Acta Scientific AGRICULTURE (ISSN: 2581-365X)

Volume 6 Issue 10 October 2022

Research Article

Metabolic Processes and Chlorophyll Biosynthesis Affected by Cu and PEG 6000 in Maize

Mamta Hirve and Meeta Jain*

School of Biochemistry, Devi Ahilya University, Takshashila Campus, Indore, India

***Corresponding Author:** Meeta Jain, School of Biochemistry, Devi Ahilya University, Takshashila Campus, Indore, India.

DOI: 10.31080/ASAG.2022.06.1188

Received: May 18, 2022

Published: September 23, 2022

Abstract

Simultaneous exposure of plants with more than one stresses is a frequent phenomenon in the natural environment. A combination of heavy metals and water deficit stress is one of them. The present study was undertaken to investigate the effects of essential metal copper (Cu), polyethylene glycol (PEG) 6000 induced water deficit and their combination on biochemical attributes and chlorophyll biosynthesis in maize *(Zea mays* L. cv. Ganga safed-2) seedlings. Results of the study indicate a slight increase in the DNA content of roots with 10% PEG and $CuSO_4$ together. A similar pattern was obtained for total RNA in roots and the effect was substantial and significant with 10% PEG along with 100 µM $CuSO_4$. An increase in protein content and band intensities in protein profile by SDS PAGE was observed, especially, with 100 µM $CuSO_4$, 10% PEG treatment, and its combination with 10 and 100 µM $CuSO_4$. Both the stresses were found to decrease the contents of chlorophylls, carotenoids, and δ-amino levulinic acid (ALA), as well as, ALA synthesizing activity, δ-aminolevulinic acid dehydratase (ALAD), and porphobilinogen deaminase (PBGD) activities. More Cu was accumulated in roots than in shoots. Increased protein content in maize root and shoot tissues by $CuSO_4$, PEG, and their combined treatments may involve the synthesis of new proteins under stress conditions. Unaltered chlorophyll content and enzyme activities involved in chlorophyll biosynthesis by the supply of $CuSO_4$ may be because of balance maintained between formation and utilization of chlorophyll biosynthetic precursors in the presence of essential metal Cu. Inhibition of ALA synthesizing and PBGD activities as well as a decrease in ALA content by PEG, indicates the inhibition of early steps of chlorophyll biosynthetic pathway to be responsible for decreasing the chlorophyll content.

Keywords: Maize; Chlorophyll Biosynthesis; Heavy Metals; Water Deficit

Introduction

Plant growth is affected by several environmental barriers, involving temperature variations (heating, chilling, freezing etc.), climatic obstruction (flooding, drought etc.) or presence of some interfering agents (salinity, heavy metal toxicity etc.). Among them, heavy metals are well-known ecological contaminants due to their persistence, bioaccumulation and toxic nature [1]. Copper (Cu) is an essential trace element that plays a vital role in maintaining plant's normal metabolism by participating in various physiological processes, such as, photosynthesis, oxidative stress response, respiratory electron transport chain, hormone signaling as well as in cell wall metabolism [2]. However, excess of Cu can be extremely toxic. The release of excessive Cu in the environment is mainly anthropogenic i.e. the use of bactericides, fungicides, algaecides and industrial effluents in the agricultural lands [3,4]. Plants exposed to Cu are known to produce thiol rich peptides and phytochelatins by utilizing glutathione [5]. The toxicity symptoms of Cu includes chlorosis, necrosis, inhibition of seed germination,

root and shoot length, seedling biomass, accumulation of sugars and phytochelatins as well as alteration in the pigments, protein components, thylakoid membrane structure and CO_2-fixation reactions of photosynthesis etc. [6-9].

In addition to metal toxicity, water deficit stress is a widespread threat around the world [10]. The visible symptoms of plants subjected to water deficit are leaf wilting, stunted plant height, reduction in leaf area and number, and a hindrance in the development of buds and flowers [11]. Within the cell, water insufficiency results in metabolic imbalance, generation of ROS and hence activation of plants antioxidative machinery [12]. The co-occurrence of water deficit and metal stress in field condition is an example of a combined abiotic stresses [13]. Drought and heavy metals (Ni, Cu, Co and Cr) in combination have been shown to reduce the growth of *Red maple* in an additive manner by altering xylem structure and hydraulic conductivity [14]. Combined application of excess Cu and water deficit has been shown to increase proline and abscisic acid content with decreased leaf relative water content in tobacco leaves [15]. Moreover, heavy metals affect the water uptake by roots, slowdowns the short-distance water movements in the different plant parts, decrease the flow of water in the vascular tissues and thus ultimately affect the water supply to the shoot [16]. Furthermore, it has been suggested that the heavy metals influence water delivery to the shoot via decreasing leaves size, the thickness of the lamina, intercellular spaces and affecting the stomatal size and density [16].

Chlorophyll is a predominant tetrapyrrole and the fundamental biosynthetic pathway of chlorophyll is common for all the tertapyrroles with some variations in prokaryotes and eukaryotes [17]. It is a highly complex regulated metabolic pathway involving a series of cooperative reactions catalyzed by a number of enzymes [18]. δ-Amino levulinic acid (ALA), is the common precursor molecule for synthesizing all tetrapyrrole pigments. Its formation is the first regulatory step of the pathway. δ-Aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24), catalyzes the formation of porphobilinogen by condensation of two molecules of ALA. Being a metalloenzyme, ALAD requires a variety of divalent and monovalent cations for its activity; plant enzyme uses Mg^{2+} ion for the activity [19]. Porphobilinogen deaminase (PBGD; EC 2.5.1.61) catalyzes the condensation of four porphobilinogen (PBG) molecules to form hydroxymethylbilane; the first tetrapyrrole of the pathway [20]. Inhibition of ALAD activity by heavy metals, such as, Cr, Cd, As and Hg has been reported in various plant systems [21-24]. Due to less availability of its substrate ALA, decreased ALAD activity and its gene expression has been documented in rice seedlings under water deficit stress [25]. In addition, the alleviative role of ALA against abiotic stresses has been shown in plants by supplying it exogenously [26]. Studies on combined effects of Cu and water deficit in plants, including maize are very scanty. Hence, the present study is aimed at analyzing the effects of stress induced by Cu, water deficit (induced by PEG 6000) and their combination on biochemical parameters, total chlorophyll content and key enzymes of chlorophyll biosynthesis in maize seedlings with an intention to reveal the mechanistic details.

Materials and Methods

Collection of plant material, growth conditions and treatment

Zea mays L., cv. Ganga safed-2 seeds procured from Ganga Kaveri Seeds Pvt. Ltd., Hyderabad were surface-sterilized with 0.1% $HgCl_2$ for 1-2 min followed by thorough washing with tap water and then with distilled water. Seeds were placed in 20 cm diameter Petri plates (10 seeds/plate) lined with filter paper discs. They were treated with $CuSO_4.5H_2O$ (0, 10 and 100 µM) for inducing metal stress. Water deficit conditions were generated by supplying polyethylene glycol 6000 solutions (0, 5 and 10%). For imposing combined stress (metal + water deficit) combinations used were 5% PEG + 10 µM Cu (WD1+Cu1), 5% PEG + 100 µM Cu (WD1+Cu2), 10% PEG + 10 µM Cu (WD2+Cu1) and 10% PEG + 100 µM Cu (WD2+Cu2). Seeds were allowed to germinate for five days in a light chamber under continuous light of about 40W/m^2 intensity and 25 ± 3ºC temperature. On 5th day, root and shoot tissues were separated from seedlings and analyzed for biochemical parameters. For parameters concerned with chlorophyll biosynthetic pathway, maize seedlings were germinated in complete darkness for 4 days, after that Petri plates were transferred to light for 24 h. Shoot tissues were separated from seedlings and used for analyzing chlorophyll biosynthetic parameters.

Analytical procedures

Cu content in roots and shoots were determined after digesting 500 mg of oven dried and powdered root/shoot samples in concentrated HNO_3. The digested and diluted extract was then filtered through Whatman's no. 41 filter paper and used to estimate the metal content by atomic absorption spectrophotometer. The

translocation factor (TF) i.e. the mobility of a heavy metal from root to the shoot was calculated using the following formula [27]:

$$TF = \frac{\text{Metal content of shoot (µg)}}{\text{Metal content of root (µg)}} \times 100$$

To estimate the total protein content, Lowry's method [28] was used. Treated tissues were boiled and extracted in 5.0 ml 80% ethanol. The extract was centrifuged at 5000 rpm for 10 min. and the pellet obtained was suspended in 5.0 ml of 10% TCA, incubated for 30 min and centrifuged again. Supernatant was discarded and the pellet was dissolved in 5.0 ml NaOH (0.1 N) and incubated for 1 h and centrifuged. The supernatant was then used to estimate protein content using Folin-Ciocalteu's Phenol reagent. For protein profile, root and shoot tissues were homogenized in liquid nitrogen and extracted with Tris-HCl buffer (50 mM, pH 7.4) containing Na-EDTA (10 mM), 5% β-mercaptoethanol and 10% glycerol. The homogenate was centrifuged in a refrigerated centrifuge 'Remi C-24 Plus' first at 5000 rpm for 10 min to remove cell debris and then at 10,000 rpm for 30 min to obtain clear supernatant. The supernatant was used as crude extract to perform SDS PAGE for separation of proteins according to the method of Laemmli [29]. Total RNA content was measured using Orcinol reagent by the method given by Webb and Levy [30] and for the estimation of total DNA content diphenylamine (DPA) reagent [31] was used. RNA and DNA were also extracted in 80% ethanol. Pellet obtained was mixed with 2.0 ml of 1% PCA and centrifuged. The pellet was treated with 5.0 ml of ethanol: diethyl ether: chloroform (2:2:1) mixture. To the resulting pellet residue, 3.0 ml of KOH (0.3 N) was added. The mixture was allowed to stand for about 18 h at 37°C. After incubation, the pH of the medium was adjusted to 2.0 by adding 1 N PCA. The contents were centrifuged and supernatant obtained was used for RNA estimation and pellet for DNA estimation. RNA content was estimated in diluted supernatant using Orcinol reagent. For DNA estimation, 3.0 ml of 5% PCA was added to the pellet, mixed and the mixture was then boiled for 15 min. The supernatant obtained after centrifugation was used to estimate DNA content using DPA reagent.

Chlorophyll and carotenoid contents were estimated using the equation of [32]. Shoot tissues (200 mg) of maize seedlings were homogenized in 3.0 ml 80% acetone, centrifuged, supernatant was diluted with 80% acetone and its absorbance was measured at 646, 663 and 470 nm using spectrophotometer. Chlorophyll 'a', chlorophyll 'b' and carotenoid contents were calculated using the equations:

Chlorophyll a (µg ml^{-1}) = 12.21(A_{663}) - 2.81(A_{646})

Chlorophyll b (µg ml^{-1}) = 20.13(A_{646}) - 5.03(A_{663})

Carotenoids (µg ml^{-1}) = [1000 (A_{470}) - 3.27 (Chlorophyll a) - 104 (Chlorophyll b)]/229

The ALA content was estimated using method of Tewari and Tripathy, 1998 [33]. Shoots (100 mg) were homogenized in cold in 1.0 ml of Na-acetate buffer (1 M, pH 4.6), and centrifuged in a cooling centrifuge for 10 min at 10,000 rpm at 4ºC. The supernatant thus obtained was used to quantitate ALA using modified Ehrlich reagent. For ALA synthesizing activity, treated seedlings were first incubated in phosphate buffer (50 mM, pH 6.0) containing levulinic acid (60 mM) for 4 h in light and then ALA content was measured using modified Ehrlich reagent according to the method of Tewari and Tripathy, 1998 [33].

δ-Aminolevulinic acid dehydratase activity was assayed according to the method of Jain and Gadre, 2004 [22] using modified Ehrlich reagent [34]. Shoot material (500 mg) was homogenized in 2.0 ml of Tris-HCl buffer (50 mM, pH 8.2) containing DTT (10 mM) and the extract obtained was centrifuged at 15,000 rpm at 4ºC in a cooling centrifuge for 30 min. The supernatant thus obtained was used as the source of enzyme. A reaction mixture was prepared by adding 1.35 ml Tris-HCl buffer (50 mM, pH 8.2) containing DTT (10 mM), 0.27 ml of ALA (1 mg/ml) and 0.08 ml of 20 mM $MgCl_2$. One ml of enzyme was added to this to start the reaction. After incubation, the reaction was stopped by adding 0.3 ml of 3 M TCA and enzyme activity was measured after adding modified Ehrlich reagent. Porphobilinogen deaminase activity was determined by the method of [35]. Shoot material (500 mg) was homogenized in cold with 2.0 ml of Tris-HCl buffer (0.2 M, pH 8.2) containing 0.1 M cysteine, 0.1 M $MgCl_2$ and 3 mM *o*-phenanthroline. The homogenate was centrifuged and supernatant obtained was used for the assay of enzyme. For the assay, a reaction mixture was prepared by mixing 0.1 ml PBG (100 µg), 0.5 ml Tris-HCl buffer (50 mM, pH 8.2) containing 2.5 µmol EDTA. Enzyme extract (0.4 ml) was added to this and the reaction mixture was incubated at 32°C for 45 min and the reaction was stopped by adding 0.1 ml of 3 M TCA. The mixture was then centrifuged and supernatant was mixed with 5 M HCl. After 4.5 h of incubation the urogen formed was measured at 406 nm.

Statistical analysis

Data presented in the study are an average of at least 5 replicate experiments and represented as mean ± standard error. Student's't' test was used for mean comparison and to test the significance of mean difference obtained for various treatments. Correlation coefficient (R) value was calculated using Microsoft excel.

Results

Effects of supply of $CuSO_4$ and PEG 6000 on Cu accumulation in maize seedlings

Atomic absorption spectrophotometric analysis was performed to detect the presence of Cu in maize seedlings. Copper content increased significantly in a concentration dependent manner in both root and shoot tissues with the supply of 0-1000 µM $CuSO_4$ (Table 1a). There was approximately twenty fold increase in the metal content of roots and 15 fold increase in shoots at highest Cu level (Table 1a). Highly significant correlation of $R^2 = 0.982$ for root and $R^2 = 0.971$ for shoot was obtained between Cu concentration and Cu accumulation (Figure 1). Translocation factor for Cu was calculated to know the extent of its distribution between roots and shoots. Low value of translocation factor was found with the supply of 50-1000 µM $CuSO_4$; however, a higher value was obtained for 10 µM $CuSO_4$ treated seedlings (Table 1a). When the seeds were germinated in presence of 5 and 10% PEG 6000, copper was detected in both root and shoot tissues (Table 1b). Slightly higher (non significant) Cu content was noted in shoots of PEG treated seedlings. As a result of this, the translocation factor was marginally increased (Table 1b). Further, accumulation of Cu was found significantly higher in roots than shoots in seedlings treated with combinations of PEG and Cu (Table 1c). Lower value of translocation factor was obtained in all the combined treatments (Table 1c).

$CuSO_4$ Conc. (µM)	Root Cu Content (µg g^{-1} Dry Weight)	Shoot Cu Content (µg g^{-1} Dry Weight)	Translocation Factor
0	4.9 ± 0.2 (100)	3.0 ± 0.1 (100)	62 ± 4 (100)
10	9.8 ± 0.8* (200)	6.0 ± 0.4* (200)	62 ± 1^{ns}(100)
50	13.7 ± 0.7** (280)	6.3 ± 0.6* (208)	46 ± 6^{ns} (74)
100	27.0 ± 0.9*** (550)	11.8 ± 0.3*** (391)	44 ± 1^{ns} (71)
300	38.3 ± 0.8*** (781)	16.9 ± 0.2*** (559)	44 ± 1^{ns} (72)
500	50.5 ± 1.5*** (1030)	19.5 ± 0.1*** (646)	39 ± 1^{ns} (63)
1000	98.8 ± 1.0*** (2016)	43.7 ± 0.3*** (1445)	44 ± 1^{ns} (72)

Table 1a: Effect of supply of $CuSO_4$ on Cu accumulation in root and shoot tissues of maize seedlings.

Maize seeds were supplied with 0-1000 µM $CuSO_4$ in continuous light at 25 ± 3ºC for 5 days and Cu content was measured in dried and digested root and shoot tissues.

Values are mean ± SE and relative values are given in parentheses. ns = Non Significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

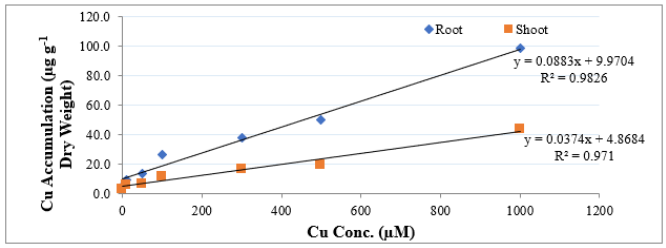


Figure 1: Correlation analysis of $CuSO_4$ concentration and Cu accumulation in root and shoot tissues of maize seedlings.

PEG 6000 Conc. (%)	Root Cu Content (µg g^{-1} Dry Weight)	Shoot Cu Content (µg g^{-1} Dry Weight)	Translocation Factor
0	4.8 ± 0.3 (100)	3.0 ± 0.1 (100)	63 ± 3 (100)
5	5.5 ± 0.1^{ns} (114)	3.6 ± 0.2^{ns} (120)	67 ± 6^{ns} (105)
10	5.2 ± 0.4^{ns} (108)	3.9 ± 0.4^{ns} (130)	75 ± 13^{ns} (119)

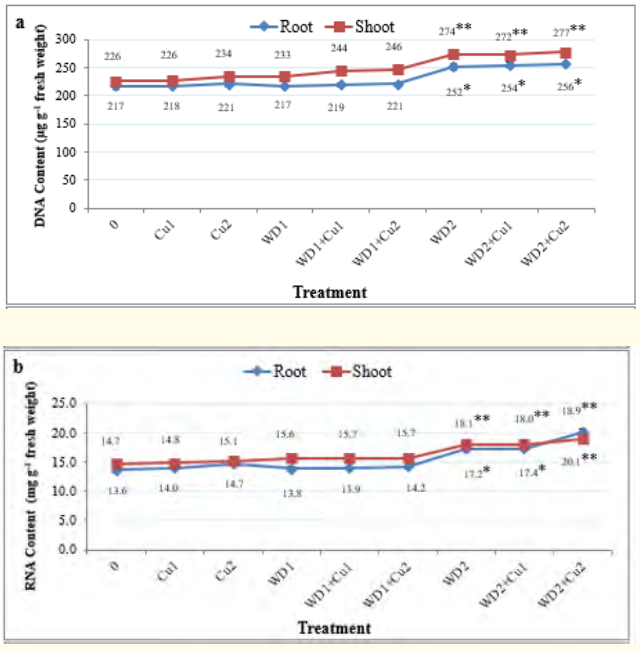
Table 1b: Effect of supply of PEG 6000 on Cu accumulation in root and shoot tissues of maize seedlings.

Maize seeds were supplied with 0-10% PEG 6000 in continuous light at 25 ± 3ºC for 5 days and Cu content was measured in dried and digested root and shoot tissues.

Values are mean ± SE and relative values are given in parentheses. ns = Non Significant.

Effects of $CuSO_4$ and PEG 6000 on DNA, RNA and total protein content in maize seedlings

The DNA content in roots and shoots of maize seedlings was found unchanged with the supply of 10 and 100 µM $CuSO_4$ and 5% PEG 6000; however, marginal increase was noticed by 10% PEG (Figure 2a). Similarly, there was no change in DNA content when 5% PEG was present in combination, while it was slightly increased by 10% PEG and $CuSO_4$ together (Figure 2a). Analogous pattern was obtained for total RNA in roots and shoots with slightly higher content at 10% PEG with 10 µM Cu and substantial and significant with 10% PEG along with 100 µM $CuSO_4$ (Figure 2b). The protein content increased slightly with the supply of 100 µM Cu and its combination with PEG in root tissue (Figure 2c). Prominent increase in protein content was observed with 10% PEG treatment and its combination with 10 and 100 µM $CuSO_4$, being highest in the latter (Figure 2c). There was minor increase (non-significant) in the total protein content of the shoot tissue by all the treatments, except, combined treatments of 10% PEG with $CuSO_4$, where slightly higher content of protein was measured (Figure 2c).



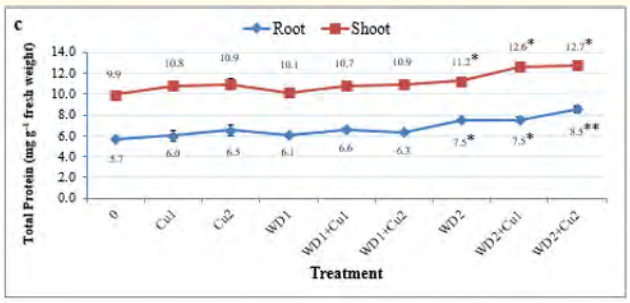


Figure 2: Effect of $CuSO_4$ and PEG 6000 on DNA, RNA and total protein content in root and shoot tissues of maize seedlings. a = DNA content, b = total RNA content and c = total protein content.

Maize seeds were supplied with CuSO4, PEG 6000 and their combinations in continuous light at 25 ± 3ºC for 5 days.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$. Values are mean ± SE and relative values are given in parentheses. ns = non significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Effects of $CuSO_4$ and PEG 6000 on protein profile in maize seedlings

SDS PAGE analysis of proteins in the root of maize seedlings revealed some distinctive changes in protein band patterns on treatment of seeds with $CuSO_4$, PEG 6000 and their combinations (Figure 3a). Increased intensities of bands near about 42, 57 and 65 kDa were seen in root samples, especially with 100 µM $CuSO_4$ treatment. Further, in 10% PEG and its combinations, the intensity of the band of about 57 kDa was higher (Figure 3a). In protein profile of shoot tissue several bands of approximately 15-100 kDa range were observed (Figure 2b). The intensities of the bands of approximately 59, 65, 68, 75 and 100 kDa were slightly higher in seedlings treated with Cu and its combination with PEG. However, among combined treatments of 10% PEG and Cu, the visibility of bands of about 75 and 100 kDa was very clear (Figure 3b). The intensity of 59 kDa band was decreased with individual 5 and 10% PEG treatment (Figure 3b).

Effect of $CuSO_4$ and PEG 6000 on chlorophyll a, chlorophyll b and chlorophyll a/b ratio in shoot tissues of maize seedlings

When dark grown maize seedlings treated with 10 and 100 µM $CuSO_4$ were transferred in light and kept for 24 h, chlorophyll 'a'

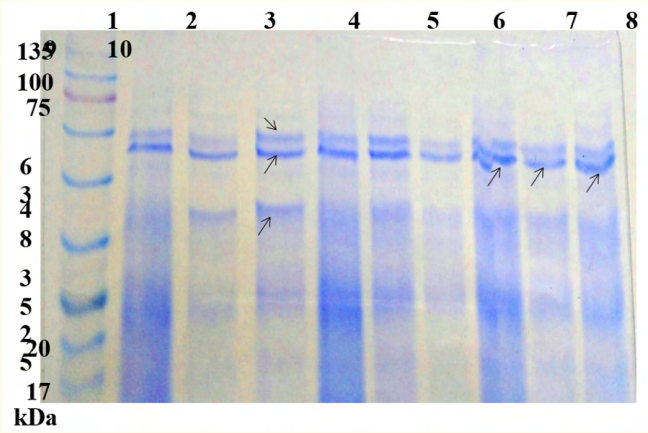


Figure 3a: Effect of $CuSO_4$ and PEG 6000 on protein profile in root tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in continuous light at 25 ± 3ºC for 5 days.

Lane 1 = Marker; Lane 2 = Control; Lane 3 = Cu1; Lane 4 = Cu2; Lane 5 = WD1; Lane 6 = WD1+Cu1; Lane 7 = WD1+Cu2; Lane 8 = WD2; Lane 9 = WD2+Cu1 and Lane 10 = WD2+Cu2.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM CuSO4 and Cu2 = 100 µM $CuSO_4$.

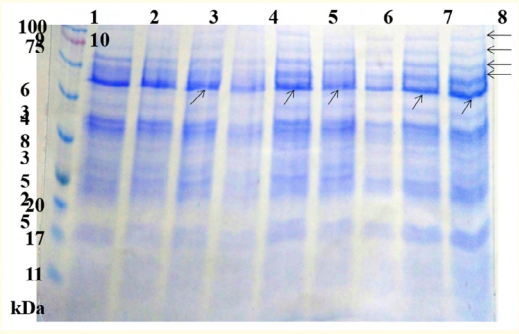


Figure 3b: Effect of $CuSO_4$ and PEG 6000 on protein profile in shoot tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in continuous light at 25 ± 3ºC for 5 days.

Lane 1 = Marker; Lane 2 = Control; Lane 3 = Cu1; Lane 4 = Cu2; Lane 5 = WD1; Lane 6 = WD1+Cu1; Lane 7 = WD1+Cu2; Lane 8 = WD2; Lane 9 = WD2+Cu1 and Lane 10 = WD2+Cu2.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$.

content of shoots remained unchanged; however, it decreased with 5 and 10% PEG and their combinations (Table 2a). Chlorophyll 'b' content was found unaffected by metal as well as water deficit stress. Chlorophyll a/b ratio was decreased with PEG treatment and its combination with $CuSO_4$ (Table 2a).

Treatment	Chlorophyll a (µg g^{-1} fresh weight)	Chlorophyll b (µg g^{-1} fresh weight)	Chlorophyll a/b Ratio
Control	149 ± 4 (100)	61 ± 1 (100)	2.4 ± 0.1 (100)
Cu1	151 ± 4^{ns} (101)	59 ± 1^{ns} (97)	2.6 ± 0.1^{ns} (105)
Cu2	151 ± 2^{ns} (101)	59 ± 1^{ns} (97)	2.5 ± 0.1^{ns} (104)
WD1	132 ± 3* (88)	59 ± 1^{ns} (98)	2.2 ± 0.1^{ns} (91)
WD1+Cu1	131 ± 2* (88)	60 ± 1^{ns} (99)	2.2 ± 0.1^{ns} (88)
WD1+Cu2	130 ± 2* (87)	60 ± 1^{ns} (98)	2.2 ± 0.1^{ns} (89)
WD2	125 ± 1* (84)	59 ± 1^{ns} (96)	2.1 ± 0.1^{ns} (87)
WD2+Cu1	125 ± 2* (84)	58 ± 1^{ns} (95)	2.2 ± 0.1* (88)
WD2+Cu2	122 ± 3** (82)	56 ± 1^{ns} (92)	2.2 ± 0.1* (89)

Table 2a: Effect of $CuSO_4$ and PEG 6000 on chlorophyll a, chlorophyll b and chlorophyll a/b ratio in shoot tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in complete darkness for a period of 4 days, after that Petri plates were transferred in continuous light at 25 ± 3ºC for 24 h.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$. Values are mean ± SE and relative values are given in parentheses. ns = non significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Effect of CuSO4 and PEG 6000 on total chlorophylls and carotenoids in shoot tissues of maize seedlings

There was no change in total chlorophylls and carotenoid contents in shoots by $CuSO_4$ supply, however, the pigment contents were significantly reduced by PEG 6000 and its combination with $CuSO_4$ (Table 2b). The decrease in carotenoids was found to a higher extent than the decrease in total chlorophylls (Table 2b).

Treatment	Total Chlorophylls (µg g^{-1} fresh weight)	Carotenoids (µg g^{-1} fresh weight)
Control	210 ± 4 (100)	24.7 ± 0.9 (100)

Cu1	210 ± 3^{ns} (100)	25.4 ± 1.2^{ns} (103)
Cu2	210 ± 2^{ns} (100)	24.6 ± 0.8^{ns} (100)
WD1	191 ± 3* (91)	19.3 ± 0.3* (78)
WD1+Cu1	191 ± 2* (91)	19.7 ± 0.4* (80)
WD1+Cu2	190 ± 2* (91)	18.7 ± 0.6** (76)
WD2	184 ± 2* (88)	18.2 ± 0.2* (74)
WD2+Cu1	183 ± 2* (87)	16.0 ± 0.3* (64)
WD2+Cu2	178 ± 2** (85)	15.8 ± 0.4** (64)

Table 2b: Effect of $CuSO_4$ and PEG 6000 on total chlorophylls and carotenoids in shoot tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in complete darkness for a period of 4 days, after that Petri plates were transferred in continuous light at 25 ± 3ºC for 24 h.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$. Values are mean ± SE and relative values are given in parentheses. ns = non significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Effect of $CuSO_4$ and PEG 6000 on ALA content and ALA synthesizing activity in shoot tissues of maize seedlings

When ALA content was measured in shoots, significant decrease was obtained with 10% PEG treatment and its combination with $CuSO_4$ (Table 3). Treatment of maize seeds with 10 and 100 µM $CuSO_4$ did not affect the ALA synthesizing activity in shoot tissues under both light and dark conditions (Table 3). However, supply of PEG decreased the activity significantly almost to the same extent in absence as well as presence of Cu under both light and dark conditions (Table 3).

Treatment	ALA Content (nmole g^{-1} fresh weight)	ALA Synthesizing Activity (nmole ALA formed h^{-1} g^{-1} fresh weight)	
		Light	Dark
Control	311 ± 4 (100)	72 ± 1 (100)	75 ± 1 (100)
Cu1	318 ± 4^{ns} (103)	71 ± 1^{ns} (98)	72 ± 1^{ns} (97)
Cu2	303 ± 6^{ns} (98)	69 ± 1^{ns} (96)	71 ± 2^{ns} (95)
WD1	301 ± 6^{ns} (97)	65 ± 1** (91)	67 ± 2** (89)
WD1+Cu1	304 ± 6^{ns} (98)	63 ± 1*** (88)	65 ± 1*** (86)
WD1+Cu2	309 ± 4^{ns} (100)	56 ± 1*** (77)	61 ± 2*** (82)
WD2	258 ± 6* (83)	51 ± 1*** (71)	60 ± 1*** (80)
WD2+Cu1	250 ± 5* (80)	51 ± 1*** (71)	59 ± 2*** (78)
WD2+Cu2	258 ± 7* (83)	50 ± 1*** (70)	58 ± 1*** (77)

Table 3: Effect of $CuSO_4$ and PEG 6000 on ALA content and ALA synthesizing activity in shoot tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in complete darkness for a period of 4 days, after that Petri plates were transferred in continuous light at 25 ± 3ºC for 24 h.

For ALA synthesizing activity, light treated seedlings were incubated in phosphate buffer (50 mM, pH 6.0) containing 60 mM levulinic acid at 25 ± 3ºC for 4 h in light and dark.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$. Values are mean ± SE and relative values are given in parentheses. ns = non significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Effect of $CuSO_4$ and PEG 6000 on ALAD and PBGD activities in shoot tissues of maize seedlings

Inclusion of $CuSO_4$ during germination of maize seeds did not influence ALAD and PBGD activities in shoot tissues (Table 4). Water deficit stress induced by PEG supply inhibited the enzyme activities slightly in absence and presence of Cu (Table 4). Further, the inhibition of PBGD activity was comparatively higher by 10% PEG and its combination with Cu (Table 4).

Treatment	ALAD Activity (nmole PBG formed h^{-1} g^{-1} fresh weight)	PBGD Activity (nmole urogen formed h^{-1} g^{-1} fresh weight)
Control	92 ± 2 (100)	3.3 ± 0.02 (100)
Cu1	91 ± 2^{ns} (99)	3.3 ± 0.01^{ns} (100)
Cu2	90 ± 2^{ns} (97)	3.2 ± 0.02^{ns} (98)
WD1	87 ± 2^{ns} (94)	3.1 ± 0.01** (94)

WD1+Cu1	85 ± 3^{ns} (92)	2.9 ± 0.01** (90)
WD1+Cu2	87 ± 2^{ns} (94)	3.0 ± 0.01** (91)
WD2	83 ± 4^{ns} (90)	2.7 ± 0.01*** (81)
WD2+Cu1	82 ± 2^{ns} (89)	2.5 ± 0.02*** (77)
WD2+Cu2	79 ± 3^{ns} (86)	2.6 ± 0.02*** (79)

Table 4: Effect of $CuSO_4$ and PEG 6000 on ALAD and PBGD activities in shoot tissues of maize seedlings.

Maize seeds were supplied with $CuSO_4$, PEG 6000 and their combinations in complete darkness for a period of 4 days, after that Petri plates were transferred in continuous light at 25 ± 3ºC for 24 h.

WD1 = 5% PEG, WD2 = 10% PEG, Cu1 = 10 µM $CuSO_4$ and Cu2 = 100 µM $CuSO_4$. Values are mean ± SE and relative values are given in parentheses. ns = non significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Discussion and Conclusion

Increased Cu level in the medium resulted in its higher uptake and accumulation in plant organs [36]. Roots have a potential ability to accumulate Cu without being overly sensitive towards Cu toxicity [36]. Accumulation of Cu in roots, shoots and leaves of maize seedlings with 10^{-3} and 10^{-4} M $CuSO_4$ has been reported; however, with 10^{-2} M, no accumulation of Cu was obtained in shoot and leaves [37]. Higher accumulation of Cu in root tissues than in shoots of Ganga safed-2 maize variety is in accordance with these findings (Table 1a). Similar to my results, more accumulation of Cu in root than in shoot was observed in maize hybrid 2B688Hx [38]. Further, higher content of Cu in root than in shoot among combined treatments might be due to its less translocation to shoot under water insufficiency (Table 1c).

Proteins are the macromolecules that function in cell signaling, catalysis, movement of nutrients, structural support, regulation and defense mechanisms as well as metal ion chelation and repair or removal of metal stress damaged proteins [39-41]. Increased protein content in root tissues of seedlings treated with 100 µM $CuSO_4$ and its combination with PEG in this study may involve synthesis of new proteins under the stress conditions (Figure 2a). These proteins may include cysteine-rich oligomers i.e. phytochelatins or metallothioneins, heat-shock proteins, proteins involved in metabolism of glutathione and antioxidative enzymes [42-45]. Genes that are responsible for inducing production of these proteins has also been identified. SDS PAGE analysis of root tissues of metal treated seedlings reveal increased intensity of bands with molecular weights approx. 42, 57 and 65 kDa at 100 µM $CuSO_4$ (Figure 3a). Zhang., *et al.* [46] reported increased total protein content in germinating rice embryos when treated with 50-200 µM $CuSO_4$. Their results of SDS PAGE analysis identified smaller molecular weight proteins (5-25 kDa) that were Cu-responsive. They also found increased intensities in pattern of about 12 kDa and 20 kDa proteins with increasing Cu concentrations.

Increase in total soluble protein content up to -0.15 MPa water potential in roots and up to -0.49 MPa in leaves of 704 and 301 cultivar of maize has been reported by [47]. However, in their study, reduced protein content in these tissues of both the varieties was observed at -1.03 and -1.76 MPa water potentials. The results of SDS PAGE analysis revealed the accumulation of dehydrin-like proteins of 38, 50, 57, and 65 kDa M.W. in roots and 15, 17, 20, 27, 30, 37, 54, and 59 kDa M.W. in leaves at -1.76 MPa water potential. Dehydrin proteins possibly protect cells against additional dehydration stress during water deficit. Up-regulation of other stress related proteins, such as, heat shock proteins, ascorbate peroxidase, cysteine proteinase inhibitor, glutathione transferase, peroxidase and serine/threonine-protein phosphatase has also been reported under low water conditions [48]. Genes encoding water deficit stress related proteins, such as, dehydrin and small heat shock proteins as well as modification in their expression level has also been extensively studied [49,50]. Further, the observed increase in protein content seems to be interrelated with RNA and DNA contents (Figure 2a, 2b and 2c).

Chlorophyll biosynthesis is a vital and highly coordinated metabolic process of plant's life [18]. When dark grown maize seedlings treated with 10 and 100 µM $CuSO_4$ were transferred to light, the chlorophyll content and enzyme activities involved in chlorophyll biosynthesis remained unaltered; this may be because of balance maintained between formation and utilization of chlorophyll biosynthetic precursors in presence of essential metal Cu. Increase in chlorophyll 'a', chlorophyll 'b' and total chlorophyll content has been reported in leaves of *Brassica juncea* seedlings by 10 to 100 µmol $CuSO_4$ concentrations [51]. Significant decrease in chlorophyll content by >300 µM $CuSO_4$ treatment has been shown in the leaves of *Camellia sinensis* [52]. Decrease in chlorophyll 'a',

total chlorophylls and caroteniods in shoots have been observed by the supply of 5 and 10% PEG as well as by combined stress of PEG and $CuSO_4$ could be a result of water deficit stress (Table 2a and 2b). Further, chlorophyll 'a' was found to be more affected than chlorophyll 'b' (Table 2a). Similar to this, Meher., *et al*. [53] reported that chlorophyll 'a' is more sensitive to PEG induced water deficit stress than chlorophyll 'b' in peanut leaves (*Arachis hypogaea*). However, in wheat, chlorophyll 'b' has been found to be more prone to drought stress than chlorophyll 'a' [54]. Decreased chlorophyll 'a', total chlorophylls and total carotenoids under mild water deficit condition have been reported earlier in rice leaves [55]. Carotenoids are non enzymatic components involved in scavenging ROS, especially, singlet O_2 [56]. They are found associated with photosynthetic reaction centers and water deficit induced damage of these reaction centers cause strong loss of carotenoid content [57]. Additionally, water deficit induced ROS, such as, $^1O_2$ and H_2O_2 causes lipid peroxidation and therefore chlorophyll destruction [53]. These ROS also causes chloroplast damage that ultimately results in decrease in chlorophyll content under water deficit [58]. In the present work, the decline in caroteniods under water deficit condition is most prominent (Table 2b). Thus, it seems that the protective role offered by caroteniods against free radicals/photo inhibition is not operating well under water deficit stress and hence, inhibiting chlorophyll biosynthesis/promoting chlorophyll degradation.

ALA formation is a rate limiting step in tetrapyrrole biosynthesis, including synthesis of chlorophyll. In this study, decreased ALA content observed by 10% PEG might be due to limited availability of precursors for ALA synthesis (Table 3). The correlation between total chlorophylls and ALA content was found strong with correlation coefficient value of R = 0.829. Decline in ALA content due to less availability of substrate GSA and inactivation of the enzyme GSAT involved in ALA biosynthesis under water deficit conditions in rice seedlings has been demonstrated [25]. Decreased ALA synthesizing activity (almost to same extent) in both light and dark conditions reflect the sensitivity of both chloroplastic and mitochondrial ALA synthesis towards water deficit stress (Table 3). This assumption is further supported by strong correlation of total chlorophylls with ALA synthesizing activity in both light (R = 0.893) and dark (R = 0.987) with PEG treatments. Furthermore, activity of enzyme PBGD is significantly decreased by 5% ($p < 0.01$) as well as 10% ($p < 0.001$) PEG treatment (Table 4), this enzyme inhibition may contribute in decreasing chlorophyll content. Correlation coefficient value of R = 0.914 for PBGD with total chlorophylls supports these assumption. Decreased chlorophyll content and intermediates of chlorophyll biosynthetic pathway, such as, GSA, ALA and protochlorophyllide has been reported in *Oryza sativa* under water deficit stress conditions [25]. Authors observed down regulation in gene expression for ALA synthesizing activity, ALAD, PBGD, coproporphyrinogen III oxidase, porphyrinogen IX oxidase, Mg-chelatase and protochlorophyllide oxidoreductase activities. They suggested that under water deficit, decreased synthesis of early intermediates of chlorophyll biosynthesis, such as, ALA and GSA modulate the gene expression of the latter enzymes of the pathway and hence decreased chlorophyll pigment.

The results of the present study suggest synthesis of new proteins in root and shoot tissues of maize seedlings under abiotic stress conditions induced by the supply of $CuSO_4$, PEG 6000 and their combinations. It seems that increased protein content is interrelated with RNA and DNA contents. Further, pattern reveals cumulative effect of both the stresses in root tissues; while, it was entirely of PEG in shoots. Inhibition of ALA synthesizing and PBGD activities as well as decrease in ALA content by PEG indicates the inhibition of early steps of chlorophyll biosynthetic pathway to be responsible for decreasing the chlorophyll content. Furthermore, decrease in caroteniod content, may also contribute in inhibiting chlorophyll biosynthesis/ promoting chlorophyll degradation as the protective role offered by them against free radicals/ photo inhibition gets abolished under water deficit stress.

Statements and Declarations

Competing Interest- The authors declare that they have no conflicts of interest to report regarding the present study.

Authors' Contribution

MJ conceived and designed the experiments. MH carried out the experiments, analyzed data and wrote the manuscript with inputs from MJ.

Bibliography

1. Ali H., *et al*. "Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation". *Journal of Chemistry* (2019): 6730305.

2. Rodriguez FI., *et al*. "A copper cofactor for the ethylene receptor ETR1 from Arabidopsis". *Science* 283 (1999): 996-998.

3. Arao T., *et al*. "Heavy metal contamination of agricultural soil and countermeasures in Japan". *Paddy and Water Environment* 8 (2010): 247-257.

4. Houhou J., *et al*. "Study of copper phytotoxicity on maize plants (*Zea mays* L.) irrigated by water treated with copper sulfate as algaecide". *International Journal of Science and Research* 5 (2016): 1680-1685.

5. Cobbett C and Goldsbrough P. "Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeo-stasis". *Annual Review of Plant Biology* 53 (2002): 159-182.

6. Demirevska-Kepova K., *et al*. "Biochemical changes in barely plants after excessive supply of copper and manganese". *Environmental and Experimental Botany* 52 (2004): 253-266.

7. Maksymiec W and Krupa Z. "Effects of methyl jasmonate and excess copper on root and leaf growth". *Biologia Plantarum* 51 (2007): 322-326.

8. Gupta D and Abdullah. "Toxicity of copper and cadmium on germination and seedling growth of maize (*Zea mays* L.) Seeds". *Indian Journal of Scientific Research* 2 (2011): 67-70.

9. Contreras R A., *et al*. "Copper stress induces antioxidant responses and accumulation of sugars and phytochelatins in Antarctic *Colobanthus quitensis* (Kunth) Bartl". *Biological Research* 51 (2018): 48.

10. Soltani A., *et al*. "Seed reserve utilization and seedling growth of wheat as affected by drought and salinity". *Environmental and Experimental Botany* 55 (2006): 195-200.

11. Bhatt R M., *et al*. "Influence of pod load on response of okra to water stress". *Indian Journal of Plant Physiology* 10 (2005): 54-59.

12. Huang B., *et al*. "Exogenous melatonin alleviates oxidative damages and protects photosystem II in maize seedlings under drought stress". *Frontiers in Plant Science* 10 (2019): 677.

13. Swapna B and Rama Gopal G. "Interactive effects between water stress and heavy metals on seed germination and seedling growth of two green gram (*Vigna radiata* L. Wilzec.) cultivars". *Biolife* 2 (2014): 291-296.

14. De Silva N D G., *et al*. "Effects of combined drought and heavy metal stresses on xylem structure and hydraulic conductivity in red maple (*Acer rubrum* L.)". *Journal of Experimental Botany* 63 (2012): 5957-5966.

15. Ku H M., *et al*. "The effect of water deficit and excess copper on proline metabolism in Nicotiana benthamiana". *Biologia Plantarum* 56 (2012): 337-343.

16. Rucinska-Sobkowiak R. "Water relations in plants subjected to heavy metal stresses". *Acta Physiologiae Plantantarum* 38 (2016): 257.

17. Brzezowski P., *et al*. "Regulation and function of tetrapyrrole biosynthesis in plants and algae". *Biochimica et Biophysica Acta (BBA)- Bioenergetics* 1847 (2015): 968-985.

18. Beale SI. "Enzymes of chlorophyll biosynthesis". *Photosynthesis Research* 60 (1999): 43-73.

19. Jaffe EK. "The porphobilinogen synthase family of metalloenzymes". *Acta Crystallographica Section D* 56 (2000): 115-128.

20. Beale SI and Weinstein JD. "Tetrapyrrole metabolism in photosynthetic organisms". In: Dailey (ed.), Biosynthesis of heme and chlorophylls. McGraw-Hill, New York (1990): 287-391.

21. Vajpayee P., *et al*. "Chromium (VI) accumulation reduces chlorophyll biosynthesis, nitrate reductase activity and protein content in *Nymphaea alba* L". *Chemosphere* 41 (2000): 1075-1082.

22. Jain M., *et al*. "Inhibition of 5-aminolevulinic acid dehydratase activity by arsenic in excised etiolated maize leaf segments during greening". *Journal of Plant Physiology* 161 (2004): 251-255.

23. Sarangthem J., *et al*. "Inhibition of δ-aminolevulinic acid dehydratase activity by cadmium in excised etiolated maize leaf segments during greening". *Plant Soil and Environment* 57 (2011): 332-337.

24. Gupta P., *et al*. "Inhibition of 5- aminolevulinic acid dehydratase by mercury in excised greening maize leaf segments". *Plant Physiology and Biochemistry* 62 (2013): 63-69.

25. Dalal V K and Tripathy BC. "Modulation of chlorophyll biosynthesis by water stress in rice seedlings during chloroplast biogenesis". *Plant Cell and Environment* 35 (2012): 1685-1703.

26. Wu Y., *et al*. "5-Aminolevulinic acid (ALA) biosynthetic and metabolic pathways and its role in higher plants: a review". *Plant Growth Regulation* 87 (2019): 357-374.

27. Marchiol L., *et al*. "Phytoextraction of heavy metals by canola (*Brassica napus*) and radish (*Raphanus sativus*)". *Environmental Pollution* 132 (2004): 21-27.

28. Lowry O H., *et al*. "Protein measurement with the Folin-Phenol reagent". *Journal of Biological Chemistry* 193 (1951): 265-275.

29. Laemmli UK. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature* 227 (1970): 680-685.

30. Webb JM and Levy H B. "New developments in the chemical determination of nucleic acids". In: Glick D (ed.), Methods of biochemical analysis, InterScience, New York, 6 (1958): 1-30.

31. Gendimenico GJ., *et al*. "Diphenylamine-colorimetric method for DNA assay: a shortened procedure by incubating samples at 50ºC". *Analytical Biochemistry* 173 (1988): 45-48.

32. Lichtenthaler H K and Welburn A R. "Determination of total carotenoids and chlorophylls a and b of extracts in different solvents". *Biochemical Society Transactions* 11 (1983): 591-592.

33. Tewari A K and Tripathy BC. "Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat". *Plant Physiology* 117 (1998): 851-858.

34. Mauzerall D and Granik S. "The occurrence and determination of δ-aminoleveulinic acid dehydratase and porophobilinogen in urine". *Journal of Biological Chemistry* 219 (1956): 435-446.

35. Prasad DDK and Prasad ARK. "Porphyrin metabolism in lead and mercury treated bajra (*Pennisetum typhoideum*) seedlings". *Journal of Biosciences* 15 (1990): 271-279.

36. Manivasagaperumal R., *et al*. "Effect of copper on growth, dry matter yield and nutrient content of *Vigna radiata* (L.) Wilczek". *Journal of Phytology* 3 (2011): 53-62.

37. Benimeli CS., *et al*. "Bioaccumulation of copper by Zea mays: Impact on root, shoot and leaf growth". *Water, Air, and Soil Pollution* 210 (2010): 365-370.

38. Dias MAN., *et al*. "Uptake of seed-applied copper by maize and the effects on seed vigor". *Bragantia Campinas* 74 (2015): 241-246.

39. Ahsan N., *et al*. "Excess copper induced physiological and proteomic changes in germinating rice seeds". *Chemosphere* 67 (2007): 1182-1193.

40. Amm I., *et al*. "Protein quality control and elimination of protein waste: The role of the ubiquitin-proteasome system". *Biochimica et Biophysica Acta (BBA)- Molecular Cell Research* 1843 (2014): 182-196.

41. Hasan MK., *et al*. "Responses of plant proteins to heavy metal stress- A review". F*rontiers in Plant Science* 8 (2017): 1492.

42. Clemens S. "Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants". *Biochimie* 88 (2006): 1707-1719.

43. Emamverdian A., *et al*. "Heavy metal stress and some mechanisms of plant defense responses". *The Scientific World Journal* 756120 (2015): 18.

44. Tuna A L., *et al*. "Maize (*Zea mays* L.) plant responses to excess copper, cadmium, cobalt, lead and chromium". *Fresenius Environmental Bulletin* 24 (2015): 3996-4006.

45. Pochodylo A and Aristilde L. "Molecular dynamics of stability and structures in phytochelatin complexes with Zn, Cu, Fe, Mg, and Ca: Implications for metal detoxification". *Environmental Chemistry Letters* 15 (2017): 495-500.

46. Zhang H., *et al*. "Proteomic identification of small, copper-responsive proteins in germinating embryos of Oryza sativa". *Annals of Botany* 103 (2009): 923-930.

47. Mohammadkhani N and Heidari R. "Effects of drought stress on soluble proteins in two maize varieties". *Turkish Journal of Biology* 32 (2008): 23-30.

48. Kim S G., *et al*. "Physiological and proteomic analyses of Korean F1 maize (*Zea mays* L.) hybrids under water-deficit stress during flowering". *The Korean Society for Applied Biological Chemistry* 62 (2019): 32.

49. Godehkahriz SJ., *et al*. "Effects of water deficit on the physiological response, total protein, and gene expression of Rab17 in wheat (*Triticum aestivum*)". *Journal of Plant Process and Function* 5 (2017): 35-42.

50. Wang B., *et al*. "Effects of maize organ-specific drought stress response on yields from transcriptome analysis". *BMC Plant Biology* 19 (2019): 335.

51. Pantola RC., *et al*. "Copper scavenging potential and its effect on chlorophyll in seedlings of *Brassica Juncea* (L.) Czern". *Advanced Research Journal of Plant and Animal Sciences* 1 (2013): 014-017.

52. Dey S., *et al*. "Effect of copper on growth and chlorophyll content in tea plants (*Camellia sinensis* (L.) O Kuntze". *International Journal of Research in Applied, Natural and Social Sciences* 2 (2014): 223-230.

53. Meher Shivakrishna P., *et al*. "Effect of PEG-6000 imposed drought stress on RNA content, relative water content (RWC) and chlorophyll content in peanut leaves and roots". *Saudi Journal of Biological Sciences* 25 (2018): 285-289.

54. Shamsi K. "The effects of drought stress on yield, relative water content, proline, soluble carbohydrates and chlorophyll of bread wheat cultivars". *Journal of Animal and Plant Sciences* 8 (2010): 1051-1060.

55. Cha-um S., *et al*. "Glycinebetaine alleviates water deficit stress in indica rice using proline accumulation, photosynthetic efficiencies, growth performances and yield attributes". *Australian Journal of Crop Science* 7 (2013): 213-218.

56. Chandrasekar V., *et al*. "Physiological and biochemical responses of hexaploid and tetraploid wheat to drought stress". *Journal of Agronomy and Crop Science* 185 (2000): 219-227.

57. Efeoglu B., *et al*. "Physiological responses of three maize cultivars to drought stress and recovery". *South African Journal of Botany* 75 (2009): 34-42.

58. Smirnoff N. "Antioxidant systems and plant response to the environment." In: Environment and plant metabolism: flexibility and acclimation, N Smirnoff (ed.). Bios Scientific Publishers, Oxford, UK (1995): 217-243.