performed through detecting some of the enzymatic antioxidants (SOD, CAT, APX, and POD) and non-enzymatic antioxidants (phenolics, flavonoids, carotenoids). The outcome of the protective antioxidant defense system in depleting ROS evaluated through conducting the change in lipid peroxidation inhibition%, OH·& H_2O_2 free radicals scavenging%, and metal chelating%.

The phosphomolybdenum assay has been routinely used to evaluate the totalantioxidant potential of extracts (Prieto *et al.,* 1999). In the presence of antioxidants, Mo (VI) is reduced to Mo (V) and forms a green colored phospho-molybdenum V complex. The data showed a clear and a significant induction in total antioxidant potential as consequence of Cu toxicity (CuO and CuONP) which was more obvious under Cuo when compared to CuONP.On the other hand, the measurement of reducing power of the maize cells under Cu toxicity may serve as a significant indicator of its potential antioxidant activity. Our data showed that excess Cu (micro and nano) evacuated the reducing power of the cells as compared to control. But unlike total antioxidants data, reducing power was higher under CuONP (25, 50, 100 mg L^{-1}) in comparison to CuO bulk. This could be attributed to the difference in sensitivity between the two assays.

Statistical evaluation bypearsoncorrelation between the TAC assay and reducing power was found to be non-significant (r = 0.22). On the other hand, correlation between TAC and total phenolic contents was found to be significant (r = 0.67). Another significant correlation was observed between FRAP assay and the total phenolic contents (r = 0.65). This indicates that phenolic compounds might be a major contributor to the antioxidant capacities under Cu toxicity. A high correlation between the total phenolic content and antioxidant activity has been reported by many researchers (Chew *et al.,* 2008; Wang *et al.,* 2009). Lipids are the most susceptible biomolecules to undergo oxidation: polyunsaturated fatty acids which lead to malondialdehyde, a recognized marker of lipid oxidative decay and their levels are considered to accurately reflect the oxidative stress (Pisoschi and Pop, 2015).

Increased LOX activity, presented in this work under Cu bulk as compared to CuNPat (25 and 100 mg L^{-1}) treatments suggests higher lipolytic activity of the membranes and oxidation of membrane-bound fatty acids, which propagates higher lipid peroxidation (Tavallali *et al.,* 2010). Surprisingly, the data of lipid peroxidation inhibition%, shows higher level of membranes protection

under Cu bulk when compared to corresponding CuNP concentrations (25 &100 mg L^{-1}). This could be attributed to the non-enzymatic biosynthesis of lipid peroxides. Oxidized lipids in addition to being synthesized in a specific and controlled manner by lipoxygenase (LOX), the non-enzymatic peroxidation of lipids could be mediated by carbon- and oxygen-centered radicals. Like all radical reactions, this process can be broken down into three discrete phases: initiation, propagation, and termination (Yousri *et al.,* 2011). Yet, the substrate scope and general mechanism of lipid peroxidation is largely the same in both cases.

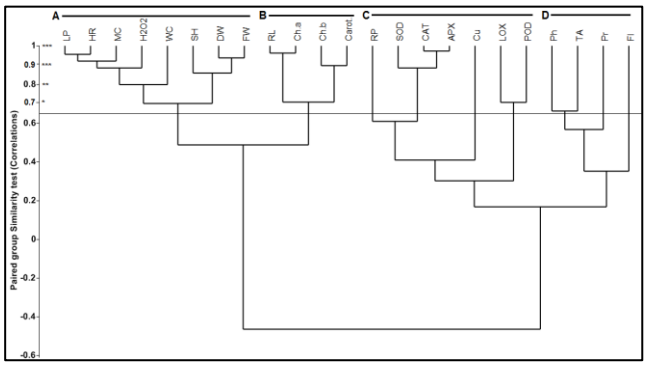

Figure 2. Cluster analysis of measured attributes show significance, * at $p \leq 0.05$, ** at $p \leq 0.01$ and *** at $p \leq 0.001$. Letters (A-D) refer to the clusters of similar trends

Nanoparticles may interfere with plant metabolism in several ways, such as by providing micronutrients (Liu and Lal, 2015), regulation of genes (Nair and Chung, 2014), or interfering with different oxidative processes in plants which results in oxidative burst (Hossain *et al.,* 2015). Some studies have demonstrated that nanoparticle exposure improves free-radical scavenging potential and antioxidant enzymatic activities and alters microRNAs expression that regulates different morphological, physiological and metabolic processes in plants (Siddiqi and Husen, 2016b). It is clear that several nanoparticles when present in excess results into ROS production, and interfere with the oxidative mechanism (Anshu *et al.,* 2017).

Redox homeostasis of the cell is ensured by its complex endogenous antioxidant defense system. Attempts were made to classify antioxidant systems from the reactivity standpoint: the so called “first line of defense” has been identified as the enzymatic antioxidant system, including superoxide dismutase which depletes superoxide radical anion $O^{\cdot}_2$, catalase and peroxidase, and also the ascorbate peroxidase that decomposes H_2O_2. The “second line of defense” is represented mainly by reduced thiols and low molecular-weight antioxidants. The latter include a broad range of molecules, both hydro- and liposoluble (tocopherols, ascorbate, polyphenols, etc.) or metabolic compounds (ascorbate and reduced glutathione), and low molecular weight scavengers, like lipoic acid (Poljsak *et al.,* 2013). These compounds impart basically the antioxidant capacity to biological media. The respective biomolecules can reach particular locations in cells affected by the oxidative attack (Tessutti *et al.,* 2013).

All measured enzymatic antioxidants (SOD, CAT, APX and POD) activities were increased in the plumules of *Zea mays* gradually with increase in bulk-CuO concentration to the double of control values at 150 mg. L^{-1}. Meanwhile, this rise was faster, at 25 and 50 mg L^{-1}, under nano-conditions. It has been already reported that these antioxidant enzymes can protect plant cells against the adverse effects of reactive oxygen species (Das and Roychoudhury, 2014). The increase of CAT activity in leaves under NPs stress suggested that its effective scavenging mechanism to remove H_2O_2 resulted from metal stress caused oxidative damage (Reddy *et al.,* 2005). While POD acts as scavenger of ROS, SOD and CAT jointly convert O_2^- and H_2O_2 to H_2O and O_2 and also reduce overall free OH· radical which suggested that this enzyme served as an intrinsic defense tool to resist CuO and CuONP induced oxidative damage in maize plants.This confirms the regulation of antioxidant system as a response to nanoparticle interaction with maize plants. It has been reported that excess Cu triggers the generation of ROS and free radicals and thus causes molecular damage to plants (Liu *et al.,* 2004). Reactive oxygen species (ROS) are represented by both free radical and non-free radical oxygenated molecules such as hydrogen peroxide (H_2O_2), superoxide ($O\dot{2}^-$), singlet oxygen (1/2 O_2), and the hydroxyl radical (OH·). Reactive nitrogen, iron, copper, and sulfur species are also encountered (Riley, 1994).

Following the outcome of the protective antioxidant defense system (OH·& H_2O_2 free radicals scavenging %, and metal chelating %), the data obtained proved the increased antioxidants' ability of maize cells under bulk Cu toxicity as compared to Nano Cu toxicity to contain the effects of reactive oxygen species activity, and delay the incidence of cell death obviously at (25 & 100 mg L^{-1}). This implies higher oxidative stress incidence under CuNPdespite of higher enzymatic antioxidants (SOD, CAT, POD and APX) and non-enzymatic antioxidants (phenolics, flavonoids). If the antioxidant produced are unable to control the ROS, the ROS oxidized the cell macromolecules (Sharma *et al.,* 2012), which ultimately results in the death of the plant.

The concept of biological antioxidant refers to any compound that, when present at a lower concentration compared to that of an oxidizable substrate, is able to either delay or prevent the oxidation of the substrate (Godic *et al.,* 2014). Antioxidant functions imply lowering oxidative stress, as well as other parameters of cell damage.

It's apparent from the data obtained herein, that both first and second line of defense could not help the plant to survive the elevating concentrations of CuNP. Nevertheless, it could be proposed that mainly at sustained free radical action, the defense system's capacity against ROS can be overwhelmed, leading to retarded growth and death occurrence. Hence, Seed germination was completely stopped under 150 and 200 mg L^{-1} CuONP. These concentrations showed the same or more drastic effect than 150 and 200 Cu bulk, and

showed that Nano sized particles of CuO proved more phytotoxicity than bulk particles.

Taking over these considerations, it is important to remind that the novel concept of oxidative stress is not restricted to free radical damage of the biomolecules, but relies on identifying perturbation of cellular redox status (Lopez-Alarcon and Denicola, 2013). Based on many studies on redox signaling pathways, on antioxidant mechanism and oxidative stress markers, Dean Jones re-defines oxidative stress as "a disruption in redox signaling and control", hence the action of the antioxidant systems is viewed as more complicated than merely blocking reactive free radicals (Jones, 2006). Higher activity of antioxidants (enzymatic and non-enzymatic) obtained herein, which accompanied with higher reducing power content could lead to more disruption in the cell redox signaling rather than blocking ROS, this disruption may not help the cell to survive under Cu toxicity stress, particularly at germination and plant establishment stage which is comprehensively regulated by multiple factors, and the presence of reactive oxygen species (ROS) at particular levels are among these factors (Schopfer *et al.,* 2001). A confirmation of this assumption could be gathered from the analysis of Principle component (PCA), that showed a reduction in growth parameters with the rise in CuONP concentrations, which in turn accompanied with increment in the activity of almost all antioxidants and reducing power.

It can be concluded that seedlingsgrowth, development and pigments followed the same trend and they were can not cope with CuOand CuONP phytotoxicity. Nano sized particles of CuO proved more phytotoxicity than bulk particles and causedcomplete germination retardation under 150 and 200 mg L^{-1} CuONP. These concentrations showed the same or more drastic effect than 150 and 200 Cu bulk. Higher enzymatic antioxidants (SOD, CAT, POD and APX) and non-enzymatic antioxidants (phenolics and flavonoids) could not sustain the survival of maize seedlings under elevating concentrations of CuNP. The capacity of defense systems against ROS could be overwhelmed at sustained free radical action, leading to growth retardation and death incidence.

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