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Cadmium stress in barley seedlings: Accumulation, growth, anatomy and physiology

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ABSTRACT

Heavy metal stress has marked effects on some growth parameters, physiology, anatomy, and genetics of plants. Among heavy metals, cadmium (Cd) is an extremely toxic one and effects living organisms at even low concentrations. The presence of Cd in air, water and soil and its accumulation in plants create significant negations such as cancer, renal failure, cardiovascular and musculoskeletal diseases in humans when taken from direct and indirect ways. The defense mechanism of the plants which is responsible from stress tolerance can be investigated to improve crop yield under Cd stress. Numerous studies have shown negative effects in plants exposed to Cd. Therefore, in this study, 0 (for control), 50, 100, 200 and 400 µM (for experimental groups) $CdCl_2$ were applied to barley (*Hordeum vulgare* L.) plants and some growth, development, physiological and anatomical parameters were measured. As a result, it has been observed that barley plants can manage stress in terms of some parameters under low Cd stress conditions, however, they are negatively affected at all Cd concentrations to a certain extent. In addition, it was observed that barley plants were adversely affected by high levels of Cd stress, although they maintained their vitality throughout the experiment.

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Introduction

Cadmium (Cd) is a heavy metal with an atomic number 48 and atomic weight 112.41 g/mol, has the density value of 7.86 g/cm^3, boiling point of 767°C and melting point of 321°C. It is a soft, workable and silver color metal which cannot be found alone in nature [1-3].

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Cd is released into the environment in various amounts, both from anthropogenic and natural sources such as volcanic eruptions, parent rocks weathering, wind-blown dust, forest fires and sea sprays [4-7]. Highest contribution to environmental Cd pollution arise form anthropogenic sources such as combustion of fossil fuel, use of phosphate fertilizers, mining, smelting and cement industries, metal ore processing, metallurgical works, sewage sludge and municipal wastes and reach almost 90% of total Cd emissions [8, 9]. In addition, it is frequently used in pigments, plastics, alloys, batteries and solar panels to increase corrosion resistance. In addition to all these, Cd can also be found in polyvinyl chloride (PVC) materials, engine oil, fungicides, vulcanized polymers and textile leach solutions [10]. Industrial processes and traffic emissions are the main responsible factors of Cd pollution in urbanized areas and soils [11]. Cd has a wide usage area (67%) in the production of Cd electroplates to be used in nickel-Cd batteries [7, 12]. While phosphate fertilizers are considered to be the major source of Cd pollution in agricultural soils, the use of contaminated manure may also result in Cd contamination/pollution in soils [7, 13].

Among phytotoxic heavy metals, Cd has a considerable importance due to high water solubility of its salts, high mobility, and significant toxic effects even at low concentrations. Features causing mutagenesis and carcinogenesis together with its high accumulation capacity in plants tissues are the main factors of the rigorous toxicity of Cd [3, 14-16]. All these properties mentioned made Cd one of the most dangerous pollutants in agricultural soils, which has a high potential to participate in the food chain of living things [17, 18] and negatively effects human health [19]. Cd is on the priority list of harmful substances by many environmental non-governmental organizations and the United States Environmental Protection Commission [20].

Although plants can regulate their metabolism under relatively higher amounts of Cd without getting adversely affected (compared to animals), excessive amounts of Cd cause negative effects on plants [21]. Above a critical threshold concentration, obvious signs of cadmium toxicity, such as brownish roots, growth retardation, chlorosis, necrosis, and even death were observed in many plant species [22-24]. Accumulation of Cd in plant tissues can intensely affects lots of physiological processes, such as the interference of respiration and photosynthesis [25], transport and absorption of mineral nutrients [26-28]. Also, diminished

water balance [29], and disturbed carbohydrate metabolism [30] are seen after Cd treatments. Cd exposure in plants, especially at high concentrations, may result in a series of toxicity-related deteriorations in metabolism, such as lipid peroxidation, enzyme inhibition, the formation of reactive oxygen species (ROS) altering the gene and protein expression and even cause DNA damage [3, 31-33].

Cd uptake in plants usually takes place from soil solutions through the root system. Therefore, the amount of Cd taken up by plants is directly related to the Cd concentration in the soil solution [34, 35]. Cd, along with many other heavy metals uses ion transporters such as Ca^{2+}, Fe^{2+} and Mn^{2+} to enter into the root system from the soil solution [36]. The amount of cadmium that plants take from the soil is affected by the pH range of the soil, salinity, humus content and the types of crops grown. Cd regulatory limit of agricultural soils is reported to be 100 mg kg^{-1} soil [37]. Moreover, primary processes such as transport efficiency of the xylem sap, uptake ability of the plant roots and final relocation within the seeds of the plants have an effect on the Cd uptake and accumulation capacity of the [3, 7].

Plant roots play a key role in absorbing heavy metals as well as water and essential nutrients. Additionally, a small portion of Cd in the soil system is adsorbed by the surface of plant roots. Therefore, the increase in the surface area of plant roots extends the plant's exposure time to toxic heavy metal and results in higher concentrations of Cd accumulation in various plant parts [38-40]. Once Cd enters into the plant roots, then transportation occurs, either through apoplastic or symplastic pathways. However, Cd from different complexes formed with various ligands such as organic acids and phyto-chelators mainly moves into the root vacuoles and/or nuclei [7, 16, 41]. Cd is mainly transported by xylem from the roots to upper plant parts such as shoots and leaves, while plasmalemma and Casparian strips also support xylem for upward metal transport within plant tissues [36, 42, 43]. The transport of Cd from roots to various plant parts is carried out by both passive (transpiration) and active (ion transport channels) mechanisms [41, 44]. However, the amount of translocated Cd depends on both the concentration of Cd in the lower parts of the plant, and the concentration of all other nutrients in the cell sap. The uptake of Cd from the soil by plants causes toxic effects on various physiological and morphological processes, adversely affecting plant growth and development [7].

Plants have developed some natural defense systems, such as using enzymatic and non-enzymatic antioxidants, osmolyte production, and synthesizing chelating agents, to protect their metabolic processes against stress caused by metals. Plant species tolerate heavy metal stress at varying rates depending on the variety of plant and the type of heavy metal [45-48]. Metal-binding ligands play important roles in plant metabolism as taking part in heavy-metal detoxification with naturally occurring ligands such as amino acids, organic acids, peptides and polypeptides [49, 50]. Phytochelatins (PCs) are glutathione-derived peptides [51] and they are responsible in decreasing free metal concentration in plant tissues [52], they support plants defense mechanism [53] and protect plant tissues from heavy metal damage. PCs support cellular detoxification by forming stable complexes with metal ions and reduce the adverse effects of heavy metal stress [54]. Metallothioneins (MTs) presumably function similar to PCS [50]. Chelators such as PCs and MTs, which have cysteine sulfhydryl groups in their structures, allow storage in the vacuole and cell wall by binding to heavy metals and forming stable complexes [3, 55].

Barley (*Hordeum vulgare* L.) is an important annual cereal species belonging to the Poaceae family such as wheat, rice and corn. It is the fourth important annual cereal product of the Poaceae family after wheat, rice and corn. In addition to being consumed as animal feed, barley is consumed by itself or mixed with other grains in the form of porridge, breakfast foods, sattu (roasted barley) and chapattis, and is also used in malt fermentation [56]. According to (USDA, 2020), barley production in Kyrgyzstan were in a period of rapid increase especially after 2014 and in 2020, it was reached 500 hundred metric tons (MT) with a Growth Rate of 13.64 compared to previous years [57]. However, recent studies conducted with plants in Kyrgyzstan showed that Cd concentrations are higher than normal limits in many plants [58-60]. Therefore, in this study, the effects of Cd on growth, development, some anatomical and physiological parameters in barley, which is increasingly important for Kyrgyzstan, were investigated by applying Cd exposures at different concentrations (50, 100, 200 and 400μM).

Materials and Methods

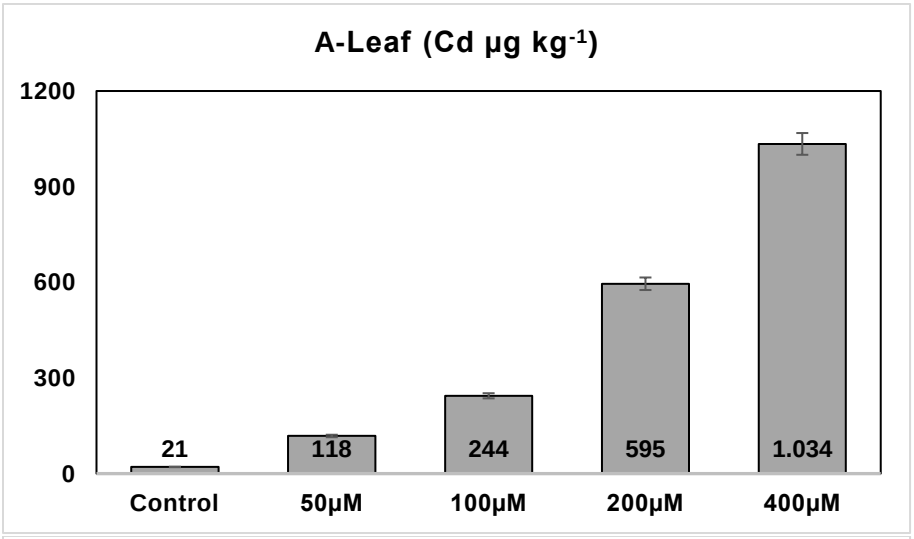
In this study, Alta variety belonging to barley (*Hordeum vulgare* L.) plant were obtained from "Kyrgyzstan State Plant Genetic Resources Center". Seeds of Alta barley variety were washed under tap water for 1 hour, and then germinated for 14 days in standard pots with 100 g of standard soil. During the germination period, the seeds were watered with the full-strength Hoagland nutrient solution [61]. Plant samples were grown under 5000 μmol m^{-2} s^{-1} fluorescent light, in a relative humidity percentage of 45-50% at 24 ± 2°C. During 14 days of germination, 16 hours light and 8 hours darkness/day of photoperiod conditions were applied to each group of 10 replicated seedlings. After the germination period, each of the experimental groups were watered with 20 ml Hoagland's solution containing 0, 50, 100, 200 and 400 μM $CdCl_2$ once per two days for 45 days in above mentioned growth conditions.

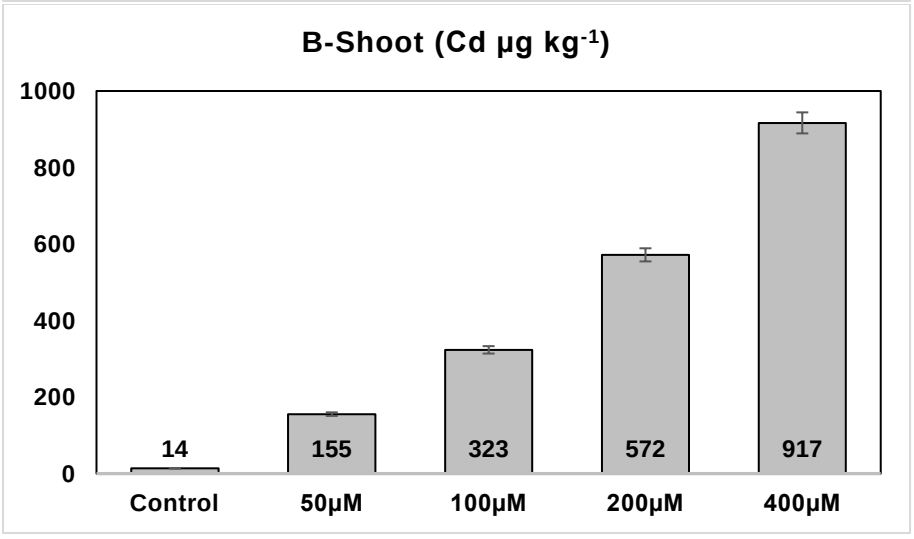
At the end of 45-days of vegetation period, leaves, shoots and roots of the harvested seedlings were oven-dried at 80ºC for 48 h. Plant parts which reached to constant weight were milled in a micro-hammer cutter and passed through a 1.5-mm sieve. For the determination of Cd accumulation level, 0.5 g sample of each plant part was placed in a Teflon vessel and 8 ml of 65% nitric acid (HNO_3) was added. Samples were digested at 160°C by using a microwave oven (CEM Mars 5). After cooling, digested samples were filtered through Whatman filter paper, and diluted to 50 ml with ultra-pure water. Cd concentrations were measured by an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES - PerkinElmer Optima 7000dv).

Parameters such as length and width of the lower and upper leaves, shoot length, and total chlorophyll amounts, were measured using calipers, millimetric rulers and chlorophyll content meter (Opti-Sciences Inc., CCM-300). In addition, stomata numbers of abaxial leaf surfaces (for each $1mm^2$ area) of control and experimental groups were counted using an Olympus digital microscope. For this purpose, cross sections were taken from the lower surfaces of the leaves using a sharp razor blade and preparations were examined directly under the microscope.

Result and Discussion

In this study, the amount of Cd accumulated in the roots, shoots and leaves of barley plants treated with various concentrations (0, 50, 100, 200 and 400 µM) of $CdCl_2$ during the 45-day experiment period was measured by an ICP-OES instrument. In addition, the length and width of the lower and upper leaves, shoot length and total chlorophyll amounts in the lower and upper leaves were measured. As a result of the experiment; cadmium accumulation values (in µg kg^{-1}) were 34, 282, 410, 1178 and 1427 in the root, 14, 155, 323, 572 and 917 in the shoot and 21, 118, 224, 595 and 1034 in the leaves of the control (0) and experimental (50, 100, 200 and 400 µM $CdCl_2$ treated) groups, respectively (Figure 1, A-C). Accordingly, the accumulation was in the order of root>leaf>shoot.





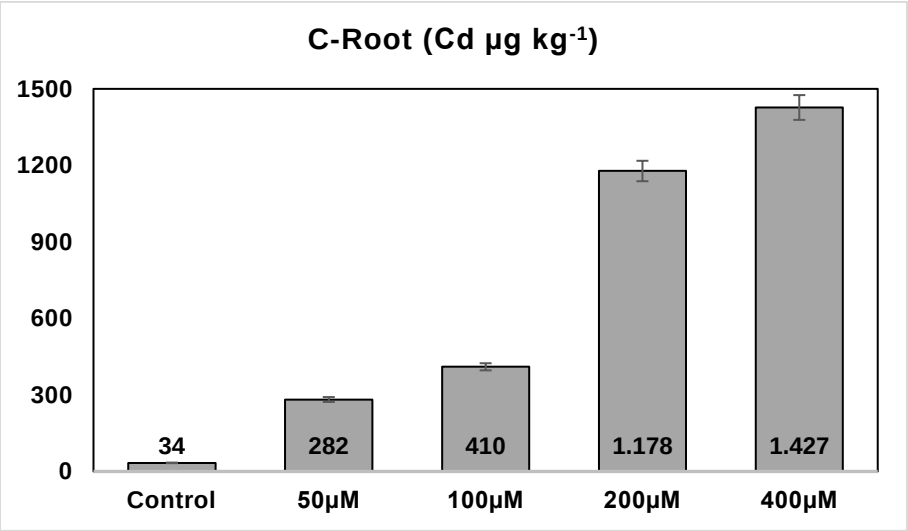


Fig 1 Cd accumulation values (µg kg^{-1}) in **(A)** leaf, **(B)** shoot and **(C)** root of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM).

In a similar study, *Bromus sterilis* plant was exposed to Cd stress at concentrations of 40 and 120 mg kg^{-1}, and Cd accumulations (in mg kg^{-1}) were determined as 114 and 463 in roots, and 65 and 75 in shoots [62]. The same researchers also observed Cd accumulation levels of 189 and 495 (roots) and 14 and 29 (shoots) after 40 and 160 mg kg^{-1} Cd applications in *Vicia sativa* plant. Furthermore, in the same research, Cd accumulations in *Apium graveolense* plant (after 40 and 120 mg kg^{-1} Cd applications) were obtained between 220 and 300 (root) and 16 to 40 (shoots). In another study, *Paspalum atratum* cv. Reyan plants were exposed to Cd at a concentration of 8 mg kg^{-1} and Cd accumulations were measured as 349 (root) and 46 (shoot) [63]. In a similar study, *Salix caprea* treated with 0.5 mg L^{-1} Cd, and Cd accumulations were 400 in roots and 340 in leaves [64]. On the other hand, in *Cynara cardunculus* plants treated with 2100 mg kg^{-1} Cd, and accumulation ranges were 10-280 (roots) and 10-260 (shoots) [65]. In 2 different studies conducted with *Zea mays*, the applied Cd concentrations were 2-100 mg kg^{-1} [65] and 40-160 mg kg^{-1} [62], and the accumulations were as 10-500 (roots), 5-130 (shoots) [65] and 199-243 (roots), 57-60 (shoots) respectively [62].

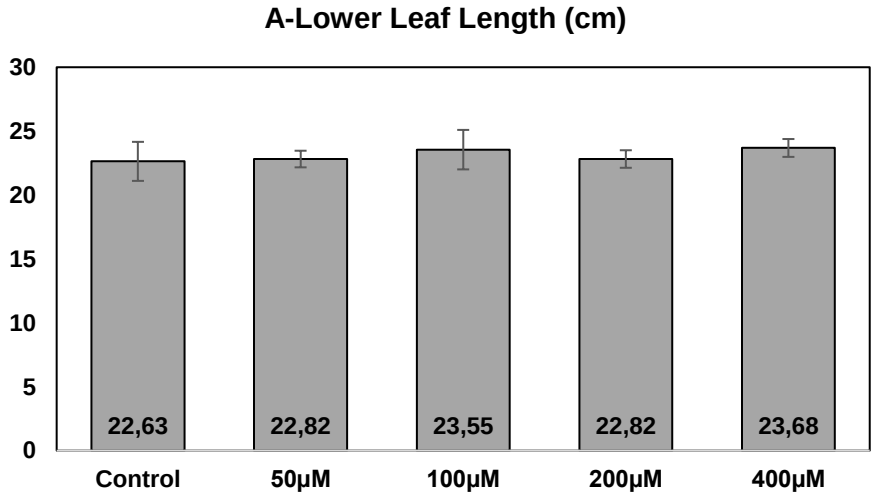
Di Baccio et al. (2014) applied lower (54.3 mg kg^{-1}) and higher (163 mg kg^{-1}) concentrations of Cd to poplar (*Populus x canadensis*) clones. Accumulation levels at lower Cd

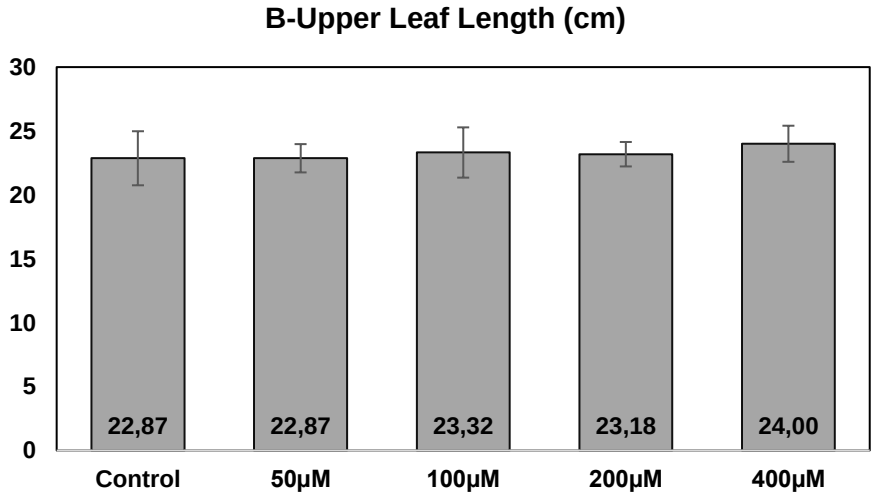
concentrations were determined as 7.6 (leaves), 4.6 (shoots) and 57 (roots), while at higher Cd concentrations accumulation values were measured as 12.7 (leaves), 15 (shoots) and 80 (roots) [66]. In another study, conducted with Kalanchoe plants, Cd accumulations (in mg ml^{-1}) increased from 0.629 to 3.164 in leaves, from 0.460 to 2.890 in shoots and from 1.327 to 5.178 in roots after 0-400 μM Cd applications [22]. In another study, the degree of Cd uptake from soil into vegetable species was given in the order of French beans, beetroots, radishes, peas, carrots, broccoli < potatoes, tomatoes, zucchini, and sweet corn < onions, leeks, parsnips, < turnips < cabbage, kale < lettuce, and spinach [18].

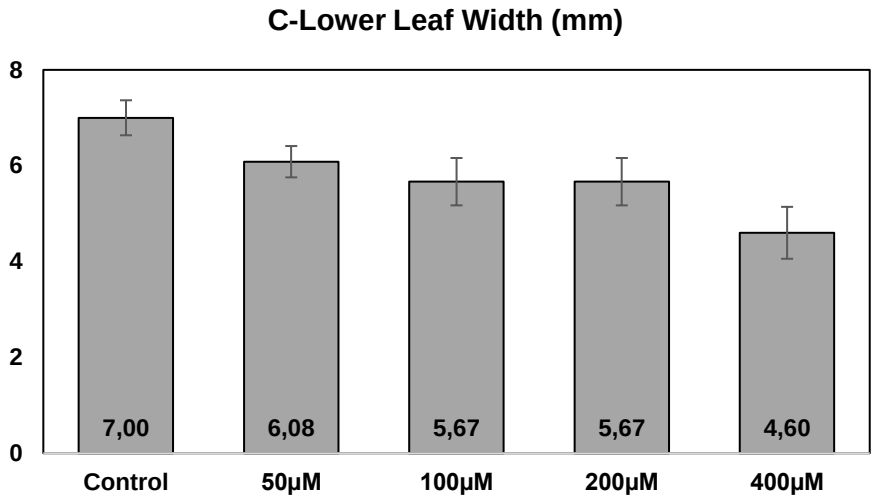
"Soil-related parameters such as pH, temperature, cation exchange capacity and particle size play an important role in the degree of Cd uptake. In addition, physiological parameters of the plants such as total root surface area, transpiration and root exudation rate are also effective in the mobilization of Cd in plant tissues. Cd accumulates predominantly in the roots in most plant species and is transferred to the shoots in very low concentrations [67, 68].

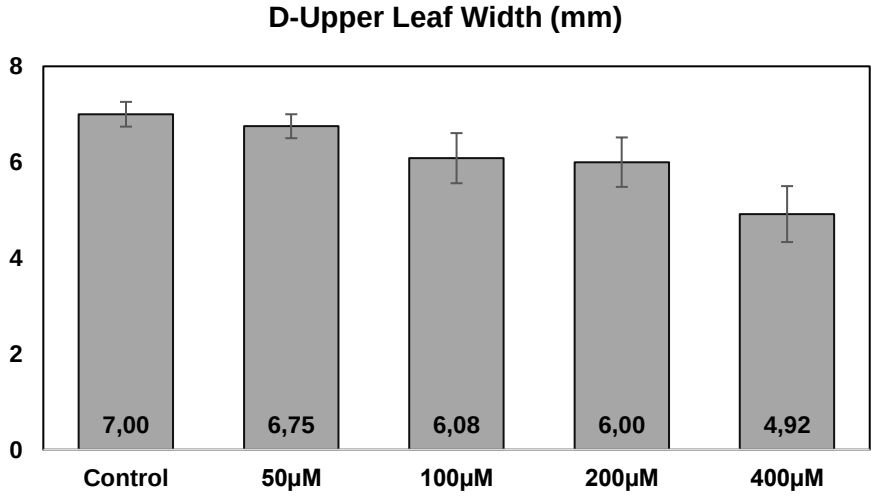
As a result of the above-mentioned Cd applications (0, 50, 100, 200 and 400 μM $CdCl_2$), the lower leaf lengths (in cm) were measured as 22.63, 22.82, 24.42, 22.82 and 23.68, while the upper leaf lengths were 22.87, 22.87, 23.32, 23.18 and 24.00, respectively (Figure 2, A and B). These results indicate that the leaf length slightly increases in barley under the effect of Cd stress. It was observed that the largest increase was 1.05 cm in lower leaves and 1.13 cm in upper leaves in the plants exposed to 400 μM $CdCl_2$. When the widths of the lower and upper leaves were examined, it was observed that the widths of lower leaves (in mm) were 7.00, 6.08, 5.67, 5.67 and 4.60, while the widths of upper leaves were 7.00, 6.75, 6.08, 6.00 and 4.92 in applied Cd concentrations, respectively (Figure 2, C and D). The greatest reduction in leaf width was observed as 2.4 mm in the lower leaves and 2.08 mm in the upper leaves in the application of 400 μM $CdCl_2$. These findings suggest that, although there was not a considerable change in leaf length, leaf width decreased significantly with increasing Cd concentrations. As a result, it can be said that Cd has a reducing effect on the leaf width in the barley plant. In this study, it was determined that the shoot length values (in cm) measured at the end of the experiment periods were 13.57, 12.03, 11.15, 10.75 and 8.65, respectively (Figure 2, E). This finding indicates that the shoot length of the barley plant is

negatively affected by the Cd stress and decreased inversely with increasing concentration. When the total chlorophyll values of the lower and upper leaves were examined, it was seen that the total amounts of chlorophyll in the lower leaves were 6.18, 3.40, 3.35, 3.01 and 2.91, while in the upper leaves were 8.55, 3.84, 3.66, 3.24 and 2.97, respectively (Figure 3, A and B). This result indicates that the total amount of chlorophyll of the barley plant was negatively affected by the Cd element and decreased inversely with increasing Cd concentrations.






C-Lower Leaf Width (mm)
8
6
4
2
0
7,00
6,08
5,67
5,67
4,60
Control
50µM
100µM
200µM
400µM


D-Upper Leaf Width (mm)
8
6
4
2
0
7,00
6,75
6,08
6,00
4,92
Control
50µM
100µM
200µM
400µM

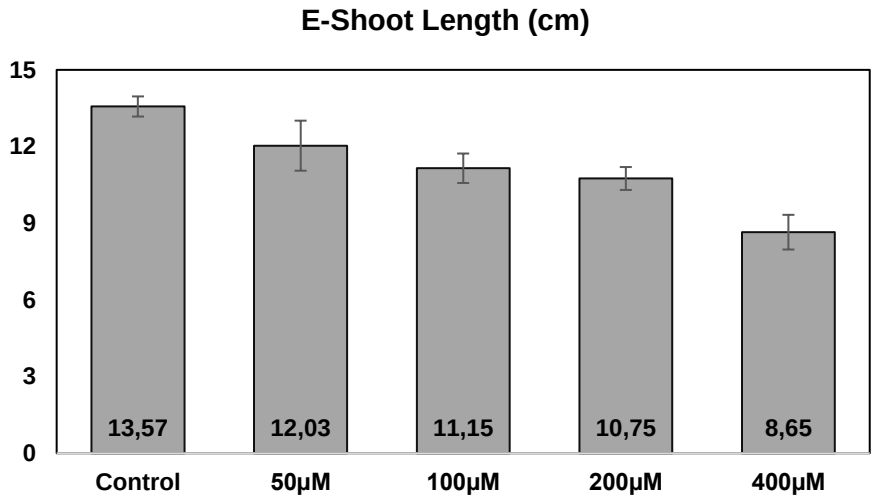
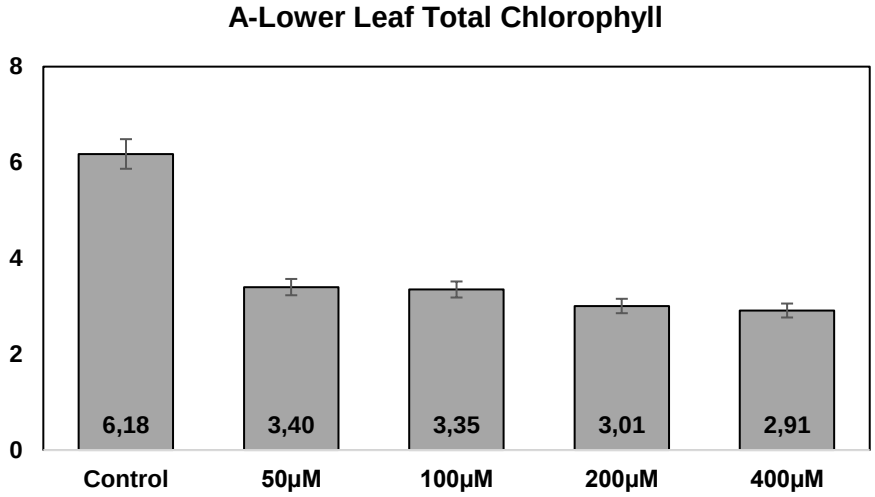


Fig 2 Some growth parameters of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM); **(A)** lower leaf length, **(B)** upper leaf length, **(C)** lower leaf width, **(D)** upper leaf width, and **(E)** shoot length.



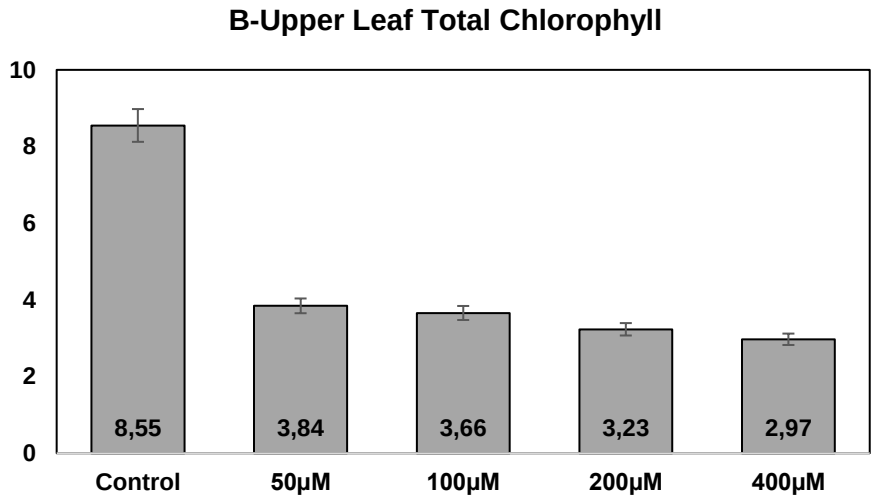


Fig 3 Total chlorophyll amounts of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM); **(A)** lower leaf and **(B)** upper leaf.

In a similar study, a decrease was observed in fresh and dry weights of roots and shoots as well as biomass, growth rate and chlorophyll content of *Tagetes patula* plant exposed to 10, 25 and 50 µM Cd stress [69]. A gradual decrease in photosynthetic pigmentation, chlorophyll *a*, *b* and total chlorophyll ratios were observed in *Groenlandia densa* (0-20 mg L^{-1} Cd application) [70]. In addition to the decreasing plant growth parameters in *Solanum tuberosum* plant treated with 1, 5, and 25 mg kg^{-1} Cd [71] and *Triticum aestivum* plant treated with 200 mg kg^{-1} Cd, reductions in chlorophyll (*a* and *b*) and photosynthetic pigment contents were also observed [72].

Li et al. (2013) observed a decrease in root and shoot lengths of two kenaf (*Hibiscus cannabinus* L.) cultivars treated with 20-120 µM Cd, as well as a decrease in root and shoot biomass [73]. In *Brassica napus* treated with 10-50 µM Cd, no adverse effects were observed up to 20 µM Cd concentration (compared to the control group), but especially at higher concentrations decreases were observed in plant height, leaf area and leaf number, root length, root diameter, root surface, root tip number and root volume [74]. Ozyigit et al. (2016) observed significant decreases in growth parameters and chlorophyll concentrations of kalanchoe (*Kalanchoe daigremontiana*) plants after 60 days of Cd (0, 50, 100, 200 and 400 µM) application. The reduction rates were ~40.57% for chlorophyll *a*, ~37.63% for

chlorophyll *b*, ~20.58% for chlorophyll *a*/*b*, and ~36.27% for total chlorophyll [22]. From the studies mentioned above, it is clearly seen that different responses (mostly negative) occur in plants depending on the type, concentration and duration of the applied Cd stress.

In another part of our study, transverse sections were taken from the lower surfaces of the leaves (Figure 4, A-E), and the stomata of the abaxial leaf surfaces (per 1mm^2 area) of the control and experimental groups were counted. As a result of the 45-day 0 (control), 50, 100, 200 and 400 μM $CdCl_2$ applications, the average number of stomata in 1 mm^2 surface area was examined. It was observed that the stoma number of 61.88 in the control was changed into 64.38, 59.25, 58.13 and 57.63 in the experimental groups, respectively (Figure 5).

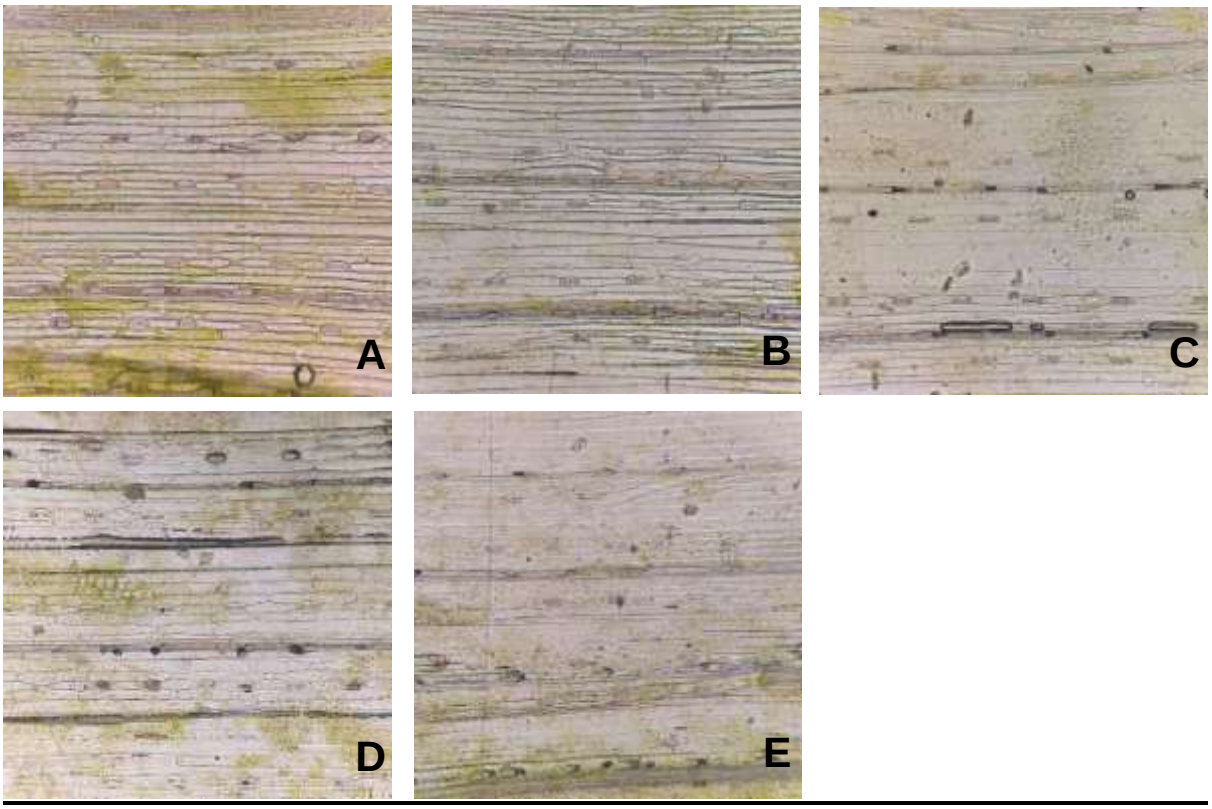


Fig 4 Anatomical images of abaxial leaf surfaces (1 mm^2) of barley treated with four different concentrations of $CdCl_2$ **(A)** 0 control, **(B)** 50, **(C)** 100, **(D)** 200 and **(E)** 400 μM.

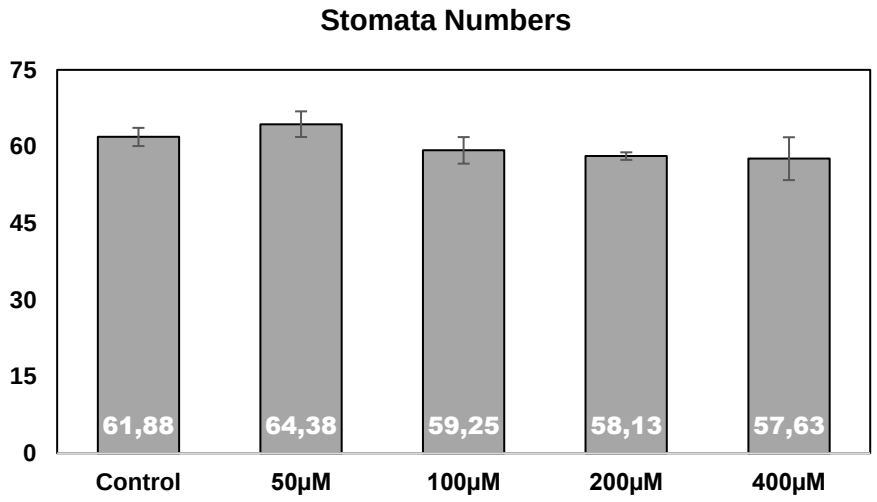


Fig 5 Stomata numbers of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM).

The results showed that 50 µM Cd stress caused a small increase in the number of stomata, but at other Cd concentrations, the stomata numbers decreased inversely related to increasing Cd concentration. This result shows that the barley plant increases stomata to manage lower concentrations of Cd stress, but this mechanism does not work at higher Cd concentrations. In a similar study conducted with 0, 1, 5, 25 mg kg^{-1} Cd applied barley plant, stomata numbers on the adaxial and the abaxial leaf surfaces (in 1 mm^2 area) were calculated as 59, 53, 59, 60 and 51, 58, 63, 46 respectively [75]. Similar to ours, researchers observed a fluctuating change due to the applied Cd concentrations instead of a sharp decrease. Stomata numbers of the lemon balm plant exposed to 0, 10, 20, 30 mg kg Cd concentrations were determined as 3.5, 2.6, 1.6, and 0.8 for adaxial and 5.8, 4.5, 3.3, and 1.6 for abaxial leaf surfaces, respectively [76]. Stomata number increased from 189.86 to 291 in *Biscutella auriculata* leaf exposed to 0 and 125 µM Cd concentrations [77]. Similarly, in our study, it was observed that 50 µM Cd stress caused an increase in the number of stomata, but this increase occurred at a lower rate. In *Cenchrus ciliaris* leaf, stomata numbers in 0, 30, 60 mg L^{-1} Cd applications were determined as 80.25, 69.12 and 60.23 respectively [78]. Stomata numbers in *Trigonella foenum graecum* Linn. plant exposed to 0, 5, 15, 30, 50 µg g^{-1} Cd concentrations were determined as 33.20, 29.21, 27.20, 25.19 and 22.17, respectively [79]. In *Cajanus cajan* plant exposed to 0, 5, 10, 15, 25 and 50 µg g^{-1} Cd concentrations, stomata numbers were

determined as 26.40, 20.64, 19.04, 18.60, 18.40, and 17.20, respectively [80]. When the above studies and the obtained data from our study are evaluated together, it is concluded that the plants show different responses depending on the species, the severity and duration of the Cd stress applied. In our study, while a slight increase was observed in the number of stomata in lower (50 μM) Cd application, there was a slight decrease in applications at other concentrations. However, when compared to some other studies, they have emerged with sharper increases and decreases in stomata numbers. All these findings were the results of different stress responses that vary from plant to plant.

Cadmium is one of the most toxic metals that prevail in agricultural soils, and negatively affects plant growth. Different sources increase the Cd concentrations in the soil day by day, and this causes water and nutritional imbalance in agricultural soils. On the one hand, the stress caused by Cd pollution causes contamination in agricultural products, and adversely affects the growth and development processes of plants by affecting the physiological and molecular mechanisms resulted with plant productivity decrease. Today, the most suitable option can be applied is to obtain new tolerant/resistant agricultural varieties using classical or biotechnological methods that can grow in contaminated soils, and to manage the nutritional imbalance in the soil in order to maintain the quantity and quality of the crops grown. According to the findings of our study and previous studies, plants (except some hyperaccumulator species) are negatively affected by Cd. Although field crops manage low concentrations of Cd, they are adversely affected by Cd above a certain concentration. Future studies on this subject should be aimed at producing safe products in contaminated soils. It is obvious that applying a single method will not be sufficient enough to increase the product yield and production quality in Cd contaminated soil. A diverse and integrated approach will be developing strategies to reduce Cd uptake and migration into the food chain by agricultural crops. Use of combined approaches such as biotechnological studies, soil amendments, plant rotation and metal immobilization, can help with sustainable agriculture and food security in metal contaminated areas worldwide.

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