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Effects of different doses of cadmium on physiological, biochemical, and phytoextraction potential of mustard (*Brassica juncea* L.)

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Abstract

This study investigated the physiological and biochemical tolerance and response, cadmium (Cd) accumulation capacity of the mustard plant (*Brassica juncea* L.) to different doses of Cd (0.0 (control)-, 25-, 50-, 100-, 200-, and 300 ppm) under greenhouse conditions. After harvesting the mustard plant, physiological parameters (plant length, plant fresh and dry weight, roots fresh weight and dry weight), and biochemical parameters such as chlorophyll a (Chl a), and chlorophyll b (Chl b), carotenoids, proline, malondialdehyde (MDA), antioxidant enzymes such as peroxidase (POX), and catalase (CAT) were examined. Cd content was measured in leaves and roots to determine phytoextraction capacity. Cd stress decreases plant and root fresh weight (Fwt) and dry weight (Dwt). Chl a-Chl b, and carotenoid contents 100 ppm of Cd decrease Cd doses increase $p \leq 0.05$. The osmolyte molecule proline increased to 100 ppm Cd dose and then declined to 300 ppm. Accumulation of MDA (2.9 to 33.8 nmol g^{-1} Fwt), H_2O_2 (2.9 to 30.4 µmol g^{-1} Fwt), and antioxidant enzymes (POX and CAT) showed an increasing trend with increasing Cd doses, $p \leq 0.05$. Cd accumulation in leaves (0.0 to 53.8. mg kg^{-1}) and roots (0.0 to 67.7. mg kg^{-1}) increased depending on the applied Cd concentration. The highest Cd accumulation was determined at 300 ppm Cd level. These findings suggest that mustard plants can accumulate high levels of Cd in both leaves and roots, indicating that they are hyperaccumulators. As a result, mustard plants can be utilized as phytoremediation plants in Cd-contaminated soils.

Keywords: Phytoremediation, Phytoextraction, Mustard, Heavy metal

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INTRODUCTION

Rapid population growth has reduced agricultural area and increased environmental pollution (Özyürek, 2016; Maja et al., 2021). Soil pollution resulting from heavy metal contamination has risen significantly in recent years. Heavy metals are one of the natural components of soils in trace amounts (Rahimzadeh et al., 2017; Priya et al., 2023). There are heavy metals known as essential nutritional elements including iron (Fe), copper (Cu), cobalt (Co), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn), which are necessary for plant growth and physiological functions (Farooq et al., 2016; Daulta et al., 2022). In comparison, non-essential heavy metals such as arsenic (As), mercury (Hg), cadmium (Cd), and lead (Pb) are not required by plants for their physiological functions (Bortoloti and Baron, 2022). Several heavy metals have a long history of accumulating in soil through industrial waste and wastewater disposal, including Fe, Mn, Cu, Ni, Co, Cd, Zn, and Hg. Excessing some of these metals can affect plant growth, metabolism, physiology, and aging, although they are essential micronutrients that support many regular processes in plants (Peng and Shahidi, 2021).

Heavy metal stress in plants causes the formation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), leading to the oxidation of proteins, lipids, and nucleic acids in the cell and peroxidation in cell membranes, protein denaturation and oxidative changes in the structure of DNA (Shoaib and Javaid, 2021). Due to its high mobility and bioavailability, the accumulation of Cd

in plants poses a significant threat to the ecosystem. Cadmium is mainly found in soils bound to solid phases, and it is rapidly released into the soil and becomes available for plant uptake (Ondrasek et al., 2020). Cadmium is released into the environment by the Zn, Pb, and Cu industries, used in phosphate fertilizers, urban composting, wastewater irrigation, and metal processing industries. It is one of the most toxic and major environmental pollutants in the world and represents a serious problem in agriculture due to its detrimental effects on crops. When plants grow in soils contaminated with Cd, their roots uptake the heavy metal, accumulate in various organs, and ultimately reduce plant growth (Bruno et al., 2017). Cadmium toxicity induces oxidative stress, damages cell membranes and electron transport, inhibits enzymes, and impairs nucleic acids, photosynthesis, and growth (Lu et al., 2013; Waheed et al., 2022).

Various methods are available for treating soil contaminated with heavy metals. Physical, chemical, and biological processes can be used to remove and control pollutants. Although these methods are effective, they have negative aspects such as high cost, long time, and environmental damage. Phytoremediation, which has recently been widely used due to its low cost, is a method for removing pollutants from contaminated environments. This technique involves growing hyperaccumulator plants in areas contaminated with heavy metals (Laghlimi et al., 2015; Karakas et al., 2021). Phytoextraction is a soil treatment method classified under phytoremediation. It removes heavy metals that cause soil and water pollution from the environment by storing the pollutants in their roots, stems, or leaves using hyperaccumulator plants (Dağhan et al., 2012; Aybar ve ark., 2023).

The mustard plant (*Brassica juncea*) is considered an effective species for the phytoremediation of soils contaminated with heavy metals such as cadmium (Cd). Research has shown that mustard plants can absorb Cd through their roots and accumulate it in their upper parts, such as leaves and stems. This characteristic enhances their potential for cleaning Cd-contaminated soils. In particular, their high Cd tolerance and substantial biomass make them a preferred species in phytoremediation studies (Doe ve Smith, 2023).

This study aimed to evaluate the plant's phytoremediation capacity by exposing *Brassica juncea* to different concentrations of cadmium (0 [control], 25, 50, 100, 200, and 300 ppm), a significant heavy metal pollutant. Additionally, it assessed the plant's growth, physiological and biochemical responses, tolerance levels, and phytoextraction potential in leaves and roots**.**

MATERIALS AND METHODS

Experimental design

The mustard plants were grown with different doses of Cd (0.0 (control)-, 25-, 50-, 100-, 200-, and 300 ppm) as $CdNO_3$ in 8 L pots or a randomized block design with 5 replicates under greenhouse conditions. The pots were filled with air-dry soil (clay texture), and mustard plant seeds were sown and thinned, leaving 10 plants in a pot. After four weeks, the pots were irrigated three times per week at pot capacity with irrigation water containing Cd at different doses. The mustard plants were harvested after a 12-week growing period. Physiological and biochemical analyses were made to determine the responses and tolerance of mustard plants. The Cd content of the leaves and roots was also determined to evaluate the phytoextraction potential of the plant (Figure 1).

Figure 1. The mustard plant growth with different Cd doses.

Physiological parameters

After harvest, physiological measurements (plant length, shoot fresh and dry weights, and root fresh and dry weights) were taken on plants. Plant length was measured from the soil surface to the top of the plant shoot. Shoot and root fresh weights were determined using a sensitive scale. When the samples were dried in an oven at 70 ° C until constant weight and dry weights were determined.

Biochemical parameters

Mustard plant chlorophyll-a (Chl *a*) and chlorophyll-b (Chl *b*) contents were determined according to Arnon (1949). Carotenoids were determined via the method suggested by Rajput and Patil (2017) Fresh leaf samples (0.5 g) were homogenized in 10 mL 80% acetone: water (80:20, v:v), then filtered were read for Chl *a*, Chl *b*, and

carotenoid contents at 663, 645, 480, and 510 nm, respectively in a UV microplate spectrophotometer (Epoch, SN: 1611187, manufactured in the USA). The results were calculated mg g^{-1} Fwt.

Proline content was determined according to the method of Bates et al. (1973). Fresh leaf tissue (0.5 g) was homogenized in 3% w/v sulfosalicylic acid and the homogenate was filtered through Whatman No. 1 filter paper. Then, 2 mL of filtrate, 2 mL of acid-ninhydrin reagent (1.25 g of ninhydrin in 30 mL of glacial acetic acid, and 20 mL of 6 mol L^{-1} phosphoric acid) were mixed in a tube and boiled at 100 °C for 1 hour. The mixture was completed in an ice bath. Then 5 ml of toluene was added to the mixture. The solution was then shaken thoroughly for 20 seconds and then left at room temperature for 20 minutes to achieve a two-layer separation. Then the absorbance of the solution was read at 515 nm using a toluene blank. L-proline prepared in different toluene concentrations was used for a standard curve. The results were reported as mol g^{-1} Fwt.

MDA contents were measured using the method of Sairam and Saxena (2000). A fresh leaf sample (0.5 g) was homogenized with 0.1% (w/v) trichloroacetic acid (TCA). After the homogenate was centrifuged at 10,000 g for 5 min, 1 mL of the supernatant was mixed with 4 mL of 20% v/v TCA containing 0.5% v/v thiobarbituric acid (TBA). The mixture was heated in boiling water for 30 minutes, after which the process was stopped by immersing the tubes in an ice bath. The mixture was measured at 532 and 600 nm.

Hydrogen peroxide (H_2O_2) content was determined using the method of Velikova et al. (2000). Fresh leaf tissue (0.5 g) was extracted with 5 mL of 0.1% (W: V) trichloroacetic acid (TCA) and centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant (0.5 ml) was added to 0.5 ml of 10 mmol L^{-1} potassium phosphate buffer (pH 7.0) and 1 ml of 1 mol L^{-1} potassium iodide. The reaction was measured at 390 nm in a UV microplate spectrophotometer (Epoch, SN: 1611187, USA). The H_2O_2 content was expressed in mol g^{-1} Fwt.

Peroxidase (POX) enzyme activity (E.C.1.11.1.7) was determined using the method of Cvikrova et al. (1994). Fresh leaf samples (0.5 g) were homogenized in 10 mL of 50 mmol L^{-1} Na-phosphate buffer solution (pH 7.0). Then, 10 µL of the supernatant was added to 290 µL of the reaction mixture containing 5 mmol L^{-1} H_2O_2, 13 mmol L^{-1} guaiacol, and 50 mmol of L^{-1} Na-phosphate. Thereafter, the oxidation of guaiacol was carried out by increasing the absorbance at 470 nm using a UV microplate spectrophotometer (Epoch, SN: 1611187, manufactured in the USA) at intervals of 1 to 3 minutes. One unit of POX enzyme activity is defined as the activity that results in an increase in absorbance of 0.1 units per minute at 25°C. The activity is expressed as enzyme unit g^{-1} Fwt.

Catalase (CAT) enzyme activity (EC 1.11.1.6) was determined according to the method of Aebi (1984). For analysis, 5 µL of the homogenate (as obtained above) was added to 300 µl of the reaction mixture containing 50 mmol L^{-1} Na-phosphate buffer, 10 mmol L^{-1} H_2O_2, and 4 mmol L^{-1} Na_2EDTA. Reading with a UV microplate spectrophotometer (Epoch, SN: 1611187, USA) at 240 nm for 30 s. One CAT unit (U) was defined as a 0.1 increase in absorbance at 240 nm. The activity is expressed as enzyme unit g^{-1} Fwt.

The Cd content in leaves and roots was determined according to Kacar and Inal's method (2004). Inductively coupled plasma (ICP, Perkin Elmer) was used to measure the extract obtained after filtration.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) (version 26.0) using Duncan's SPSS software package. Duncan's Multiple Range Test was used to differentiate the treatment means for each measured parameter at a significance level of $P \leq 0.05$. Correlation Hierarchical cluster analysis (HCA) and Heatmap of Pearson's Coefficient (r) heatmap were also used to observe the relation's parameters.

RESULTS AND DISCUSSION

Impact of Cd application on physiological parameters of the mustard plant

Cd was not applied to control plants, and their physiological development was not negatively affected. However, as Cd dose applications increased, signs of stress became evident in the plants, which was reflected in their physiological development. Cd applications at 25, 50, 100, 200, and 300 ppm Cd doses reduced plant length by (6.3%, 10.2%, 14.1%, 22.7%, and 32.8%), plant Fwt by (21.8%, 41.6%, 47.2%, 53.9%, and 61.2%,) and the root Fwt by (29.3%, 34.9%, 40.0%, 40.5%, and 46.3%), respectively compared to a control (0.0 ppm Cd). The lowest plant and root DW were found at a Cd dose of 300 ppm to be 0.6 g $plant^{-1}$ and 0.3 g $plant^{-1}$, respectively (Table 1).

We found that Cd stress negatively affected the physiological growth of mustard plants. Cd toxicity reduces root development in plants and causes growth recession **(**Waheed ve ark., 2022**).** The study, we determined that increasing Cd levels inhibited root development and restricted overall plant growth. The same results were found for *Eruca sativa* (Waheed et al., 2022), *Lantana camara* (Liu et al. 2019), and soybean (Xue et al., 2013).

Impact of Cd application on biochemical parameters of the mustard plant

In plants, responses of stress markers such as biochemical parameters chlorophyll (Chl *a* and Chl *b*), carotenoid, proline, MDA, H_2O_2, POX, and CAT antioxidant enzymes were determined in the harvested mustard plant. Cadmium stress causes a decrease in chlorophyll levels in plants by suppressing chlorophyll synthesis, increasing its degradation, and inducing oxidative stress. This leads to chlorosis, reduced photosynthetic capacity, and growth retardation. The study showed that Cd stress significantly decreased Chl a, Chl b, and carotenoid levels with a Cd dose of 100 ppm. The highest reductions in Chl a, Chl b, and carotenoid contents were 48.4%, 50.0%, and 30.1%,

respectively, at 300 ppm Cd (P≤0.05, Figure 1). The content of chlorophyll a, chlorophyll b, and carotenoids decreased with increasing Cd dose. Numerous studies have reported the reduction of chlorophyll and carotenoid under Cd stress in barley, tomato, maize, *Lepidium sativum*, *Gossipium hirsutum*, strawberry, and *Carpobrotus acinaciformis* (Vassilev et al., 2002; Ammar et al., 2008; Ekmekci et al., 2008; Gill et al., 2012; Karanlık et al., 2013; Muradoglu et al., 2015; (Karakas et al., 2021).

Table 1. Physiological properties of the mustard plant at different Cd doses.

Cd Doses (ppm)	plant length (cm plant^{-1})	Plant Fwt (g plant^{-1})	Plant DW (g plant^{-1})	Root Fwt (g plant^{-1})	Root DW (g plant^{-1})
Control (0.0)	25.6±0.9a	16.5±0.71a	1.6±0.06a	4.7±0.14a	0.5±0.02a
25	24.0±0.4a	12.7±0.47b	1.2±0.04b	3.3±0.08b	0.4±0.01b
50	23.0±0.3b	9.6±0.39c	1.1±0.02c	3.0±0.04c	0.4±0.02c
100	22.0±0.5c	8.68±0.58d	1.0±0.02d	2.8±0.06d	0.3±0.01d
200	19.8±0.9d	7.6±0.20d	0.9±0.03d	2.8±0.04d	0.3±0.01d
300	17.2±0.6e	6.4±0.30e	0.6±0.01e	2.5±0.02e	0.3±0.01e

*Different letters (a,b,c,d, and e) indicate different means in the same column. P<0.05

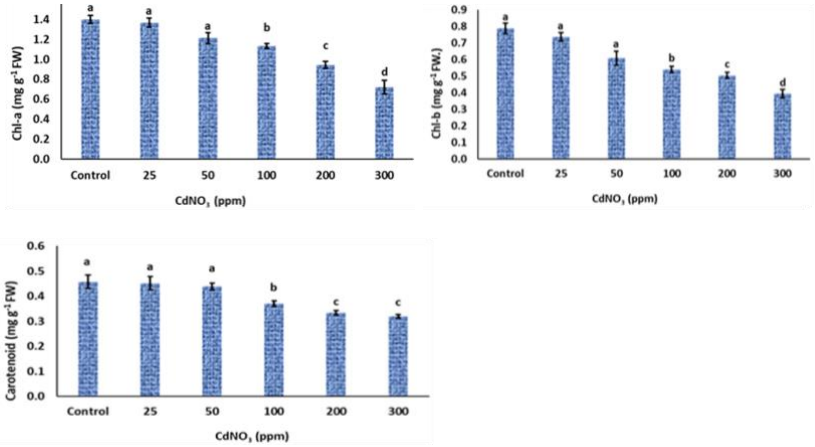

Figure 1. Chl-a, Chl-b, and carotenoid contents in the mustard plant at Cd applications.

The proline content increased significantly at a Cd concentration of 100 ppm. However, no significant increase in proline content was observed at Cd doses of 200 and 300 ppm. The MDA and H_2O_2 contents increased significantly with increasing Cd doses, 12 and 10 times, respectively, at 300 ppm Cd applications (P≤0.05, Figure 2).

In our study, increasing Cd stress resulted in increased proline, MDA, and H_2O_2 concentrations in the mustard plant. Similar findings were seen in other studies such as strawberry (Doğan et al., 2022), *Arachis hypogaea* (Dinakar et al., 2008), *Lantana camara* (Liu et al., 2019) under Cd conditions.

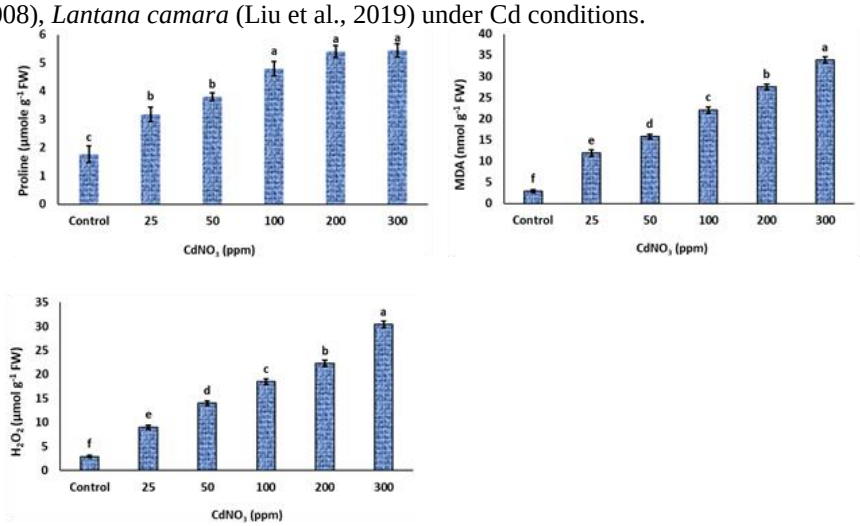

Figure 2. Proline, MDA, and H_2O_2 contents in the mustard plant at Cd applications.

POX and CAT increased significantly with increasing Cd exposure. The content of antioxidant enzymes POX and CAT were found to be 13 and 14 times higher, respectively, at the highest dose of 300 ppm Cd compared to the control plant. (P≤0.05, Figure 3).

Cd stress causes oxidative stress in plants. The effect of this stress is caused by radical oxygen species (ROS). Plant cells can be protected from the harmful effects of ROS by antioxidant enzymes (Lakhdar et al., 2010). Catalase antioxidant enzyme plays an important role in the control of hydrogen peroxide, one of the ROS types caused by Cd in the environment in the plant (Martins et al., 2011). Boysan Canal et al. (2018) determined that Cd application increased CAT enzyme activity in lettuce plants due to the effect of stress. Yu et al., (2013) stated that increasing Cd applications increased CAT enzyme activity in rice (*Oryza sativa* L.) plants. Similar findings were obtained in this study.

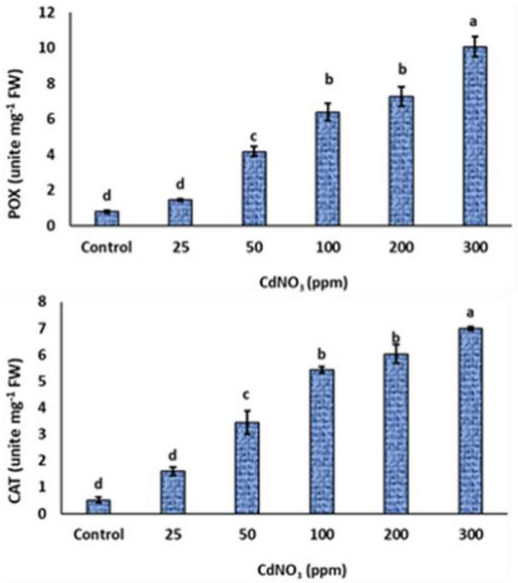

Figure 3. POX and CAT antioxidants enzymes in the mustard plant at Cd applications.

The Cd content increased with increasing Cd content in leaves and roots. The highest amount of Cd was found in leaves and roots at 53.78 and 67.71 mg kg^{-1} DW, respectively, when mustard plants were exposed to 300 ppm Cd (P≤0.05, Figure 4).

Cd hyperaccumulation refers to plant species that are capable of accumulating more than 100 mg kg^{-1} Cd DW in plants (Baker et al., 2000). The mustard plant can be used as a phytoremediation plant in Cd-polluted soils as a hyperaccumulator plant because it accumulates large amounts of Cd in its leaves and roots.

Heavy metal ions can accumulate in the roots, leaves, and stems of the plant or be excreted from the leaves through transpiration (Ximenez et al., 2002). The roots of the plant act as a barrier to heavy metal transport and could be a potential tolerance mechanism operating in the roots (Bonnet et al., 2010). It has been reported that increased Cd content leads to an increase in the amount of Cd in the leaves and roots, and a large part of the Cd absorbed by the plant is stored in the roots while a very small part is transported to the green parts of the plant (Tiryakioglu et al., 2006). Cd hyperaccumulation refers to plant species that can accumulate more than 100 mg kg^{-1} Cd in dry weight in plant parts (leaves and stems) (Baker et al., 2000). In this study, the mustard plant is a good hyperaccumulator plant for the toxic Cd element by accumulating Cd in its leaves and roots.

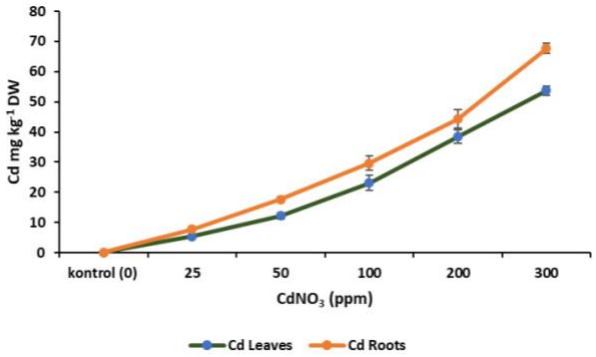

Figure 4. Leaves and roots Cd content in mustard plants with Cd applications.

Hierarchical cluster analysis (HCA) and Heatmap of Pearson's Correlation Coefficient (r) the heatmap were performed using physiological, biochemical, and Cd accumulation determinations (Figure 5). There were negative correlations between Cd content and biochemical components such as chlorophyll and carotenoid while there were negative correlations with stress parameters such as plant weight, proline, MDA, Cd accumulation in roots and leaves, and H_2O_2 suggesting that Cd stress triggers oxidative damage in plant cells. The positive correlation between Cd content and POX and CAT reflects the potential of plants to combat Cd stress. These analyses help us understand the effects of Cd on mustard and the plant's responses

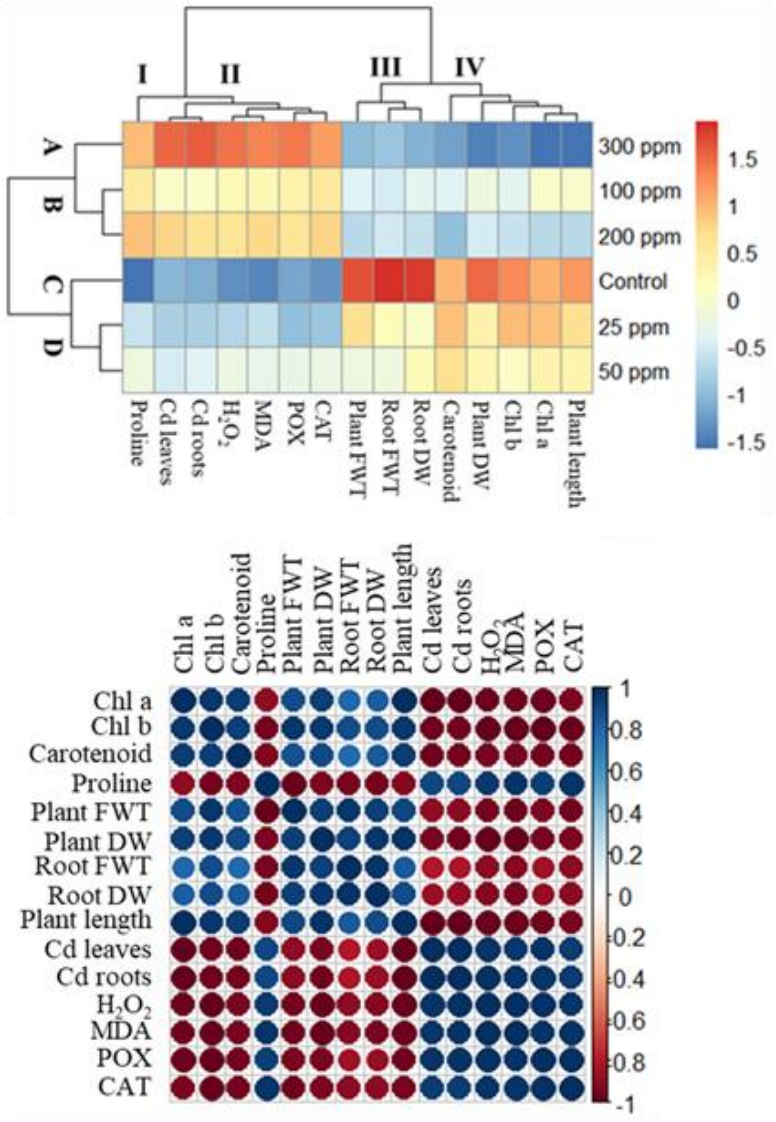

Figure 5. Hierarchical clustering analysis (HCA) and Heatmap of Pearson's Correlation Coefficient (r) showing the scores for physiological, biochemical, and Cd accumulation in mustard plants grown under different Cd levels (control, 25-, 50-, 100-, 200-, and 300 ppm).

CONCLUSION

This study demonstrated that the mustard plant (*Brassica juncea* L.) exhibited strong potential for the phytoremediation of Cd-contaminated soils, with significant relationships observed between cadmium exposure and key physiological parameters. Among these, chlorophyll content, antioxidant enzyme activity, and biomass accumulation emerged as critical indicators for tolerance and phytoextraction efficiency. The plant accumulated Cd in its leaves and roots, leading to decreases in Fwt, DW, and chlorophyll content compared to the control plants. However, despite exposure to high Cd levels, the plant survived, highlighting its tolerance to cadmium stress and ability to withstand harsh environmental conditions. In response to Cd-induced oxidative stress, proline, MDA, and H_2O_2 levels increased while the activities of antioxidant enzymes such as CAT and POX were elevated, suggesting an adaptive defense mechanism against ROS. The significant accumulation of Cd in mustard leaves and roots further emphasized that the plant has a strong phytoremediation potential. By effectively accumulating

Cd from the soil while maintaining its tolerance to this heavy metal, the mustard plant emerged as a promising candidate for the remediation of Cd-polluted lands, offering an eco-friendly and sustainable method of soil detoxification. Our future study, therefore, will focus on optimizing the growth conditions of the plant to enhance Cd uptake, exploring the molecular mechanisms underlying metal tolerance, and investigating genetic modification changes to improve phytoremediation efficiency further. The cultivation of Mustard and the improvement of methods such as the use of amino acids, plant growth regulators, and signaling molecules are also on our agenda.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The author has no conflict of interest to declare.

Author contribution

Conceptualization, R.A., S.K., M.D., and F.U.; methodology, S.K., R.A.; software, S.K., and F.U; validation, R.A., S.K., F.U., and M.D.; formal analysis, R.A., and S.K.; investigation, R.A., and S.K.; resources, R.A., and S.K.; writing original draft preparation, S.K.; writing review and editing, R.A., F.U., and M.D., supervision, S.K.; project administration, S.K. All authors have read and agreed to the last version of the manuscript.".

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