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

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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  $\mu\text{M}$  increased the growth, and thereafter a decline was observed. No considerable alteration in MDA,  $\text{H}_2\text{O}_2$ , bound phenolics, flavonoids, ascorbate, and copper content was observed with the existence of 25  $\mu\text{M}$  copper, then levels of these parameters were raised with rising copper concentrations. Similarly, 25  $\mu\text{M}$  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  $\mu\text{M}$  didn't considerably reduce amino acids and soluble proteins, whereas higher concentrations reduced these parameters. Copper treatments reduced the soluble carbohydrates accumulation; only 75  $\mu\text{M}$  enhanced this accumulation. Copper at 25  $\mu\text{M}$  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 sterilized 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  $\alpha$ -naphthalene acetic acid (NAA) and various concentrations of Cu (0, 25, 50, 75 and 200  $\mu$ M) 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  $\mu$ M m<sup>-2</sup> S<sup>-1</sup> irradiance), and the temperature was adjusted at 25  $\pm$  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 -80°C for physiological parameters analysis. Another callus of freshly harvested was oven-dried at 60 °C for 48 h so as to outline the dry weight (DW).

$$\text{Water content} = \text{FW} - \text{DW}$$

### 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  $\mu$ Mg<sup>-1</sup> FW, utilizing an extinction coefficient (155 mM<sup>-1</sup> cm<sup>-1</sup>).

The H<sub>2</sub>O<sub>2</sub> 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 °C. 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<sub>234</sub> mg protein<sup>-1</sup> min<sup>-1</sup>).

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<sub>480</sub> mg protein<sup>-1</sup> min<sup>-1</sup>).

Aebi [13] technique was followed to estimate the CAT activity (EC 1.11.1.6). The consuming of H<sub>2</sub>O<sub>2</sub> for one min was examined at 240 nm (DA<sub>240</sub> mg protein<sup>-1</sup> min<sup>-1</sup>).

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<sup>-1</sup> min<sup>-1</sup>).

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<sup>-1</sup> min<sup>-1</sup>).

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<sup>-1</sup> min<sup>-1</sup>).

### **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  $\mu\text{g/g}$  FW. Fresh calli tissues (0.5 g) were extracted in 50% methanol for 90 min at 80°C 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  $\mu\text{l}$ ) 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%  $\text{Na}_2\text{CO}_3$ . 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 °C. 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  $\text{Cu}_2\text{SO}_4 \cdot 5\text{H}_2\text{O}$  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  $\text{H}_2\text{SO}_4$  ( $D = 1.84$ ) 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  $\text{HClO}_4$  (60%) and concentrated  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  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

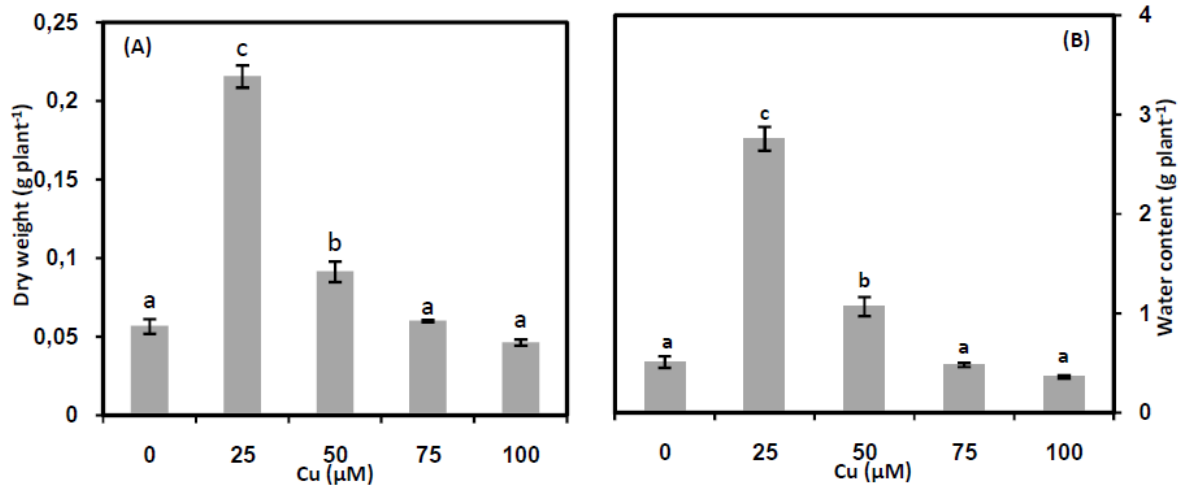
Appropriate levels of micronutrients might rely on plant type, the hyperaccumulator plants might need a greater concentration of micronutrients than MS medium. Cu up to 75  $\mu\text{M}$  increased DW and WC of *S. nigrum* callus, and thereafter a decline was observed (Fig. 1A-B). The most enhancing in callus DW was around 279.8% at 25  $\mu\text{M}$ , over the control. Therefore, 25  $\mu\text{M}$  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).

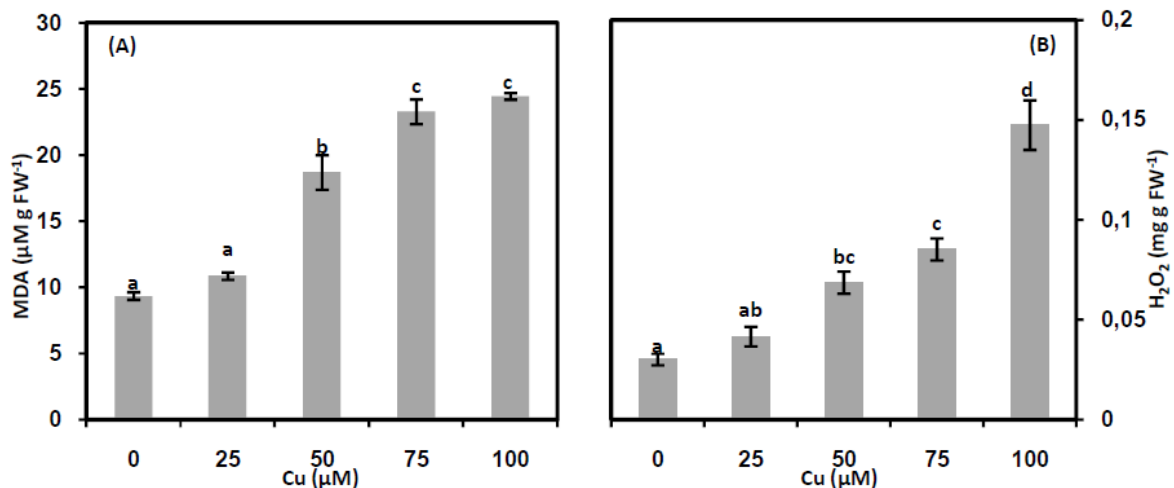
### 3.2. Lipid peroxidation

MDA and  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  contents of calli was observed with the existence of 25  $\mu\text{M}$  Cu in the medium, then levels of both (MDA and  $\text{H}_2\text{O}_2$ ) raised with rising Cu levels in media.

It is important to notice that MDA and  $\text{H}_2\text{O}_2$  contents were non-significantly and negatively correlated with the Cu content in calli (-0.524, -0.474, respectively). Further, correlations between MDA and  $\text{H}_2\text{O}_2$  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  $\pm$  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 (H<sub>2</sub>O<sub>2</sub>; B) content of *S. nigrum* callus grown under different concentrations of Cu. The data are means  $\pm$  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  $\mu$ M failed to cause an identical response when compared with their absolute control (Fig. 3B).

Concerning the CAT activity (Fig. 3C) at 25  $\mu$ M 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.

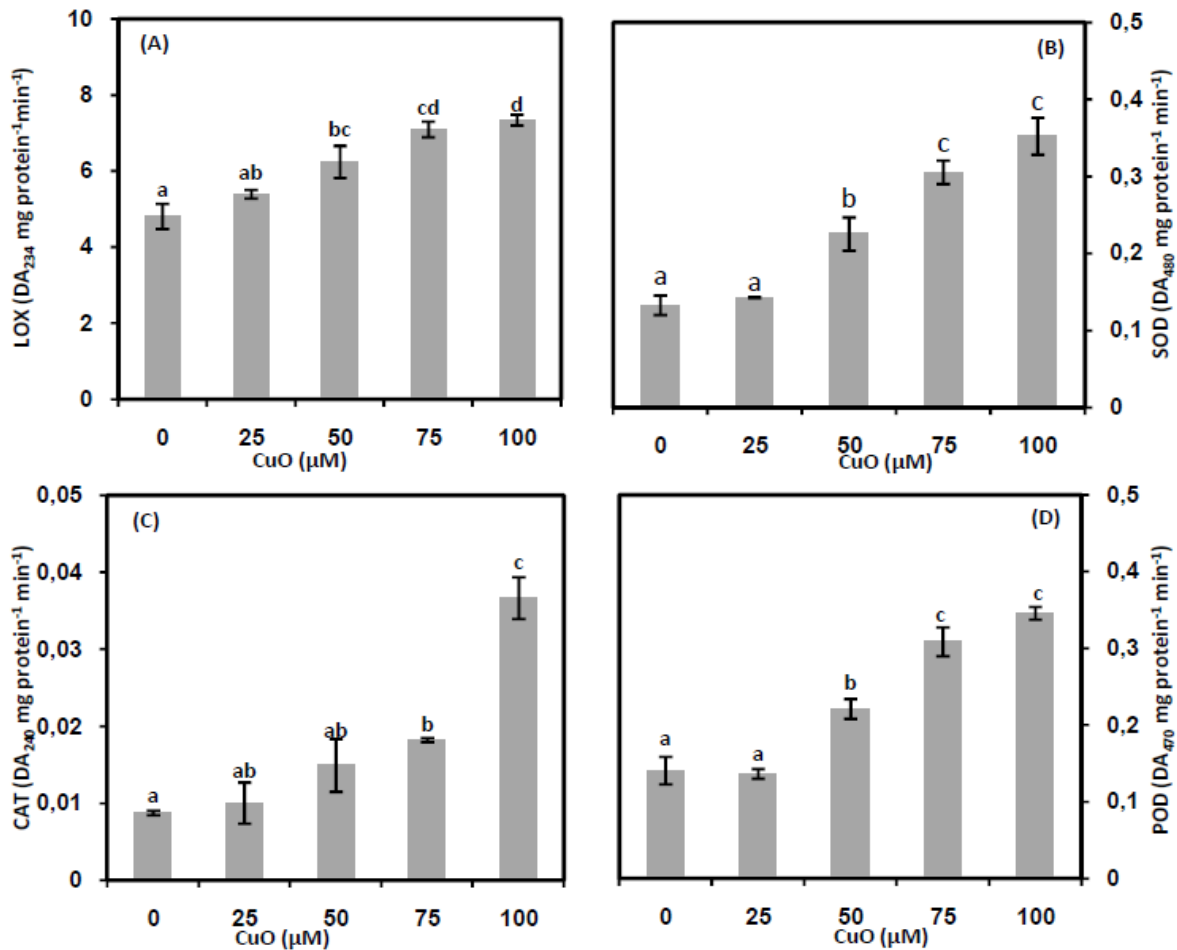
Similarly, Cu applied to calli at 25  $\mu$ M failed to display any significant modification in the POD activity compared with that of the untreated control (Fig. 3D). The high increase in POD activity was recorded over the control when Cu was supplied at high concentrations (50-100  $\mu$ M).

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  $\mu\text{M}$ ), and thereafter its activity gradually raised by rising Cu concentrations (75 and 100  $\mu\text{M}$ ).

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).

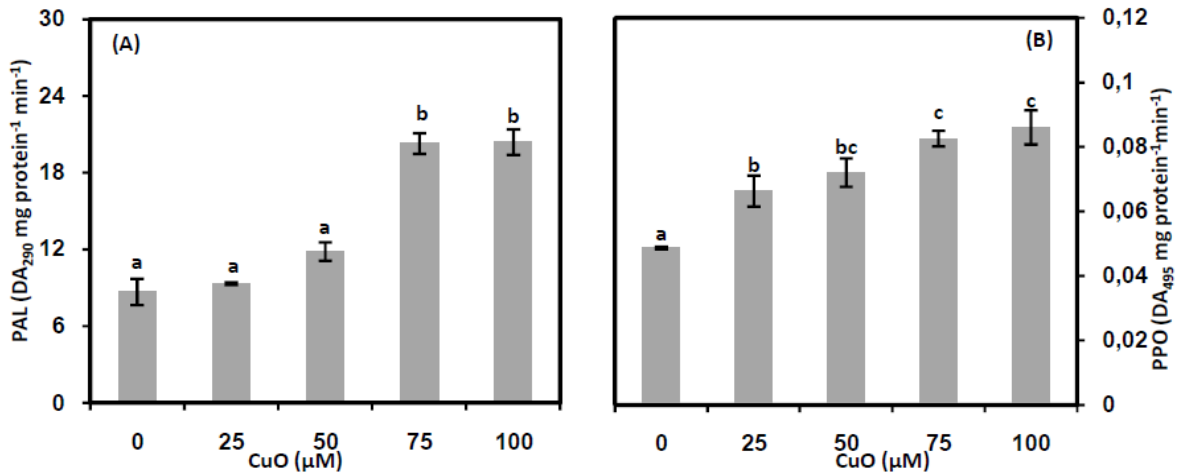


**Figure 3.** Lipoxygenase (LOX; A), superoxide dismutase (SOD; B), catalase (CAT; C), peroxidase (POD; D) activities of *S. nigrum* callus grown under different concentrations of Cu. The data are means  $\pm$  SD (n = 4). Different letters indicate statistically significant differences according to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

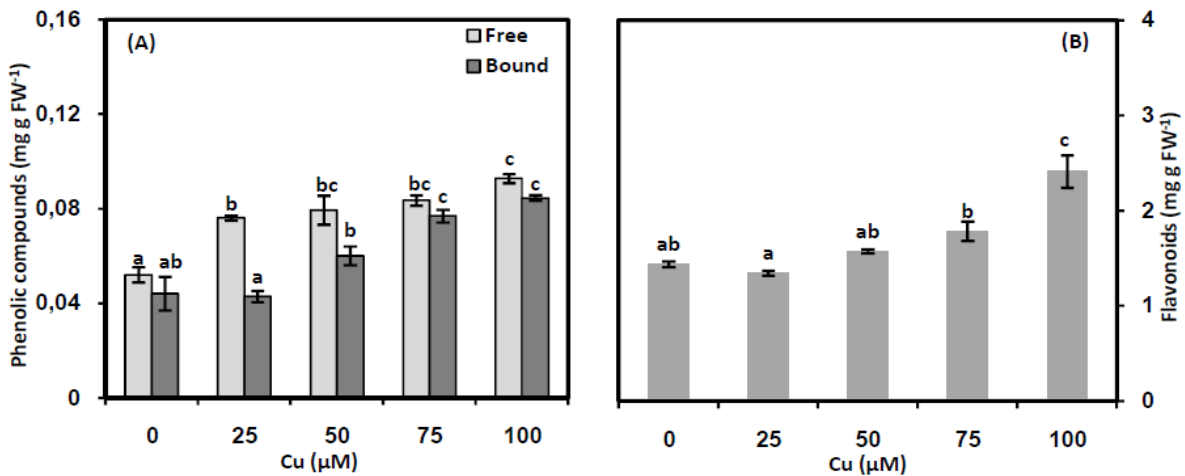
### 3.4. Free and bound phenolic compounds and flavonoids

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  $\mu\text{M}$  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  $\pm$  SD ( $n = 4$ ). Different letters indicate statistically significant differences according to the Tukey's HSD test ( $p < 0.05$ ).



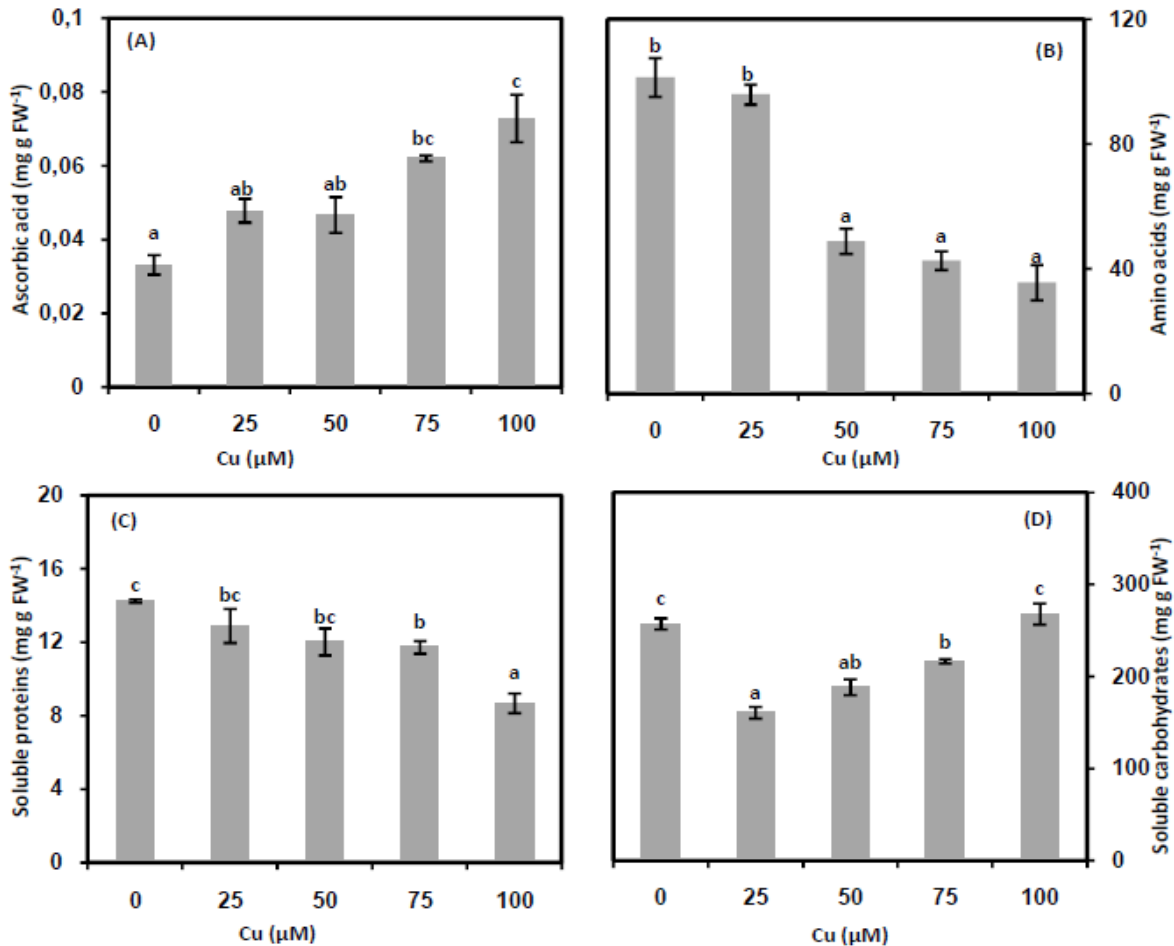
**Figure 5.** The content of free and bound phenolic compounds (A) and flavonoids (B) of *S. nigrum* callus grown under different concentrations of Cu. The data are means  $\pm$  SD ( $n = 4$ ). Different letters indicate statistically significant differences between different treatments according to 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  $\mu\text{M}$  Cu when compared with controls (Fig. 6A). Further, higher concentrations (75 and 100  $\mu\text{M}$ ) 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  $\mu\text{M}$ ), whereas 25  $\mu\text{M}$  Cu failed to induce the same response, compared with their control (Fig. 5B). Moreover, the data appeared that the amino acids concentration negatively correlated 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  $\pm$  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  $\mu\text{M}$ ) didn't significantly reduce soluble proteins in *S. nigrum* calli, whereas higher concentrations (75 and 100  $\mu\text{M}$ ) 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

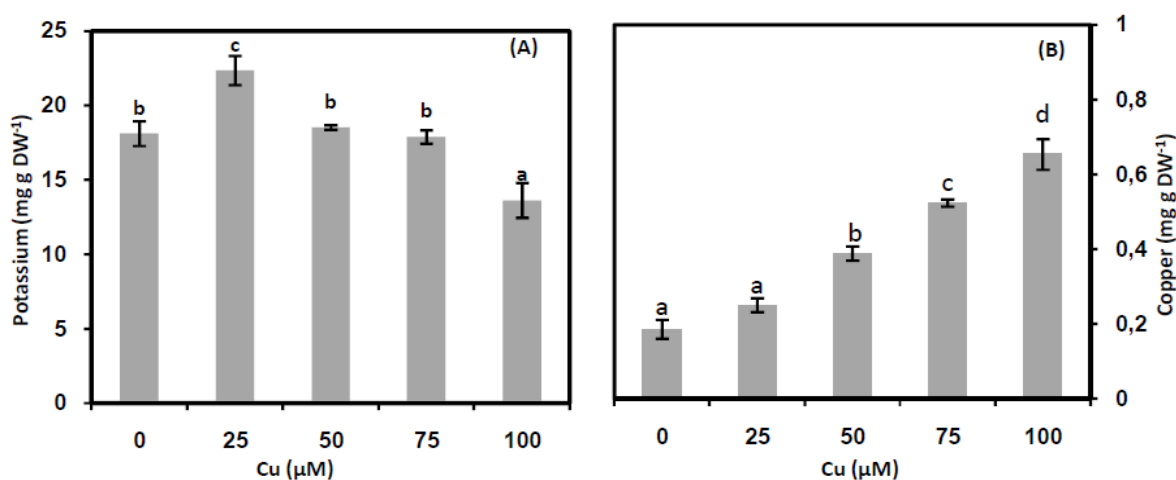
Based on the data presented in Fig. 6D, it can be observed that Cu reduced the soluble carbohydrates accumulation in *S. nigrum* calli, as compared with the control; only 100  $\mu\text{M}$  increased its accumulation. Further, it is detected that the correlation between soluble carbohydrates and the 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  $\mu\text{M}$  Cu was recorded to be 123.4%. Further, moderate concentrations (50 and 75  $\mu\text{M}$ ) failed to reduce the K content over their corresponding control, whereas 100  $\mu\text{M}$  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  $\mu\text{M}$  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  $\pm$  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  $\mu\text{M}$  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  $\mu\text{M}$ ) tested, 25  $\mu\text{M}$  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  $\text{H}_2\text{O}_2$  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  $\mu\text{M}$ ) on MDA and  $\text{H}_2\text{O}_2$  content in *S. nigrum* calli, and thereafter a rise was observed. These effects suggested that Cu at 25  $\mu\text{M}$  might be the optimum concentration and did not have toxic impacts. The obtained data herein indicated that MDA and  $\text{H}_2\text{O}_2$  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  $\mu\text{M}$  Cu, whereas 50-100  $\mu\text{M}$  Cu treatments exhibited an improvement relative to the control. This result confirmed MDA and  $\text{H}_2\text{O}_2$  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 strengthens 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  $\mu\text{M}$ ) 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 be 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 its 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  $\mu\text{M}$ , the rise was non-significant. The rise in free phenolic compounds at 25  $\mu\text{M}$  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  $\mu\text{M}$ ) 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  $\mu\text{M}$ ) 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  $\mu\text{M}$ ) 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  $\mu\text{M}$  Cu increased the K accumulation in calli, while 100  $\mu\text{M}$  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<sup>-</sup> concentration has been related with increased K efflux and decreased K concentration in the plant under Cu stress.

The double chemistry of Cu (Cu<sup>2+</sup> and Cu<sup>+</sup>) 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  
H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide  
K: Potassium  
LOX: lipoxygenase  
MDA: malondialdehyde  
MS: Murashige and Skoog  
NAA:  $\alpha$ -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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