www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK
ACTA BIOLOGICA CRACOVIENSIA Series Botanica 64/2: 49–64, 2022
DOI: 10.24425/abcsb.2022.143382

PAN
POLSKA AKADEMIA NAUK
ODDZIAŁ W KRAKOWIE

Alleviating Effect of Melatonin on Zinc-Copper Stressed Seedlings of Hemp (*Cannabis sativa* L.)

Hakimeh Oloumi[1], Ali Zamani[1], Hossein Mozaffari[1], Seyyed Mohammad Javad Arvin[2], Hassan Salari[1]

[1]*Department of Ecology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran*

[2]*Plant Products Division, Shahid Bahonar University, Kerman, Iran*

Received August 28, 2022; revision accepted January 25, 2023

The study was carried out to investigate the interactive effects of exogenous melatonin and excess amounts of zinc and copper on the growth and physiological parameters, antioxidant defense system and nutritional balance of cannabis seedlings. *Cannabis sativa* L. plants, grown under a completely randomized design, were irrigated with complete Hoagland's nutrient solution. $CuSO_4$ (0, 50 and 150 µM) and $ZnSO_4$ (0, 50 and 100 µM) and their combinations were supplied to 21-day-old seedlings for 2 weeks. During the second week, melatonin was added to the nutrient solution at 100 µM. Zn and Cu stress led to reduced growth and physiological parameters, it promoted oxidative stress, changes in antioxidant enzymes activity and imbalance of mineral nutrients in cannabis seedlings. However, melatonin alleviated the growth retardation and physiological disorders of seedlings under normal conditions and heavy metal stress. The content of reduced glutathione and the activity of antioxidant enzymes such as glutathione reductase and ascorbate peroxidase were improved by melatonin. Excess amounts of zinc and copper changed the pattern of nutritional elements distribution in cannabis seedlings. Cu and Zn caused reduced content of Fe, Ca and K ions in shoots and roots. Melatonin treatment was able to adjust the nutrients content in metal-stressed seedlings up to the level of the control. Exogenous melatonin reduced toxic levels of Cu and Zn in seedlings overloaded with copper and zinc. MT also raised K, Ca and Fe concentrations in roots and shoots of seedlings under stress. Our results support the idea that melatonin acts as a powerful antioxidant, it can also be considered as a potent regulator of ion homeostasis in cannabis seedlings under heavy metal toxicity. Further studies still need to investigate the alleviatory effects of melatonin under field conditions.

Keywords: antioxidant enzymes, cannabis, mineral nutrition, plant growth stimulator

INTRODUCTION

Nowadays, as a result of deposition of wastewater, mining activities, metal smelting, industrial activities, pesticide applications and unnecessary use of chemical fertilizers, copper (Cu) and zinc (Zn) are introduced into the environment in large quantities (He et al., 2005). Zn and Cu as micronutrients are used in various biochemical and physiological pathways in plants. Zn and Cu ions act as activators and cofactors of some vital enzymes in plant cells (Hänsch and Mendel, 2009). However, there is much evidence suggesting that plants express symptoms of heavy metal toxicity when exposed to excessive levels of Zn and Cu (Nagajyoti et al., 2010). Chlorosis, necrosis, root system damage (Alaoui-Sossé et al., 2004), photosynthesis inhibition, and cell membrane damage

* Corresponding author, e-mail: h.oloumi@kgut.ac.ir

PL ISSN 1898-0295

(Narula et al., 2005) are among common toxic effects of Cu and Zn in plants. Thus, exposure of plants to high concentrations of Zn and Cu considerably triggers oxidative burst and a wide range of physiological alterations (Schutzendubel and Polle, 2002). Zn ions cause oxidative stress through disruption of the electron transport chain, interaction with the antioxidant defense system, and induction of lipid peroxidation (Bray and Bettger, 1990). Excessive amounts of Cu stimulate the oxidative stress in plants through the generation of free radicals and production of high levels of Reactive Oxygen Species (ROS) (Thounaojam et al., 2012). High amounts of ROS lead to electron leakage, lipid peroxidation, and subsequent membrane injury, as well as nucleic acid and protein damages. Plants have evolved antioxidant defense mechanisms to counteract ROS toxicity, including antioxidant enzymes (e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX)) and non-enzymatic compounds (e.g., glutathione, ascorbic acid, α-tocopherol, and carotenoids) (Cao et al., 2019). Toxic amounts of zinc and copper in plants also lead to nutritional imbalance of micro- and macro-nutrients in plant tissues and organs. Zn and Cu ions at higher contents may interfere with the uptake, transport and accumulation of other nutritional elements. It has been proposed that a toxic amount of Zn decreases the absorption or translocation rate of essential nutrients into plants, which leads to mineral imbalances. High levels of Zn in soil considerably change the absorption and translocation of plant nutrients like Fe, Mg, K, P and Ca. Moreover, excessive amounts of Cu alter the absorption of other nutrients. Plants exposed to overload of Cu are highly susceptible to the deficiency of other nutritional elements, thus suffering a disturbance of essential metabolic processes (Cayton et al., 1985). Ke et al. (2007) reported that with an increasing content of Cu in the nutrient solution, the concentra tions of Ca^{2+} and Mn^{2+} would decline in shoots. Also, the studies by Bosnić et al. (2019) showed deficiencies of Fe, Zn and Mn, following Cu toxicity in plants.

N-acetyl-5-methoxytryptamine known as melatonin (MT) is an evolutionarily highly conserved compound and a secondary messenger (Dubbels et al., 1995) in plant tissues involved in a wide range of plant developmental processes. MT plays a vital role in coping with various abiotic and biotic stressors in plants (Sharif et al., 2018) such as cold, drought, radiation, extreme temperatures, and chemical stresses. Positive effects of MT on reducing oxidative damage induced by water deficit have also been reported for *Cucumis sativus* (Zhang et al., 2013), *Glycine max* (Ren et al., 2019), *Lupinus albus* (Hernández-Ruiz and Arnao, 2008) and *Vitis vinifera* (Meng et al., 2017). Melatonin modulates stress responsive gene expressions and thereby regulates plant responses to abiotic stress. Furthermore, melatonin modulates ROS/RNS function as a secondary messenger and, therefore, activates downstream signaling transduction pathways during plant growth under a stress response (Arnao et al., 2021).

Melatonin regulates antioxidant-related genes which are involved in different hormone signaling pathways. Transcriptome analysis showed that melatonin improves plant tolerance to abiotic stressors through the regulation of downstream signaling component and the expression genes involved in ABA-dependent pathways and DREB/CBF, HSF, SOS mechanisms (Zhang et al., 2013; Murch and Saxena, 2002; Shi et al., 2016). Melatonin has been shown to have protective effects against stress conditions through the hormonal cross-talk between melatonin and other plant hormones such as auxin, abscisic acid, salicylic acid, ethylene and jasmonic acid. Moreover, melatonin treatment enhances the expressions of heat shock proteins (HSP), leading to heat stress tolerance. On the other hand, melatonin is able to interact with nitric oxide (NO), leading to production of superoxide anion ($O_2^{\bullet-}$) and peroxynitrite anion ($ONOO^-$) (Tiwari et al., 2022). Melatonin, as a bio-stimulator, has also protective effects against biotic stresses and pathogen attacks, probably through the activation of mitogen-activated proteinase kinases and heat shock proteins (Wang et al., 2018).

Among all plant growth regulators, MT has the highest antioxidant capacity and is recognized as a biological stimulant with very intense antioxidant activities (Korkmaz et al., 2017). MT and its derivates regulate biological systems not only through the direct elimination of free radicals, but also through limiting the chemical activity of toxic metals (Flora et al., 2013; Romero et al., 2014). MT and its precursors have been reported to be able to bind to several toxic metals such as Al, Cd, Cu, Pb and Fe (Limson et al., 1998) and thereby prohibit heavy metal toxic actions in plant tissues. Electrochemical studies have shown that

MT binds to both Cu^{2+} and Cu^{1+}, and protects plant cells against free radical damage (Parmar et al., 2002). The application of MT to the cauliflower plant enhanced its vegetative growth and survival when exposed to high Cu concentrations (Zhang et al., 2017). Pomyk et al. (2008) found that MT pretreatment protects red cabbage seedlings against the toxicity of Cu ions. According to Cao et al. (2019), Cu toxicity in cucumber plants could be alleviated by MT through improved Cu sequestration, carbon metabolism, and ROS .

Based on the existing reports, we assumed that the exogenous application of MT might be effective in improving the heavy metal tolerance of cannabis seedlings. Cannabis (*Cannabis sativa*), belonging to the family Cannabinaceae, has been used as a hyper accumulator species for different toxic trace metals such as Pb, Cd, Mg, Cu, Cr, and Co (Raman et al., 2017). However, the decontamination capacity of hemp plants requires verification. Although numerous studies have examined the effects of MT on heavy metal stress in plants, few have investigated the MT effects on the plant nutritional balance under heavy metal stress in horticultural crops. The mineral nutrition of higher plants is of fundamental importance to agriculture. To provide an insight into the role of MT in nutrients balance adjustment under heavy metal excess, in this research we estimated the contents of Cu, Zn, Fe, Mg, Mn, Ca and K in both shoots and roots of cannabis seedlings. The influence of MT on the growth and physiological parameters of cannabis seedlings subjected to excess amounts of Zn and Cu, was also studies. The antioxidant responses of seedlings such as glutathione, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase were investigated by MT treatment.

MATERIALS AND METHODS

This research was conducted at the Graduate University of Advanced Technology. The experiment was carried out on a completely randomized factorial design with three replications. Seeds of cannabis (*Cannabis sativa* L.) cv. Mashhad provided from Pakan Bazr Company (Isfahan, Iran) were sterilized by immersing in 0.1% (w/v) sodium hypochlorite solution for five min and rinsed extensively with distilled water. Cannabis seeds were planted in pots (17.5 cm diameter × 15 cm height) filled with acid-washed sandy soil under greenhouse conditions. The plants were irrigated with complete Hoagland's nutrient solution on alternate days until they grew enough to withstand high levels of copper and zinc. Three weeks after seed germination and the growth of the seedlings, $CuSO_4$ (0; as control, 50 µM; as a mild stress, and 150 µM; as a severe stress), $ZnSO_4$ (0; as control, 50 µM; as a mild stress, and 100 µM, as a severe stress) and $ZnSO_4$+$CuSO_4$ combinations at all concentrations were added into irrigation nutrient solutions and the treatment was continued for two weeks. Melatonin was applied into irrigation solutions at 0 and 100 µM (MT solved in distilled water) to treat the cannabis seedlings during the second week of metal treatment. One of the seedlings in each pot was harvested and used for measurements of growth parameters such as fresh weight, dry weight, root length and shoot length. A fully expanded third leaf of the samples was used to measure relative water content, ion leakage and other physiological and biochemical parameters.

MICRO- AND MACRO-NUTRIENT MEASUREMENTS

The dried leaves were ground to powder using a mortar and pestle. Ground samples (0.5 g per replicate) were made up to 10 ml with nitric acid. After 24 h this solution was boiled to remove any acidic gases, then filtered into a 50 ml volumetric flask and filled up to 50 ml with deionized water. Ions were determined in these sample solutions using Atomic absorption spectroscopy equipped with the flame technique. All equipment was obtained from Varian Techron Pty. Limited, Mulgrave, Victoria, Australia. Standard solutions for Cu, Zn, Fe, Mg, Mn, Ca and K were used to calculate calibration curves. All the chemicals used were of analytical reagent grade.

RELATED WATER CONTENT (RWC)

To establish RWC, leaf fresh weight, turgid weight (held 5 hours in deionized water) and the leaf dry weight (72 hours at 70°C) were measured. Relative water content was calculated using the following formula (Mullan and Pietragalla, 2012):

$$\frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Torgid weight} - \text{Dry weight}} \times 100$$

CELL MEMBRANE STABILITY INDEX (MSI)

We used the electrolyte leakage technique to evaluate the membrane stability index of cells. The percentage of ion leakage was measured according to Lutts et al. (1996), using the following equation. L1 in the equation is ion leakage recorded for 0.3 g leaf fresh weight kept in 20 ml distilled water at 25°C for 24 hours. After addition of 20 ml distilled water to the former aliquot and autoclave at 120°C for 20 min, the ion leakage of cooled samples was recorded as L2.

$$\text{MSI}\ (\%) = 1\ - (\frac{\text{L1}}{\text{L1} + \text{L2}} \times 100)$$

PHOTOSYNTHETIC PIGMENTS

To measure Chl_a and Chl_b and their ratio, 0.2 g of frozen leaves were extracted in 15 ml of 80% acetone, based on the method described by Lichtenthaler (1987). The absorbance of the infiltrate solute was read by a spectrophotometer at 646.8, 663.2 and 470 nm. Using the following equations, Chl_a and Chl_b contents were measured based on µg/g fresh weight, and the Chl_a/Chl_b ratio was recorded in this study.

Chl_a = 12.25 A663.2 - 2.79 A646.8
Chl_b = 21.21 A646.8 - 5.1 A663.2

HYDROGEN PEROXIDE

The hydrogen peroxide content was measured using the method described by Velikova et al. (2000). 0.5 g frozen leaf tissue was homogenized in 0.1% trichloroacetic acid (TCA) in an ice bath. The extract was centrifuged (Centrifuge 5804R, Germany from Eppendorf) at 1500 ×g for 10 min. 0.5 ml supernatant was added to 0.5 ml potassium buffer (10 mM, pH 7) and 1 ml KI 1 M. The absorbance was read at 390 nm. The hydrogen peroxide concentration was calculated using the extinction coefficient 0.28 $M^{-1}cm^{-1}$.

LIPIDS PEROXIDATION

The content of malondialdehyde (MDA) was measured as the lipid peroxidation index, according to Hodges et al. (1999). 0.2 g of fresh leaf tissues was extracted in 5 ml of 0.1% TAC and centrifuged in 5000 ×g for 5 min. 5 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA acid was added to 1 ml supernatant. The mixture was heated at 95°C for 30 min. Then test tubes were immediately cooled in an ice bath and centrifuged at 5000 ×g for 10 min. The absorbance of the solutes was read at 523 nm. The absorbance of non-specific pigments was determined at 600 nm, deducted from the former value. The extinction coefficient of 1.55×105 M^{-1} cm^{-1} was used to measure MDA content. The results were expressed based on micro molar MDA in fresh weigh.

TOTAL, OXIDIZED AND REDUCED GLUTATHIONE

Griffith's (1980) method was used to measure the amount of total, oxidized and reduced glutathione. For glutathione extraction, 0.5 g of the plant's aerial parts was first thoroughly ground in a mortar containing 2 ml of 2% metaphosphoric acid. The homogenate was then centrifuged at 5500 ×g at 4°C for 20 min. 100 µl supernatants was added to the test tube containing 700 µl NADPH (0.3 mM), 100 µl DTNB (6 mM) and 100 µl distilled water. After 3–4 min, 10 µl glutathione reductase was added. The sample absorption was read at 412 nm and the total glutathione content was recorded in milligrams per gram fresh weight, calculated using the glutathione standard curve.

To measure the oxidized glutathione content, 100 µl of the extract was added to the test tube containing 2 µl 2-vinyl pyridine and kept at room temperature for one hour. Afterwards, 700 µl NADPH (0.3 mM), 100 µl DTNB (6 mM) and 10 µl glutathione reductase were added to the aliquot and the sample absorption was read at 412 nm. Oxidized glutathione was calculated using a standard curve and recorded in milligram per gram fresh weight. The amount of reduced glutathione was obtained by subtracting the oxidized glutathione from the total glutathione content (Griffith, 1980).

ANTIOXIDANT ENZYME EXTRACTION AND ACTIVITY DETERMINATION

For enzyme extraction and activity assays, frozen samples were extracted in 50 mM potassium phosphate buffer (pH = 7) containing 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mM sodium ethylene di-amine tetra-acetic acid (Na_2EDTA), and 1% (m/v) polyvinyl pyrrolidone (PVP). Leaf extracts were centrifuged at 15000 ×g at 4°C for 15 min, and the supernatants were used for estimation of the protein content and enzyme activ-

ities. The total protein content was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

CATALASE

The catalase (CAT; E.C. 1.11.1.6) activity was measured according to the modified method of Dhindsa et al. (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 100 µl of enzyme extract. The rate of H_2O_2 decomposition was followed by measuring the decrease in absorbance at 240 nm. The activity of enzyme was expressed as enzyme unit per milligram of protein. One CAT unit decomposes 1 mM H_2O_2 within a min (Velikova et al., 2000).

GUAIACOL PEROXIDASE

The guaiacol peroxidase (GPX; E.C. 1.11.1.7) activity was determined in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.3% (v/v) H_2O_2, 1% (v/v) guaiacol and 200 µl of the enzyme extract by a method derived from Plewa et al. (1991). Using the spectrophotometric method, the guaiacol oxidation followed at 470 nm for three min (Plewa et al., 1991). An extinction coefficient of 25.5 $mM^{-1}cm^{-1}$ was used for estimation of the tetraguaiacol content in the samples. One unit of GPX oxidizes one micromole of guaiacol for one min.

ASCORBATE PEROXIDASE

The ascorbate peroxidase (APX; E.C. 1.11.1.11) activity was assayed by monitoring absorbance decline due to ascorbic acid oxidation at 290 nm (Nakano and Asada, 1987). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 150 µl enzyme extract. The activity of APX was calculated using an extinction coefficient 2.8 $mM^{-1}cm^{-1}$. One unit of APX activity was defined as the amount of enzyme that decomposed one micromole ascorbic acid per min.

GLUTATHIONE REDUCTASE

The activity of the glutathione reductase (GR; E.C. 1.6.4.2) was determined following the decrease in absorbance at 340 nm associated with the oxidation of NADPH (Foyer and Halliwell, 1976). The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.8), oxidized glutathione (GSSG) 0.5 mM, NADPH 0.1 mM and 50 µl of enzymatic extract. One unit of GR was defined as the amount of enzyme that oxidized 1 µM of NADPH per min.

STATISTICAL ANALYSIS

Significant differences in the growth characteristics, physiological and biochemical parameters, and macro- and micro-nutrients were analyzed following a 3-way analysis of variance. The means of groups were compared by Duncan's test using the statistical software SPSS version 18.0. All statistical tests were screened for significant variations at $P \leq 0.05$.

RESULTS

Three-way ANOVA confirms the significant effects of MT and metal factors separately and their interactions on almost all parameters studied in this research project (except for leaf calcium concentrations) at the significance level of 5% and 1%.

MINERAL NUTRIENT CONTENTS

Figures 1 and 2 illustrate mean comparison of mineral concentrations in shoots and roots of seedlings, respectively, in the presence of MT.

The results of Cu and Zn impact on mineral nutrition showed that excess amounts of zinc and copper disturb mineral compositions in shoots and roots of cannabis seedlings. The effect of Cu and Zn on root and shoot systems of cannabis seedlings can be briefly and comprehensively presented as follows. Cu stress at both concentrations caused higher Cu accumulation but lower concentrations of Zn, Fe, Mg, Ca and Mn (at Cu 150 µM) in shoots. On the other hand, Cu exposure caused higher Cu, Zn, Mg, Ca contents and decreased amounts of Fe, K and Mg (at Cu 150 µM) in roots of cannabis seedlings. Cu 50 µM and Cu 150 µM resulted in 29% and 23% reduction in the calcium content of shoots, respectively (Fig. 1f).

Zn stress exhibited a different impact on mineral composition of seedlings. Zn exposure at both excess levels caused higher Zn and Cu ions and also decreased the amounts of Fe, Mg, Mn, and K in cannabis shoots. However, Zn exposure reduced Fe and K contents and raised Cu, Zn, Mg, Mn, and Ca accumulations in roots. Zn 50 µM and

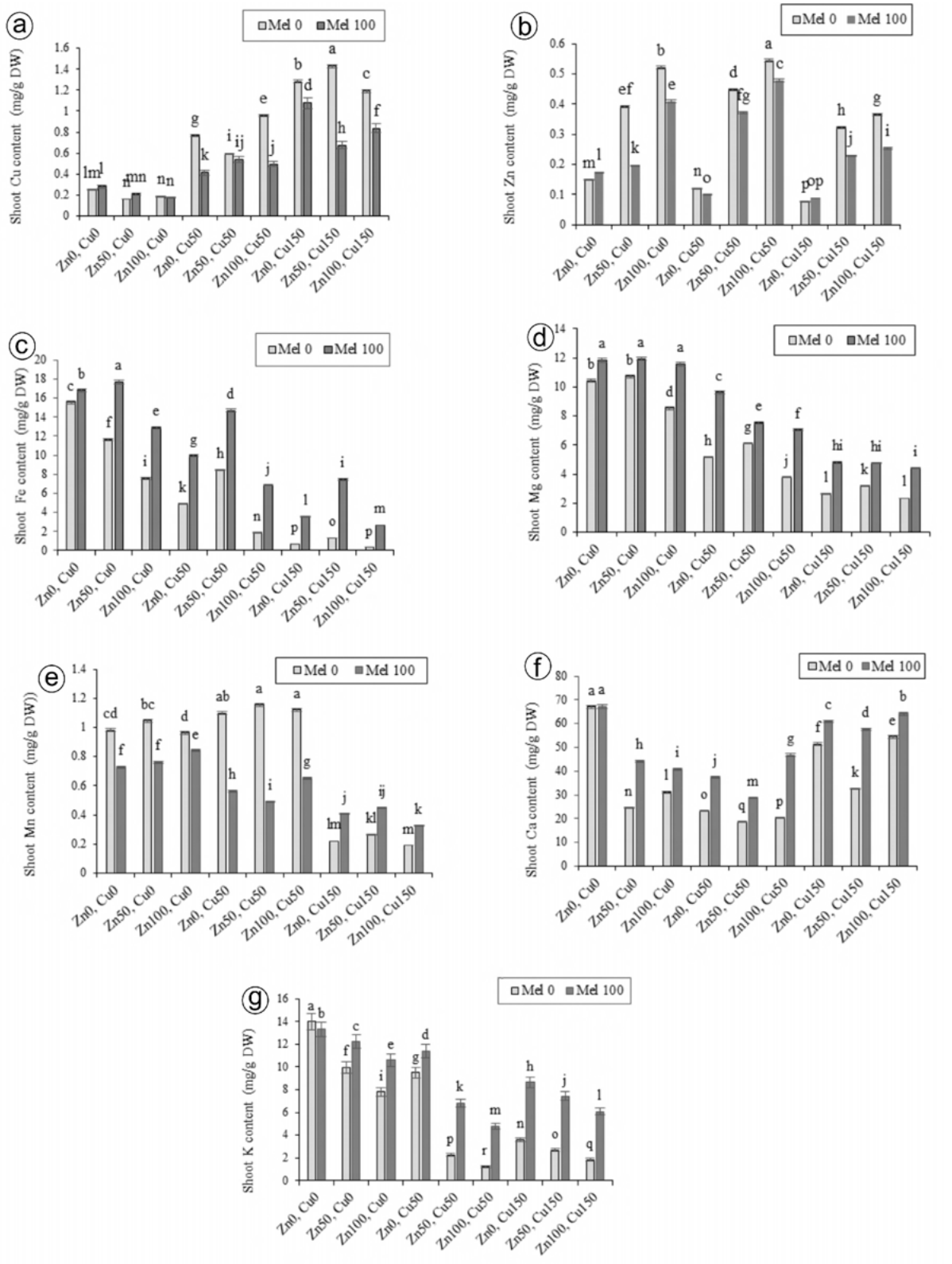

Fig. 1. Effects of MT, Cu and Zn interactions on Cu (**a**), Zn (**b**), Fe (**c**), Mg (**d**), Mn (**e**), Ca (**f**) and K (**g**) accumulation in shoots of cannabis seedlings. Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

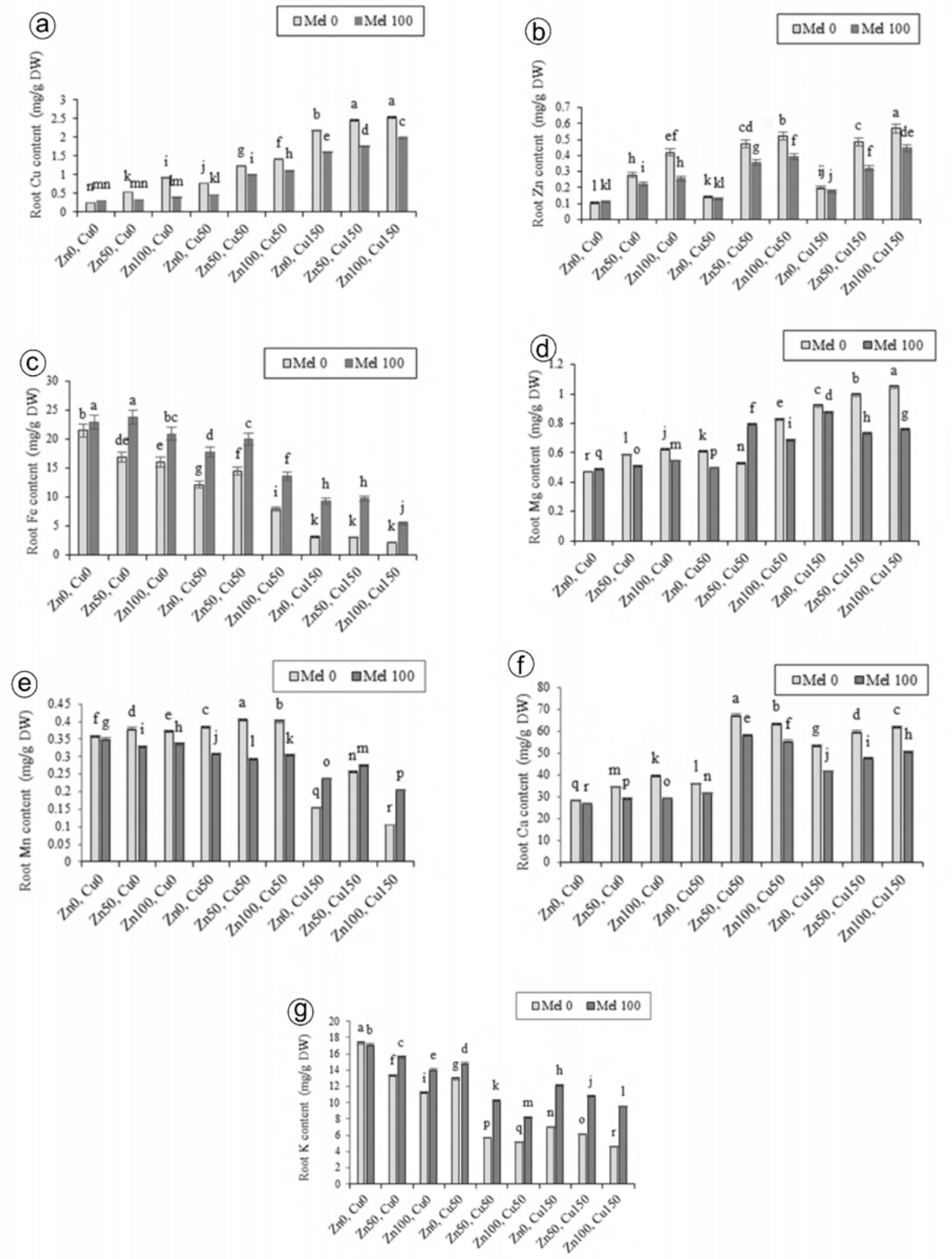

Fig. 2. Effects of MT, Cu and Zn interactions on Cu (**a**), Zn (**b**), Fe (**c**), Mg (**d**), Mn (**e**), Ca (**f**) and K (**g**) accumulation in roots of cannabis seedlings. Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

100 µM treatment reduced Ca concentration 2.7 and 1.2-fold compared to the control (Fig. 1f). Excess amounts of Zn and Cu, when applied together, caused a higher accumulation of Cu and Zn in shoots and roots while their combination reduced the amount of Fe, Mn, and K in both roots and shoots. Our results also showed that Cu 50 µM and 150 µM reduced Mg concentration in cannabis shoots 2-fold and 3.9-fold, respectively. On the other hand, 150 µM Cu + 100 µM Zn reduced the magnesium accumulation in the cannabis shoot approximately 4.4-fold (Figs. 1 and 2).

Interestingly, MT treatment showed a regulatory effect on mineral composition of seedlings exposed to Cu and Zn. As it was mentioned before, Cu accumulation significantly increased in roots of seedlings exposed to different concentrations of both Cu and Zn, when used separately and together. However, in the presence of MT, Cu accumulation was significantly reduced in shoots. MT reduced the shoot Cu concentration in the seedlings exposed to 150 µM Cu + 50 µM Zn 2-fold (Fig. 1a). MT treatment also decreased Zn accumulation in shoots and roots of the seedlings exposed to extra amounts of Zn and Zn + Cu (Figs. 1b and 2b). Moreover, MT treatment significantly adjusted iron content in roots and shoots up to control plants, particularly in seedlings exposed to simultaneous excess amounts of Cu and Zn (Figs. 1c and 2c). MT treatment caused elevated amount of Mg in shoots under Cu and Zn + Cu exposure, while it reduced Mg accumulation in roots in these treatment groups (Figs. 1d and 2d). Likewise, MT application inverted the effects of metal exposure on Mn concentrations in shoots and roots. MT caused a reduction of Mn concentrations in roots and shoots of seedlings exposed to 50 µM Cu, 50 µM Zn, 100 µM Zn and their interactions, but it also enhanced Mn concentration in seedlings receiving 150 mM Cu and Cu 150 mM combined with Zn overloads (Figs. 1e and 2e). MT treatment significantly increased Ca and K accumulation in cannabis shoots receiving extra amounts of Cu and Zn (Figs. 1 and 2f). Therefore, similar to other minerals such as Mg, Mn and Fe, melatonin treatment was able to neutralize toxic effects of Zn and Cu on mineral composition of Ca and K ions.

GROWTH PARAMETERS

The results presented in Table 1, compare growth parameters of seedlings exposed to excess amounts of Cu, Zn and their interactions before and after MT treatment. Our results showed that Zn 100 µM and Cu 50 µM and 150 µM and concomitant use of Cu and Zn significantly reduced growth indices including seedlings' fresh weight, seedlings' dry weight, and root and shoot length, confirming the toxic effect of the applied metal concentrations.

TABLE 1. Effects of MT, Cu and Zn interactions on shoot fresh weight, shoot dry weight, shoot length and root length. Different letters show significant differences based on Duncan's test ($p \leq 0.05$).

Root length (cm/plant)	Shoot length (cm/plant)	Shoot FW (g/plant)	Shoot DW (g/plant)	Melatonin (µM)	Treatment (µM)
36.80±0.62 f	27.43±1.20 h	4.87±0.35 cd	1.21±0.07 c	**MT 0**	**Zn 0 Cu 0**
47.16±0.30 a	60.63±1.20 a	8.13±1.13 a	2.34±0.42 a	**MT 100**	
42.90±0.70 c	30.53±1.02 g	4.72±0.19 d	1.21±0.07 c	**MT 0**	**Zn 50 Cu 0**
44.93±0.41 b	55.80±2.19 b	6.85±0.41b	1.92±0.05 b	**MT 100**	
23.73±0.35 l	20.23±1.65 jk	1.65±0.10g	0.46±0.03 e	**MT 0**	**Zn 100 Cu 0**
40.83±0.85 d	40.83±1.23 d	5.36±0.42 cd	1.35±0.12 c	**MT 100**	
26.10±0.43 k	18.16±1.13 kl	1.54±0.05 g	0.38±0.00 e	**MT 0**	**Zn 0 Cu 50**
43.66±0.35 c	47.06±2.48 c	5.63±0.31 c	1.40±0.06 c	**MT 100**	
19.70±0.55 n	17.83±0.60 l	1.46±0.02 g	0.36±0.01ef	**MT 0**	**Zn 0 Cu 150**
30.33±0.65 i	21.43±0.77 j	2.79±0.49 f	0.79±0.04 d	**MT 100**	
32.86±0.66 h	20.50±0.91 j	1.99±0.27g	0.52±0.07 e	**MT 0**	**Zn 50 Cu 50**
39.33±1.46 e	36.66±0.70 e	5.24±0.75 cd	1.31±0.21 c	**MT 100**	

Root length (cm/plant)	Shoot length (cm/plant)	Shoot FW (g/plant)	Shoot DW (g/plant)	Melatonin (µM)	Treatment (µM)
19.66±0.40 n	16.13±0.25 lmn	1.40±0.01 g	0.33±0.01 ef	**MT 0**	**Zn 100 Cu50**
34.93±0.65 g	33.93±1.90 f	5.02±0.18 cd	1.24±0.06 c	**MT 100**	
16.50±0.88 o	15.20±0.81mn	1.33±0.03 g	0.31±0.00 ef	**MT 0**	**Zn 50 Cu 150**
27.90±0.49 j	24.73±1.60 i	3.61±0.49 e	0.91±0.10 d	**MT 100**	
14.36±0.66 p	14.33±0.65 n	1.28±0.03 g	0.12±0.16 f	**MT 0**	**Zn 100 Cu 150**
22.46±0.98 m	16.96±0.45 lm	1.69±0.20 g	0.30±0.01 ef	**MT 100**	

Fresh weight and dry weight of seedlings receiving excess amounts of Cu or Zn were considerably increased by MT treatment. MT also caused 38% longer shoots and 36% longer roots in seedlings which were treated with both 150 µM Cu and 50 µM Zn. However, contrary to higher metal concentrations, it seems that MT functions better on growth parameters under mild or moderate stress conditions (Table 1).

PHYSIOLOGICAL PARAMETERS

Excess amounts of Cu, Zn and their concomitant application, significantly influenced cell membrane integrity in terms of electrolyte leakage (EL) and relative water content (RWC) of leaves. Cu 50 and 150 µM caused 88% and 126% reduction in relative water content, respectively, when compared to the control. MT application caused improvement of physiological parameters (Fig. 3a-c). MT significantly caused higher membrane stability of cells and the leaf water content in seedlings exposed to excess amounts of Cu and Zn. With MT application, MSI increased almost two-fold in seedlings exposed to 50 µM Cu and 50 µM Zn. MT treatment increased the ratio of Chl_a/Chl_b in seedlings exposed to metal treatments.

OXIDATIVE STRESS INDICES

Excessive amounts of Cu and Zn increased H_2O_2 content in cannabis seedlings ($p \leq 0.01$). 150 µM Cu increased hydrogen peroxide accumulation and lipid peroxidation by 46% and 22%, respectively. The accumulation of H_2O_2 and the level of MDA were also elevated by 33% and 8%, respectively, in response to Zn 100 µM exposure. Hydrogen peroxide and lipid peroxidation levels were reduced by the application of MT in control seedlings as well as Cu and Zn treatment (Fig. 4).

ANTI-OXIDATIVE RESPONSES

As it is shown in Fig. 5, different concentrations of Cu and Zn, separately and their combination, increased the total and reduced glutathione content, compared to the control ($p \leq 0.01$). However, MT application caused even a higher total glutathione content in seedlings receiving extra amounts of Cu and Zn ($p \leq 0.01$). MT reduced oxidized glutathione by 17% in seedlings exposed to Cu 150 µM + Zn 50 µM (Fig. 5).

Statistical analysis showed that CAT and GPX activities increased by overloads of Cu and Cu + Zn (Fig. 6a). However, Zn 50 µM and 100 µM reduced GPX activity comparing to the control. Based on Duncan's test, MT treatment significantly ($p \leq 0.05$) reduced catalase and GPX activities in seedlings under excessive amounts of Zn and higher levels of Cu (Fig. 6b). The activity of APX and GR, two main enzymes involved in ascorbate-glutathione cycle, was also influenced by Cu and Zn and their interactions. Both Zn and Cu stress enhanced APX and glutathione reductase activities ($p \leq 0.01$) in cannabis seedlings, as compared to the control (Fig. 6c,d). A combination of Cu 150 µM + Zn 100 µM increased the activity of GR 4.8-fold, compared to the control. However, MT treatment had also an enhancement effect on the activity of APX in seedlings affected by Zn and Cu stress. Likewise, MT treatment increased the GR activity in both control and metal-treated seedlings (Fig. 6d).

DISCUSSION

Cannabis sativa is reported to be a high biomass-producing plant with the capability of heavy metals accumulation in roots and shoots, therefore it was introduced as a good candidate for phytoremediation of metal contaminations (Bona et al.,

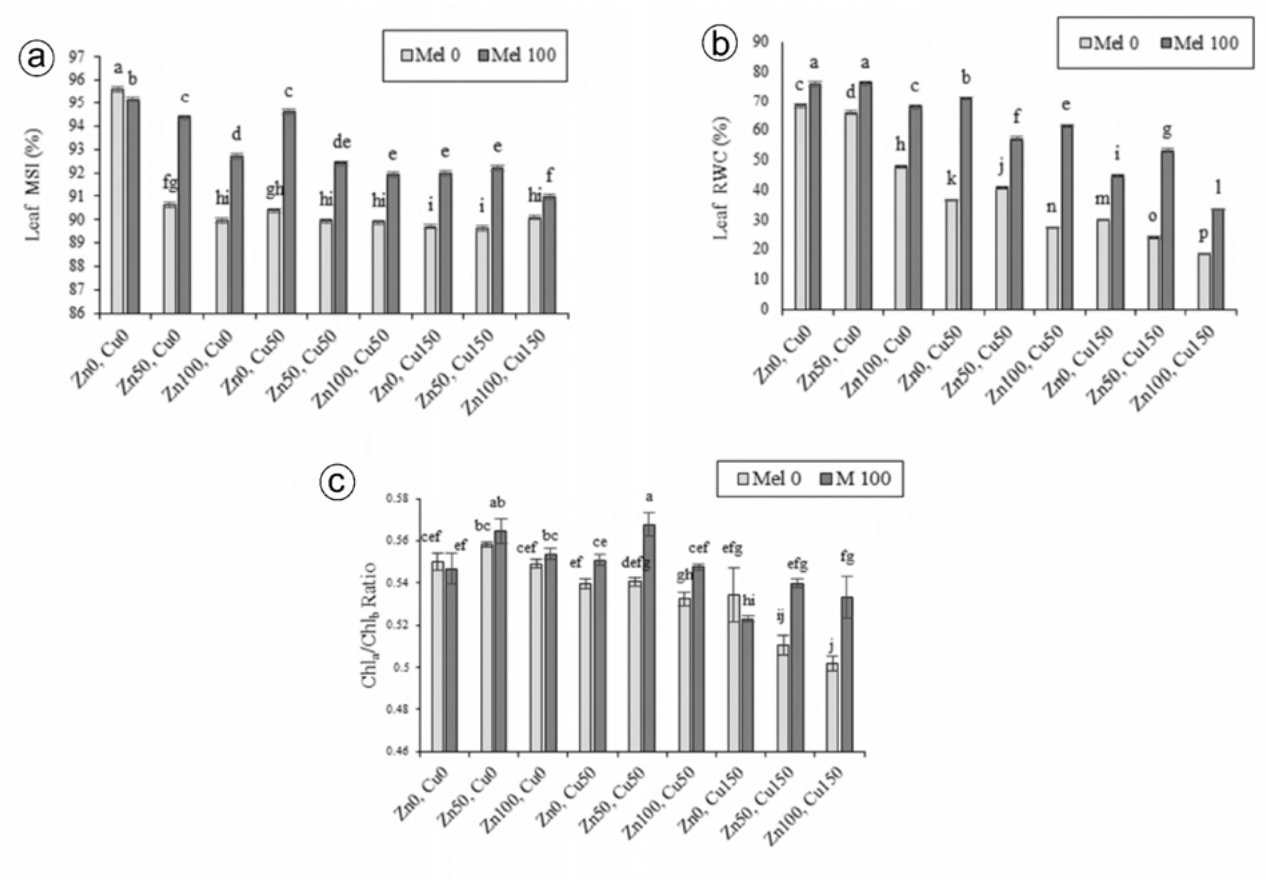

Fig. 3. Effects of MT, Cu and Zn interactions on membrane stability index (MSI) (**a**), relative water content (RWC) (**b**), and chlorophyll$_a$/chlorophyll$_b$ ratio (**c**). Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

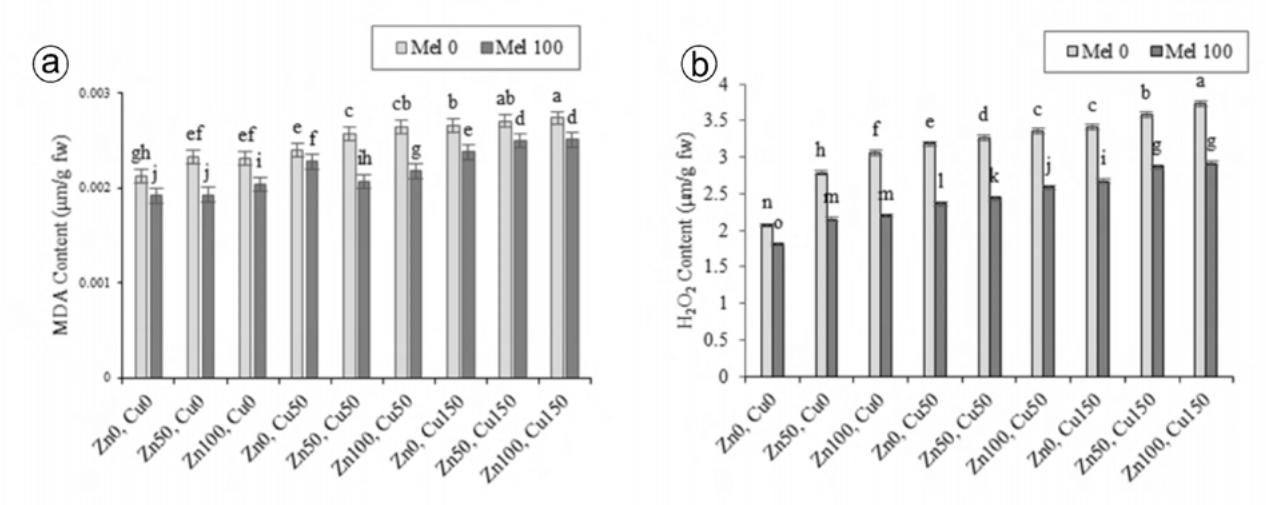

Fig. 4. Effects of MT, Cu and Zn interactions on MDA content (**a**), and H_2O_2 content (**b**). Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

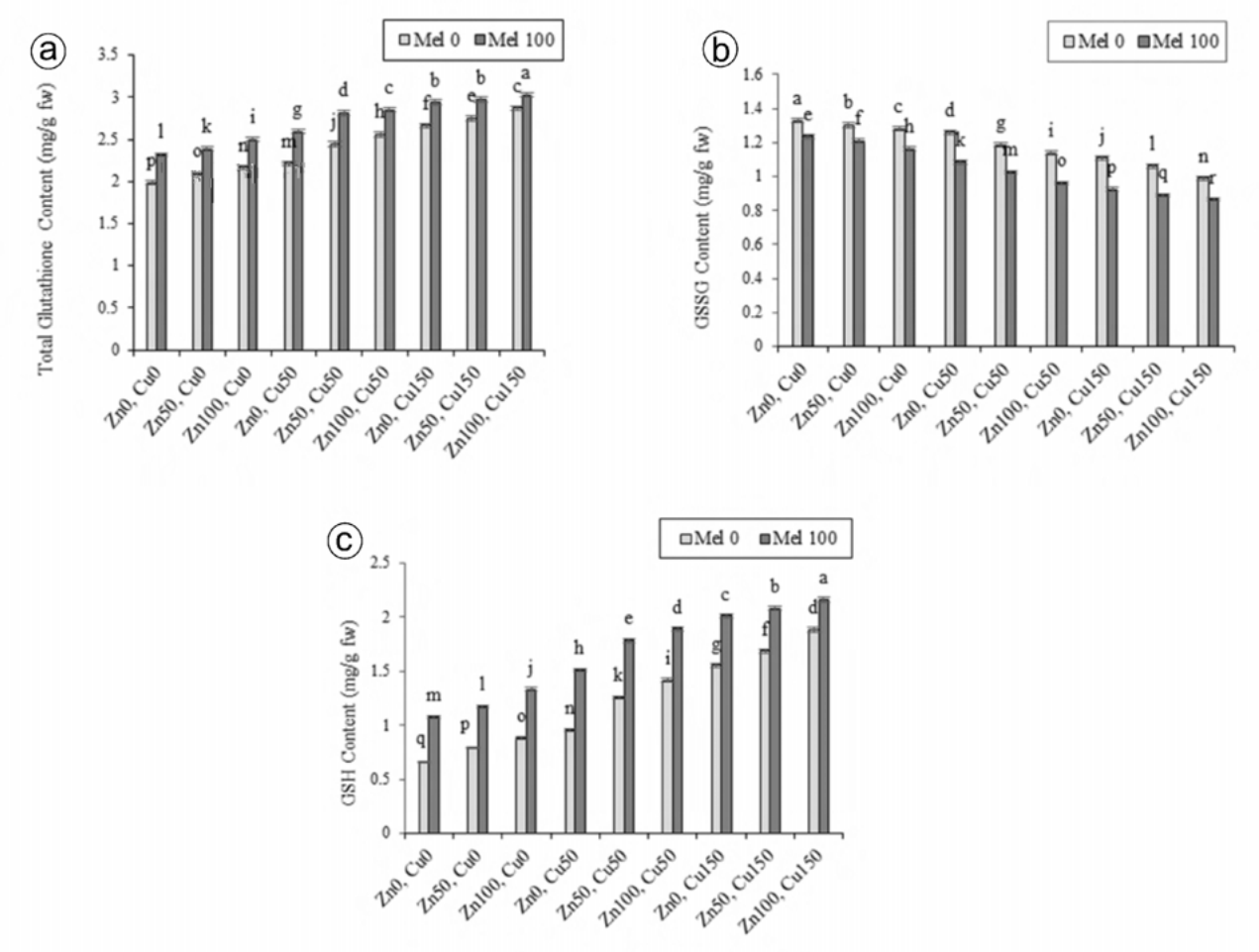

Fig. 5. Effects of MT, Cu and Zn interactions on total glutathione (**a**), oxidized glutathione (**b**), and reduced glutathione (**c**) of cannabis leaves. Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

2007). In this research, we studied the interactive effects of exogenously applied MT with excess amounts of Zn and Cu on the growth, mineral nutrition, and antioxidant responses in cannabis (*C. sativa* L.) seedlings. Copper (Cu) and zinc (Zn) are essential nutrients when present in lesser amounts. These elements play roles in various metabolic and physiological processes in plants, although in excessive amounts these exert detrimental effects. Cu and Zn toxicity in plants might be due to the induction of oxidative stress combined with pronounced disturbances in the nutrient absorption (Yruela, 2009). Our results showed that Cu and Zn accumulation significantly increased in roots and shoots of seedlings exposed to different concentrations of Cu and Zn. However, it seems that MT has an important influence on both nutrient content and partitioning in shoots and roots. MT treatment considerably decreased Cu and Zn accumulation in shoots and roots of the seedlings exposed to extra amounts of these elements.

Excess amounts of Cu and Zn had considerable impact on accumulation of other nutrients in shoots and roots. Both Zn and Cu exposure decreased Fe, Mg, Mn, Ca and K content in shoots and Fe, Mn, and K content in roots, while increased Ca content in roots.

It has been reported that the inhibitory effect of zinc and copper ions on micro- and macro-nutrients uptake and translocations could be due to the competition for the same sites for absorption into the plant root. It has been shown that Zn toxicity leads to iron (Fe) deficiency, due to prevention of Fe transfer from the root to the shoot. However, the effect of MT on the balance of micro-

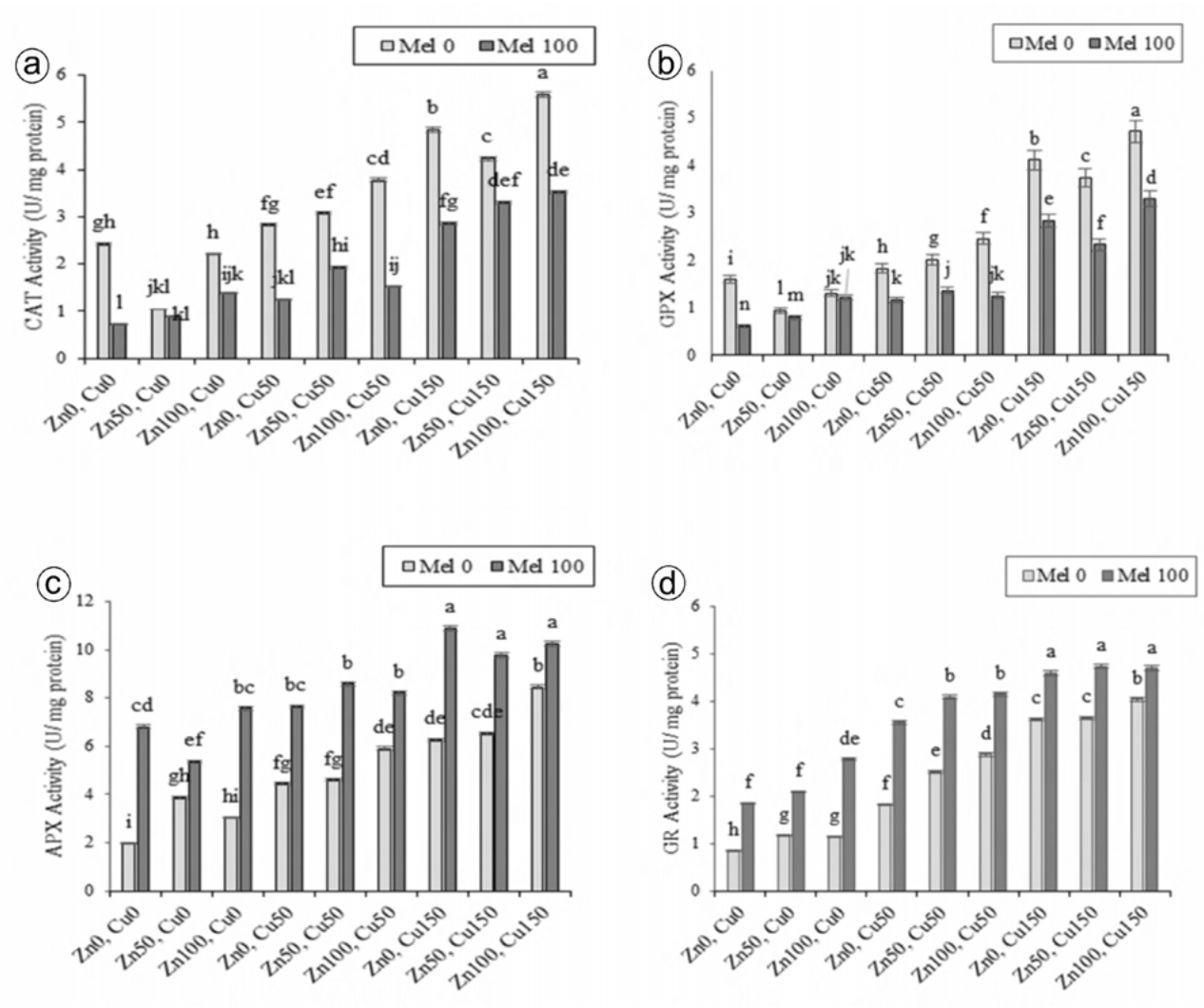

Fig. 6. Effects of MT, Cu and Zn interactions on CAT (**a**), GPX (**b**), APX (**c**), and GR (**d**) activities. Columns with the same letters do not show significant differences based on Duncan's test ($p \leq 0.05$). The vertical bar represents the standard error of the mean.

and macro-nutrient was observed in this study. Based on our results, it seems that MT, when applied exogenously, can counteract the negative effect of toxic amounts of zinc and copper on micro- and macro-nutrients accumulation in cannabis seedlings. Since the contents of Cu, Zn and Mn were increased by Cu and Zn stress, MT treatment reduced Cu, Zn, Mn concentrations in seedlings' shoots under the same conditions. Moreover, MT caused an increase of Fe, Mg, Ca and K contents in seedlings' shoots under Cu and Zn stress. MT treatment also significantly elevated magnesium accumulation under excess amounts of Cu and Zn + Cu. Furthermore, MT reduced Cu, and Ca contents and increased Fe and K contents in roots of cannabis exposed to extra amounts of Cu and Zn. The ability of the hemp plant to tolerate heavy metals is assumed to be related to the activation of genes involved in stress tolerance (Ahmad et al., 2016). The phytoremediation capability of cannabis plants has been reported earlier, however based on our results, it cannot be claimed that in cannabis this capability is strong. Interestingly, our results showed that MT application regulates nutrients partitioning between roots and shoots of cannabis. Therefore, the results of the present study suggest that MT application could be considered as a candidate for phytoremediation strategies. The melatonin-mediated improvement of mineral uptake, and

MT-induction of the gene expression of mineral transporters were reported before (Arnao and Hernández-Ruiz, 2014). However, to confirm this hypothesis in cannabis plants, further investigation and additional studies are needed. Hodžić et al., (2019) observed changes in Ca and Mg concentrations of the pre-treated lemon balm (*Melissa officinalis* L.) plants with exogenous 0.1 mM melatonin and increased levels of zinc and cadmium. Their observations referring to MT impact on Cd and Zn uptake and translocation by lemon balm plants made them propose that melatonin can be used for phytoremediation purposes (Hodžić et al., 2019). It has been reported that K accumulation is higher in MT-treated Bermuda grass plants compared with those of non- MT treated plants (Chen et al., 2017). The induced transcripts of K^+ transporting genes by exogenous MT treatment in Bermuda grass were described as the main reason for K accumulation (Chen et al., 2017). Potassium plays an important role in plant development including osmotic regulation, activation of numerous enzymes, and protection of electrical potential gradients in cell membranes. Potassium also protects water content in plants (Wang et al., 2013). Thus, besides induction of oxidative stress and lipid peroxidation, the impaired MSI and reduced RWC observed in this study might be as a result of K depletion under Cu and Zn toxic conditions.

Calcium is known as an important factor for physiological and biochemical processes in plant cells, such as integrity of membranes and cell walls, organization of photosynthetic organelles in photosystem II, as well as the stomata movement adjustment (Klimecka and Muszyńska, 2007). Therefore, improved RWC and biomass production could be a resultant of MT impact on calcium accumulation and balance.

An overall reduction of plant biomass, inhibition of root growth, chlorosis, bronzing, and necrosis are usually reported symptoms of Cu and Zn stress, due to increased production of reactive oxygen species and harmful interactions at the cellular level (Yruela, 2009). The results of the present research showed that excessive amounts of Cu and Zn significantly affect almost all growth parameters in cannabis seedlings confirming the toxicity of the applied concentrations. Moreover, the combined exposure to the two examined metals showed synergistic toxicity symptoms on several parameters (e.g., ratio of chlorophylls, RWC and hydrogen peroxide content), at higher doses. These results could be due to Cu and Zn competition for uptake of nutritional elements such as Ca in shoots, but not always at a similar rate. However, 100 µM MT application significantly reduced the susceptibility of cannabis seedlings to damages caused by Cu and Zn excess. Exogenous MT application considerably increased plant biomass and longitudinal growth of the seedlings. Similar to our results, MT applications on barley, wheat, sweet cherry, and rice, have been reported to improve the growth parameters such as root and shoot growth and also seed germination, to enhance redox homeostasis, rhizogenesis and root formation, as well as leaf growth (Sharif et al., 2018). The foliar application of melatonin on apple caused increment of the photosynthetic rate, mono-saccharides, sucrose, starch, and sorbitol (Arnao et al., 2021). These results generally support the idea that MT acts as a signaling molecule which is responsible for physiological responses stimulating plant growth, through improvement of metabolic pathways and photosynthesis, under stress conditions (Debnath et al., 2019).

The results obtained from measurements of physiological parameters generally support the influence of excess concentrations of Zn and Cu, used in this study. However, our results showed higher Chl_a/Chl_b ratio in MT treated plants. These results indicate that exogenously applied MT is able to alleviate Zn and Cu toxic effects on photosynthesis, probably through chlorophyll maintenance, which in turn causes improved growth parameters. The chlorophyll-protective efficacy of melatonin was also reported in seedlings of pistachio subjected to cold stress (Barand et al., 2020). There is some evidence that supports the idea that MT mediated enhancement in photosynthesis is accompanied by the reduced catabolism of chlorophyll molecules and down-regulation of genes favoring the process of senescence (Sharma and Zheng, 2019). Therefore, melatonin-mediated regulation of genes involved in photosynthetic pigments biosynthesis could be one of the reasons for the changes in Chl*a*/Chl*b* ratio, which was observed in our study. However, further studies are still needed to draw more accurate conclusions about MT impact on metabolism of photosynthetic pigments. It seems that MT can protect chlorophyll molecules as antioxidants since our results showed a reduced MDA content, membrane leakage, and hydrogen peroxide content in MT-treated plants under Cu and Zn stress.

Generally, in this study, MT-treated plants performed better resistance against Cu and Zn stresses, probably due to adjustment of antioxidant enzyme activities, which could be relevant to reduced H_2O_2 content and MDA production in plants treated with MT. The high activities of antioxidant enzymes under stress are necessary for plants to defend themselves against ROS damage. Antioxidant enzymes in plants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GP). Plants use this defense system to counteract the effects of oxidative stress caused by heavy metals. The SOD enzyme converts superoxide radicals to H_2O_2. However, high H_2O_2 content is toxic to cells, so it is necessary to remove it from them. CAT, APX, and GPX are involved in detoxifying processes of H_2O_2 conversion into water and oxygen. These processes entail maintaining a balance between the antioxidant systems and ROS content. Melatonin is a strong antioxidant compound which has the ability to adjust free radicals such as superoxide anion, hydrogen peroxide, and malondialdehyde, leading to membrane stability in plants under normal and stress conditions. MT increased glutathione levels and induction of gene expression of antioxidant enzymes such as SOD, CAT, guacol and ASC peroxidases(Arnao et al., 2021). Hodžić et al. (2019) reported that MT pretreatment of leaves in *Valeriana officinalis* induced alternation in SOD iso-enzyme profiles and activities as well as POD activity in plants treated with Zn and Cd.

Our data on antioxidant enzyme activities confirm the previous reports which stated that MT has significant roles in scavenging of reactive oxygen and in enhancing activities of antioxidant enzymes, mainly glutathione reductase and glutathione peroxidase (Fischer et al., 2013). Melatonin and some of its metabolites are known as endogenous free radical scavengers and antioxidants (Zhang, 2013), which may directly scavenge H_2O_2. Presumably, one important function of melatonin, along with CAT, GPX and APX, may be maintaining intracellular H_2O_2 concentrations at steady-state levels (Zhang et al., 2017). H_2O_2 accumulation was inhibited by exogenous melatonin, which may have resulted from direct ROS scavenging by melatonin and enhanced CAT, GPX and APX activities. Therefore, these results confirm the role of melatonin as a free-radical scavenger and an antioxidant. These findings also show that MT can stimulate antioxidant enzymes to protect plant tissues from oxidative damage. However, the physiological and molecular mechanism by which exogenous melatonin enhances heavy metal tolerance of plants needs further research.

Glutathione (GSH) turns into glutathione disulfide (GSSG), through the activity of glutathione peroxidase and decomposition of H_2O_2 (Dringen and Hamprecht, 1997, Zhao et al., 2016). In the current study, total and reduced glutathione were significantly increased by MT. It seems that MT makes a balance between antioxidant enzymes activity and active radical production that reduces MDA content and maintains MSI and relative water content in cells of cannabis seedlings. In accordance with our results, Cen et al. (2020) reported that MT pretreatment caused decreasing activity of glutathione peroxidase while the relative expression levels of antioxidant enzymes such SOD, CAT and APX genes were up-regulated under normal conditions and in salt-stressed alfalfa plants. It should be noted that the exact mechanism of MT impact on the antioxidant defense system still requires further studies. These data confirm the role of MT in protecting photosynthesis in cannabis plants.

CONCLUSIONS

Our results demonstrate that cannabis has a great potential to regulate Cu, and Zn content when their amounts are excessive. The results show that MT treatment caused alleviated growth retardations and oxidative stress of cannabis seedlings under conditions of excessive amounts of Cu and Zn by adjusting ion homeostasis, scavenging ROS, increasing of GSH and regulating antioxidant enzyme activity. Therefore, MT may potentially be used to protect cannabis plants against Zn and Cu stress. Besides, considering the remarkable role of MT in nutritional balance and ion homeostasis of nutritional elements, this compound can be applied as a bio-stimulator and also a homeostasis factor of nutritional elements in plants. However, there are still many questions about the role of melatonin in the mineral nutrient uptake, metal transporter gene expression and their content, and its regulatory effect on nutrient partitioning in plant cells. The role of MT as an active component in modulation of the nutrient content and phytoremediation technology still need more consideration.

AUTHORS' CONTRIBUTIONS

The authors confirm contribution to the paper as follows: Hakimeh Oloumi: study conception, design and performance of experiments, data analysis, wrote the article. Ali Zamani: performed experiments and co-wrote the paper. Hossein Mozaffari and Seyyed M. Javad Arvin: study conception and design, analysis and interpretation of results. All authors reviewed the results and approved the final version of the manuscript. The authors declare no conflicts of interest.

ACKNOWLEDGMENT

Authors acknowledge Plant Antioxidant Center of Excellence (PACE), University of Isfahan for their help and support.

REFERENCES

Ahmad R, Tehsin Z, Malik ST, Asad SA, Shahzad M, Bilal M, Shah MM, and Khan SA. 2016. Phytoremediation potential of hemp (*Cannabis sativa* L.): identification and characterization of heavy metals responsive genes. *CLEAN–Soil, Air, Water* 44: 195–201.

Alaoui-Sossé B, Genet P, Vinit-Dunand F, Toussaint M-L, Epron D, and Badot P-M. 2004. Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Science* 166: 1213–1218.

Arnao MB, and Hernández-Ruiz J. 2014. Melatonin: plant growth regulator and/or biostimulator during stress? *Trends Plant Science* 19: 789–797.

Arnao MB, Hernández-Ruiz J, Cano A, and Reiter RJ. 2021. Melatonin and carbohydrate metabolism in plant cells. *Plants* 10: 1917.

Bona E, Marsano F, Cavaletto M, and Berta G. 2007. Proteomic characterization of copper stress response in *Cannabis sativa* roots. *Proteomics* 7: 1121–1130.

Bosnić D, Bosnić P, Nikolić D, Nikolić M, and Samardžić J. 2019. Silicon and iron differently alleviate copper toxicity in cucumber leaves. *Plants* 8: 554.

Bray TM, and Bettger WJ. 1990. The physiological role of zinc as an antioxidant. *Free Radical Biology and Medicine* 8: 281–291.

Cayton MTC, Reyes E, and Neue H. 1985. Effect of zinc fertilization on the mineral nutrition of rices differing in tolerance to zinc deficiency. *Plant and Soil* 87: 319–327.

Cen H, Wang T, Liu H, Tian D, and Zhang Y. 2020. Melatonin application improves salt tolerance of alfalfa (*Medicago sativa* L.) by enhancing antioxidant capacity. *Plants* 9: 220.

Chen L, Fan J, Hu Z, Huang X, Amombo E, Liu A, Bi A, Chen K, Xie Y, and Fu J. 2017. Melatonin is involved in regulation of Bermudagrass growth and development and response to low K+ stress. *Frontiers in Plant Science* 8: 2038.

Debnath B, Islam W, Li M, Sun Y, Lu X, Mitra S, Hussain M, Liu S, and Qiu D. 2019. Melatonin mediates enhancement of stress tolerance in plants. *International Journal of Molecular Sciences* 20: 1040.

Dringen R, and Hamprecht B. 1997. Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. *Brain Research* 759: 67–75.

Dubbels R, Reiter R, Klenke E, Goebel A, Schnakenberg E, Ehlers C, Schiwara H, and Schloot W. 1995. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *Journal of Pineal Research* 18: 28–31.

Fischer TW, Kleszczyński K, Hardkop LH, Kruse N, and Zillikens D. 2013. Melatonin enhances antioxidative enzyme gene expression (CAT, GPx, SOD), prevents their UVR-induced depletion, and protects against the formation of DNA damage (8-hydroxy-2'-deoxyguanosine) in *ex vivo* human skin. *Journal of Pineal Research* 54: 303–312.

Flora S, Shrivastava R, and Mittal M. 2013. Chemistry and pharmacological properties of some natural and synthetic antioxidants for heavy metal toxicity. *Current Medicinal Chemistry* 20: 4540–4574.

Foyer CH, and Halliwell B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* 133: 21–25.

Griffith OW. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry* 106: 207–212.

Hänsch R, and Mendel RR. 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Current Opinion in Plant Biology* 12: 259–266.

He ZL, Yang XE, and Stoffella PJ. 2005. Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine and Biology* 19: 125–140.

Hernández-Ruiz J, and Arnao MB. 2008. Melatonin stimulates the expansion of etiolated lupin cotyledons. *Plant Growth Regulation* 55: 29–34.

Hodges DM, Delong JM, Forney CF, and Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207: 604–611.

Hodžić E, Balaban M, šuškalo N, Galijaševic S, Hasanagić D, Kukavica B. 2019. Antioxidative response of *Melissa officinalis* L. and *Valeriana officinalis* L. leaves exposed to exogenous melatonin and excessive zinc

and cadmium levels. *Journal of the Serbian Chemical Society* 84: 11-25.

Ke W, Xiong Z-T, Chen S, and Chen J. 2007. Effects of copper and mineral nutrition on growth, copper accumulation and mineral element uptake in two *Rumex japonicus* populations from a copper mine and an uncontaminated field sites. *Environmental and Experimental Botany* 59: 59–67.

Klimecka M, and Muszyńska G. 2007. Structure and functions of plant calcium-dependent protein kinases. *Acta Biochimica Polonica* 54: 219–233.

Korkomaz A, Karaca A, Kocacinar F, and Cuci Y. 2017. The effects of seed treatment with melatonin on germination and emergence performance of pepper seeds under chilling stress. *Journal of Agricultural Sciences* 23: 167–176.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology.* Elsevier.

Limson J, Nyokong T, and Daya S. 1998. The interaction of melatonin and its precursors with aluminium, cadmium, copper, iron, lead, and zinc: an adsorptive voltammetric study. *Journal of Pineal Research* 24: 15–21.

Lutts S, Kinet J, and Bouharmont J. 1996. NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Annals of Botany* 78: 389–398.

Meng J-F, Shi T-C, Song S, Zhang Z-W, and Fang Y-L. 2017. Melatonin in grapes and grape-related foodstuffs: a review. *Food Chemistry* 231: 185–191.

Mullan D, and Pietragall J. 2012. Leaf relative water content. In: *Physiological Breeding II: a field guide to wheat phenotyping,* 25–27. CIMMYT, Mexico.

Murch SJ, and Saxena PK. 2002. Melatonin: a potential regulator of plant growth and development? *In Vitro Cellular. Developmental Biology – Plant,* 38: 531–536.

Nagajyoti PC, Lee KD, and Sreekanth T. 2010. Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters* 8: 199–216.

Narula A, Kumar S, and Srivastava P. 2005. Abiotic metal stress enhances diosgenin yield in *Dioscorea bulbifera* L. cultures. *Plant Cell Reports* 24: 250-254.

Parmar P, Limson J, Nyokong T, and Daya S. 2002. Melatonin protects against copper-mediated free radical damage. *Journal of Pineal Research* 32: 237–242.

Plewa MJ, Smith SR, and Wagner ED. 1991. Diethyldithiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhibiting tobacco cell peroxidase. *Mutation Research / Fundamental and Molecular Mechanisms of Mutagenesis* 247: 57–64.

Posmyk MM, Kuran H, Marciniak K, and Janas KM. 2008. Presowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentrations. *Journal of Pineal Research* 45: 24–31.

Raman V, Lata H, Chandra S, Khan IA, and Elsohly MA. 2017. Morpho-anatomy of marijuana (*Cannabis sativa* L.). *Cannabis sativa L. – Botany and Biotechnology.* Springer.

Ren S, Jiang G-L, and Rutto L. 2019. Melatonin priming enhances symbiotic nitrogen fixation in soybean, *Glycine max* L. *Journal of Biotech Research* 10: 136–144.

Romero A, Ramos E, De Los Rios C, Egea J, Del Pino J, and Reiter RJ. 2014. A review of metal-catalyzed molecular damage: protection by melatonin. *Journal of Pineal Research* 56: 343–370.

Schutzendubel A, and Polle A. 2002. Plant responses to abi,otic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* 53: 1351–1365.

Sharif R, Xie C, Zhang H, Arnao MB, Ali M, Ali Q, Muhammad I, Shalmani A, Nawaz MA, and Chen P. 2018. Melatonin and its effects on plant systems. *Molecules* 23: 2352.

Sharma A, and Zheng B. 2019. Melatonin mediated regulation of drought stress: physiological and molecular aspects. *Plants* 8: 190.

Shi H, Chen K, Wei Y, and He C. 2016. Fundamental issues of melatonin-mediated stress signaling in plants. *Frontiers in Plant Science* 7: 1124.

Thounaojam TC, Panda P, Mazumdar P, Kumar D, Sharma GD, Sahoo L, and Sanjib P. 2012. Excess copper induced oxidative stress and response of antioxidants in rice. *Plant Physiology and Biochemistry* 53: 33–39.

Tiwari RK, Lal MK, Kumar R, Mangal V, Altaf MA, Sharma S, Singh B, and Kumar M. 2022. Insight into melatonin-mediated response and signaling in the regulation of plant defense under biotic stress. *Plant Molecular Biology* 109: 385–399.

Velikova V, Yordanov I, and Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science* 151: 59–66.

Wang M, Zheng Q, Shen Q, and Guo S. 2013. The critical role of potassium in plant stress response. *International Journal of Molecular Sciences* 14: 7370–7390.

Wang Y, Reiter RJ, and Chan Z. 2018. Phytomelatonin: a universal abiotic stress regulator. *Journal of Experimental Botany* 69: 963–974.

Yruela I. 2009. Copper in plants: acquisition, transport and interactions. *Functional Plant Biology* 36: 409–430.

Zhang N, Zhao B, Zhang HJ, Weeda S, Yang C, Yang ZC, Ren S, and Guo YD. 2013. Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (*Cucumis sativus* L.). *Journal of Pineal Research* 54: 15–23.

Zhang R, Sun Y, Liu Z, Jin W, and Sun Y. 2017. Effects of melatonin on seedling growth, mineral nutrition, and nitrogen metabolism in cucumber under nitrate stress. *Journal of Pineal Research* 62: e12403.

Zhao H, Ye L, Wang Y, Zhou X, Yang J, Wang J, Cao K, and Zou Z. 2016. Melatonin increases the chilling tolerance of chloroplast in cucumber seedlings by regulating photosynthetic electron flux and the ascorbate-glutathione cycle. *Frontiers in Plant Science* 7: 1814.