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Optimization of copper for the improvement of *in vitro* plant tissue growth of *Solanum nigrum*

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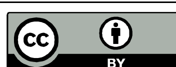

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ABSTRACT: Here was investigated the incorporation of copper in MS medium on growth, and metabolic activities of *Solanum nigrum* callus. Copper up to 75 µM increased the growth, and thereafter a decline was observed. No considerable alteration in MDA, H_2O_2, bound phenolics, flavonoids, ascorbate, and copper content was observed with the existence of 25 µM copper, then levels of these parameters were raised with rising copper concentrations. Similarly, 25 µM copper didn't induce a considerable change in lipoxygenase, superoxide dismutase, catalase, peroxidase, phenylalanine ammonia lyase, and polyphenol oxidase activities, however, high levels stimulated these enzymes. Copper at 25 µM didn't considerably reduce amino acids and soluble proteins, whereas higher concentrations reduced these parameters. Copper treatments reduced the soluble carbohydrates accumulation; only 75 µM enhanced this accumulation. Copper at 25 µM significantly increased the potassium accumulation, whereas higher concentrations reduced this accumulation. From these results, it might be contemplated the optimum effect concerning copper.

Keywords: Amino acids; Antioxidant enzymes; Carbohydrates; Copper; Lipid peroxidation; Potassium; Proteins.

1. INTRODUCTION

Black nightshade (*Solanum nigrum*) is a shrub belonging to the family Solanaceae and contains valuable medicinal components. It is a rapidly and highly output plant under normal and stressful ecological cases [1]. Recently, above its useful medicinal components, it is classified as hyperaccumulation plant.

Tissue culture technique has different implementations in enhancement and regeneration of plant, and nutrients concentrations in media have deep effects on calli outgrowth and regeneration [2]. An outgrowth of plant tissues under *in vitro* status is broadly controlled by the construction of the culture medium. In Murashige and Skoog [3] medium that is used as a tissue culture medium for many plants, levels of essential inorganic ions are based mostly for the tobacco tissue. These ion levels that were applicable for tobacco tissue culture might not enough be most favorable for the culture of different plants like *S. nigrum* [4]. The primary step towards the improvement of these crops could also be the optimization of the essential ions levels.

Cu is an elementary ion that is needed for plain outgrowth and evolution of plant [5, 6]. It's a vital ion concerned in many activities, comprising the respiration, photosynthesis [7]. Nevertheless, surplus Cu can produce injury at the cellular level, results in the inhibition of plant growth [8].

The current investigation aimed to optimize of Cu level as a fundamental micronutrient, therefore, to elevate the growth of *S. nigrum*. This research was focused on effects of various Cu concentrations on the growth, lipid peroxidation, antioxidative responses, K and Cu content.

2. MATERIALS AND METHODS

2.1. Plant tissue culture and CuO treatment

Leaves of the wild vegetation of *S. nigrum* that is grown in Assuit governorate (27°11'00"N 31°10'00"E), were used as explants to set up *in vitro* cultures. Leaves were washed under running water for ~20 min, carried to a laminar air flow chamber and treated with 50% commercial bleach containing few drops of Tween-20 for 7 min and then pursued by washing 4 times with sterile distilled water. Sterilized leaf was cut into ~1.5 cm segments and placed on sterilizes nutrient Murashige and Skoog (MS) media [3]. The MS medium consisted of 4.4 g/l MS, 30 g/l sucrose and 3 g/l gelrite, 1 mg/l of α-naphthalene acetic acid (NAA) and various concentrations of Cu (0, 25, 50, 75 and 200 µM) as CuO. The pH of the medium was buffered to 5.7 before adding gelrite and autoclaved for 15 min at 121°C temperature and 105 kPa pressure. Leaf segments (3) were transferred into the sterilized MS media. The cultures were transmitted to a cultivation room under a 16/8 h photoperiod with the cool white fluorescent light (30 µM m^{-2} S^{-1} irradiance),and the temperature was adjusted at 25 ± 1°C with 50-60% relative humidity. Each treatment contained 20 replicates (jars) and the whole experiment was repeated twice.

After 4 weeks the callus was quickly weighed for fresh weight (FW) determination, quickly frozen in liquid nitrogen and stored at -80ºC for physiological parameters analysis. Another callus of freshly harvested was oven-dried at 60 ºC for 48 h so as to outline the dry weight (DW).

Water content = FW – DW

2.2. Physiological and biochemical analysis

2.2.1. Lipid peroxidation

Lipid peroxidation in calli was estimated to consider the membrane injury. The thiobarbituric acid (TBA) test that defines malondialdehyde (MDA) as an ending output of lipid peroxidation, was measured [9]. MDA was measured as μMg^{-1} FW, utilizing an extinction coefficient (155 mM^{-1} cm^{-1}).

The H_2O_2 content of the callus samples was calorimetrically measured as represented by Mukherjee and Choudhuri [10].

2.2.2. Enzymes assay

Frozen calli (0.5 g) were crushed to a soft powder in liquid nitrogen and mixed with 5 ml of phosphate buffer (50 mM, pH 7.8) inclusive 5 mM DTT (Dithiothreitol), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 g polyvinylpyrrolidone (PVP). The mixture was purified by cheesecloth and underwent to centrifugation for 10 min at 18,000 rpm at 4 ºC. The supernatant used for the evaluating the enzymes.

The lipoxygenase (LOX; EC 1.13.11.12) activity was estimated as stated by following the Minguez-Mosquera et al. [11] technique. The precise activities were measured as changes in absorbance per mg protein per min (DA_{234} mg $protein^{-1} min^{-1}$).

The superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by following the autoxidation of epinephrine (adrenochrome) as described by Misra and Fridovich [12]. The specific activity was studied as changes in absorbance per mg protein per min (DA_{480} mg $protein^{-1}$ min^{-1}).

Aebi [13] technique was followed to estimate the CAT activity (EC 1.11.1.6). The consuming of H_2O_2 for one min was examined at 240 nm (DA_{240} mg $protein^{-1}$ min^{-1}).

The POD activity (EC 1.11.1.7) was determined in a reaction mixture spectrophotometrically following the Zaharieva et al. [14] method. The specific activity was measured as changes in absorbance per mg protein per min. (DA_{470} mg protein^{-1} min^{-1}).

The phenylalanine ammonia lyase (PAL; EC 4.3.1.5) activity was tested as the method described by Havir and Hanson [15]. The specific activity was expressed as changes in absorbance per mg protein per min (DA_{290} mg protein^{-1} min^{-1}).

The polyphenol oxidase (PPO; EC 1.14.18.1) activity was assayed by the procedure of Kumar and Khan [16]. The specific activity was calculated as changes in absorbance per mg protein per min (DA_{495} mg protein^{-1} min^{-1}).

2.2.3. Free and bound phenolic compounds

Free and bound phenolic compounds were measured by following the Kofalvi and Nassuth [17] method. Phenolics concentration was estimated from gallic acid standard curve and calculated as µg/g FW. Fresh calli tissues (0.5 g) were extracted in 50% methanol for 90 min at 80ºC and centrifuged at 14000 rpm for 15 min and the supernatant was used for free phenolics determination. The pellet was mixed with 2 ml of 0.5 N NaOH for 24 h at room temperature to release the bound phenolics, neutralized with 0.5 ml 2 N HCl and centrifuged at 14000 rpm for 15 min. The methanol and NaOH extracts (100 ul) were diluted to 1 ml with distilled water and mixed with 0.5 ml 2 N Folin-Ciocalteu's reagent and 2.5 ml of 20% Na2CO3. The absorbance was measured after 20 min at 725 nm.

2.2.4. Total flavonoids

The flavonoids content was determined by the aluminum chloride, colorimetric method [18]. The quercetin was used for calibration curve [19]. The reaction mixture was comprised of 1.0 ml of methanol extract, 0.5 ml of aluminum chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm.

2.2.5. Ascorbic acid

The ascorbic acid was determined according to Jagota and Dani [20]. A standard curve was prepared by various concentrations of ascorbic acid. Calli tissues (0.2 g) were ground with liquid nitrogen and suspended in 2 ml of 5% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 15 min at 4 ºC. Sample extract (0.2 ml) and 0.8 ml of 10% TCA were mixed vigorously and kept in an ice bath for 5 min and centrifuged at 3000 rpm for 5 min. The extract (0.5 ml) was diluted to 2.0 ml using bi-distilled water and after 0.2 ml of Folin's reagent. After 10 min, the absorbance of the blue color was measured.

2.2.6. Amino acids

Amino acids concentration was measured with the Moor and Stein [21] technique. The concentration was calculated from the glycine standard curve. Enzyme extract (0.2 ml) and 1 ml stannous chloride reagent [4 g stannous chloride in 10 ml citrate buffer and 10 ml ninhydrin reagent (0.25 g ninhydrin in 100 ml methanol)] were incubated in a boiling water bath for 20 min then after cooling measured at 570 nm.

2.2.7. Soluble proteins

Soluble proteins were measured by Folin reagent dependent on Lowry et al. [22] technique. The data were calculated through bovine serum albumin (BSA) calibration curve. Enzyme extract (0.1 ml) was added to 5 ml of the alkaline reagent solution and allowed to stand at room temperature for 10 min after that, 0.5 ml of Folin-Ciocalteu's reagent mixed rapidly. The alkaline reagent was freshly prepared by mixing 50 ml of

reagent A (2 g sodium carbonate in 100 ml 0.1 N sodium hydroxide) with 1 ml of reagent B (0.5 g $Cu_2SO_4.5H_2O$ in 100 ml 1% sodium potassium tartrate). The extinction was read after 30 min at 750 nm.

2.2.8. Soluble carbohydrates

The anthrone sulphuric acid method [23-24] was used for the determination of soluble carbohydrates. The concentration was calculated from the glucose standard curve. Fresh tissue (0.5 g) was homogenized using liquid nitrogen; suspended in 5 ml distilled water and boiled in a water bath for two hours, then centrifuged at 18000 rpm for 10 min and the supernatants collected and completed to definite volume. Sample extract (0.2 ml) and 4.5 ml freshly prepared anthrone reagent were thoroughly mixed and boiled in a water bath for 7 min, after which it was directly cooled under tap water. Anthrone reagent consisted of 0.2 g anthrone, 30 ml distilled water, 8 ml absolute ethyl alcohol, and 100 ml concentrated H_2SO_4 (D =1.84) were respectively mixed under continuous cooling in an ice bath. The absorbance of the developed blue-green color was determined at 620 nm against a blank containing only water and anthrone reagent.

2.2.9. Copper and potassium

Dried calli materials were powdered and digested in a mixture of acids including $HClO_4$ (60%) and concentrated HNO_3 and H_2SO_4 acids (1: 3: 1). Digested samples were utilized to assay K by following the Havre [25] method by using the Carl Zeiss flame photometer. Copper was determined in the digested samples by atomic absorption spectrophotometry (Buck model 210 Vgp - the USA).

2.3. Statistical Analysis

Statistical programme package SPSS (version 22) was utilized to estimate the data through one-way followed by Tukey's multiple ranges posthoc tests ($p < 0.05$). Pearson's correlation was used to understand the relationship between the average values of different parameters of examining plants.

3. RESULTS

3.1. Growth

Appropriate levels of micronutrients might rely on plant type, the hyperaccumulator plants might need a greater concentration of micronutrients than MS medium. Cu up to 75 µM increased DW and WC of *S. nigrum* callus, and thereafter a decline was observed (Fig. 1A-B). The most enhancing in callus DW was around 279.8% at 25 µM, over the control. Therefore, 25 µM Cu may designate as an optimum concentration for maximum growth of *S. nigrum*.

It is worthy to mention that correlations between growth parameters (DW and WC) and Cu content were non-significant (-0.486, -0.496, respectively).

3.2. Lipid peroxidation

MDA and H_2O_2 contents that were reported to occur at a low level in response to Cu toxicity were determined in *S. nigrum* calli subjected to various Cu treatments to assess the degree of membrane damage (Fig. 2A-B). No considerable alteration in MDA and H_2O_2 contents of calli was observed with the existence of 25 µM Cu in the medium, then levels of both (MDA and H_2O_2) raised with rising Cu levels in media.

It is important to notice that MDA and H_2O_2 contents were non-significantly and negatively correlated with the Cu content in calli (-0.524, -0.474, respectively). Further, correlations between MDA and H_2O_2 and Cu content in calli were considerably positive (0.966**, 0.948**, respectively).

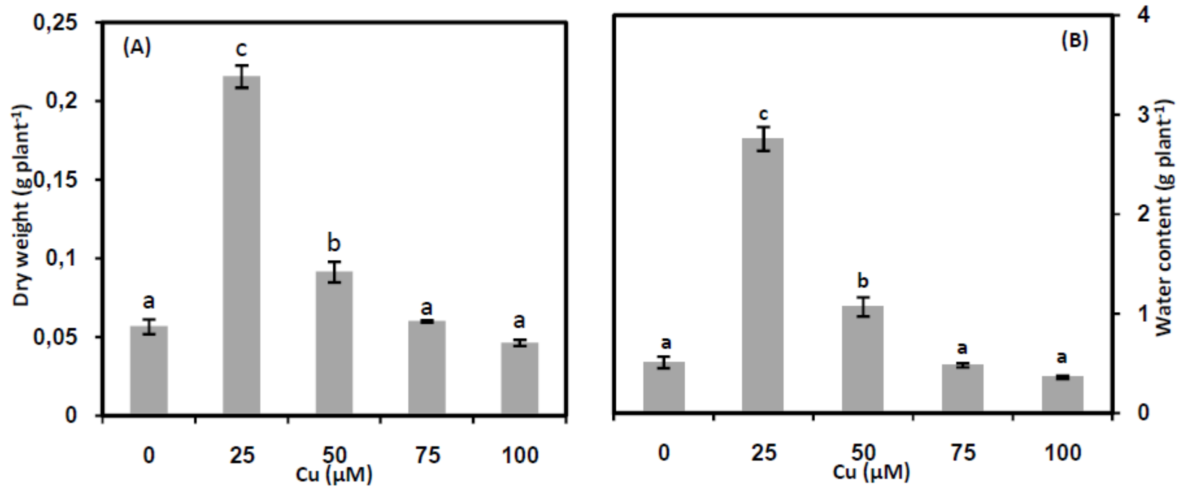

Figure 1. Dry weight (A) and water content (B) of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 20). Different letters indicate statistically significant differences according to the Tukey's HSD test (p< 0.05).

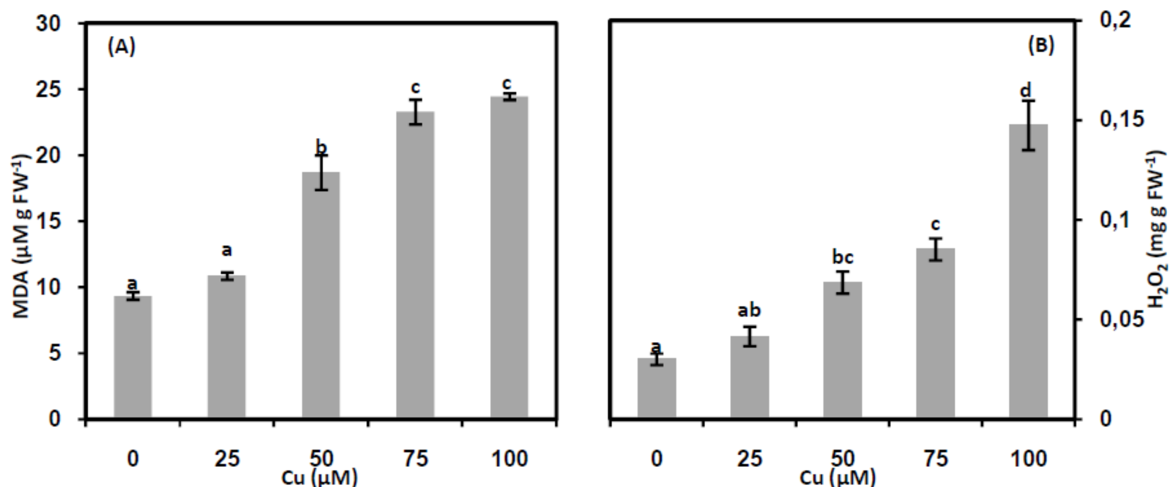

Figure 2. Malondialdehyde (MDA; A) and hydrogen peroxide (H2O2; B) content of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences according to the Tukey's HSD test (p< 0.05).

Several assays were else carried out to consider the efficiency of a variety of antioxidant enzymes under Cu treatments. Cu treatments had significant stimulatory influences on the SOD activity at high levels of Cu, while 25 µM failed to cause an identical response when compared with their absolute control (Fig. 3B).

Concerning the CAT activity (Fig. 3C) at 25 µM Cu, the minimum increase in the CAT activity was around 3.7%, whereas the maximum increase was 212.3% at the highest concentration, over the control.

Similarly, Cu applied to calli at 25 µM failed to display any significant modification in the POD activity compared with that of the untreated control (Fig. 3D). The high increase in POD activity was recorded over the control when Cu was supplied at high concentrations (50-100 µM).

The result pertaining to the influence of Cu on the activity of PAL, which has been known as the key enzyme in the biosynthesis of various protective-related secondary compounds, activity in *S. nigrum* was demonstrated in Fig. 4A. The data exhibited that PAL activity in calli was non-significant stimulated by low

Cu levels (25 and 50 µM), and thereafter its activity gradually raised by rising Cu concentrations (75 and 100 µM).

Regarding the PPO activity that has been assumed as the defense enzyme, its activity gradually increased with increasing Cu concentrations in nutrient media by 36.2, 47.8, 69.4, and 76.6%, respectively, more than the control.

With regard to the correlations between LOX, SOD, CAT, POD, PAL, PPO activities and Cu content in the *S. nigrum* callus were considerably positive (0.956**, 0.988**, 0.964**, 0.974**, 0.947**, 0.896**, respectively).

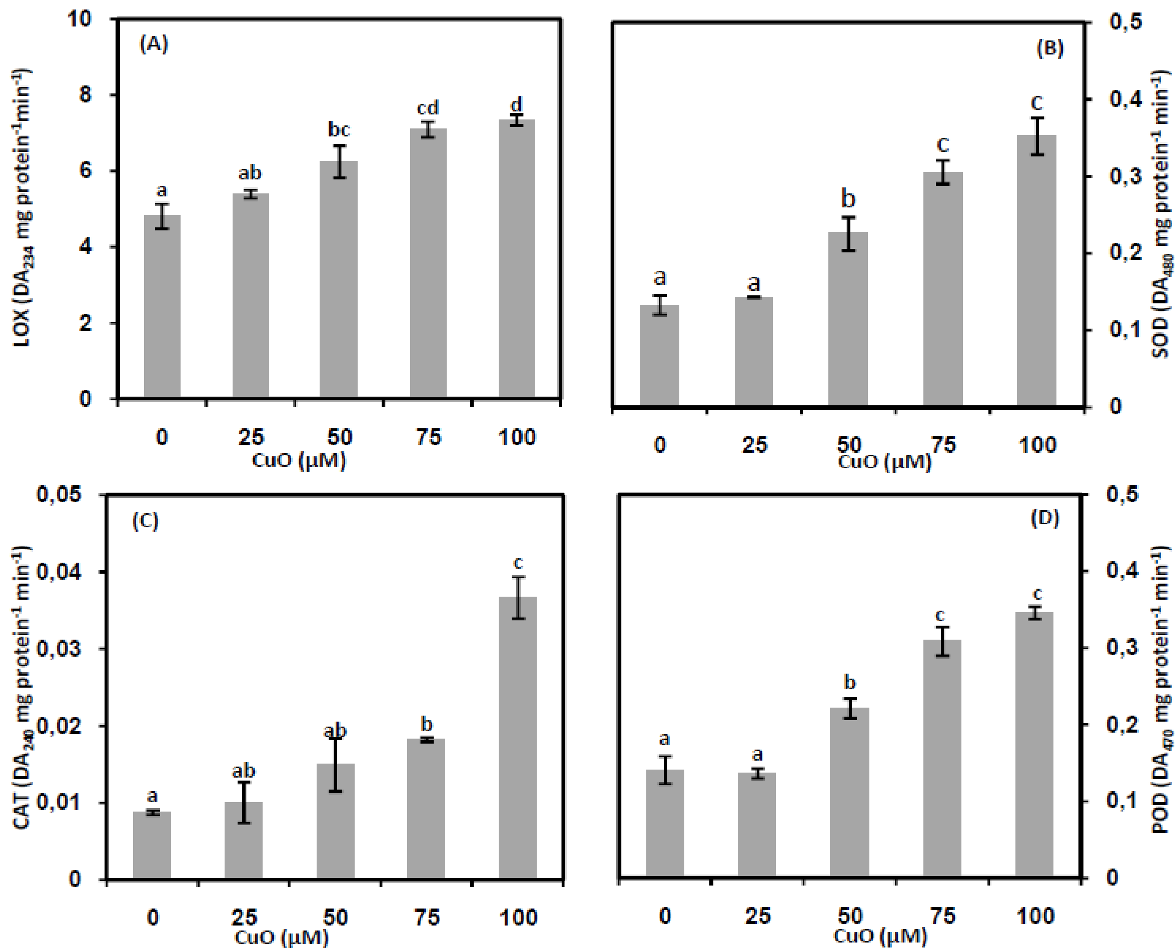

Figure 3. Lipoxygenase (LOX; A), superoxide dismutase (SOD; B), catalase (CAT; C), peroxidase (POD; D) activities of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences according to the Tukey's HSD test ($p < 0.05$).

3.4. Free and bound phenolic compounds and flavonoids

Phenolics that have been known as plant secondary metabolites playing important roles in plants resistance, were also carried out to estimate the impact of Cu treatments on *S. nigrum* calli. As revealed in Fig. 5A, phenolic compounds (free and bound) of *S. nigrum* calli increased gradually, in most cases, with the increase of the Cu level, and the highest phenolics were consistently found in calli grown at the highest Cu level. Moreover, the result exposed that there is a considerable favorable correlation between free and bound phenolics and Cu concentration in the callus (0.861** and 0.971**, respectively).

The data presented in Fig. 5B showed that Cu treatments non-significantly increased the flavonoids content in *S. nigrum* calli; only 100 µM considerably increased it. The outcome of that study indicated that the flavonoids content represented a significant correlation (0.915**) with the rise in Cu concentration in the medium.

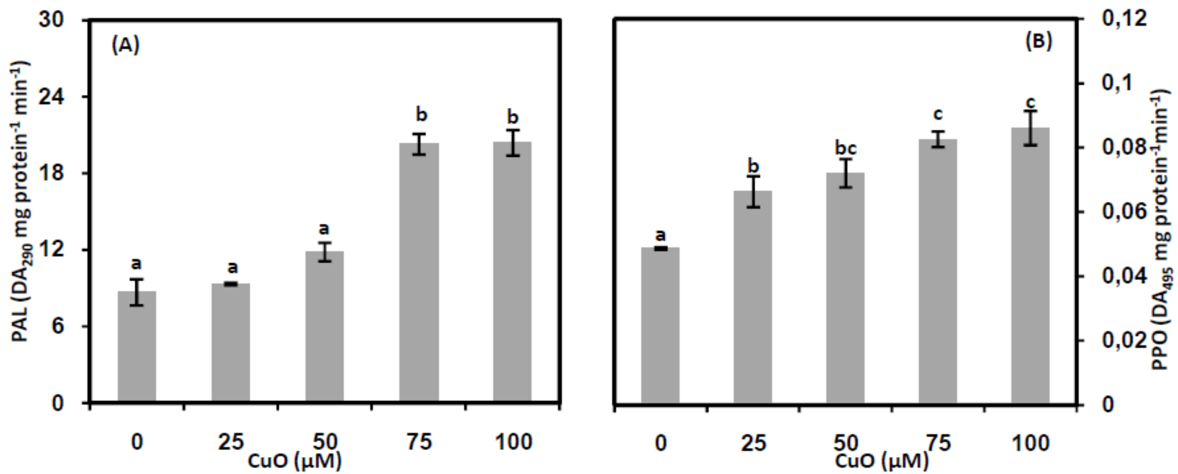

Figure 4. Phenylalanine ammonia lyase (PAL; A) and polyphenol oxidase (PPO; B) activities of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences according to the Tukey's HSD test (p< 0.05).

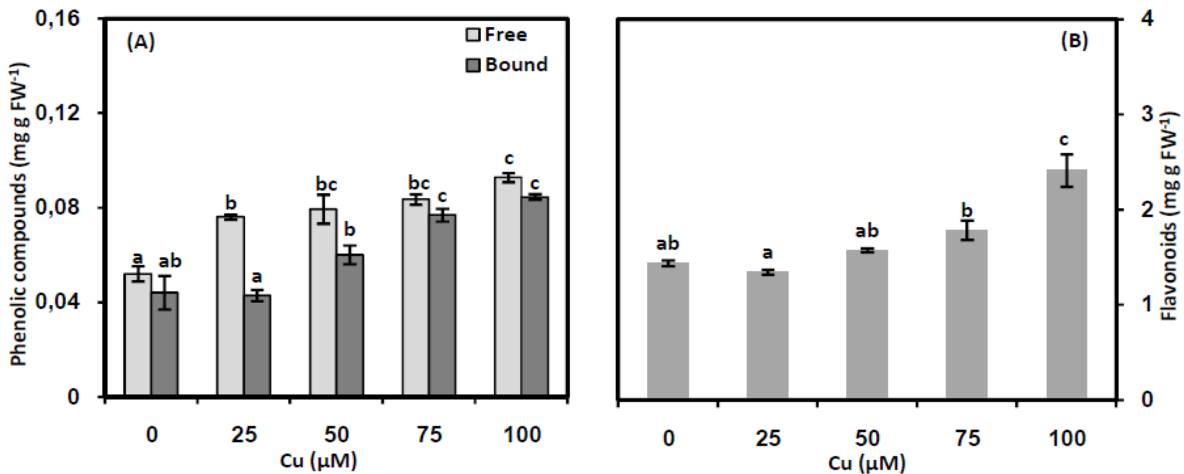

Figure 5. The content of free and bound phenolic compounds (A) and flavonoids (B) of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences between different treatments according to the Tukey's HSD test (p < 0.05).

3.5. Ascorbic acid

Results of the AsA in *S. nigrum* calli, the antioxidant molecule, revealed that the AsA failed to exhibit a significant stimulation at 25 and 50 µM Cu when compared with controls (Fig. 6A). Further, higher concentrations (75 and 100 µM) caused 87.2 and 119.9% an enhancement in the AsA of calli, respectively, over than controls. Interestingly, the Cu application revealed a considerable positive correlation (0.916**) between AsA.

3.5. Amino acids

Cu treatments had inhibitory results on amino acids concentration of *S. nigrum* calli at high Cu levels (50-100 µM), whereas 25 µM Cu failed to induce the same response, compared with their control (Fig. 5B). Moreover, the data appeared that the amino acids concentration negatively correlative with the Cu concentration in calli (-0.918**).

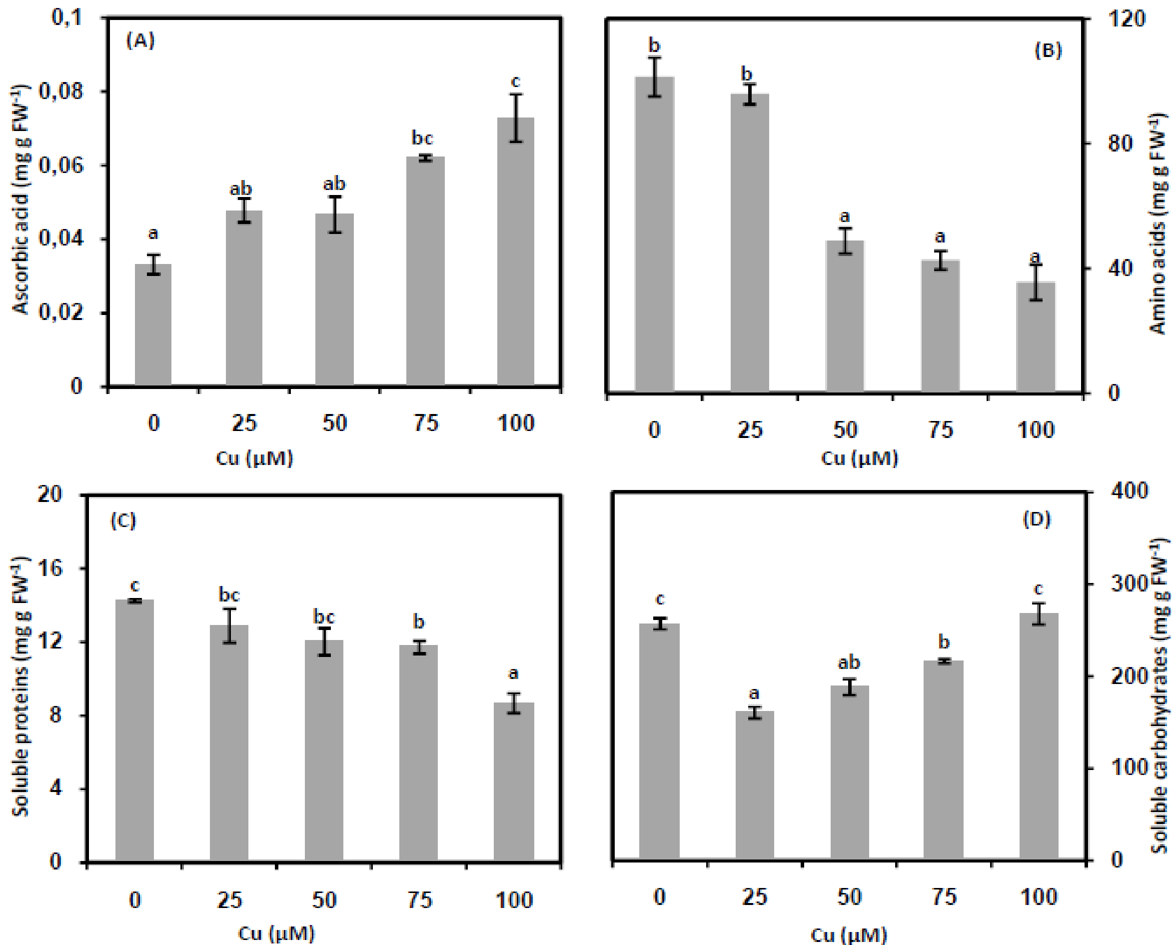

Figure 6. Ascorbic acid (A), amino acids (B), soluble proteins (C), and soluble carbohydrates (D) of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences according to the Tukey's HSD test ($p < 0.05$).

3.7. Soluble proteins

Cu at low levels (25-50 µM) didn't significantly reduce soluble proteins in *S. nigrum* calli, whereas higher concentrations (75 and 100 µM) induced a regarding 17.6 and 39% reduction in soluble proteins, respectively, in comparing with controls (Fig. 6C). The treatment with Cu displayed a strong negative correlation between soluble proteins (-0.918**) and increment in the Cu content.

3.8. Soluble carbohydrates

Based on the data presented in Fig. 6D, it can be observed that Cu reduced the soluble carbohydrates accumulation in *S. nigrum* calli, as compared with the control; only 100 µM increased its accumulation. Further, it is detected that the correlation between soluble carbohydrates and the Cu content was non-significantly (0.360).

3.9. Potassium and copper

The data on K concentration, the superabundant cation in plants, in *S. nigrum* calli are expressed in Fig. 7A. Compared to the control, stimulation of K accumulation with application 25 µM Cu was recorded to be 123.4%. Further, moderate concentrations (50 and 75 µM) failed to reduce the K content over their corresponding control, whereas 100 µM reduced it. The correlation between contents of K and Cu was negatively significant (-0.721*).

The outcomes of this study (Fig. 7B) disclosed that the Cu content of *S. nigrum* calli increased gradually with increasing the Cu level in nutrient media. The highest accumulation of Cu in the callus was consistently found in plants grown in culture supplying with 100 µM Cu. Interestingly to notice that applying of the lowest concentration of Cu failed to induce a considerable raise within the Cu content in calli.

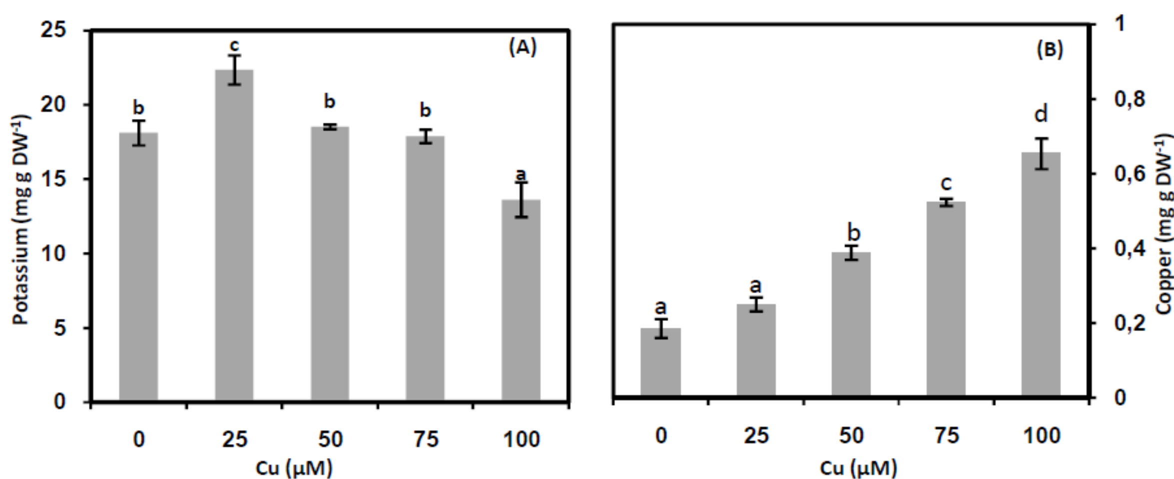

Figure 7. Potassium (A) and copper (B) content of *S. nigrum* callus grown under different concentrations of Cu. The data are means ± SD (n = 4). Different letters indicate statistically significant differences according to the Tukey's HSD test ($p< 0.05$).

4. DISCUSSION

Micronutrients confer main roles in metabolism and in the conservation of tissue function [26]. Cu is a main micronutrient required for enzymes and proteins, which involve in plant metabolism. In this investigation, the consequences of Cu, as a micronutrient, on the metabolism of *S. nigrum*, in turn of its potential use for optimum growth *in vitro*, were studied.

4.1. Growth parameters

The Cu application up to 75 µM increased growth parameters of *S. nigrum* calli. An additional increase in Cu beyond optimal levels conferred adverse impacts on the callus growth. Of various levels of CuO (25-100 µM) tested, 25 µM was found the optimum to induce the highest callus growth (3.8-fold DW, compared to control) after 4 weeks of culture. Non-significant correlations between growth parameters and Cu concentration in calli confirmed this result. It has been considered that the optimization of Cu in the MS medium might display an important function in getting the highest callus growth. Similar growth stimulation by supplied Cu, over than MS medium, has been recorded for various plant species under *in vitro* situation [27-29].

4.2. Lipid peroxidation

In order to analyze whether Cu treatments affected the induction of MDA and H_2O_2 that have been

known to be induced by the Cu toxicity, these parameters were analyzed. Data reported here displayed non-considerable influences of the best growth concentration (25 µM) on MDA and H_2O_2 content in *S. nigrum* calli, and thereafter a rise was observed. These effects suggested that Cu at 25 µM might be the optimum concentration and did not have toxic impacts. The obtained data herein indicated that MDA and H_2O_2 content were enhanced under high used concentrations of Cu that are widely known to stimulate membranes injury [30, 31].

4.3. Enzymes

Under toxic Cu levels, the lipid peroxidation of membranes is probably a reaction induced by the LOX activity [32], additionally by the reactive oxygen species (ROS) created by the Fenton reaction under toxic Cu [33]. The present results clarified a non-significant increase in the LOX activity at 25 µM Cu, whereas 50-100 µM Cu treatments exhibited an improvement relative to the control. This result confirmed MDA and H_2O_2 results, which can reveal that excess Cu than optimum resulting in lipid oxidation. Recently, Hippler et al. [34] found a remarkable rise in the *LOX1* gene expression in Arabidopsis roots with rising Cu concentrations in the culture medium, which refers that such oxylipins were linked with a Cu-stimulated response.

These results would possibly confirm previous results, which improved activities of these enzymes at the supra-optimal concentration may be helpful for responses of calli to Cu stress, which the excess Cu is able to stimulate the antioxidant systems related to these enzymes that further strengths the *S. nigrum* plant to face up excessive Cu stress. Similar activation in antioxidant enzymes by Cu toxicity has been demonstrated for various plant species under Cu toxicity stress conditions [30, 35, 36].

The activity of PAL is increased in plant development stages and additionally by (a)biotic stresses [37]. In the current study, Cu at high levels caused a notable stimulation of the PAL activity, whereas low levels (25 and 50 µM) did not exert a significant stimulation. This result agreed with Ibrahim et al. [38] who reported that a rise in PAL activity under an excess of Cu could because of limitation in proteins content that produced due to restrictions in nitrogen pool under Cu toxicity.

Enhancement of the PPO activity clearly under (a)biotic stresses indicates the involvement of PPO in plant defence against different stressors [39]. In this investigation, the PPO activity augmented progressively with rising Cu concentrations. We speculated that the PPO is a Cu-containing enzyme and thus it is activity increased with rising the Cu content within nutrient media. The present result is consistent with previous findings of Dalfard et al. [40]. However, the activation role of Cu in the PPO capacity in the plant is not yet clear and needs further investigations.

4.4. Free and bound phenolic compounds and flavonoids

As *S. nigrum* plants are rich with phenolic compounds, phenolics have considerable pharmacological characteristics and thus any amendment in environmental conditions can have an effect on the quantity or construction of chemical compounds. Cu plays a remarkable role in the biosynthesis of phenolics and its shortage can reduce phenolics in the plant [41]. The data obtained herein implied that free phenolics were markedly increased in *S. nigrum* calli with the increasing Cu content within the nutrient medium. Similarly, bound phenolics were considerably increased in Cu-treated calli, except at 25 µM, the rise was non-significant. The rise in free phenolic compounds at 25 µM Cu without damage of plants might be important in medicative plants. Correspondingly, Gautam et al. [42] pointed out that free phenolics increased under excess Cu in safflower variety PBNS-12 seedlings that were cultivated *in vitro*. The strong correlations between free and bound phenolics and Cu concentration in calli may confirm earlier study of Jung et al. [43] who referred that OH and COOH groups of phenolics help in binding with heavy metals such Cu. Moreover, Iwasaki et al. [44] reported that oxidized Cu(II) is reduced to reduced form Cu(I) by phenolics and the re-oxidation of Cu(I) to Cu(II) is accompanied by the formation of ROS.

Flavonoids are related to a wide vision of health-boosting impact and are necessary compounds in a diversity of nutraceutical, pharmaceutical, medicinal and cosmetic applications [45]. The present data cleared that Cu treatments non-significantly increased the flavonoids content, except the highest used level that considerably increased it. The significant correlation between flavonoids and Cu content may demonstrate the function of these secondary metabolites in the defence mechanisms towards excess Cu. That effect is supported by the result shown by Gautam et al. [42], which demonstrated that flavonoids are established to be increased with the rise in Cu in the nutrient medium. Flavonoids have the antioxidant activity that scavenges the ROS *via* limiting and neutralizing radicals previous they damage the cell [46].

4.5. Ascorbic acid

It is supposed that apoplastic AsA contents might be pivotal for ecological stressor perception as an instantaneous connect and after engaged within the following downstream stresses signaling and response in plant [47, 48]. It was clear that low Cu treatments (25 and 50 µM) did not cause a significant stimulation in the AsA content in calli, whereas higher Cu concentrations induced a considerable increase in the AsA content. The rise in AsA at the high concentration would possibly assign as an antioxidant for trapping ROS that synthesized under Cu stress. Recently, López-Vargas et al. [49] found that the utilization of Cu nanoparticles remarkably increased the AsA content, which consequently increases tomato yield and quality.

4.6. Amino acids

Amino acids have an effect on varied physiological processes and help in withstand (a)biotic stresses, additionally to their role as proteins constituent [50]. Within the current study, the reduction in the biosynthesis of amino acids at high used Cu concentrations and additionally their negative correlation with Cu concentration might be associated with the attack of ROS to biomolecules [51]. Otherwise, the low used Cu concentration (25 µM) failed to change the amino acids concentration would possibly because of this ideal concentration for calli growth.

4.7. Soluble proteins

Excess heavy metals affect cellular proteins *via* interfering with their folding process [52]. In this research, raised concentrations of Cu (75 and 100 µM) considerably reduced soluble proteins, whereas low levels non-significantly reduced their content in *S. nigrum* calli. These results could confirm [8] results who concluded that Cu can be bound irreversibly with SH groups, resulting in stimulate protein degradation. At low Cu levels, it was clear that there is no proteins degradation and this might help in the stimulation of calli growth.

4.8. Soluble carbohydrates

Carbohydrates count as the master supply of energy in plants and Cu is a substantial trace element in their metabolism. The obtained outcomes revealed that Cu decreased the soluble carbohydrates content in *S. nigrum* calli, only the highest concentration failed to minimize its accumulation. This reduction in soluble carbohydrates suggested that consumption of soluble carbohydrates could favor augmentation of cell population *via* enhancing the callus growth. In this context, Wu et al. [53] reported that carbohydrates act as a signal molecule and glucose has a pivotal function in plants growth by interacting with phytohormones [54].

4.9. Potassium and copper

Excess Cu disrupts cations flux like Ca and K [55, 56], modify membranes stability and permeability [58], and launch a stress response, which includes an imbalance in the ROS production and scavenging leading to tissues damage [55, 58]. The current study referred that application of 25 µM Cu increased the K accumulation in calli, while 100 µM concentration reduced its accumulation. This increase in K content may

be also required to enhance the callus growth. The decrease in K content under the highest Cu used level may confirm Palm et al. [56] results who concluded that increased OH^- concentration has been related with increased K efflux and decreased K concentration in the plant under Cu stress.

The double chemistry of Cu (Cu^{2+} and Cu^+) permits to a wide interaction with various molecules, particularly proteins, to boost chemical reactions [59]. In the current study, Cu content increased considerably in *S. nigrum* calli, and this increase was more pronounced under the highest used level, except at the lowest level there was a non-significant increase. This increase in Cu content under Cu stress was previously elucidated in other plants [60].

5. CONCLUSION

Improvement *S. nigrum* growth the medicinal and phytoaccumulator plant is important. The manipulated concentration of inorganic ions demonstrated that each plant has a specific demand for nutrients. This study refers that optimization of Cu in the nutrient medium plays a pivotal role in getting maximum callus growth in *S. nigrum*. The outcomes of this research referred that growth of *S. nigrum* calli improved after the usage of optimum concentration of Cu. The stimulatory impact of the optimum concentration of Cu accompanied by non-significant changes in lipid peroxidation parameters, antioxidant enzymes, phenolic compounds, flavonoids, AsA, amino acids, soluble proteins, and Cu content. Otherwise, the optimum Cu level caused a reduction in soluble carbohydrates and increase in the K content. Therefore, it might be suggested that at this Cu used level there is no toxicity impact. Additional analysis is needed to explore the optimum role of other ions in plants.

Abbreviations:

ANOVA: analysis of variance
AsA: ascorbic acid
BSA: serum albumin
CAT: catalase
CuO: copper oxide
DTT: dithiothreitol
DW: dry weight
EDTA: ethylenediaminetetraacetic acid
FW: fresh weight
H2O2: hydrogen peroxide
K: Potassium
LOX: lipoxygenase
MDA: malondialdehyde
MS: Murashige and Skoog
NAA: α-naphthalene acetic acid
PAL: phenylalanine ammonia lyase
POD: peroxidase
PPO: polyphenol oxidase
PVP: polyvinylpyrrolidone
ROS: reactive oxygen species
SOD: superoxide dismutase
S. nigrum: *Solanum nigrum*
TBA: thiobarbituric acid
WC: water content

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REFERENCES

1. Rehman MZ, Rizwan M, Ali S, Ok YS, Ishaque, Saifullah W, et al. Remediation of heavy metal contaminated soils by using *Solanum nigrum*: A review. Ecotoxicol Environ Saf. 2017; 143: 236-248.
2. Ramage CM, Williams RR. Mineral nutrition and plant morphogenesis. In Vitro Cell Develop Biol Plant. 2002; 38: 116-124.
3. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962; 15: 473-497.
4. Dahleen LS. Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell Tissue Organ Culture. 1995; 43: 267-269.
5. Li S, Zhang G, Gao W, Zhao X, Deng C, Lu L. Plant growth, development and change in GSH level in safflower (*Carthamus tinctorius* L.) exposed to copper and lead. Arch Biol Sci. 2015; 67: 385-396.
6. Adhikari T, Sarkar D, Mashayekhi H, Xing B. Growth and enzymatic activity of maize (*Zea mays* L.) plant: Solution culture test for copper dioxide nano particles. J. Plant Nutr. 2016; 39: 99-115.
7. Adrees M, Ali S, Rizwan M, Ibrahim M, Abbas F, Farid M, et al. The effect of excess copper on growth and physiology of important food crops: a review. Environ Sci Pollut Res. 2015; 22: 8148-8162.
8. Bouazizi H, Jouili H, Geitmann A, El Ferjani E. Copper toxicity in expanding leaves of *Phaseolus vulgaris* L.: antioxidant enzyme response and nutrient element uptake. Ecotoxicol Environ Saf. 2010; 73: 1304-1308.
9. Madhava Rao KV, Sresty TV. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. Plant Sci. 2000; 157: 113-128.
10. Mukherjee SP, Choudhuri MA. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Physiol Plant. 1983; 58: 166-170.
11. Minguez-Mosquera MI, Jaren-Galan M, Garrido-Fernandez J. Lipoxygenase activity during pepper ripening and processing of paprika. Phytochemistry. 1993; 32: 1103-1108.
12. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247: 3170-3175.
13. Aebi H. Catalase in vitro. Methods Enzymol. 1984; 105: 121-126.
14. Zaharieva T, Yamashita K, Matsumoto H. Iron deficiency induced changes in ascorbate content and enzyme activities related to ascorbate metabolism in cucumber roots. Plant Cell Physiol. 1999; 40: 273-280.
15. Havir EA, Hanson KR. L-phenylalanine ammonia-lyase. II. Mechanism and kinetic properties of the enzyme from potato tubers. Biochemistry. 1968; 7: 1904-1914.
16. Kumar KB, Khan PA. Peroxidase and polyphenol oxidase in excised ragi (*Eleusine corocana* cv PR 202) leaves during senescence. Indian J Exp Biol.1982; 20: 412-416.
17. Kofalvi S, Nassuth A. Influence of wheat streak mosaic virus infection on phenylpropanoid metabolism and the accumulation of phenolics and lignin in wheat. Physiol Mol Plant Pathol. 1995; 47: 365-377.
18. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002; 10: 178-182.

19. Ordoñez AA, Gomez JG, Vattuone MA, Isla MI. Antioxidant activities of *Sechium edule* (Jacq.) Swart extracts. Food Chem. 2006; 97: 452-458.
20. Jagota SK, Dani HM. A new colorimetric technique for the estimation of vitamin C using Folin phenol reagent. Anal Biochem. 1982; 127: 178-182.
21. Moore S, Stein WH. Photometric ninhydrin method for use in the chromatography of amino acids. J Biol Chem. 1948; 176: 367-388.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
23. Fales FW. The assimilation and degradation of carbohydrates by yeast cells. J Biol Chem. 1951; 193: 113-118.
24. Schlegel HG. Die Verwertung organischer Säuren durch *Chlorella* im licht. Planta. 1956; 47: 510-526.
25. Havre GN. The flame photometric determination of sodium, potassium and calcium in plant extracts with special reference to interference effects. Anal Chim Acta. 1961; 25: 557-566.
26. Shenkin A. The key role of micronutrients. Clin Nutr. 2006; 25: 1-13.
27. Jain P, Kachhwaha S, Kothari SL. Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* (Bert.) by using high copper levels in the culture medium. Sci Horticul. 2009; 119: 315-319.
28. Fatima N, Ahmad N, Anis M. Enhanced *in vitro* regeneration and change in photosynthetic pigments, biomass and proline content in *Withania somnifera* L. (Dunal) induced by copper and zinc ions. Plant Physiol Biochem. 2011; 49: 1465-1471.
29. Shahid A, Ahmad N, Anis M, Alatar AA, Faisal M. Morphogenic responses of *Rauvolfia tetraphylla* L. cultures to Cu, Zn and Cd ions. Rendiconti Lincei Scienze Fisiche Naturali. 2016; 27; 369-374.
30. Liu JJ, Wei Z, Li JH. Effects of copper on leaf membrane structure and root activity of maize seedling. Bot Stud. 2014; 55: 47.
31. Chen J, Shafi M, Li S, Wang Y, Wu J, Ye Z, et al. Copper induced oxidative stresses, antioxidant responses and phytoremediation potential of Moso bamboo (*Phyllostachys pubescens*). Sci Rep. 2015; 5: 13554.
32. Cuypers A. The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. J Plant Physiol. 2011; 168: 309-316.
33. Yruela I. Copper in plants: acquisition, transport and interactions. Funct Plant Biol. 2013; 36: 409-430.
34. Hippler FW, Mattos-Jr D, Boaretto RM, Williams LE. Copper excess reduces nitrate uptake by *Arabidopsis* roots with specific effects on gene expression. J Plant Physiol. 2018; 228: 158-165.
35. Karimi P, Khavari-Nejad RA, Niknam V, Ghahremaninejad F, Najafi F. The effects of excess copper on antioxidative enzymes, lipid peroxidation, proline, chlorophyll, and concentration of Mn, Fe, and Cu in *Astragalus neo-mobayenii*. Sci World J. 2012; 2012: 615670.
36. Islek C, Unal BT. Copper toxicity in *Capsicum annuum*: superoxide dismutase and catalase activities, phenolic and protein amounts of *in-vitro*-grown plants. Polish J Environ Stud. 2015; 24: 2441-2445.
37. Zhang X, Liu CJ. Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol Plant. 2015; 8: 17-27.
38. Ibrahim MH, Chee Kong Y, Mohd Zain NA. Effect of cadmium and copper exposure on growth, secondary metabolites and antioxidant activity in the medicinal plant Sambung Nyawa (*Gynura procumbens* (Lour.) Merr). Molecules. 2017; 22: E1623.

39. Boeckx T, Winters AL, Webb KJ, Kingston-Smith AH. Polyphenol oxidase in leaves: is there any significance to the chloroplastic localization? J Exper Bot. 2015; 66: 3571-3579.
40. Dalfard AB, Khajeh K, Soudi MR, Naderi-Manesh H, Ranjbar B, Sajedi RH. Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium. Enzyme Microb Technol. 2006; 39: 1409-1416.
41. Dicko HM, Gruppen H, Traore AS, Voragen AG, Berkel WJ. Phenolic compounds and related enzymes as determinants of sorghum for food use. Biotechnol Mol Biol Rev. 2006; 1: 21-38.
42. Gautam S, Anjani K, Srivastava N. *In vitro* evaluation of excess copper affecting seedlings and their biochemical characteristics in *Carthamus tinctorius* L. (variety PBNS-12). Physiol Mol Biol Plant. 2016; 22: 121-129.
43. Jung C, Maeder V, Funk F, Frey B, Sticher H, Frossard E. Release of phenols from *Lupinu salbus* L. roots exposed to Cu and their possible role in Cu detoxification. Plant Soil. 2003; 252: 301-312.
44. Iwasaki Y, Hirasawa T, Maruyama Y, Ishii Y, Ito R, Saito K, Umemura T,Nishikawa A, Nakazawa H. Effect of interaction between phenolic compounds and copper ion on antioxidant and pro-oxidant activities. Toxicol In Vitro. 2011; 25: 1320-1327.
45. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016; 5: e47.
46. Boguszewska D, Zagdańska B. ROS as signaling molecules and enzymes of plant response to unfavorable environmental conditions. InTech Rijeka Croatia. 2012; 341-362.
47. Horemans N, Foyer CH, Asard H. Transport and action of ascorbate at the plant plasma membrane. Trend Plant Sci. 2000; 5: 263-267.
48. Akram NA, Shafiq F, Ashraf M. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. Front Plant Sci. 2017; 8: 613.
49. López-Vargas ER, Ortega-Ortíz H, Cadenas-Pliego G, Romenus K, Fuente MC, Benavides-Mendoza A, Juárez-Maldonado A. Foliar application of copper nanoparticles increases the fruit quality and the content of bioactive compounds in tomatoes. Appl Sci. 2018; 8: 1020.
50. Hildebrandt TM, Nesi AN, Araújo WL, Braun HP. Amino acid catabolism in plants. Mol Plant. 2015; 8: 1563-1579.
51. Zhang FQ, Wang YS, Lou ZP, Dong JD. Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere. 2007; 67: 44-50.
52. Hasan MK, Cheng Y, Kanwar MK, Chu XY, Ahammed GJ, Qi ZT. Responses of plant proteins to heavy metal stress - a review. Front Plant Sci. 2017; 8: 1492.
53. Wu QS, Srivastava AK, Zou YN. AMF-induced tolerance to drought stress in citrus: A review. Sci Horticul. 2013; 164: 77-87.
54. Mishra BS, Singh M, Aggrawal P, Laxmi A. Glucose and auxins signaling interaction in *Arabidopsis thaliana* seedling root growth and development. PloS One. 2009; 4: e4502.
55. Rodrigo-Moreno A, Andrès-Colas N, Poschenrieder C, Gunse B, Penarrubias L, Shabala S. Calcium- and potassium permeable plasma membrane transporters are activated by copper in Arabidopsis root tips: linking copper transport with cytosolic hydroxyl radical production. Plant Cell Environ. 2013; 36: 844-855.
56. Palm E, Nissim WG, Giordano C, Mancuso, S, Azzarello E. Root potassium and hydrogen flux rates as potential indicators of plant response to zinc, copper and nickel stress. Environ Exp Bot. 2017; 143: 38-50.

57. Demidchik V, Sokolik A, Yurin V. Characteristics of non-specific permeability and H^+-ATPase inhibition induced in the plasma membrane of *Nitella flexilis* by excessive Cu^{2+}. Planta. 2001; 212: 583-590.
58. Demidchik V, Straltsova D, Medvedev SS, Pozhvanov GA, Sokolik A, Yurin V. Stress induced electrolyte leakage: the role of K^+-permeable channels and involvement in programmed cell death and metabolic adjustment. J Exp Bot. 2014; 65: 1259-1270.
59. Festa RA, Thiele DJ. Copper: an essential metal in biology. Curr Biol. 2011; 21: R877-R883.
60. Da Costa M, Sharma P. Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in *Oryza sativa*. Photosynthetica. 2016; 54: 110-119.