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Bacterial Detoxification of Copper and Its Impacts on Germination Indices of Barley and Mung Bean

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ON ACCOUNT of incessant human activities, copper is accumulated in the environment at elevated concentrations that induce harmful influences on all kinds of living organisms. An oxic bioreactor was employed to transform copper ions into copper particles using volatile metabolites of *Escherichia coli* culture. SEM and EDX analysis of the transformed copper showed the formation of elongated particles with 1-5 µm in length comprising of copper, sulfur, carbon, oxygen and nitrogen elements. Mung bean seeds and barley grains exposed to ionic copper demonstrated low germination and apparent decline of seedlings growth parameters while higher germination and growth rates were recorded with those treated with copper particles. At the same time, an enhanced POD activity was noticed with all Cu treatments, CAT activity seemed to be induced in response to ionic Cu only meanwhile APX activity was markedly affected with both types of Cu. Furthermore, seedlings subjected to Cu particles showed higher protein contents. Toxicity reduction of copper treated with *E. coli* volatiles was ascribed to the decrease of the mobile copper concentration as a result of interaction with vaporized chelators that reduce bioavailability of copper.

Keywords: Copper - Detoxification - *E. coli* - Antioxidant enzymes - *Hordeum vulgare* - *Vigna radiata*.

At the moment, there is an extensive raise in the discharge of industrial effluents into the environment, primarily soil and water that generally leads to the accumulation of heavy metals. Heavy metals pollution is a major health concern since they are non-degradable and have long-lasting effects on the ecosystem. Heavy metals discharge from industrial activities is considered common source of heavy metal pollution such as fertilizer production, electroplating and plastics manufacturing in addition to mining and metallurgical processes (Zouboulis *et al.*, 2004). Most heavy metals such as arsenic, cadmium, chromium, copper, lead, mercury, nickel, silver, zinc are toxic to all living organisms even at very low concentrations. Toxic consequences of excess levels of heavy metals in

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plants include cellular damage and production of elevated amounts of reactive oxygen species (ROS)-led to oxidative stress, and cellular metabolic arrest (Gill and Tuteja, 2010). At relatively low concentrations, a number of heavy metal such as Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mo^{2+}, Ni^{2+}, and Zn^{2+} are recognized as essential trace elements for living cells as they act as cofactors of metalloproteins and some enzymes (Jansen *et al.*, 1994). These metals can also modulate plant ROS-metabolizing/scavenging system that comprising of enzymatic such as catalase, guaiacol peroxidase, glutathione sulfotransferase, ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase , dehydroascorbate reductase and non-enzymatic such as ascorbate, glutathione, carotenoids, tocopherols, and phenolics (Anjum *et al.*, 2012).

Although copper is required for normal cell growth, it is considered as a common soil contaminant. It is growing environmental problem due to the continuity of human activities leading to copper pollution. These activities include mining, sewage sludge application to soils and copper containing fungicides (Mackie *et al.*, 2012 and Ruyters *et al.*, 2013).

The ionic form of Cu^{2+} caused root growth alterations in durum wheat at low concentration; 1.0 μM and high Cu concentrations (200 and 500 μM) may reduce the growth of both root and shoots (Michaud *et al.*, 2008 and Thounaojam *et al.*, 2014). Toxicity of Cu is affected by bioavailability of metal in the soil and by the concentration of the metal as well as pH of the soil (Wang *et al.*, 2009). High concentration of Cu^{2+} are toxic and leads to inhibition of plant growth, disturbance of the mitosis, inhibition of root elongation, damage to root cell membranes (Ouzounidou *et al.*, 1995). Cu tends to be accumulated in the root tissue with little translocate to the shoots (Marschner, 1995).

The capability of microbial cells to stay alive in the occurrence of elevated doses of heavy metals could be assigned to the occurrence of various strategies to accommodate with these fatal pollutants. Of these mechanisms is the aptitude of the microbial cell to discharge non-specific extracellular molecules that are engaged in the attenuation of heavy metals toxicity through altering the physicochemical conditions around microbial biomass. Such these modifications induce the bioprecipitation of these pollutants in the microbial surroundings (Fomina *et al*., 2008; Dupraz *et al.*, 2009 and Jang *et al*., 2015).

It is well known that plants produce root exudates containing an assortment of organic substances which have considerable consequences on the development of microbial communities and their activities. Therefore, the interaction between plant roots and soil microbes can influence on the mobilization and immobilization of metals in the soil (Seshadri *et al.*, 2015). Protection of plants from the toxicity of heavy metals such as copper, zinc and lead occurs through microbial changing metal speciation into biologically unavailable species (He and Yang, 2007; Huang *et al.*, 2005 and Dixit *et al*., 2015). Thus the aim of the present study was to evaluate the impact of ionic

copper and copper treated with the volatile metabolites of *E. coli* on seed germination and metabolism of barley and mung bean seedlings.

Materials and Methods

Treatment of $CuSO_4$ by bacterial volatiles

Two concentrations of copper sulfate ($CuSO_4.5H_2O$) solutions (0.2 mM and 0.4 mM) were prepared from stock solution (1.0 M $CuSO_4$). The bacterial strain, *Escherichia coli*, (Essa, 2012) was used in this study. It was grown aerobically in Luria Broth medium on shaking incubator (200 rpm) for 24 hr at 30 ^oC. Bacterial growth was monitored by measuring the optical density at 600 nm. Two sets of the $CuSO_4$ solutions, (200 ml of each) were exposed to the *E. coli* biogases (OD ≈ 0.8) for 24 hr in an oxic bioreactor according to Essa *et al.* (2012). Then one set of the treated copper solutions were used to study their effect on seed germination and seedlings growth criteria while the other set was subjected for centrifugation at 10000 rpm for 15 min. The supernatant was discarded and precipitates were washed with 20 ml deionized water followed by centrifugation as before. The last step was repeated three times and Cu-precipitate were collected and dried at 30 ^oC. Cu-precipitates were examined with a JEOL JSM 5900 scanning electron microscope with the elemental composition determined by energy dispersive X-ray microanalysis (EDX) using an Oxford Link ISIS System according to Essa and Khallaf (2014).

Plant materials and treatments

Mung bean (*Vigna radiata; Fabaceae*) seeds and barley (*Hordeum vulgare*; *Poaceae*) grains were surface sterilized by immersion in ethyl alcohol (70%) for 2 min followed by rinsing three times with sterilized deionized water, 5 minutes each. Surface sterilized seeds or grains were germinated in 10 cm plastic Petri dishes containing sterilised filter papers witted with 20 ml of treatment solutions, 10 seeds or grains in per dish where control dishes contained 20 ml of sterilized water. Triplicates of each treatment were done and all dishes were kept in the dark at 18 ± 20 °C.

The treatments were carried out as following:

1) Control: sterilized deionized water.
2) Cu-IL: Ionic Cu solution ($CuSO_4.5H_2O$) at low concentration (8 µM).
3) Cu-NL: Cu-particles solution prepared via *E. coli* volatile metabolites at low concentration (8 µM).
4) Cu-IH: Ionic Cu solution ($CuSO_4.5H_2O$) at high concentration (16 µM).
5) Cu-NH: Cu-particles solution prepared via *E. coli* volatile metabolites at high concentration (16 µM).

Analysis of growth parameters

The percentage of germinated seeds or grains, length of seedlings (in centimetres), fresh weight and dry weight of seedlings were determined to express metal toxicity in both ionic and non-ionic Cu forms.

Antioxidant enzymes

All extraction procedures were carried out at 4 ºC, where one gram of fresh seedlings was grinded in 5 ml of phosphate buffer pH 7.0 followed by centrifugation at 14000 *g* at 10 °C for 15 min. The supernatants were used for determination of enzyme activity.

Peroxidase activity (POD)

For determination of POD (EC 1.11.1.7) activity, supernatants (0.1 ml) were mixed with assay mixture (3 ml) and the development of the brown color was monitored. The assay mixture for POD activity contained 40 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 5 mM guaiacol, 0.3 mM H_2O_2. The increase in the absorbance due to oxidation of guaiacol (Extinction factor = 26.2 mM cm^{-1}) was measured spectrophotometrically at 470 nm. POD activity was calculated in terms of µmol of guaiacol oxidized min^{-1} g^{-1} Fresh weight at 25 ± 2 °C (MacAdam *et al.*, 1992; Zhang, 1992).

Catalase activity (CAT)

By monitoring the disappearance of H_2O_2, CAT (EC 1.11.1.6) activity was measured according to the method of Chandlee and Scandalios (1984). The disappearance of H_2O_2 was measured by the decrease in absorbance at 240 nm (E= 0.036 mM^{-1} cm^{-1}) of a reaction mixture (3 ml) consisting of 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract (0.1ml). One CAT unit is the amount of enzyme necessary to decompose 1 µmol min^{-1} H_2O_2 under the above-mentioned assay conditions.

Ascorbate peroxidase activity (APX)

APX (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The supernatants were mixed with the assay medium for testing the activity of APX. The assay medium consists of 3 ml containing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.3 mM ascorbate, 0.06 mM H_2O_2 and 0.1 ml enzyme extract. The decrease in ascorbate concentration was followed by decline in absorbance at 290 nm and activity was calculated using the extinction coefficient (E =2.8 mM^{-1} cm^{-1}) for ascorbate.

Protein content

Soluble, insoluble and total protein contents of seedlings were determined according to Lowry *et al.* (1951). Dry tissues were extracted in 10 ml distilled water for 2 hr at 90 °C for analysis of soluble protein. Moreover, the total proteins were extracted in 10 ml NaOH (0.1 N) for 2 hr at 90 °C. The extracts were centrifuged and the supernatants were collected. One ml of extract was added to 5 ml of alkaline reagent (50 ml of 2% Na_2CO_3 prepared in 0.1 N NaOH and 1 ml 0.5% of $CuSO_4.5H_2O$ prepared in 1% sodium potassium tartrate), mixed thoroughly and then allowed to stand for 10 min. Folin reagent diluted 1:1 (v/v) was then added and mixed immediately. After 30 min, the extinction against appropriate blank was measured at 700 nm. Results were expressed as milligrams per gram dry weight. Insoluble proteins were calculated as the

difference between the amounts of total and water-soluble proteins. Bovine serum albumin was used for calibration curve.

Total free amino acids content

According to Moore and Stein (1948), total free amino acids were extracted and determined. Dry tissue samples were extracted in distilled water by heating in water bath at 90 ^{o}C for 2 hrs. The extracts were then centrifuged and the supernatants were collected. Supernatant (0.1 ml) was added to 1 ml of ninhydrin solution with stannous chloride. The tubes were heated in boiling water bath for 20 min till a purple color was developed. Five milliliter of the diluents were added and mixed well. After 15 min, the intensity of the color against a reagent blank was measured in a colorimeter at 570 nm. The free amino acids concentrations were calculated as mg/g dry matter.

Statistical analyses

The resulted data were tested by using the ANOVA test for significance. Means were compared by least significant differences (LSD) test at levels $P < 0.05$ and $P < 0.01$. All statistical tests were carried out using SPSS (v. 16.0) software (Garth, 2008).

Results

Effect of E. coli biogas on $CuSO_4$ solution

Data in Table 1 showed that changes in the optical density (OD), pH and color of $CuSO_4$ solutions in the precipitation chamber of the bioreactor as a result of exposing to the volatiles produced aerobically by *E. coli.* There was a gradual increase in the OD and pH values of copper solution by increasing the exposure time where maximum OD was recorded after 12 hr with pH value 8.2. After 24 hr exposure time, the pH value reached the highest value (8.8) and a pale blue precipitate was formed. At the same time, there was a marked change in the color of $CuSO_4$ solution by increasing the exposure time until 24 hr where pale blue precipitate was formed. Scanning electron microscope analysis of the Cu-precipitate (Fig. 1) showed the formation of elongated particles with 1 - 5 µm in length. At the same time, electron dispersive X-ray analysis of these particles elucidated their elemental composition. They comprised of 61.71% copper, 8.44% sulfur, 4.03% carbon, 22.19% oxygen and 1.98% nitrogen (Fig. 1).

Analysis of growth parameters

The germination of both mung bean seeds and barley grains under the effect of Cu treatment was presented in Fig. 2. Compared to control, all treatments could reduce the growth of seedlings at variable levels. The effect of Cu toxicity was more obvious in radicals than plumules. In case of mung bean seedlings, radicals become reduced and totally inhibited in barley seedlings. Non-ionic Cu form seemed to be less toxic than the ionic form of Cu in both low and high Cu concentrations.

TABLE 1. Influence of exposure time of *E. coli* volatiles on the physical characters of copper sulfate solution in the precipitation chamber of the oxic bioreactor.

Exposure time (hrs)	$O.D_{600}$	pH	Color
0	0.000	6.3	Blue
2	0.069	6.5	Blue
4	0.113	7.4	Turbid blue
8	0.437	7.8	Cloudy blue
12	0.792	8.2	Light blue precipitate
24	0.547	8.8	Pale blue precipitate

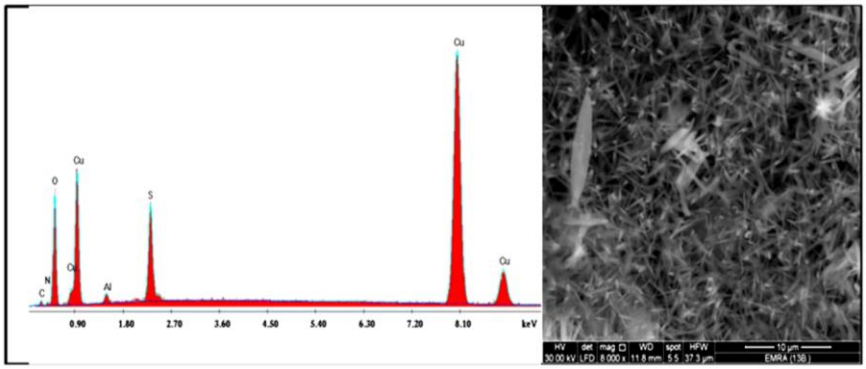

Fig. 1. Scanning electron microscope (SEM) and energy dispersive X-ray (EDX) analysis of copper particles produced in the bioreactor through the interaction of volatile metabolites of *E. coli* culture with copper sulfate solution.

The percentage of seed or grain germination was affected by Cu application in either ionic or non-ionic form (Fig. 3). Cu could lower the percentage of germination and the reduction depended on Cu concentration, the higher Cu concentration the lower the percentage of germination. Different percentages of germination were obtained in different forms of Cu. In mung beans, the percentage of germination at low Cu were 53% and 73% in case of ionic and non-ionic Cu forms, respectively. Almost similar responses were detected in barely grains germinated in ionic and non-ionic Cu forms. Results concerning the percentage of germination revealed that, the toxicity of Cu was lower in case of non-ionic Cu for the same concentration in both plants.

The length of seedlings produced in all Cu treatments was reduced (Fig 3). The severity of reduction was concentration and Cu-form dependent. In all concentrations, the non-ionic Cu form was less toxic to both mung bean and barely seedlings. Compared to the control, low concentration caused reduction of 57 % and 90% in cases of mung bean treated with low and high concentration of ionic Cu, respectively. Barley seedlings grown on high non-ionic Cu were 65 %

longer than those germinated on high ionic Cu. Moreover, in case of mung bean seedlings, high ionic and non-ionic Cu concentrations reduced seedling lengths to 92% and 47% less than control, respectively.

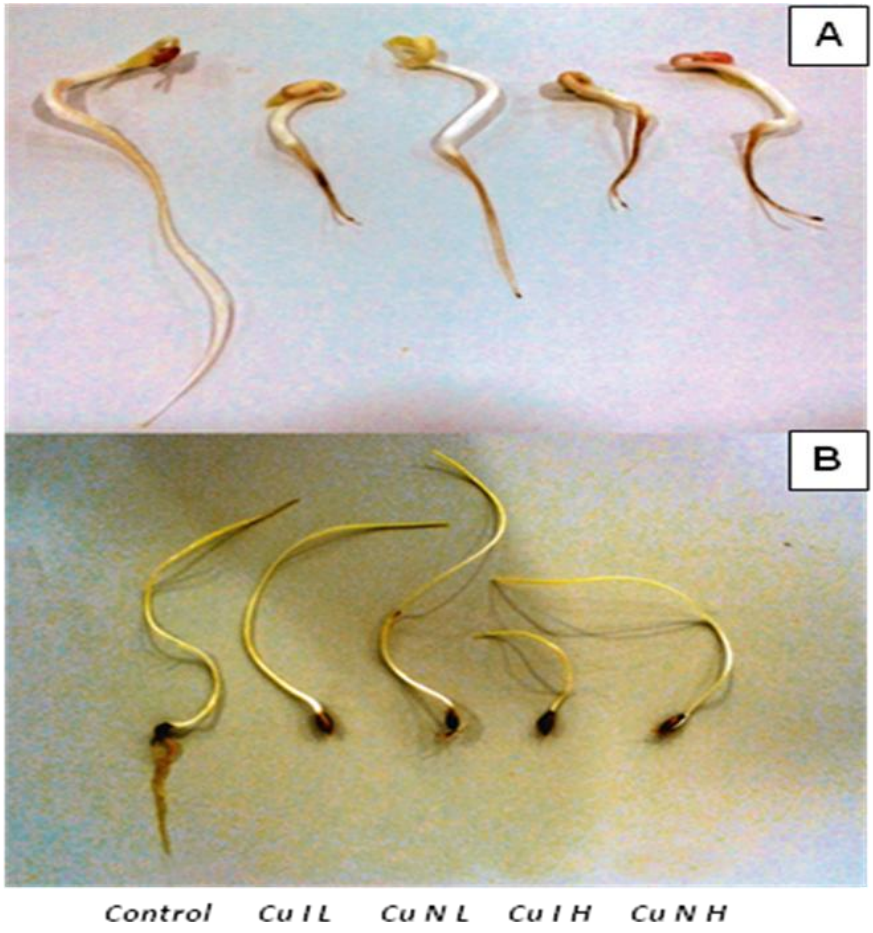

Fig. 2. Impact of ionic and non-ionic copper on the germination of mung bean seeds (*Vigna radiate*; A) and barley grains (*Hordeum vulgare*; B).

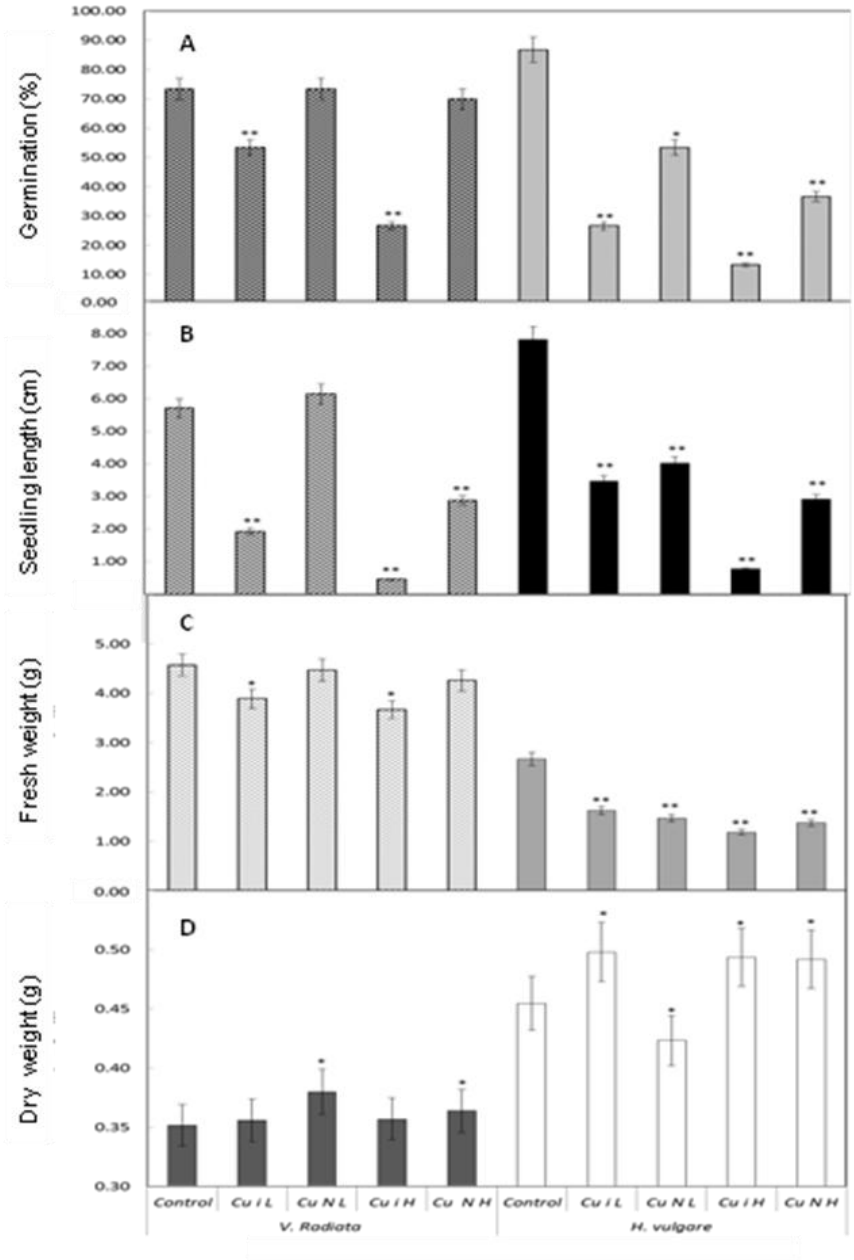

Fig. 3. Growth parameters of mung bean seeds (*Vigna radiata*) and barley grains (*Hordeum vulgare*) germinated on ionic and non-ionic Cu-solutions where (A) is germination percentage, (B) is seedling length, (C) is fresh weight and (D) is dry weight.

Comparing the weights (fresh and dry) of the produced seedlings under stressful conditions with the corresponding controls, the obtained results demonstrated lower weights of seedlings due to Cu treatments. Similarly, Cu toxicity leads to reduced fresh and dry weights in case of ionic Cu in low and high concentrations in both plants. On the other hand, non-ionic Cu showed less of no toxicity in mung bean where the fresh and dry weights were almost similar to controls.

Antioxidant enzymes analyses

The analyzed antioxidant enzymes peroxidase (POD), Catalase (CAT) and Ascorbate peroxidase (APX) extracted from both mung bean and barely seedling grown for 10 days on different Cu treatments were tested for their activities. Alterations in enzyme activities were detected in response to different forms of Cu as well as different plants (Table 2). Comparing the controls, POD extracted from barely was 10 fold more active than mung bean POD. Peroxidase activity was enhanced in seedlings grown on all Cu treatments. High concentration of non-ionic Cu could induce the activity of POD to be 390 % and 102% more than control for mung bean and barely, respectively. Peroxidase activity was induced with lesser amounts in non-ionic Cu treatments compared with the corresponding ionic forms. For high concentration of non-ionic Cu, POD activity increased with percentages of 50 % and 89% for mung bean and barley, respectively. Cu in non-ionic form could lower the POD activities compared with the corresponding ionic forms but still the values higher than those of the controls.

Catalase activity was lowered by subjection to Cu in most treatments. In comparison to control, low concentration of ionic Cu could lower the activity of CAT to be 98% and 49% for mung bean and barely, respectively. In case of non-ionic Cu, CAT activity increased by 9% and 7% for low and high concentrations in mung bean. On the other plant, the increase reached 6% and 15% above control in case of low and high concentration of non-ionic Cu applied to barley seedlings. Compared to the other analyzed enzymes, the effect of Cu treatments on CAT activity was less in both plants.

Ascorbate peroxidase (APX) activity showed similar behavior as peroxidase in its response towards Cu in its different forms. In details, high concentration of non-ionic Cu induced the activity of APX to be 176% and 332% above controls in case of mung bean and barely, respectively. Non-ionic Cu form reduced the activity of APX to be 9% in mung bean and double folded the activity in barely compared with their controls. Generally, non-ionic Cu had less effect on the activity of *APX* than ionic Cu.

Proteins and amino acids

Soluble, insoluble and total proteins were determined for mung bean and barley seedlings (Table 3). The obtained data revealed that soluble, insoluble and total proteins were lowered with all Cu treatments in both plants. On exception, low non-ionic Cu could increase the soluble, insoluble and total proteins content

in barely seedlings while high non-ionic Cu form increased the soluble protein only. It seemed that non-ionic Cu could increase the protein contents compared with ionic Cu. Meanwhile, soluble and total proteins were induced by non-ionic Cu in barely seedlings where the values reached 130% and 121% for soluble and total proteins, respectively when seedlings were germinated in low concentration of non-ionic Cu. There are slight differences between the effect of low and high concentrations of ionic Cu on total protein contents in mung bean whereas in barely it was found that the higher the concentration of ionic Cu the lower the total proteins content.

TABLE 2. Effect of ionic and non-ionic Cu treatments on antioxidant enzymes (Unit g^{-1} FW) of *Vigna radiata* and *Hordeum vulgare* seedlings. Values are means (M) of four replicates ± standard deviation (SD).

Plants	Treatments	*POD*			*CAT*			*APX*		
		M	SD	%	M	SD	%	M	SD	%
V. radiata	Control	19.34 ± 4.58		100.00	25.80 ± 8.40		100.00	19.97 ± 2.05		100.00
	Cu-IL	35.37** ± 7.29		182.89	25.20 ± 7.85		97.67	12.15* ± 0.82		60.87
	Cu-NL	29.77** ± 1.82		153.95	28.20* ± 9.00		109.30	13.76** ± 0.41		68.90
	Cu-IH	94.85** ± 2.62		490.46	26.20 ± 1.79		101.55	55.27** ± 6.14		276.81
	Cu-NH	28.94** ± 7.61		149.67	27.60 ± 2.16		106.98	16.08** ± 2.05		80.43
H. vulgare	Control	188.10 ± 3.13		100.00	19.50 ± 4.95		100.00	9.84 ± 4.09		100.00
	Cu-IL	320.55** ± 9.56		170.41	19.30 ± 0.71		98.97	19.97** ± 0.41		202.94
	Cu-NL	300.64** ± 6.72		159.82	20.56 ± 4.24		105.44	16.94* ± 4.09		70.59
	Cu-IH	379.58** ± 3.76		201.79	25.73** ± 5.66		131.95	42.53** ± 4.50		432.35
	Cu-NH	353.88** ± 3.97		188.13	22.38* ± 1.41		114.77	20.25** ± 6.55		205.88

Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*.

TABLE 3. Effect of ionic and non-ionic Cu treatments on soluble, insoluble and total proteins (mg g^{-1} FW) of *Vigna radiata* and *Hordeum vulgare* seedlings. Values are means (M) of four replicates ± standard deviation (SD).

Plants	Treatments	Soluble proteins			Insoluble proteins			Total proteins		
		M	SD	%	M	SD	%	M	SD	%
V. radiata	Control	127.73 ± 7.02		100.00	171.93 ± 3.73		100.00	299.67 ± 5.70		100.00
	Cu-IL	94.53** ± 6.74		74.01	108.80** ± 2.32		63.28	203.33** ± 8.04		67.85
	Cu-NL	107.73* ± 9.98		84.34	107.27** ± 8.41		62.39	215.00** ± 3.61		71.75
	Cu-IH	94.27** ± 3.14		73.80	118.40** ± 4.74		68.86	212.67** ± 3.32		70.97
	Cu-NH	112.93* ± 4.80		88.41	89.73** ± 2.59		52.19	202.67** ± 4.39		67.63
H. vulgare	Control	58.67 ± 9.26		100.00	146.00 ± 3.07		100.00	204.67 ± 2.82		100.00
	Cu-IL	54.27 ± 6.65		92.50	137.40 ± 6.36		94.11	191.67* ± 7.32		93.65
	Cu-NL	75.73** ± 1.52		129.09	171.27** ± 5.72		117.31	207.00 ± 8.54		120.68
	Cu-IH	96.53** ± 3.89		164.55	62.13** ± 4.66		42.56	158.67* ± 7.50		77.52
	Cu-NH	65.87** ± 1.59		112.27	107.47* ± 4.69		73.61	173.33* ± 3.50		84.69

Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*.

Total free amino acids of control and Cu treated seedlings of mung bean and barley after 10 days of germination were shown in Table 4. Free amino acids were increased in most of Cu treatments by 5 - 40% above the corresponding controls. The ionic forms of Cu could increase the total free amino acids in both mung bean and barley. In case of mung bean, the increase caused by low and high ionic Cu was 10 and 40%. Similarly in barley seedlings, low and high concentrations of ionic Cu increased the total free amino acids by 15 and 19% more than controls. Non-ionic Cu could keep the content of total free amino acids more or less than controls in both mung bean and barley seedlings. The values were 89 and 103% in case of mung bean and barley at high concentration of non-ionic Cu. It can be concluded that ionic forms could induce more contents of free amino acids while non-ionic Cu form could keep the values almost similar to those of the corresponding controls.

TABLE 4. Effect of ionic and non-ionic Cu treatments on total free amino acids (mg g^{-1} FW) of *Vigna radiata* and *Hordeum vulgare* seedlings. Values are means (M) of four replicates ± standard deviation (SD).

Plants	Treatments	Total free amino acids			
		M		SD	%
V. radiata	Control	29.96	±	4.10	100.00
	Cu-IL	33.1*	±	2.76	110.48
	Cu-NL	31.74	±	3.63	105.94
	Cu-IH	41.82**	±	7.76	139.59
	Cu-NH	26.92*	±	1.25	89.85
H. vulgare	Control	8.60	±	0.47	100.00
	Cu-IL	9.90*	±	0.21	115.12
	Cu-NL	8.44	±	1.12	98.14
	Cu-IH	10.24*	±	1.18	119.07
	Cu-NH	8.90	±	1.03	103.49

Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*.

Discussion

Although Copper is considered essential for physiological processes within plant cells, it persuades an obvious toxicity depending on its concentration and availability in soil. The present study showed the influence of ionic Cu and non-ionic Cu on seed germination and antioxidant enzymes' activities of mung bean and barely. Non-ionic Cu particles were prepared by a bioprocess using *E. coli* volatile gases in a specific bioreactor. EDX analysis revealed that Cu-particles comprised copper, sulfur, nitrogen, oxygen and carbon but with no measurable phosphorus that confirms the nonexistence of contamination by bacterial cells. According to our preceding studies (Essa *et al.*, 2005 and Macaskie *et al.*, 2007), the bacterial biogas included sulfur-based and nitrogen-based gaseous metabolites such as amines and dimethyl disulfide that are discharged during the excessive metabolic activity of the bacterial culture. Bacterial volatile sulfur molecules hold immense affinity to complex metal ions into insoluble metallo-sulfur compoundsd

(Brummett *et al*., 2015; Essa and Khallaf, 2016). At the same time, the incidence of ammonia in *E. coli* biogas (confirmed using Nessler's solution) is in charge for altering pH value of copper solution in the direction of alkalinity (Table 1). Biogenic ammonia beside other amines liberated from *E. coli* culture have vital task in the complexation of copper ions into less soluble Cu-particles. The complete chemical identification of Cu-particles was not available in this investigation but EDX-analysis verified the incidence of nitrogen and sulfur in addition to carbon elements in the transformed Cu-particles. These results are in agreement with our previous study (Essa and Mostafa, 2011; Essa *et al*., 2012) where some organo-sulfur and amine groups such as thiocarbonyl, disulfide and amine functional groups were spotted by Fourier-Transform Infrared Spectroscopy in the metal complexes resulting from the treatment of heavy metals with gaseous metabolites of oxic bacterial culture.

The obtained results revealed less toxicity of non-ionic Cu particles compared with the ionic Cu form in both low and high concentrations. The percentage of seed germination as well as all growth parameters analyzed; seedling lengths, fresh and dry weights, confirmed less toxicity of Cu particles. Inhibition of root elongation caused by heavy metals may be due to metal interference with cell division, chromosomal aberrations and abnormal mitosis (Jain *et al.*, 2010 and Liu *et al.*, 2003). Moreover, reduced seedling growth in metal treatments could be as a result of the reduction in meristematic cells (Kabir *et al.*, 2008). The obtained results in this investigation revealed acceptable growth patterns with non-ionic Cu treatments indicating less toxicity of Cu in its non-ionic Cu form and hence Cu detoxification. Furthermore, in the present work, the antioxidant enzyme activities were significantly altered in response to Cu ions and non-ionic Cu. For example, all antioxidant enzymes had higher activities in seedlings treated with ionic forms of Cu. Moreover, non-ionic Cu forms could lower the POD and APX activities compared with the corresponding ionic forms but still the values higher than those of the controls. Similar to the present results, Nekrasova *et al.* (2011) reported decrease in CAT activity in *E. densa* at higher concentrations of copper ions. This can be explained by enzyme inactivation, might be, due to substitution of Fe^{2+} ion in its active center with Cu^{2+} ion (Hou *et al.*, 2007; Mallick, 2004). Clearly, Cu ions inhibited the antioxidant enzymes more strongly, compared to non-ionic Cu particles (Nekrasova *et al.*, 2011). In the present, a reason behind the APX inhibition in some cases of Cu treatments might be the lack of ascorbate due to presence of high amounts of Cu ions. It was reported previously that, copper ions promote rapid loss of ascorbate (Packer, 2001).

Several mechanisms were suggested by which the plant cells resist the toxic effects of heavy metals in particles form. One of those mechanisms is that plant cell walls present in natural settings, are primarily composed of carbon hydrate polymers, and are semi permeable. Thus, exotics need to penetrate through the cell wall prior to the membrane invagination. Limited size of pores in plant cell walls prevents larger molecules from free passing through cell wall (Carpita

et al., 1979). Moreover, creating an apoplastic pool of Cu as a way for Cu detoxification affects chemical and structural changes in cell wall under the influence of Cu excess. Increasing the permeability of plant cell walls by metal exposure lead to create "holes", and then enter into the cells by penetrating through the "holes". After entering the cells, the non-ionic Cu particles are able to transport between cells via plasmodesmata which are microscopic channels of plants traversing the cell walls and enabling transport and communication between cells. Plasmodesmata or intercellular bridges were reported to be cylindrical channels with 40nm in diameter (Tilney *et al.*, 1991). These plasmodesmata allow particles to pass from cell to another depending on their size causing less toxic effects than ionic Cu. Furthermore, several investigations excluded dissolution from the main mechanisms regulating the toxicity of metal-based non-ionic particles (Nel *et al.*, 2006). Even though the dissolution of non-ionic Cu to cupric ions has a negligible effect in plant agar media, some amount of non-ionic Cu may dissolve to become cupric ions within the cell. Most toxic action results from the non-ionic Cu because of the presence of particles or aggregated nanoparticles within the cell (Lee *et al.*, 2008). Another mechanism is changing the permeability of plasma membrane. Previously reported, damaging the plasma membrane through formation of OH· radicles which can be achieved by traces of transition metal ions such as iron and copper (Packer, 2001). Reduction in seed germination can also be attributed to alterations of selection permeability properties of cell membrane. Alteration in plasma membrane permeability might be due to changes in membrane protein channels leading to metal ions passage and hence metal toxicity. It is well known that, cells become active produce the hydrolytic enzymes they begin to digest the stored food which is converted into soluble form and transported to the primary root and shoot tips for enzyme amylase which converts starch into sugar and proteases act on proteins. When the activities of hydrolytic enzymes are affected, the food does not reach to the primary root and shoot, thereby affecting the seedling growth (Kabir *et al.*, 2008).

Another mechanism of Cu toxicity is through the generation of reactive oxygen intermediates (H_2O_2, O_2^-, OH, superoxide etc.) and then oxidative stress. Previously reported, exposure to excessive Cu ions leads to generation of oxidative stress in plant system (Gaetke and Chow, 2003). A suggested strategy to avoid toxicity of heavy metals is through the induction of antioxidant enzyme system and increase in the non-enzymatic antioxidants to reduce the oxidative damage related to excessive ROS formation caused by heavy metals (Verma and Dubey, 2003). In the present work, induced antioxidant enzyme activities were noticed in both ionic and non-ionic Cu treatments with different rations.This supports the theory of detoxification through management of oxidative stress. Antioxidant enzyme activities were highly induced in case of ionic Cu indicating more stressful conditions. Presence of ROS causes oxidative damage to biomolecules such as lipids, proteins, nucleic acids, etc. (Radwan *et al.*, 2010 and Radwan, 2012). The transition metals such as Cu can act as specific cofactors for numerous metalloproteins because of their physical and chemical

properties. It is involved in the maintenance of the functional and structural integrity of plant cells. Heavy metals exercise a detrimental influence on cells by binding to vital proteins and manage protein/enzyme functioning. In this experiment, reduction of soluble and total proteins contents was noticed in both ionic and non-ionic Cu treatments except for barley soluble protein contents. Comparing the contents of proteins, plants treated with non-ionic Cu had higher contents than those exposed to ionic Cu. Obviously, Cu could reduce the total protein contents in mung bean and barley. Packer (2001) reported that copper cations are bound to proteins leading to their degradation. Cu toxicity is determined by binding to SH-groups in proteins, thereby inhibiting enzyme activity or protein function (Cohu and Pilon, 2010).

In the present work, a noticeable decrease in the amino acids content with Cu stress that might be involved in detoxification of copper (ionic or non- ionic). Organic acids, amino acids, or peptides are potential ligands for phytochelation of metals (Clemens, 2001). Moreover, amino acids such as citric, malic, and histidine are potential ligands for heavy metals and so could play a role in tolerance and detoxification (Clemens, 2001). In the cytoplasm, metals can either bind to free amino acids, the non proteinogenic nicotianamine, protein ligands that are rich in Cys residues, such as metallothioneins, metallochaperones, phytochelatins, and low molecular weight thiols (Callahan *et al.*, 2006; Trampczynska *et al.*, 2010).

Conclusions

This work highlights the effect of ionic and non-ionic Cu on germination and metabolism of barley grains and mung bean seeds. The non-ionic Cu was prepared using a bioprocess through bacterial biogases in a specific bioreactor. Barley and mung bean subjected to non-ionic Cu particles showed significantly lower toxicity than those treated with ionic Cu. The reduced toxicity of non-ionic copper was attributed to the decline of copper capability to penetrate plant cells. Further studies are required to investigate the impact of this bioprocess on the toxicity of other metals in order to understand the collaborative impact of bacteria in diminishing heavy metals toxicity against plants in heavily polluted environment.

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إستخدام البكتيريا فى التخلص من سمية النحاس و أثر ذلك على مؤشرات إنبات الشعير و فول المانج

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يحدث تراكم أيونات النحاس في البيئة بتركيزات مرتفعة كنتيجة للأنشطة البشرية المختلفة والتي ينجم عنها الكثير من التأثيرات الضارة على جميع أنواع الكائنات الحية. إن الهدف من هذه الدراسة هو تقييم تأثير أيونات النحاس على إنبات ونمو بادرات الشعير و فول المانج مقارنة بجزيئات النحاس التى تم تحضيرها بإستخدام النواتج المتطايرة المصاحبة لنمو بكتيريا *Escherichia coli*. لقد أظهرت نتائج تحليل الميكروسكوب الالكتروني الماسح SEM و تحليل تشتت الأشعة السينية الطيفي EDX أن أيونات النحاس قد ترسبت على شكل جزيئات مستطيلة يتراوح طولها من ١-٥ ميكرون و التي تتركب من عناصر النحاس والكبريت والكربون والأكسجين والنيتروجين. و عند إختبار تأثير النحاس بالصورة الأيونية والغير أيونية علي إنبات ونمو بادرات الشعير وفول المانج أوضحت النتائج أن البذور التي تعرضت للنحاس بصورته الأيونية أظهرت إنخفاض واضح في نسبة الإنبات وكافة معايير النمو المختلفة للبادرات في حين سجلت معدلات أعلى للإنبات والنمو مع المعالجة بجزيئات النحاس الغير أيونية. وفي الوقت نفسه لوحظ زيادة في نشاط إنزيمات البيروكسيديز والاسكوربات بيروكسيديز مع جميع معاملات النحاس بينما كانت المعاملة بالنحاس في الصورة الأيونية فقط محفزة لنشاط إنزيم الكاتاليز. علاوةعلى ذلك فإن تعرض البادرات للنحاس في الصورة الغير أيونية سجلت محتويات أعلى من البروتينات بالمقارنة مع تلك المعالجة بالنحاس في الصورة الأيونية. وقد أرجعت الدراسة إنخفاض سمية جزيئات النحاس المعالج الى تراجع معدل ذوبان النحاس نتيجة لتفاعل أيونات النحاس مع بعض المركبات المخلبية المتواجدة فى الأبخرة المصاحبة لنمو بكتيريا *E. coli* والتي بدورها تقلل من إتاحية أيونات النحاس فى بيئة نمو النباتات.