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ORIGINAL PAPER

MITIGATION OF NEGATIVE IMPACTS OF CADMIUM STRESS ON PHYSIOLOGICAL PARAMETERS OF CURLY LETTUCE (*LACTUCA SATIVA* VAR. *CRISPA*) BY PROLINE TREATMENTS*

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Abstract

This study was conducted on a variety of lettuce curly (*Lactuca sativa* var. *Crispa*) called Caipira. The purpose was to elucidate the effects of cadmium stress on the plant's metabolic processes, response to cadmium stress and adaptation mechanisms developed against cadmium stress with the use of proline treatments. Hydroponic experiments were conducted in a climate chamber supplied with a controlled atmosphere (15±2°C 13 h dark, 22±2°C 11 h light and 70% relative humidity). When the seedlings formed new roots and had 4-5 leaves, initially 5 mM, 10 mM, 15 mM proline solutions were sprayed on the leaves of a group of seedlings 4 days before applying cadmium stress, then the plants were exposed to 40 ppm cadmium. The other group was sprayed with 5 mM, 10 mM, 15 mM proline solutions just before applying cadmium stress. Yet another group of plants was subjected only to cadmium stress without proline treatments. Therefore, 8 different treatments were tested: Control (0 ppm cadmium), only cadmium (40 ppm), proline (5 mM, 10 mM, 15 mM) 4 days before the stress + cadmium, proline (5 mM, 10 mM, 15 mM) + cadmium stress together. The present findings revealed that 5 mM proline dose was relatively effective in mitigation of negative effects of Cd stress on plant physiological and metabolic processes. Proline treatments at this dose were found to be effective in alleviating the negative effects of Cd stress on growth parameters of curly lettuce plants. It was also concluded that such effects of external proline treatments varied with the treatment dose and timing.

Keywords: antioxidant enzyme, *Lactuca sativa* var. *Crispa*, heavy metal, malondialdehyde, proline.

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INTRODUCTION

Cadmium (Cd) is a heavy metal with various negative effects on biological processes. Heavy vehicle traffic may increase the cadmium content of agricultural soils close to roadsides. Cd found in pesticides and fungicides is incorporated into soils through spraying (Kacar, Inal 2008). Cd is mostly transferred from industrial processes and phosphorus fertilizers into the food chain (Sandalio et al. 2001). Cd has strong phytotoxic effects and it may result in serious disruption of many physiological and metabolic functions (Shao et al. 2007). Cd toxicity recesses leaf growth, accelerates leaf ageing, and excessively increases peroxidase (RNA, DNA) activity (Barcelo, Poschenrieder 1990). Therefore, negative effects of heavy metals on plants are highly related to the growth stage in which plants were exposed to cadmium. Cd toxicity damages the photosynthesis mechanism by inhibiting chlorophyll biosynthesis, rubisco activity in the Calvin cycle and photosynthetic enzyme activity (Tıryakıoglu et al. 2006, Uzal, Yasar 2017).

An increase in the internal proline level is a vital physiological response of plants against the harmful effects of environmental stresses. Previous studies reported positive correlations of proline accumulation with environmental stresses and stress tolerance of the plants. Accumulation of soluble substances is a general protective mechanism under several abiotic stress conditions, such as salinity, drought, cold, heat and heavy metals (Hossaın et al. 2014, Yaısh 2015). Thus, Bıan et al. (1988) indicated that proline was a water-soluble amino acid generally released under stress conditions and providing plant resistance against stress conditions, hence it could serve as an indicator of a stressor.

Abiotic stresses create reactive oxygen species (ROS) in cells. ROS cause oxidation in the DNA, lipids and proteins. Among various types of osmolytes, proline has been reported as the only molecule that protects cells from oxidative damage through scavenging OH-like reactive oxygen species. It was indicated that proline acted as a non-enzymatic antioxidant in plant cells under stress conditions. Matysık et al. (2002) indicated that proline had quite an effective role in scavenging reactive oxygen species and played a vital role in increasing antioxidant levels under abiotic stress conditions. Alıa et al. (2001) stated that proline scavenged ROS through converting then into single oxygen ($^1O_2$) superoxide anion.

This study was planned in the light of the information given above on a potential role of proline in alleviating effects of abiotic stress factors. Curly lettuce, which can be grown in open fields and undercover, is adversely affected by cadmium stress. Cadmium stress results in serious yield losses. Different treatments should be experimented with to improve cadmium tolerance and thus to minimize such yield losses in curly leaf lettuce. Such treatments should eliminate or diminish the cadmium stress and negative impacts. In this sense, initially the stress mechanism of curly lettuce should

be clarified or well-comprehended. Therefore, this study was encouraged by the information cited above on a potential role of proline in mollifying negative impacts of abiotic stress factors. This study was conducted primarily to elucidate the effects of cadmium stress on the plant's metabolic processes, its response to cadmium stress and the adaptation mechanisms developed against cadmium stress with the use of proline.

MATERIAL AND METHOD

A variety of curly lettuce (*Lactuca sativa* var. *Crispa*) called Caipira was used as the plant material of the present study. Hydroponic experiments were conducted in a climate chamber supplied with a controlled atmosphere (15±2°C 13 h dark, 22±2°C 11 h light and 70% relative humidity). Curly lettuce seedlings of the Caipira variety with 2-3 leaves were grown for about a week using 7-liter tubs each and 3 cm thick styrofoam plates to keep the plants afloat. To place the seedlings on styrofoam plates, 4 cm holes were opened at 18-cm inter-row spacing and 14-cm in-row plant spacing. The seedlings were placed inside these holes and immobilized with cotton. Styrofoam was placed on top of the tubs so as to have the plant roots immersed in the nutrient solution. Aeration was ensured by immersing thin plastic hoses connected to an aquarium pump into the nutrient solution.

The experiments were conducted in a split-plot design with 3 replications. Each plot had 6 plants, thus each treatment included 18 plants. The nutrient solution in the growing medium was kept at 4 liters. Chemical compounds and their quantities used in the nutrient solution are given in Tables 1 and 2. The solution's pH (Hoagland, Arnon 1938) was adjusted to 5.8.

Table 1

Chemical compounds used in the nutrient solution

Macro-elements	Micro-elements
$Ca(NO_3)_2 \cdot 4H_2O$ KNO_3 KH_2PO_4 $MgSO_4$	$C_6H_5FeO_7 \cdot 4H_2O$ $MnCl_2$ H_3BO_3 $ZnCl_2$ $CuCl_2 \cdot 2H_2O$

Proline solutions were prepared at 5, 10 and 15 mM concentrations and Twin 20 was dropped into the solutions. The prepared solutions were covered with aluminum foil to avoid light and were kept in a fridge. When the seedlings formed new roots and had 4-5 leaves, initially 5 mM, 10 mM, 15 mM proline solutions were sprayed on the leaves of a group of seedlings 4 days before applying cadmium stress, then the plants were exposed to 40 ppm cadmium stress. The other groups were sprayed with 5 mM, 10 mM, 15 mM proline solutions just before applying cadmium stress. Yet another group

Table 2

Content of elements in the nutrient solution (ppm)

Elements	ppm
Nitrogen (N)	176
Phosphorus (P)	31
Potassium (K)	146
Magnesium (Mg)	24
Calcium (Ca)	200
Sulfur(S)	34
Iron (Fe)	3.3
Manganese (Mn)	0.050
Boron (B)	0.457
Copper (Cu)	0.015
Zinc (Zn)	0.055

of plants was subjected only to cadmium stress without proline treatments. Thus, 8 different treatments were tested: Control, only cadmium (40 ppm), proline (5 mM, 10 mM, 15 mM) 4 days before the stress + cadmium, proline (5 mM, 10 mM, 15 mM) + cadmium together.

At the end of the experiments, biochemical analyses were conducted to determine total chlorophyll content, lipid peroxidation, proline content, total soluble protein content and SOD, CAT, GR antioxidant enzyme activity.

Experimental data were subjected to analysis of variance with the use of Statgraphics statistical analysis software. Significant means were compared with the use of the Duncan's test at a 5% significance level.

Determination of root and crown weight

The root and crown weight was determined on 1/10.000 precision digital scales in 4 repetitions.

Chlorophyll analysis

Leaf segments, either fresh or frozen at -40°C, were placed in 5 ml of 80% ethanol and heated in a water bath at 80°C for 20 min. Total chlorophyll was evaluated in the alcohol extracts from absorbance readings, using the appropriate extinction coefficient. Chlorophyll content (mg g^{-1} fr wt) was calculated as 1000 × A654/(39,8 x sample fr. wt), according to Luna et al. (2000).

Malondialdehyde analysis

The method defined by Lutts et al. (1996) was employed for measuring the amount of malondialdehyde, which is produced as a result of the lipid peroxidation that causes stress-induced damage to cellular membranes.

Malondialdehyde (MDA) concentration was determined by using an "extinction" coefficient, which is 155 mM cm^{-1}, expressed as µmol/g fresh weight. The following equation was used in the calculation: MDA = (A 523 - A 600) x volume of the extract (ml) / (155 mM cm^{-1} x sample amount)

Total soluble protein content

The freshly weighted material was extracted in pH 7 phosphate buffer in cold mortar. In this method, the coloring property of Coomassie brilliant blue G-250, an organic dyestuff, in protein was used. Coomassie brilliant blue G-250 is a negatively charged dye that binds to (+) charged groups of the protein. The dye exists in red (Amax=465 nm) and blue (Amax=595 nm) forms. The red form is the form in a solution, and when the dye binds to the protein, a blue color is formed. The reaction is rapid and highly reproducible. The color develops within two minutes and remains stable for up to one hour (Robyt, White 1987). The following procedures were used for protein determination with the use of Bradford method.

Preparation of Coomassie Brilliant Blue G-250 solution: Initially, 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95% ethanol, then supplemented with 100 ml 85% phosphoric acid and the final volume was completed to 1 L with distilled water. The resultant solution was kept at dark overnight, then passes through filter papers and made ready for analyses.

BSA (bovine serum albumin): To generate a BSA standard curve from the stock solution, 1 ml of standard solution (BSA) with known concentrations was placed into the tubes instead of samples and the same procedures were applied. Absorbance readings were performed at 595 nm and an absorbance vs concentration graph was drawn. For the blank solution, 1 mL distilled water was placed into the tubes instead of a sample and again the same procedures were applied. About 100 µL of samples was supplemented with 5 mL of Bradford solution and left for 5 minutes. Total protein quantities were then determined from the absorbance values with the use of standard measurements.

Proline content

Freshly weighed leaf samples (10 ml) were homogenized in 3% sulphosalicylic acid (Bates et al. 1973).

Acid-ninhydrin preparation: About 30 ml glacial acetic acid was supplemented with 1.25 g ninhydrin, then with 20 ml 6M phosphoric acid. The resultant solution was shaken and kept in a cool place (4°C) for 24 hours.

Frozen plant material was supplemented with 3% aqueous sulfosalicylic acid (0.01 g 0.5 ml^{-1}) and centrifuged at 12 000 g and 10°C for 20 minutes. Then, 1 ml of homogenized tissue was reacted with 1 ml of acid-ninhydrin and 1 ml of glacial acetic acid in a test tube at 100°C for 1 h and the reac-

tion was terminated with an ice bath. The reaction mixture was extracted with 2 ml toluene, stirred vigorously and stirred at room temperature for 30 minutes until two phases were separated. The proline concentration was determined from a standard curve using L-proline. The reaction medium was supplemented with 1 ml toluene, and toluene was used as a blank and the supernatant was taken into tubs and measured at 520 nm. L-proline was used as the standard and the results were expressed in µmol proline g^{-1} FW.

Spectrophotometric enzyme activities

Typically, 1 g leaf material without the main midrib was homogenized in 10 mL 25 mm EPPS buffer (pH 7.8) containing 0.2 mm EDTA and 2% PVP. The centrifuges obtained after homogenized samples had been centrifuged at 15000 g for 15 min were used in enzyme analysis. Samples for determining enzyme activities were kept at +4°C until the measurement was taken on a spectrophotometer (Analytic Jena 40 model). SOD activity, according to the method of reduction of NBT (nitro blue tetrazolium chloride) by O_2^- under light, GR activity at 340 nm (E = oxidation of 6.2 mM cm^{-1}) and NADPH were determined by measuring CAT, H_2O_2 degradation rate at 240 nm (E = 39.4 mM cm^{-1}) – Cakmak, Marschner 1992.

RESULTS

The experiments were conducted on curly lettuce plants in order to elucidate the effects of proline treatments against cadmium stress, plant response to cadmium stress and adaptation mechanisms developed against cadmium stress with the use of proline treatments. At the end of the experiments, root and plant weights were measured, chlorophyll content, lipid peroxidation, proline content, total soluble protein content and glutathione reductase enzyme activity were determined.

Root and plant weight

As compared to the control, decreased root weights were observed in all treatments submitted to cadmium stress (WS). The greatest average root weight (4.91 g) was obtained from the control group, followed respectively by BS 5 mM proline+Cd and WS 5 mM proline+Cd treatments. The lowest average plant fresh root weight (2.578 g) was measured in only Cd-treated plants (Table 3). In terms of root weights, significant differences were not observed between proline treatments submitted to cadmium stress and increases were observed in root weights as compared to only Cd-treated plants.

In terms of crown weights, significant decreases were encountered in Cd stress treatments. The greatest decrease was seen in proline treatments submitted to Cd stress. The greatest crown weight (44.217 g) was obtained from

Table 3

Effects of proline treatments on root and plant weight of curly lettuce plants under cadmium stress

Treatments	Root weight (g)	Crown weight (g)
Control	4.914±1.363 *a*	44.217±10.407 *a*
Only Cd	2.578±0.457 *d*	16.510±3.459 *cd*
BS 5 mM proline+Cd	3.564±0.702 *b*	20.031±3.967 *b*
BS 10 mM proline+Cd	2.753±0.517 *cd*	19.821±2.751 *bc*
BS 15 mM proline+Cd	2.941±0.514 *cd*	17.577±3.133 *b-d*
WS 5 mM proline+Cd	3.677±0.682 *b*	15.225±3.133 *d*
WS 10 mM proline+Cd	3.266±0.518 *bc*	14.843±3.646 *d*
WS 15 mM proline+Cd	3.232±0.746 *bc*	14.677±2.979 *d*
P value	0.0190	0.000

The means followed by the same small letters in the same column are not significantly different at $P \leq 0.05$; BS – before stress, WS – with stress

the control group and the lowest (14.667 g) from WS 15 mM proline+Cd treatments. However, significant differences were not observed between proline doses exposed to cadmium stress (Table 3).

Chlorophyll content and lipid peroxidation (MDA)

In terms of the chlorophyll content of curly lettuce plants with proline treatments exposed to cadmium stress, the greatest value (0.426 μmol g^{-1}) was obtained from WS 5 mM proline+Cd treatments and the lowest value (0.284 μmol g^{-1}) was obtained from only Cd-treated plants. Significant increases were seen in the chlorophyll content in proline treatments (Table 4).

Table 4

Effects of proline treatments on chlorophyll content and lipid peroxidation (MDA) values of curly lettuce plants under cadmium stress

Treatments	Chlorophyll (μmol g^{-1})	MDA (μg g^{-1} FW)
Control	0.375±0.030 *ab*	5.641±1.173 *d*
Only Cd	0.284±0.034 *c*	12.086±1.387 *b*
BS 5 mM proline+Cd	0.399±0.049 *ab*	6.241±1.319 *d*
BS 10 mM proline+Cd	0.328±0.061 *bc*	7.049±1.153 *cd*
BS 15 mM proline+Cd	0.367±0.037 *ab*	14.721±1.772 *a*
WS 5 mM proline+Cd	0.426±0.027 *a*	8.956±0.305 *c*
WS 10 mM proline+Cd	0.363±0.036 *ab*	11.713±1.406 *b*
WS 15 mM proline+Cd	0.329±0.042 *bc*	14.512±1.223 *a*
P value	0.0190	0.000

The means followed by the same small letters in the same column are not significantly different at $P \leq 0.05$; BS – before stress, WS – with stress

There were significant differences in the MDA content of proline treatments submitted to cadmium stress. The greatest values were obtained from BS 15 mM proline+Cd (14.721 μg g^{-1} FW) and WS 15 mM proline+Cd (14.512 μg g^{-1} FW) treatments. The lowest value (5.641 μg g^{-1} FW) was obtained from the control group. The BS 5 mM proline+Cd treatments yielded the closest values to control treatments (Table 4).

Total soluble protein and proline contents

In terms of the total soluble protein content of curly lettuce plants with different proline treatments exposed to cadmium stress, there were significant differences between the experimental treatments.

Significant increases were observed in the soluble protein content in proline treatments as compared to the control. The greatest value was obtained from BS 5 mM proline+Cd treatments (70.311 μg ml^{-1}) and the lowest value (34.799 μg ml^{-1}) from the control plants (Table 5).

Table 5

Effects of proline treatments on total soluble protein (μg ml^{-1}) and proline (μmol g^{-1} FW) content of curly lettuce plants under cadmium stress

Treatments	Protein	Proline
Control	34.799±5.532 *c*	3.286±1.245 *d*
Only Cd	62.239±1.816 *b*	21.667±1.198 *b*
BS 5 mM proline+Cd	70.311±6.013 *a*	32.571±5.331 *a*
BS 10 mM proline+Cd	62.501±6.542 I	12.238±2.818 *c*
BS 15 mM proline+Cd	62.799±6.573 *b*	8.287±1.080 *cd*
WS 5 mM proline+Cd	68.644±12.014 *a*	22.578±6.148 *b*
WS 10 mM proline+Cd	60.549±3.081 *b*	7.524±2.021 *cd*
WS 15 mM proline+Cd	58.775±3.739 *b*	7.429±1.220 *cd*
P value	0.002	0.000

The means followed by the same small letters in the same column are not significantly different at $P \leq 0.05$; BS – before stress, WS – with stress

In terms of the proline content, there were significant differences between experimental treatments. Significant increases were seen in the proline content in proline treatments as compared to the control treatments. The greatest value was obtained from BS 5 mM proline+Cd treatments (32.571 μmol g^{-1} FW) and the lowest value (3.286 μmol g^{-1} FW) was obtained from the control plants (Table 5).

Antioxidant enzyme activity (SOD, CAT, GR)

Leaf samples taken from proline-treated curly lettuce plants under cadmium stress were analyzed for SOD (superoxide dismutase), CAT (catalase) and GR (glutathione reductase) enzyme activity and the results are provided in Table 6.

Table 6

Effects of proline treatments on SOD, CAT and GR enzyme activity of curly lettuce plants under cadmium stress

Treatments	SOD (U min^{-1} mg^{-1} FW)	CAT (μmol min^{-1} mg^{-1} FW)	GR (μmol min^{-1} mg^{-1} FW)
Control	33.213±1.251 *e*	0.633±1.461 *e*	25.233±1.461 *e*
Only Cd	42.08±1.011 *d*	0.711±1.211 *de*	24.01±1.211 *e*
BS 5 mM proline+Cd	57.1±2.703 *b*	0.82±4.703 *b*	49.0±4.703 *b*
BS 10 mM proline+Cd	35.77±1.851 *e*	0.681±1.351 *de*	27.717±1.351 *e*
BS 15 mM proline+Cd	34.47±1.260 *e*	0.695±2.260 *de*	26.407±2.260 *e*
WS 5 mM proline+Cd	78.63±1.251 *a*	0.99±1.151 *a*	70.613±1.151 *a*
WS 10 mM proline+Cd	46.80±2.992 *c*	0.788±3.992 *c*	38.890±3.992 *c*
WS 15 mM proline+Cd	41.93±1.557 *d*	0.733±2.557 *d*	33.993±2.557 *d*
P value	0.000	0.000	0.000

The means followed by the same small letters in the same column are not significantly different at $P \leq 0.05$; BS – before stress, WS – with stress

Significant differences were not observed in SOD, CAT and GR enzyme activities of the samples taken from the leaves of only cadmium-treated and the control samples, but proline treatments applied together with cadmium stress, especially BS 5 mM proline+Cd and WS 5 mM proline+Cd treatments, significantly increased enzyme activities as compared to the control plants. The other proline doses yielded enzyme activities close to only Cd-treated plants.

In terms of the SOD (superoxide dismutase) activity of curly lettuce plants with different proline treatments applied under cadmium stress, the greatest SOD activity was observed in WS 5 mM proline+Cd treatment (78.630 U min^{-1} mg^{-1} FW), followed by BS 5 mM proline+Cd treatment (57.1 U min^{-1} mg^{-1} FW) and the lowest value was seen in the control plants (33.213 U min^{-1} mg^{-1} FW) – Table 6.

The greatest CAT (catalase) enzyme activity was obtained from WS 5 mM proline+Cd treatment (0.990 μmol min^{-1} mg^{-1} FW), followed by BS 5 mM proline+Cd treatment (0.82 μmol min^{-1} mg FW) and the lowest value was obtained from the control plants (0.633 μmol min^{-1} mg^{-1} FW) – Table 6.

In terms of GR (glutathione reductase) enzyme activity of the plants, the greatest value was obtained from WS 5 mM proline+Cd treatment (70.613 μmol min^{-1} mg FW) and the lowest value was obtained from only Cd-treated plants (24.10 μmol min^{-1} mg FW) – Table 6.

DISCUSSION

Proline is a water-soluble amino acid accumulating generally under stress conditions (Matysık et al. 2002) and it acts as an indicator for the plant's ability to withstand stress (Saıram et al. 2002). Besides serving as an osmolyte, it acts as an efficient organic compound in the stabilization of cells, regulation of cytosolic pH and hydroxyl radicals. Free proline accumulation occurs in response to stress in many plants exposed to heavy metals (Alıa, Saradhı 1991).

Excessive accumulation of heavy metals in plant tissues and organs generates serious problems in the development of generative and vegetative organs (Okcu et al. 2009). Plant roots uptake heavy metal ions from the soil solution. To a lesser extent, heavy metals can also be taken up through leaves (Keser 2005). It was reported that especially the vegetables with edible leaves were able to accumulate greater quantities of heavy metals than other plants, and almost 70% of Cd encountered in a human body came from these vegetables (Jı et al. 2018). Previous studies pointed out the significance of breeding less Cd-accumulating vegetables and developing technical methods to be used in reduction of plants' Cd content for the mitigation of Cd-induced health problems (Avıla et al. 2017, Tang et al. 2018).

Previous researchers indicated that heavy metal stress reduced plant weights. It was also reported in studies conducted on different plant species and varieties that cadmium stress negatively influenced plant growth, and high cadmium doses limited plant growth and root development (Finger-Teixeira et al. 2010, Muradoğlu et al. 2020). It was reported that 10 μM cadmium treatments in a hydroponic culture reduced the root length, surface area and number of roots of pepper plants (Huang et al. 2015). In a similar study, significant decreases were reported in the fresh root weight, stem weight, leaf area and leaf weight of pepper plants exposed to 2 and 10 μM low cadmium doses (Leon et al. 2002). Schutzendubel et al. (2001) indicated that plants responded physiologically to cadmium (Cd) stress with their roots, and excessive cadmium doses reduced root lengths. In present study, cadmium stress had negative effects on the development of curly lettuce plants, and the efficiency of proline treatments in mitigating these negative effects varied with treatment doses and application periods.

Cadmium has strong phytotoxic effects and is destructive to several physiological and metabolic functions when reaching plant cells (Shao et al. 2007). Cd toxicity damages photosynthesis mechanism through inhibiting chlorophyll biosynthesis, rubisco activity in the Calvin cycle and photosynthetic enzyme activities (Tıryakıoglu et al. 2006). It was stated that Cd accumulated the most in lettuce leaves (Pıllay et al. 2007). The direct toxic effect is manifested by a decline in chlorophyll synthesis and photosynthesis due to a decrease in the biomass volume (Padmaja et al. 1990). Salt et al. (1995) conducted a study on mustard plants to elucidate the transport and accumu-

lation mechanism of cadmium, and reported that Cd and chlorosis were initially encountered in young leaves. In another study on pea (*Pisum sativum* L.) seedlings, 50 µM Cd slowed down the transpiration rate and decreased the photosynthesis rate and chlorophyll content (Sandalıo at al. 2001). Parallel to previous findings, in the present study, decreases were seen in the chlorophyll content of curly lettuce plants treated only with cadmium. Such decreases were mainly attributed to the inhibition of chlorophyll biosynthesis (Van Assche, Clıjsters 1990) and replacement of magnesium in the center of chlorophyll with heavy metals after plants were exposed to heavy metal ions (Kupper et al. 1996). Proline treatments had positive effects on the chlorophyll content.

It was reported in several studies that biotic and abiotic stress factors resulted in lipid peroxidation in plants. The malondialdehyde (MDA) level is commonly used as an indicator of lipid peroxidation. Dere, Dogan (2020) reported that different Pb concentrations resulted in membrane damage in peanut seedlings, which were diagnosed through increasing MDA levels. Kabakci (2018) reported increasing Tbars (MDA) levels with increasing Cd stress levels, and indicated that such increases were more remarkable under 200 µM Cd stress. It was reported that GLA treatments significantly increased TBARS levels of wheat seedlings, and such increases were remarkably greater (335.3%) in Cd2+GLA1 treatments than in single stress treatments. In the present study, Cd-induced membrane damage was also diagnosed by increased MDA levels. Significantly increased MDA levels could have resulted from the damage generated by free radicals formed in cell membranes due to cadmium stress. The optimum proline dose to reduce such damage was identified as 5 mM. MDA levels increased with increasing proline doses, but the increasing doses were not effective in reducing membrane damage.

Cadmium toxicity induces lipid peroxidation and thus leads to oxidative damage in plants. It also induces the formation of reactive oxygen species (ROS), thereby affecting other biomolecules of cells (Muradoglu et al. 2015). Plants develop various types of defense mechanisms against heavy metal stress conditions. Such mechanisms include an increased antioxidant enzyme content and activity, as well as higher production and accumulation of proline, trehalose, polyamines, phenolic compounds, glycine betaine and tocopherols-like low-molecular compounds (Szafranska et al. 2011). Cd stress applied to sunflower decreased GR and CAT activity (Saıdı et al. 2014). Cd stress applied to wheat also decreased GR and CAT enzyme activity. Such a decrease in GR activity was prevented by external GLA treatments. The greatest increase in GR activity was obtained in Cd1+GLA2 treatments, where it was 1.9-fold higher than in single stress treatments (Kabakcı 2018). Bayat et al. (2013) investigated the effects of proline treatments applied to young zucchini (*C. pepo* L. and *C. moschata* Poir.) plants under salt stress on antioxidative enzyme activities, and reported increased enzyme activity

of plant leaves with proline treatments. As compared to the control, the greatest increase in catalase, superoxide dismutase and glutathione reductase activity of the plants in this study was achieved in BS 5 mM proline+Cd and WS 5 mM proline+Cd treatments. It was determined that exogenous proline treatments stimulated the antioxidative enzyme system of curly lettuce plants.

An increase in the intrinsic proline level is a vital physiological reaction of plants against harmful effects of environmental stresses. Positive correlations were reported between proline accumulation and stress tolerance of plants. Proline accumulation was reported to regulate cellular homeostasis by responding to environmental stresses, such as non-optimal temperatures and heavy metals (Hossaın et al. 2014).

It was reported that biotic and abiotic stressors stimulated proline accumulation in plants (Hare, Cress 1997). The majority of plants could increase proline concentrations 100 times above the normal level under stress conditions (Aziz et al. 1998). An increasing proline content has been reported in aquatic and terrestrial plants under metal stress. Under cadmium stress conditions, an increasing proline content has been determined in *Ceratophyllum demersum* parallel to increasing Cd concentrations; as compared to control, a rise in the proline content of this macrophyte due to the influence of 0.01, 0.1 and 1 mg L^{-1} Cd concentrations was identified as 15.9% ($p>0.05$), 53.3% ($p<0.01$) and 35.7% ($p<0.05$), respectively (Dogan, Demırors Saygıdeger 2009). Similarly, Dogan, Demırors Saygıdeger (2009) reported increasing proline in wheat seedling under Pb stress. Under heavy metal stress, proline may play a role in protein denaturation, regulation of intracellular pH and (NADP+/NADPH) ratios, nitrogen source, use of carbon and elimination of reactive oxygen species (Sharmıla, Pardha Saradhı 2002). In the present study, increases were encountered in the proline content of Cd stress-treated plants, and the greatest proline content was obtained from WS 5 mM proline+Cd stress treatments. In brief, the proline content of curly lettuce plants increased under cadmium (Cd) toxicity.

Dere, Dogan (2020) reported decreases in the protein content of seedlings under Pb stress. It was emphasized that Pb might have inhibited protein synthesis in plants or induced proteolysis triggered by reactive oxygen species produced under oxidative stress. In the present study, increases were seen in the total soluble protein content of the plants subjected to cadmium stress, and the greatest total soluble protein content was obtained from WS 5 mM proline+Cd stress treatments. Accordingly, although qualitative and quantitative changes in total proteins should be analyzed in detail, it can be concluded that cadmium might have increased protein synthesis in plant. Synthesis of new proteins was also reported in some plants under stress conditions (Mahboobı et al. 2000, Arı Baykal, Oncel 2006).

CONCLUSION

Toxicity of heavy metals may vary based on form (organic compound, metal, ion and etc.), concentration of the metals, plant species, effect or exposure duration, location and time. It was concluded, based on the present findings, that low and high concentrations of proline, treatment time and type influenced plant tolerance to stress factors. The impact of Cd on some physiological parameters required for stress tolerance could be reduced by the application of proline at an appropriate concentration. Further physiological and biochemical research (including different proteins, hormones, amino acids and sugars) is recommended for better comprehension of the effects of external proline treatments on defense mechanisms of curly lettuce under Cd stress.

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