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**Mechanistic insights of copper treatment on
the physiological processes of barley plants**

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Mechanistic insights of copper treatment on the physiological processes of barley plants

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Purpose: This current research is motivated by the activation of copper metals and their harmful effects on the economic crop barley 132 (*Hordeum vulgare*). **Methods:** Grains were grown for 21 days in pot experiments under different concentrations of copper (control, 5, 10, 15, and 25 mM). To test the impact of copper, we estimated its morphological and physiological traits. **Results:** Growth parameters were enhanced with all copper concentrations except 25 mM, which showed a decreasing trend. The best concentration for this activation was 15 mM compared to the control. Copper treatments showed greenish enlarged leaves and elongated roots and shoots. As associated with plants irrigated with H₂O, soluble sugar, soluble protein, and total protein in shoots increased with copper supplementation. Additionally, cellulose and phenols were improved markedly with increased copper concentrations. Besides, catalase and peroxidase enzymes were enhanced at 10 and 15 mM concentrations. Catalase and peroxidase profiles showed three varied bands at control, 10, and 25 mM. The protein pattern at a concentration of 10 mM displayed mostly similarity to the control, while at 25 mM concentration, a significant aggregation of protein bands was observed. **Conclusion:** Copper acts as an activator at low concentrations, while higher concentrations act as inhibitors. Results provided a baseline for further research on the mechanism of copper activation in the barley crop.

Keywords: Enhancement; Heavy metal; Hormesis; Morphological traits; Physiological parameters; Toxicity

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INTRODUCTION

Because barley plants provide nutrient-dense human nourishment, they were regarded as one of Egypt's most significant crop plants. Barley is one of the main cereal grains used in bread, drinks, and other cuisines across all cultures. It is still one of the most extensively consumed grains in the world and a primary food source (Lukinac and Jukić, 2022). Heavy metals and salinity negatively impact soil fertility, which has been identified as one of the most significant factors limiting plant growth and productivity as well as soil health (Wang *et al.* 2012). This is a significant environmental barrier to most cash crop productivity in the world's arid and semiarid regions, with annual global costs estimated to be US \$11000 million (FAO, 2011). Due to human activity connected to agricultural methods, these impacted soils have a "man-made" origin (FAO, 2000). Large areas of arable lands are, therefore, mostly or entirely unproductive. According to Lacolla *et al.* (2008), barley is a cereal crop that can withstand the highest levels of saline, heavy metals, and moderate droughts without experiencing any changes in growth. Because barley dilutes and eliminates salt, it is frequently employed in soil reclamation (UC SAREP, 2006). It is believed that barley can extract zinc, copper, and lead from the soil (Ebbs *et al.* 1998). Barley aids in reducing soil erosion due to its robust root system, which reaches depths of 1.8–2.1 meters.

Copper is essential for the formation of cell walls, respiration, photosynthesis, the antioxidant system,

signal transduction metabolism, signaling to transcription and protein synthesis, oxidative phosphorylation, iron mobilization, and molybdenum cofactor biogenesis (Abd El-Samad *et al.* 2018; Ochoa *et al.* 2018; Chen *et al.* 2022; Ramadan, 2023). However, Cu²⁺ has negative impacts on several physiological processes, including antioxidant activity, germination, plant growth, photosynthesis, and anatomical and ultra-structural changes that frequently result in the synthesis and escalation of ROS (Abd El-Samad, 2017; Abd El-Samad *et al.*, 2017; Díaz *et al.* 2017; El-Shazoly, 2019; Chen *et al.* 2022). Toxicologists use the term "hormesis" to describe a biphasic dose response to environmental chemical stress agents, involving both a high dose inhibitory or toxic effect and a low dose stimulating or positive effect (Mattson, 2008).

Soil accumulation and consequent contamination by Cu from various sources have been reported due to mining, former wood treatment sites, deposits of metal scraps, organic residues, atmospheric deposition (dust fallout), and agricultural practices (mineral fertilizers, manure, pesticides, sewage sludge, and crop applications of Cu-based fungicides) (Oorts, 2013). Copper mainly occurs in a divalent state (Cu²⁺) and has a high affinity for binding to organic matter. Soils containing high amounts of clay minerals and organic matter have high natural Cu. Background copper concentrations in soil typically vary between 2 and 50 mg Cu kg⁻¹, while plant shoots typically range between 4 and 15 mg Cu kg⁻¹ dry matter (Oorts, 2013).

Culicov *et al.* (2022) studied two kinds of copper salts (CuSO_4 and $\text{Cu}(\text{NO}_3)_2$), which were added in two dissimilar amounts of soil (150 mg/kg and 300 mg/kg). Depending on the stage of plant growth, plants treated with copper salts linked to the control exhibited increases or decreases in pigments, total polyphenols, and antioxidant activity, respectively. In agriculture, cupric nitrate is either employed as a fungicide or as a herbicide, whereas copper sulfate (cupric sulfate) is used as a pesticide, fungicide, and device for microbial activity to protect plants against bacterial disease (Munene *et al.* 2017). On the other hand, excessive copper levels disrupt several aspects of plant metabolism, including producing dry matter, chlorophyll, water content, and the equilibrium between macro- and micronutrient levels. Moreover, they interfere with photosynthetic and mitochondrial electron transport, nitrogen assimilation, and cell wall metabolism. Copper toxicity depends on the progress platform, the physiological condition of plants, metal concentration, and duration of contact (Merlin *et al.* 2012; Shariat *et al.* 2017).

Numerous enzymes, including laccase, cytochrome oxidase, polyphenol oxidase, copper/zinc superoxide dismutase (Cu/Zn-SOD), amino oxidase, and phycocyanin, require copper as a cofactor (Leng *et al.* 2015; Nazir *et al.* 2019; Zhang *et al.* 2019; Gong *et al.* 2021; Chen *et al.* 2022). Copper elicits and plays a vital part in the accumulation and production of phenolic compounds (Darki *et al.* 2019), and its shortage may cause a decline in plant phenolic levels (Munene *et al.* 2017). In Zarad *et al.* (2021) elicitation, the maximum total phenolic synthesis (8.75 mg/g dry weight) was observed at 2 mg/l of CuSO_4 . However, the 5 mg/l CuSO_4 -elicited culture showed the highest significant percentage of antioxidant activity (90.85%).

There are recommendations for future research directions that offer advice for relevant studies. Several research studies examining Cu^{2+} toxicity approachable proteins have been published. Most significant research has been intensive on herbaceous plants, including rice (Zhang *et al.* 2009), wheat (Li *et al.* 2013), *Allium cepa* (Qin *et al.* 2016), and sorghum (Roy *et al.* 2017). Copper concentrations, plant species, cultivars, and plant tissues affect how hazardous Cu^{2+} is to plant proteome, as demonstrated by previous research (Li *et al.* 2013; Roy *et al.* 2016). The effects of excess Cu^{2+} doses on the Cu-binding proteins in the growing radicles of rice seeds and the immobilization of Cu^{+2} affinities due to oxidative stress were studied using mass spectroscopy, two-dimensional electrophoresis, and chromatography.

Quantitative imaging analysis revealed that 26 protein spots exhibited significant differences (1.5 times) compared to the control group when exposed to Cu^{2+} (Zhang *et al.* 2017; Prusly *et al.* 2022).

The production of excess reactive oxygen species (ROS) at elevated Cu^{2+} treatment levels could have a significant impact on the distribution of these reactive oxygen species and the activity of antioxidant enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) (Liu *et al.* 2014; Kumbhakar *et al.* 2019). The antioxidant enzymes CAT and POD activity in plant tissues were unquestionably increased up to a treatment of 10 mM Cu^{2+} , after which they somewhat decreased (Shariat *et al.* 2017; Rajput *et al.* 2021). These outcomes show that eucalypts have a proficient mechanism to withstand Cu^{2+} overloads, as indicated by the accumulation of osmoprotectants and antioxidative enzymes. Thus, this study aimed to gather information about copper's beneficial or harmful effects on barley plants' metabolomics, growth, protein pattern, and antioxidant enzymes.

MATERIALS AND METHODS

During the winter season (December 2022), a seedling growth experiment was conducted in the Plant Physiology laboratory of the Faculty of Science at Minia University. Barley grains were brought from the International Research Center of Agriculture. The grains were subjected to a three-minute immersion in a 96% ethanol and 11% H_2O_2 mixture, followed by multiple washes with sterile distilled water to achieve surface sterilization. Five grains were placed in a small plastic pot containing 200 g of clay soil in two duplicates, and 100 ml of the desired solutions were applied to reach field capacity (Figure 1). The copper concentrations were chosen after initial experiments; copper was added as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Grains were treated with dissimilar concentrations of Cu (control, 5, 10, 15, and 25 mM) solutions. The plants were cultivated under normal conditions for 21 days.

Growth parameters

At the end of the experimental period, the fresh matter of different plant organs (roots and shoots) was estimated, and the dry matter of these organs was determined by drying the definite fresh weight in an oven at 80°C for 48 h. The length of roots and shoots was measured, and the leaf area was calculated using the formulas provided by McKee (1974), Bonhomme *et al.* (1974), and Norman and Campbell (1994). This was achieved by measuring the

length of the leaf and its maximum width. The succulence index was determined as the water content per unit area of leaves (Romero-Aranda and Syvertsen, 1996). The formulae provided by Lichtenthaler (1987) were used to compute the amounts of the photosynthetic pigments (carotenoids, chlorophyll a, and chlorophyll b).

Biochemical analysis

Soluble sugars in water-extracted root and shoot samples were assessed using the anthrone-sulfuric acid method (Fales, 1951). Furthermore, water-extracted samples were analyzed for total and soluble protein content following Lowry et al.'s (1951) procedure. Proline levels were measured according to Bates et al.'s (1973) methodology, while cellulose content was determined using Updegraff's (1969) method.

Antioxidant system

Non-enzymatic antioxidants: Non-enzymatic antioxidants were evaluated using Kofalvi and Nassuth's (1995) method, which detects free and cell wall-bound phenolic compounds.

Assay of antioxidant enzymes: Sample preparation was designated according to Mukherjee and Choudhuri (1983). Catalase enzyme activity was assessed spectrophotometrically and via polyacrylamide gel electrophoresis (PAGE) isoenzyme profiling following protocols from Chan et al. (1978), Havir et al. (1996), Dinçer and Aydemir (2001), and Yoruk et al. (2005). Peroxidase activity was measured spectrophotometrically and through PAGE isoenzyme profiling using the protocol outlined by Putter (1974). The SE 600 vertical slab gel device was utilized for conducting electrophoresis on vertical polyacrylamide gels. Isoenzyme analysis was carried out using polyacrylamide gel electrophoresis with 7.5% acrylamide, following the method outlined by Laemmli (1970).

Protein analysis by electrophoresis: Proteins can be separated based on their mass using the SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) technique. Gel electrophoresis is the initial method for separating the target protein, with SDS-PAGE being the predominant type used in western blots. Western blotting is an analytical method to detect a specific protein within a complex protein mixture. The electrophoresis was conducted using the "SE 600, vertical slab gel" device with vertical polyacrylamide gels. Protein analysis was performed following Laemmli's (1970) method, utilizing 1.0% SDS (sodium dodecyl sulfate) and 12%

acrylamide. Protein profiles were scanned using a densitometer (GS 300, Hoffer), and a protein marker ranging from 10 to 250 kDa was employed to determine the molecular weight of protein bands. The analysis was carried out using the GS 365 electrophoresis data system program version 3.01 (Microsoft Windows version).

Statistical analysis

The experimental data sets were subjected to one-way analysis of variance (ANOVA) using SPSS version 11.0 to measure and evaluate the sources of variation. The means were subsequently differentiated by the least significant differences (L.S.D.) at a significance level of 0.05% as per the method outlined by Steel and Torrie (1960). Correlation coefficients were calculated using Statgraphics 5.0.

RESULTS

Growth parameters

The data presented in Table 1 and Figure 2A indicate a significant improvement in both fresh and dry matter yields of roots and shoots with increasing copper intensity up to a concentration of 15 mM Cu²⁺. At a concentration of 15 mM Cu²⁺, the percentage increase in fresh and dry matter yields of roots and shoots reached maximum values of 180%, 186.1%, 135.9%, and 185.7%, respectively, compared to normal plants. Subsequently, a significant reduction was observed at 25 mM Cu²⁺, with percentage reductions in fresh and dry matter of roots and shoots at 93.3%, 94.4%, 76.9%, and 92.8%, respectively. Regarding root and shoot lengths, the highest activation was recorded at 10 mM Cu²⁺, with percentage values of 123.5% and 128.5%, respectively, while the percentage reduction in roots and shoots at 25 mM Cu²⁺ was 29.4% and 85.7%, respectively (Table 1 and Figure 2B). Additionally, there was an enhancement effect on leaf area, reaching twice that of control plants at 10 mM Cu²⁺ concentrations; however, a reduction was observed at 25 mM concentrations (Table 1 and Figure 2B). Leaf succulence exhibited a marked decrease at lower and moderate Cu concentrations, whereas, at a high treatment level (25 mM), leaf succulence increased significantly compared to the control and other corresponding concentrations. This observation suggests an opposite trend to the growth parameters (Table 1 and Figure 2B). Additionally, copper application led to an enhancement in pigment contents with increasing Cu²⁺ concentrations. The pigments Chl. a, Chl. b, Chl. a + Chl. b, and carotenoids

showed the greatest percentage increases at 15 mM Cu^{2+} concentrations, with 168.5%, 204.2%, 173.5%, and 172.8%, respectively. However, these contents were reduced at 25 mM Cu^{2+} concentrations compared to the previous copper concentrations. Nevertheless, photosynthetic pigments were enhanced at 25 mM compared to the control, with increases of approximately 111.8%, 123.4%, 113.5%, and 115%, respectively (Figure 3A and 3B).

Biochemical analysis:

The results presented in Table 2 and Figure 4 demonstrate a marked decrease in soluble sugar contents in barley plant roots at all Cu^{2+} concentrations, with percentage reductions of 97.1%, 89.4%, 81.5%, and 76.9%, respectively. In contrast, an increase was observed in soluble sugar content in shoots at 10 mM, 15 mM, and 25 mM Cu^{2+} concentrations, with percentages of 106.8%, 139.8%, and 148.08%, respectively, compared to control plants (100%). The response of soluble protein contents varied with increasing Cu^{2+} concentrations. In roots, an increase was only noted at 10 mM and 15 mM, while in shoots, a significant enhancement was observed at all copper concentrations (Table 2 and Figure 4). The highest values of soluble protein in both roots and shoots were recorded at 15 mM, with percentages of 182.8% and 257.1%, respectively. Furthermore, there was a substantial accumulation in total protein content in barley plant shoots with increasing copper concentration, reaching 264.7% at 25 mM Cu^{2+} compared to the control (Table 2 and Figure 4). Copper treatment significantly declined root proline content by 39.08% at the 25 mM level. However, this content remained unchanged in shoots up to 15 mM Cu^{2+} , followed by a slight reduction of 93.4% at 25 mM Cu^{2+} concentration (Table 2 and Figure 4).

Non-enzymatic antioxidants and cellulose contents:

The free and bound phenols levels showed a significant increase with increasing Cu^{2+} concentration, reaching maximum values at 15 mM for free phenols and 25 mM for bound phenols, with percentage increases of 205.5% and 174.3%, respectively (Figure 5A). Furthermore, adding Cu^{2+} increased cellulose content, reaching a maximum percentage of 146.3% at 25 mM Cu^{2+} (Figure 5B).

Antioxidant enzymes:

Catalase activity gradually increased with increasing copper concentration, reaching its highest value at 25 mM Cu^{2+} with a percentage of 154.02%. Similarly,

peroxidase activity showed a similar trend, with the highest value observed at 25 mM Cu^{2+} with a percentage of 159.4% compared to control plants (Table 3). The catalase profile revealed three bands at the control (25 kDa, 50 kDa, and 55 kDa). At 10 mM Cu^{2+} concentration, three additional bands were recorded (45 kDa, 55 kDa, and 150 kDa), while at 25 mM, three bands were observed (30 kDa, 55 kDa, and 80 kDa). Notably, the 55 kDa band was present in all three Cu^{2+} concentrations, and its staining intensity and size decreased with increasing Cu^{2+} concentrations compared to the control (Figure 6A). On the other hand, the peroxidase profile exhibited three bands (36 kDa, 46 kDa, and 90 kDa) at the control level, and at 10 mM Cu^{2+} , three additional bands appeared (44 kDa, 46 kDa, and 120 kDa). Finally, at 25 mM, there were three bands (36 kDa, 46 kDa, and 66 kDa). Interestingly, the size of the 46 kDa bands decreased with increasing Cu^{2+} concentrations, and the 36 kDa band was more prominent at 25 mM compared to the control (Figure 6B).

Protein analysis by electrophoresis

Four protein bands were observed in the control barley plants with molecular weights of 30 kDa, 40 kDa, 50 kDa, and 55 kDa. However, at 10 mM Cu^{2+} concentration, there were five bands with molecular weights of 20 kDa, 30 kDa, 40 kDa, 50 kDa, and 55 kDa. The protein band with a molecular weight of 20 kDa distinguished the 10 mM Cu^{2+} concentration and the control. At 25 mM Cu^{2+} concentration, the synthesis of 14 bands was induced, with the disappearance of protein bands with molecular weights of 55 kDa and 50 kDa present at the control and 10 mM Cu^{2+} concentration. Notably, the 30 kDa and 40 kDa bands were observed in all Cu^{2+} concentrations and the control (Table 4 and Figure 7).

DISCUSSION

Growth parameters

The results demonstrated that Cu^{2+} stimulated the fresh matter, dry matter, root and shoot lengths, leaf area, and photosynthetic pigments of barley plants. Lower and moderate doses of Cu^{2+} (5, 10, and 15 mM) stimulated these growth parameters, with the 15 mM level showing the highest values for fresh matter, dry matter of roots and shoots, and pigments (Chl. a, Chl. b, Chl. a + Chl. b, and carotenoids) compared to other copper doses. The percentage increases at 15 mM Cu^{2+} were approximately 180%, 186.1%, 135.9%, 185.7%, 168.5%, 204.2%, 173.5%, and 172.8%, respectively. Conversely, the higher copper dose (25

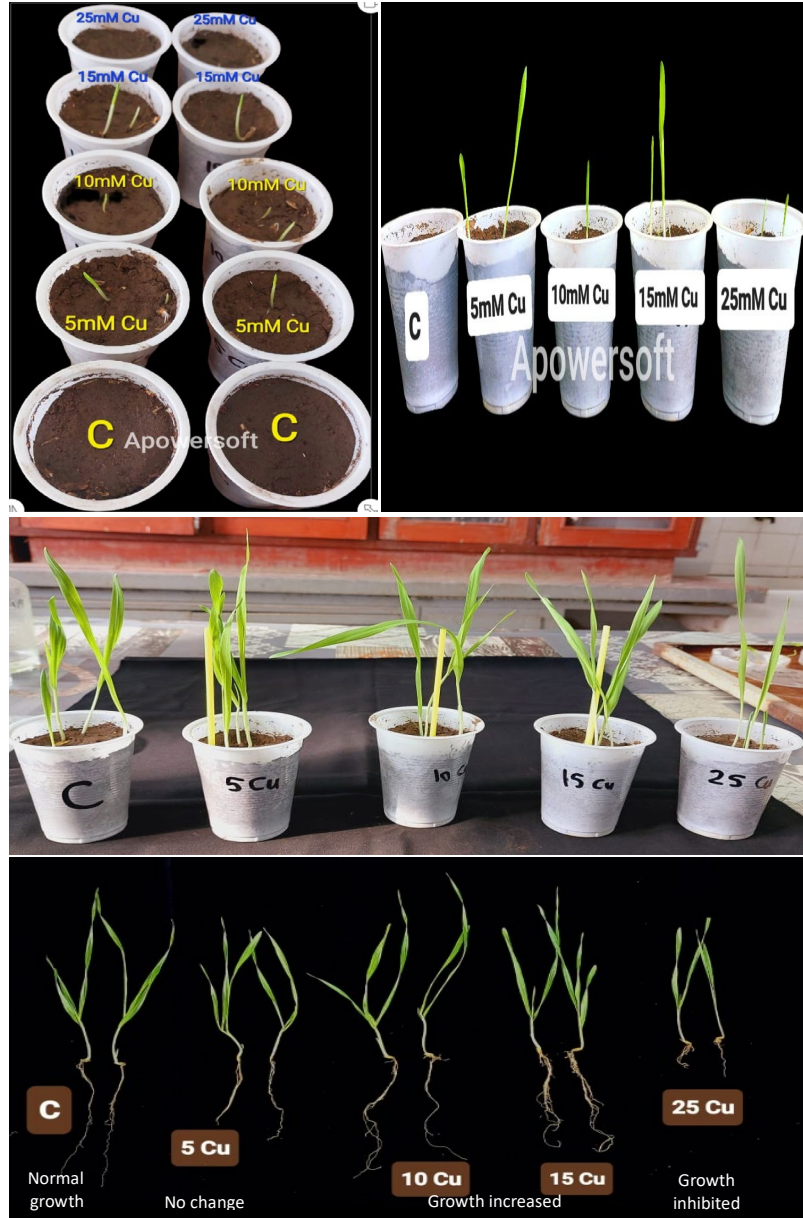


Figure 1. Barley plants grow for 21 days under different concentrations of copper.

Table 1. Fresh and dry matter of roots and shoots (g plant^{-1}), length of roots and shoots (cm), leaf area (cm^2) and succulence index of barley plants grown under different concentrations of copper

Treatment	Roots		Shoots		Length		Leaf area	Leaf succulence
	F. m.	D. m.	F. m.	D. m.	Roots	Shoots		
control	0.15	0.036	1.39	0.14	17.0	21	7.12	12.6
5 mM Cu^{+2}	0.25	0.064	1.51	0.15	13*	20	9.6*	9.38*
10 mM Cu^{+2}	0.26	0.066	1.62	0.17	21*	27*	14.7*	6.08*
15 mM Cu^{+2}	0.27	0.067	1.89*	0.26*	19*	25*	11.9*	7.24*
25 mM Cu^{+2}	0.14	0.034	1.07*	0.13	5*	18*	5.2*	16.8*
L. S. D. 0.05%	0.11	0.05	0.19	0.09	1.04	1.04	0.16	0.6

*The mean difference is significant at the 0.05 level

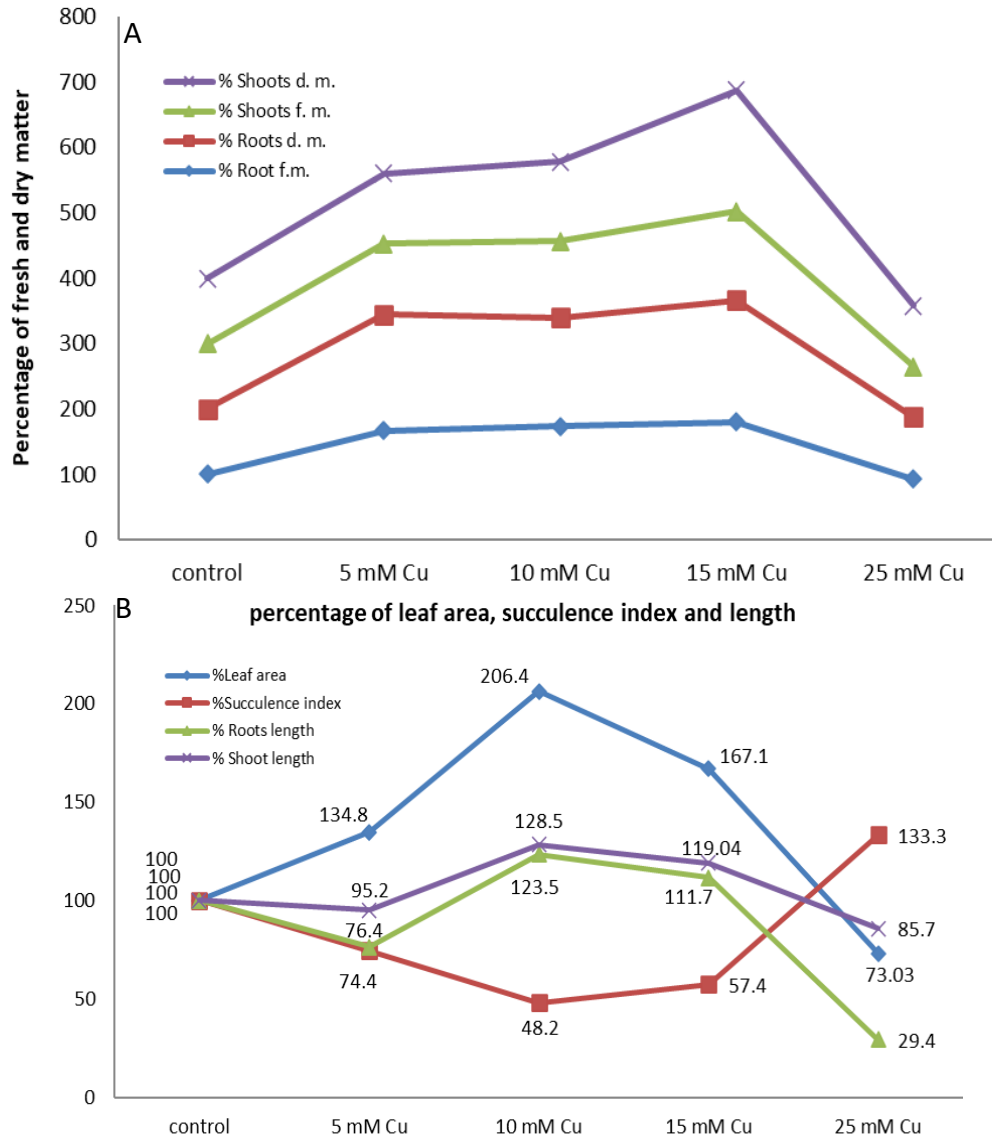


Figure 2. Percentage of fresh and dry matter of roots and shoots (g plant⁻¹) (A), leaf area (cm²), succulence index and length of roots and shoots (cm) (B) of barley plants grown under different concentrations of copper.

Table 2. Soluble sugar (mg g⁻¹ d. m.), soluble protein (mg g⁻¹ d. m.), shoot total protein (mg g⁻¹ d.m.) and proline (mg g⁻¹ d. m.) of barley plants grown under different concentrations of copper

Treatments	Soluble sugar		Soluble protein		Total protein	Proline	
	Roots	Shoots	Roots	Shoots	Shoots	Roots	Shoots
control	36.8	29.28	26.25	21.0	25.56	0.87	0.76
5 mM Cu ⁺²	35.76	28.24	24.8	25.25*	32.4*	0.65*	0.76
10 mM Cu ⁺²	32.9*	31.28*	28.0*	24.5*	42.57*	0.60*	0.77
15 mM Cu ⁺²	30.0*	40.96*	48.0*	54.0*	61.2*	0.54*	0.79
25 mM Cu ⁺²	28.32*	43.36*	21.5*	41.5*	67.68*	0.34*	0.71
L.S.D. 0.05%	1.94	1.95	1.91	1.89	1.8	0.19	0.16

*The mean difference is significant at the 0.05 level

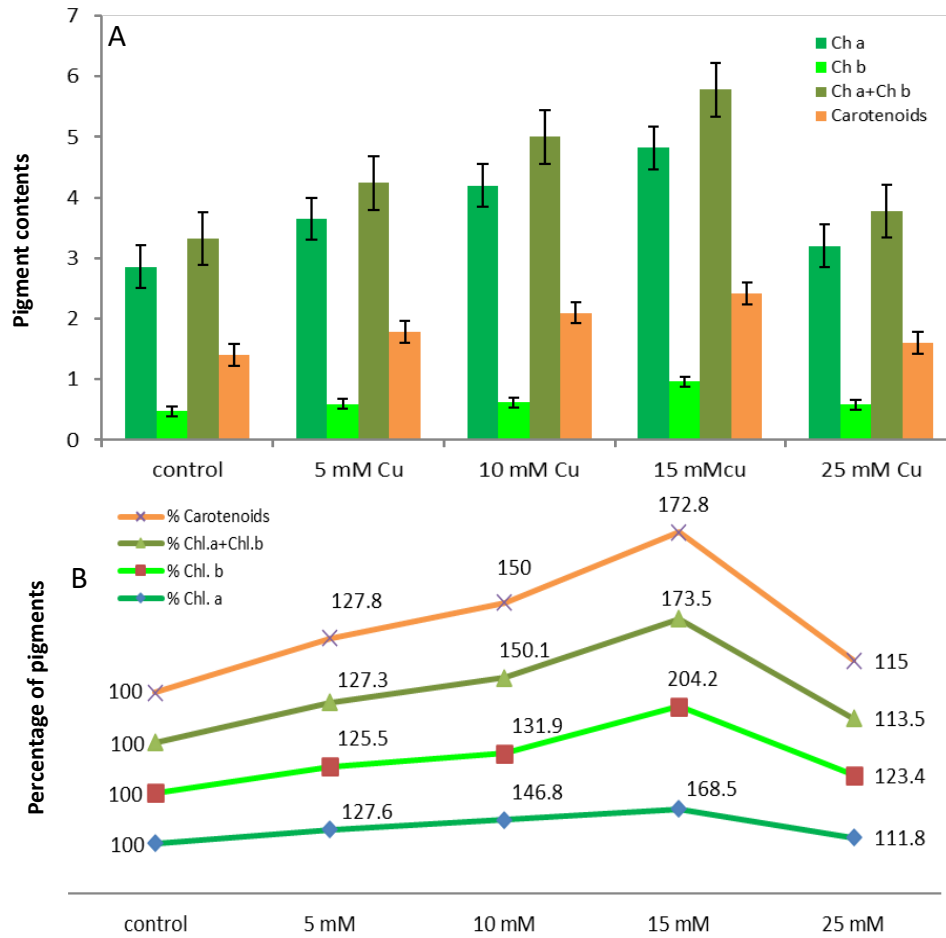


Figure 3. Pigment contents (A) and percentage (B) of (Ch a, Ch b, Ch a + Ch b and carotenoids) (mg g⁻¹ f. m.) of barley plants grown under different copper concentrations.

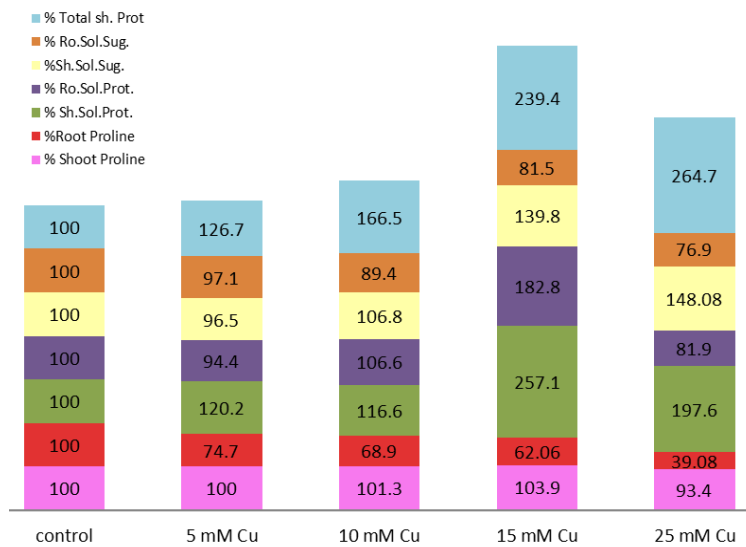


Figure 4. Percentage of soluble sugar (mg g⁻¹ d. m.), soluble protein (mg g⁻¹ d. m.), shoot total protein (mg g⁻¹ d. m.), and proline contents (mg g⁻¹ d. m.) of barley plants grown under different concentrations of copper.

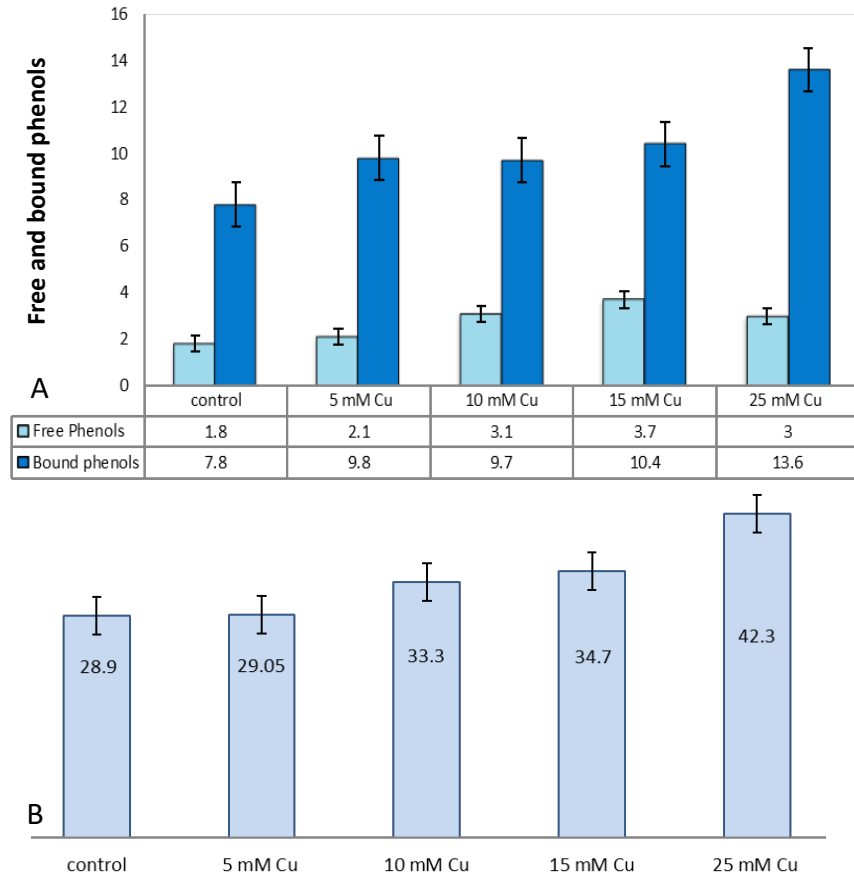


Figure 5. Free and bound phenols (A) (mg g⁻¹d. m.) and cellulose contents (B) (mg g⁻¹d. m.) of shoots of barley plant grown under different copper concentrations

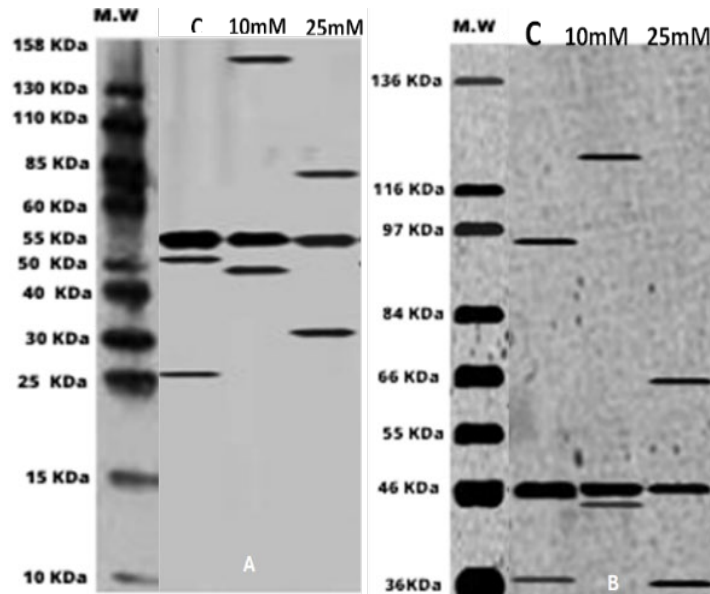
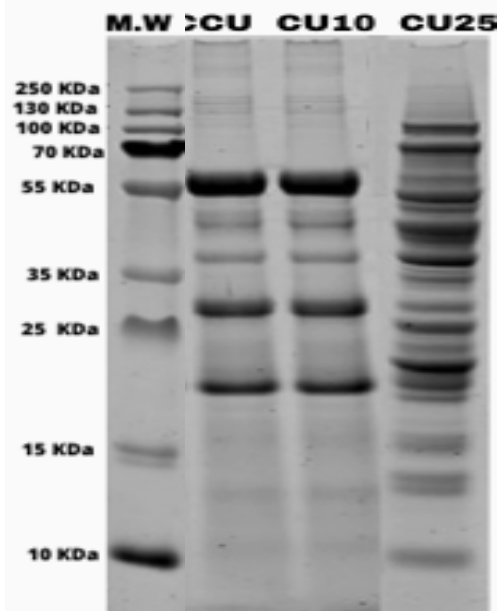


Figure 6 Catalase (A) and peroxidase (B) enzymes of barley plant under different concentrations of copper (0.0, 10 mM and 25 mM).



Protein SDS-PAGE

Figure 7. Polyacrylamide gel electrophoresis of soluble protein profiles separated from barley plant treated with 0.0, 10 mM and 25 mM copper concentrations.

Table 3. Spectrophotometric detection of catalase (mg g⁻¹ f.m) and peroxidase enzymes (µg g⁻¹ f.m) of barley plants grown under different concentrations of copper.

Treatments	Catalase	Peroxidase
control	2.98	3.03
5 mM Cu ²⁺	3.04	3.35
10 mM Cu ²⁺	3.11	3.67
15 mM Cu ²⁺	3.85	4.25
25 mM Cu ²⁺	4.59	4.83

Table 4. Protein pattern of barley plant treated with 10 mM and 25 mM copper concentrations as compared with control plants.

MW	CCU	CU10	CU25
250	-	-	-
130	-	-	-
100	-	-	-
95	-	-	+
70	-	-	+
55	+	+	-
53	-	-	+
50	+	+	-
45	-	-	+
40	+	+	+
35	-	-	+
30	+	+	+
25	-	-	+
23	-	-	+
20	-	+	+
18	-	-	+
15	-	-	+
13	-	-	+
12	-	-	-
10	-	-	+

mM) decreased plant growth parameters (fresh and dry matter in roots, shoots, and pigments) compared to the previous copper concentrations. However, there was an enhancement in photosynthetic pigments at 25 mM Cu²⁺ compared to the control, with increases of about 111.8%, 123.4%, 113.5%, and 115%, respectively.

Furthermore, the length of roots, shoots, and leaf area experienced significant enhancement with the addition of Cu²⁺, reaching maximum values at 10 mM with increases of 123.5%, 128.5%, and 206.4%, respectively. However, a decline was observed at 25 mM Cu²⁺, with decreases of 29.4%, 85.7%, and 73.03%, respectively, compared to control plants. Conversely, leaf succulence decreased notably at lower and moderate Cu²⁺ concentrations but increased significantly at a high treatment level (25 mM) compared to the control and other corresponding concentrations. This suggests an opposite relationship between leaf succulence and growth parameters. Succulence might hinder growth and act as a dilution mechanism for harmful ions. The barley cultivar may have expended considerable metabolic energy transitioning from a state of growth to succulence for survival, resulting in increased succulence at high Cu²⁺ concentrations at the expense of growth.

According to Khan *et al.* 2000 a, b; Gupta *et al.* 2013, succulence is suggested to help in ion regulation by expanding the vacuolar volume available for ion accumulation. Sucre and Suárez (2011), in agreement with Flowers and Yeo (1986), reported that adding salt increased leaf succulence compared to other treatments. To investigate the effects of Cu²⁺ treatments on barley plants, the succulence index can be utilized as a suitable selection criterion. Furthermore, Daood (2012) emphasized that succulence is a more appropriate selection criterion for salt tolerance than tissue water content.

Besides, other investigators have observed the biphasic role of Cu²⁺ (Abd El-Samad, 2017; Song *et al.*, 2017; Htwe *et al.*, 2020; Liu *et al.*, 2021). According to Manivasagaperumal *et al.* (2011), plant growth, dry matter output, and nutritional content were all significantly reduced at higher copper concentrations (100–250 mg kg⁻¹), while growth and dry matter yield increased at low concentrations (50 mg kg⁻¹). The observed growth enhancement is likely attributed to the essential trace quantity of copper required by plants (Reichman, 2002) or the possibility that the

tested soil was deficient in Cu^{2+} elements to meet the minimum plant requirements (Abd El-Samad et al., 2017, 2018). Conversely, the inhibitory effects of excessive copper at a 25 mM Cu^{2+} level on plant growth parameters may stem from reduced cell division and the toxic effects of heavy metals on photosynthesis, respiration, and protein synthesis. These factors significantly contribute to the delay in normal growth (Manivasagaperumal et al. 2011; Htwe et al. 2020; Cruz et al. 2022). The poisonous doses of Cu^{2+} were documented to deviate among species and varieties. Plant length was dramatically reduced in rice plants treated with 100 mg Cu/kg and above during the tillering stage or 200 mg Cu/kg and above during the panicle stage. A significant adverse relationship between grain yield and Cu^{2+} contents in the soil was distinguished, indicating that increased Cu^{2+} levels in the soil resulted in reduced plant growth (Htwe et al., 2020). Survival of plants with toxic levels of Cu^{2+} was established on the reaction of physiological, biochemical, and nutritional variables (Abd El-Samad, 2017; Pietrini et al., 2019; Cruz et al., 2022).

Biochemical Analysis

The impact of Cu^{2+} on the accumulation of soluble sugar and soluble protein in shoots was predominantly observed at all levels, with the highest effects noted at 25 mM concentration. However, at this high Cu^{2+} level, plant growth parameters declined significantly, while soluble sugar and soluble protein were increased in shoots by 148.08% and 197.6%, respectively. Soluble protein accumulation in roots was observed only at 10 mM and 15 mM Cu^{2+} , reaching a maximum value at 15 mM Cu^{2+} with an increase of 182.8%. This coincides with the stimulatory effect of Cu^{2+} on growth parameters at this level. Conversely, a reduction in soluble sugar and soluble protein in roots was observed at 25 mM Cu^{2+} concentration, which is consistent with the reduction in growth parameters of barley plants at this concentration. At toxic levels, Cu^{2+} diminishes water absorption and mineral nutrients, induces oxidative stress, and affects photosynthesis, reducing growth and plant production (Adrees et al. 2015; Pietrini et al. 2019).

Therefore, while Cu^{2+} is essential for normal plant growth and development, excessive levels can inhibit root growth. This detrimental effect on root organs is accompanied by a decrease in soluble sugar accumulation, leading to a reduction in dry matter formation, as observed in studies by Yuan et al. (2013)

and Díaz et al. (2017). This is supported by the presented results, which showed a 76.9% decrease in soluble sugar in the roots of barley plants at 25 mM Cu^{2+} compared to untreated plants. Abd El-Samad (2017) demonstrated that the positive impact of Cu^{2+} on shoot organs was associated with increased soluble sugar and soluble protein levels, leading to improved dry matter production. This accumulation can effectively enhance water uptake by wheat plants, supporting the notion that Cu^{2+} enhances carbohydrate and nitrogen metabolism and thereby increases growth parameters. This is further supported by Xiong et al. (2021), who reported that low concentrations of copper significantly altered 160 metabolites in *S. miltiorrhiza*, impacting pathways such as the pentose phosphate pathway, amino acid metabolism, and carbon assimilation in plants.

Applying varying copper concentrations to *Arabidopsis* seedlings demonstrated that the proper copper concentration increased the seedlings' meristem size (Song et al. 2017). Although copper is essential for plant development, most researchers have shown that elevated copper levels are hazardous to plants because they produce reactive oxygen species (ROS), which can oxidize macromolecules like lipids, DNA, and RNA and deactivate enzymes (Andre et al. 2010; Song et al. 2017). Exposure to excessive Cu^{2+} levels may lead to the death of rice plants due to the accumulation of reactive oxygen species (ROS) concentrations in rice radicles (Zhang et al. 2017). Furthermore, the decrease in electron transport frequency was related to the activity of some enzymes involved in CO_2 fixation.

High concentrations of Cu^{2+} have been found to decrease primary metabolites, growth, and biomass content due to altered metabolism and changes in biochemical parameters, as indicated by studies conducted by Soni and Thanki (2014) and Duan et al. (2020). Proline content serves as a good indicator of stress tolerance in many plants; plants with high-stress tolerance tend to accumulate high levels of proline, which is regulated by transport, synthesis, and degradation processes, as noted by Yan et al. (2020) and Furlan et al. (2020). However, other researchers consider proline accumulation in stressed plants as a marker of sensitivity, as highlighted in studies by Abd Al-Samad (2016), Eissa and Abeed (2019), and Abd El-Samad and Taha (2022b, 2023).

In plants treated with Cu^{2+} , proline levels tended to decrease in roots with increasing Cu^{2+} concentrations, reaching a reduction of 39.08% at 25 mM Cu^{2+} .

However, increasing Cu^{2+} concentrations did not affect proline contents in shoots, indicating that proline may not play a role in the enhancement effect of copper on dry matter production. In contrast to these findings, other studies have shown evident proline accumulation under heavy metals, such as *Cinnamomum camphora*, in response to Cu^{2+} (Karimi et al., 2012; Gong et al., 2021). According to Siripornadulsil et al. (2002) and Abd El-Samad et al. (2018), proline production is considered one of the earliest metabolic responses to stress and serves as an osmoregulator, metal chelator, and scavenger of hydroxyl radicals.

Non-enzymatic antioxidants and cellulose contents

The enhancement effect of copper on the accumulation of both free and bound phenols in barley plants aligns with the observed enhancement effect of Cu^{2+} on growth parameters and metabolites, such as soluble sugar and soluble protein in shoots. Plants deficient in copper may exhibit lower levels of phenolic compounds because copper is essential for phenolic compound synthesis (Rezazadeh et al., 2012). This is further supported by Isick and Unal (2015), who reported an increasing effect of Cu^{2+} on phenolic compounds. Additionally, Culicov et al. (2022) illustrated that total polyphenols may increase or decrease in plants treated with copper salts compared to control conditions.

The activation of phenol contents is directly related to the improvement trend in cellulose levels, which play a crucial role in cell wall biosynthesis. Inducing the expression of specific functional genes and producing antioxidants contributes to detoxification and tolerance to Cu^{2+} in plants (Kapoor et al., 2019). Under Cu^{2+} stress, plants generate a significant amount of ROS, disrupting ROS homeostasis. Zhang et al. (2020) outlined three strategies to maintain ROS homeostasis and regulate Cu^{2+} levels: using plant root exudates to chelate or precipitate Cu^{2+} ions, promoting Cu^{2+} absorption and transport-related gene expression to reduce intracellular Cu^{2+} ions and regulate ROS production, and utilizing various antioxidants to eliminate excess ROS and enhance plant resistance against Cu^{2+} -induced stress.

In barley plants treated with Cu^{2+} , there was a significant increase in cellulose contents across all levels of copper concentrations. This enhancement effect of Cu^{2+} on cellulose synthesis indicates cell wall biosynthesis. Tugbaeva et al. (2022) demonstrated that the upregulation of specific gene transcripts led

to thickening and lignification of cell walls in the metaxylem vessels of roots under Cu^{2+} treatment.

Printz et al. (2016) highlighted the roles played by various Cu-proteins and Cu-dependent processes in shaping the characteristics of the cell wall, which is a major site for Cu accumulation in plants (Burkhead et al., 2009). The cell wall acts as an ion sequester crucial for heavy metal response, but its synthesis and composition can be significantly affected in return (Parrotta et al., 2015). A positive correlation was observed between the enhancement effects of Cu^{2+} on the production of free and bound phenols and the increase in cellulose content, especially at Cu^{2+} supplements up to 15 mM, coinciding with the activation effect of copper on growth yield.

Despite the inhibition effect of Cu^{2+} on dry matter production of barley plants at 25 mM, the activation of antioxidants can be viewed as a sign of the role of non-enzymatic defense compounds in protecting plants from the oxidative damaging effects of Cu^{2+} at this level. This is supported by Hanafy et al. (2017), who found a significant increase in phenol content in tomato leaves under Cu^{2+} stress at varying copper concentrations. These compounds act as metal chelators and reduce ROS levels, providing defense against oxidative stress (Ullah et al., 2019).

Antioxidant enzymes (CAT and POD)

Plants activate antioxidant defense systems to counteract the oxidative damage caused by excess Cu^{2+} accumulation in cells. The enzymatic system, particularly CAT and POD, plays a crucial role in scavenging ROS and helps plants adapt to unfavorable environments (Zhang et al., 2022). The catalase activity gradually increased with rising copper concentrations, peaking at 25 mM Cu^{2+} with a percentage increase of 154.02%. Similarly, peroxidase activity showed a similar trend, reaching its highest value at 25 mM Cu^{2+} with a percentage increase of 159.4% compared to control plants.

Regarding catalase bands, new bands were observed at 10 mM and 15 mM Cu^{2+} levels, coinciding with the enhancement effects of Cu^{2+} on growth and metabolic constituents. However, at 25 mM Cu^{2+} , new bands appeared associated with the inhibition effects of Cu^{2+} on growth and metabolic constituents such as soluble sugar and soluble protein in roots. Similarly, peroxidase profiles exhibited changes with Cu^{2+} treatments, with new bands appearing at different Cu^{2+} concentrations. The disappearance of certain bands and the appearance of new ones indicate

positive changes in barley metabolic pathways at lower Cu^{2+} concentrations, while higher Cu^{2+} levels led to negative impacts on barley development and metabolism. The findings regarding peroxidase activity improving in stems and leaves due to excess Cu^{2+} accumulation is supported by other studies. The decrease in growth caused by Cu^{2+} accumulation may be linked to the stimulation of lignifying peroxidase (Jouili and El Ferjani, 2004). Additionally, Karimi *et al.* (2012) highlighted those plants exposed to excess copper utilize antioxidant defense mechanisms to protect themselves from injury. Similarly, Isick and Unal (2015) demonstrated that CAT activity and total phenolic compound levels increased in plants treated with Cu^{2+} compared to control plants. Plants exhibit tolerance to Cu^{2+} and activate CAT and phenolic compounds to mitigate Cu^{2+} stress damage. Antioxidant parameters can serve as potential indicators of oxidative stress associated with metal exposure, as demonstrated by Aziz *et al.* (2020) and Aziz and Butt (2020).

Total protein and protein pattern

The total proteins in the shoots of barley plants increased progressively with higher Cu^{2+} treatments, reaching the highest percentage value of 264.7% at 25 mM. This increase is reflected in the protein pattern, where a notable difference was observed between control plants and those irrigated with 10 mM Cu^{2+} concentration, with 14 protein bands recorded at 25 mM. The accumulation of protein bands at this level may indicate a response to damaging stress or an adaptation mechanism for barley to tolerate and overcome copper-induced injury. Amini and Ehsanpour (2005) and Abd El-Samad *et al.* (2017) suggested that protein accumulation in plants under saline conditions is a nitrogen storage form utilized after stress subsides. This accumulation may involve osmotin-like proteins and specific protein synthesis related to cell wall modification. Moreover, studying proteins in citrus leaves sensitive to Cu-toxicity has revealed various plant mechanisms associated with Cu-toxicity and Cu-tolerance. John *et al.* (2021) demonstrated that Cu-mediated protein accumulation supports the notion that Cu^{2+} toxicity is linked to increased protein aggregation. This aligns with our findings, where 25 mM Cu^{2+} concentration induced more protein aggregations alongside a reduction in growth factors and metabolomics. Due to their redox activity, many metalloenzymes rely on specific critical metal ions as catalytically active cofactors (Abd El-Samad *et al.*, 2017).

CONCLUSION

Copper supplementation exhibited varying effects on the growth and metabolites of barley plants. Low and moderate concentrations of copper acted as activators, while high concentrations acted as inhibitors, a phenomenon known as hormesis, which was evident in growth parameters. The accumulation of organic compatible solutes such as soluble sugar, soluble protein, total protein, phenolic constituents, and cellulose contributed to increased plant tolerance and enhanced barley adaptability. The increase in catalase and peroxidase activity, along with the appearance of new protein bands at 10 and 25 mM Cu^{2+} levels, was associated with the biphasic roles of Cu^{2+} . The rise in the number of protein bands at 25 mM Cu^{2+} can be considered a biomarker for oxidative stress, aligning with the observed inhibition in the growth and metabolism of barley plants. Therefore, in line with these findings, copper is an essential element for plants, and in cases of deficiency, Cu may be applied directly to soil and crops through a foliar spray.

CONFLICT OF INTEREST

The author has no conflict of interest.

ABBREVIATIONS

ROS: Reactive oxygen species; Ch a: Chlorophyll a; Ch b: Chlorophyll b; F.m.: Fresh matter; D.m.: Dry matter; Ro. S. Sug.: Root soluble sugar; Sh. S. Sug.: Shoot soluble sugar; Ro. Sol. Prot.: Root soluble protein; Sh. Sol. Prot.: Shoot soluble protein; Free phen.: Free phenols; Boun. phen.: Bound phenols; Sh. Tot. Prot.: Shoot total protein; CAT: Catalase; POD: Peroxidase; SDS PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD: Superoxide dismutase.

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