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Investigating the growth characteristics, oxidative stress, and metal absorption of chickpea (*Cicer arietinum* L.) under cadmium stress and *in silico* features of HMAs proteins

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Investigating the growth characteristics, oxidative stress, and metal absorption of chickpea (*Cicer arietinum* L.) under cadmium stress and *in silico* features of HMAs proteins

Abstract: Heavy metal contamination can have a strong effect on the morphological and physiological characteristics of plants. In the present study, *Cicer arietinum* L. (chickpea) was exposed to different concentrations of cadmium (control, 2, 4, 8 µg Cd g^{-1} perlite) and the effect on plant growth and antioxidant enzymes were evaluated. The observed morphological changes in chickpea plant included stunted growth, reduced root system development and plant color change. A significant increase in enzyme activity of peroxidase, superoxide dismutase, catalase, and ascorbate peroxidase was observed at 4 µg Cd g^{-1} perlite, with a subsequent decrease when concentration was increased to 8 µg Cd g^{-1} perlite in the leaves of the plants. The highest cadmium levels were determined at a concentration of 8 µg Cd g^{-1} perlite. With the addition of 2 µg Cd g^{-1} perlite, manganese uptake in the aboveground part of the plant increased significantly, but then decrease at higher cadmium concentrations. In addition, zinc and copper levels decrease in the presence of cadmium. These results indicate that chickpea has a relatively high adsorption capacity for cadmium in aboveground tissues and special precautions should be taken when growing chickpea. *In silico* analysis led to the identification of 13 heavy metal ATPases (HMAs) in chickpea. These proteins contain 130 to 1032 amino acids with 3 to 18 exons. They are involved in the transfer of cadmium and zinc and help in heavy metal detoxification of plants. Bioinformatics studies have been conducted to better understand the mechanism by which the plant is able to combat heavy metal stress.

Key words: cadmium, chickpea, HMAs, oxidative stress

Preučevanje rastnih značilnosti, oksidativnega stresa in prevzema kovin pri čičerki (*Cicer arietinum* L.) v razmerah kadmijevega stresa in *in silico* lastnosti HMAs proteinov

Izvleček: Onesnaženje s težkimi kovinami ima lahko močan učinek na morfološke in fiziološke lastnosti rastlin. V raziskavi je bila čičerka (*Cicer arietinum* L.) izpostavljena različnim koncentracijam kadmija (kontrola, 2, 4, 8 µg Cd g^{-1} perlita). Ovrednoteni so bili učinki na rast rastlin in na antioksidacijske encime. Opažene morfološke spremembe čičerke so bile zavrta rast, zmanjšan razvoj koreninskega sistema in spremembe v barvi rastlin. Značilna porast aktivnosti encimov peroksidaze, superoksid dismutaze, katalaze in askorbat peroksidaze je bila opažena pri 4 µg Cd g^{-1} perlita s posledičnim upadom, ko se je koncentracija povečala na 8 µg Cd g^{-1}perlita v listih tretiranih rastlin. Največja vsebnost kadmija je bila določena pri obravnavanju z 8 µg Cd g^{-1} perlita. Pri dodatku 2 µg Cd g^{-1} perlita se je privzem mangana v nadzemnih delih rastlin značilno povečal a se je pri večjih koncentracijah kadmija zmanjšal. Dodatno so se v prisotnosti kadmija vsebnosti cinka in bakra zmanjševale. Ti izsledki kažejo, da ima čičerka relativno veliko sposobnost privzema kadmija v nadzemna tkiva in moramo na to biti pozorni, če jo gojimo v s kadmijem onesnaženem okolju. *In silico* analize so vodile k prepoznavanju 13 ATPaz (HMAs), povezanih s težkimi kovinami. Ti proteini vsebujejo 130 do 1032 amino kislin s 3 do 18 eksoni. Vključeni so v prenos kadmija in cinka in pomogajo v rastlinah pri detoksikaciji težkih kovin. Za bolje razumevanje mehanizmov s katerimi rastline premagujejo stres težkih kovin so bile izvedene tudi bioinformacijske raziskave.

Ključne besede: kadmij, čičerka, HMAs, oksidativni stres

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1 INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a major legume crop that is consumed globally especially on the Africa and Asia continents (Kaur et al., 2022). Chickpea has a very high nutritional content and is one of the cheapest sources of protein and an important source of minerals (manganese, molybdenum, phosphorus and potassium) and vitamins (Mohanty et al., 2022), so measures need to be taken to avoid its contamination with heavy metals such as cadmium.

Cadmium (Cd) is one of the most important contaminants due to its high toxicity and high water solubility and is readily absorbed by the root system of many plants (Zulfiqar et al., 2022). High levels of Cd can have detrimental effects on plant physiological and biochemical processes, leading to reduced growth, impaired nutrient uptake, and disruption of cellular functions. Moreover, Cd toxicity inhibits plant growth by affecting cell division, cell elongation, and differentiation processes (Tuver et al., 2022). It disrupts hormone balance, leading to stunted root and shoot growth, reduced biomass production, and impaired reproductive development. Cd toxicity can interfere with the uptake and transport of essential nutrients such as iron, calcium, magnesium, and zinc (Zhou et al., 2022). It can bind to transporters, enzymes, and carrier proteins, thereby disrupting nutrient homeostasis and causing nutrient deficiencies. Furthermore, Cd toxicity negatively impacts photosynthesis, reducing the efficiency of light absorption, electron transport, and carbon assimilation (Zulfiqar et al., 2022).

Plants have evolved several mechanisms to mitigate the toxic effects of cadmium (Cd) and minimize its accumulation in their tissues. One crucial strategy is the sequestration of cadmium into vacuoles, which serves as a storage site for toxic metals (Jogawat et al., 2021). On the other hand, Cd toxicity leads to the generation of reactive oxygen species (ROS) in plant cells, causing oxidative stress (Zhang et al., 2019). Antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), scavenge and neutralize ROS, protecting cellular components from oxidative damage (Faria et al., 2022). Moreover, Plants possess transporters that can efflux Cd ions from the cytoplasm to the extracellular space or restrict their entry into specific tissues. ATP-binding cassette (ABC) transporters and heavy metal ATPases (HMAs) are involved in Cd transport across cell membranes. These transporters play a crucial role in minimizing the accumulation of Cd in sensitive tissues and facilitating its sequestration (Tian et al., 2023).

HMAs belong to the P-type ATPase superfamily and are localized in the plasma membrane or tonoplast (vacuolar membrane) of plant cells. HMAs play a crucial role in the detoxification of cadmium by actively transporting it out of sensitive cellular compartments or sequestering it into vacuoles. This process contributes to reducing the concentration of free cadmium in the cytoplasm and minimizing its toxic effects on plant growth and development. HMAs function as efflux pumps, actively transporting Cd ions out of the cytoplasm and extruding them from the cell or into specific compartments, such as the vacuole (Fang et al., 2016). By pumping Cd out of sensitive cellular regions, HMAs reduce the concentration of free cadmium in the cytoplasm and minimize its toxic effects on cellular processes (Satoh-Nagasawa et al., 2012). HMAs participate in the regulation of metal ion homeostasis in plants. They are involved in maintaining the balance between essential metals (such as zinc and copper) and non-essential heavy metals (such as Cd) (Fang et al., 2016). This regulation ensures that essential metals are properly acquired and utilized while minimizing the uptake and accumulation of toxic metals like Cd. HMAs interact with metal chelators, such as phytochelatins (PCs), which are small peptides that bind to heavy metal ions, including Cd. This process contributes to the detoxification and sequestration of cadmium in less sensitive cellular compartments (Tian et al., 2023).

The purpose of the study is to get insights how chickpea plants respond to cadmium, a harmful heavy metal that can contaminate soil and negatively affect plant health .Understanding the mechanisms of Cd toxicity in plants is crucial for developing strategies to mitigate its adverse effects. The first objective is therefore to examined the impact of Cd on the growth characteristics, activity of oxidative enzymes, Cd, zinc (Zn), copper (Cu) and manganese (Mn) content in chickpea.

The next objective of this study was to gain a better understanding of the role that HMAs play in chickpea, particularly under conditions of cadmium stress and to provide insights into how chickpea plants respond to cadmium by Bioinformatics analyses such as number of genes, proteins, gene loci, cellular location, phylogenetic relationship, three-dimensional protein structure, conserved domains, similar template and catalytic site.

2 MATERIALS AND METHOD

2.1 PROPAGATION AND CADMIUM EXPOSURE

Chickpea (*Cicer arietinum* L.) seeds were germinated in sterilized Cucupite and Perlite in a greenhouse on the photoperiod of 8 h light and 16 h darkness. Seedlings with leaves were planted in pots (diameter, 12 cm and height 15 cm) under controlled conditions and watered

with distilled water every 3 days. Cadmium chloride were added in four concentrations (control, 2, 4, and 8 µg Cd g^{-1} perlite) calculated per g of perlite. Plants were watered with Hoagland nutrient solution (Hoagland and Snyder 1933) without cadmium chloride (field capacity was considered). After 10 days of Cd treatment plants were harvested for further investigations.

2.2 GROWTH PARAMETERS

The fresh and dry mass of the roots and above ground parts were determined (mg). Plantlet height, leaf area, root length, shoot length and internode length were measured. Stomatal densities on the lower and upper epidermis were evaluated.

2.3 ENZYME ASSAYS

Enzyme extracts were prepared from fresh chickpea leaves (1 g) with phosphate potassium buffer (5 ml). Homogenous samples were prepared by pulverizing followed by centrifugation (4 °C, 25 min, 15000 rpm) and storage at -80 °C. Catalase enzyme activity was determined by mixing phosphate buffer (2.5 ml, pH 7.5) and hydrogen peroxide (1%, 0.1 ml) in an ice bath and the addition of enzyme extract (0.1 ml) and the rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240nm via spectrophotometer.

Peroxidase enzyme activity was determined based on the method by Koroi (1989). The reaction mixture consisted of acetate buffer (0.2 M, 2 ml, pH 5), benzidine (0.02 M, 100 ml), hydrogen peroxide (3 %, 200 µl) and enzyme extract (25 µl). The absorption was determined at 530 nm. Ascorbate peroxidase (EC11.1.11.1) activity was determined spectrophotometrically (Nakano and Asada, 1987). To the enzyme extract (100 µl) was added K_2HPO_4 (0.5 M, 2.5 ml), ascorbate (0.5 mM, 0.1 ml), EDTA (0.1 mM, 0.1 ml) and H_2O_2 (1 %, 0.2 ml) and the absorbance read at 290 nm. Specific enzyme activity was reported as units/g fresh mass (Nakano and Asada 1987). Total soluble protein was determined utilizing the Bradford assay with bovine serum albumin (BSA) as standard. The absorbance was read at 595 nm (Bradford, 1976).

2.4 CADMIUM AND OTHER ELEMENTS MEASUREMENT

Plant samples were oven dried (72 h, 60 °C) and the dry mass determined. Dried samples were ashed (550 °C, 8 h). The digested extract (1N HCl, 1 mL; nitric acid, 97 %, 1 ml, 1 h) was made to a final volume of 20 ml and the cadmium, zinc, copper and manganese content of the samples measured (Chellaiah, 2018) utilizing a Flame Atomic Absorption Spectrometer (GBC, SAVANTAA scientific equipment, Australia) which has a detection limit of 0.007 µg ml^{-1}. Cd (II), Zn (II), Cu (II) and Mn (II) standard solution were prepared using their nitrate salts in nitric acid. Bioconcentration factor (BCF) computed as heavy metal accumulated in each plant tissue to that dissolved in the soil medium (Bose and Bhattacharyya 2008).

Root bioconcentration factor: BCF = root/soil
Shoot bioconcentration factor: BCF = shoot/soil
TF = BCFshoot/BCFroot

2.5 BIOINFORMATICS ANALYSIS

The gene database of NCBI was searched utilizing the keyword „HMA". Gene characteristics included location, exon count and conserved domain. Protein sequences were used for localization prediction from the Localizer and protein tertiary structure predicted by Phyre2. Potential tunnels within each protein and catalytic pocket were predicted utilizing CAVER Web. The Jones-Taylor Thornton model was selected to obtain the phylogenies tree of HMAs from chickpea and *Arabidopsis* using the neighbor-joining (NJ) method, with a bootstrap test performed using 1000 iterations in MEGA5 (Tamura et al., 2007). Multiple sequence alignments were performed utilizing the muscle algorithm of mega 7 software to detect conserved residues (Kumar et al., 2016). HMAs from *Arabidopsis* were highlighted in green. Some information has been mentioned below:

XP_004509102.1: Probable cadmium/zinc-transporting ATPase HMA1, chloroplastic [*Cicer arietinum*], P_004487939: Cadmium/zinc-transporting ATPase HMA3-like isoform X1 [*Cicer arietinum*], XP_027189340: Cadmium/zinc-transporting ATPase HMA2-like isoform X2 [*Cicer arietinum*], XP_012573401: Putative inactive cadmium/zinc-transporting ATPase HMA3 [*Cicer arietinum*], XP_004488108: Cadmium/zinc-transporting ATPase HMA3-like [*Cicer arietinum*], XP_012573132: Copper-transporting ATPase HMA4-like [*Cicer arietinum*], XP_012574029: Copper-transporting ATPase HMA4-like isoform X1 [Cicer arietinum], XP_027192934: Copper-transporting ATPase HMA4-like isoform X2 [*Cicer arietinum*], XP_004500941: Cation-transporting ATPase HMA5-like [*Cicer arietinum*], XP_004511583: Probable copper-transporting ATPase HMA5 [*Cicer arietinum*], XP_004504792: Copper-transporting ATPase PAA1, chloroplastic [*Cicer arietinum*], XP_004504659:

Copper-transporting ATPase RAN1 [*Cicer arietinum*], XP_004501429: Copper-transporting ATPase PAA2, chloroplastic [*Cicer arietinum*].

Q9SH30 (Protein: Probable copper-transporting ATPase HMA5, Gene: HMA5, Organism: *Arabidopsis thaliana* (L.)Heynh., P0CW78 (Protein: Cadmium/zinc-transporting ATPase HMA3, Gene: HMA3, Organism: *Arabidopsis thaliana*, Q9SZW4 (Protein: Cadmium/zinc-transporting ATPase HMA2, Gene: HMA2, Organism: *Arabidopsis thaliana*, Q4L970 (Protein: Copper-exporting P-type ATPase, Gene: copA, Organism: *Staphylococcus haemolyticus* Schleifer & Kloos, 1975 *(*strain *JCSC1435*), O32220 (Protein: Copper-exporting P-type ATPase, Gene: copA, Organism: *Bacillus subtilis (Ehrenberg 1835)* Cohn 1872 *(*strain *168)*, Q9S7J8 (Protein: Copper-transporting ATPase RAN1, Gene: RAN1, Organism: *Arabidopsis thaliana*), Q9M3H5 (Protein: Probable cadmium/zinc-transporting ATPase HMA1, chloroplastic, Gene: HMA1, Organism: *Arabidopsis thaliana*).

2.6 STATISTICAL ANALYSES

Data analyses were performed using the SPSS 20 software package (SPSS Inc., Chicago, USA). All experimental data were presented as the mean ± SD. One-way ANOVA was used to test differences between various means followed by the post hoc Tukey test (homogeneity of variances and data normally distributed). The level of significance was set at $p < 0.05$ for all tests.

3 RESULTS

3.1 GROWTH CHARACTERISTICS IN THE ABOVEGROUND PARTS OF CHICKPEA SEEDLINGS AFFECTED BY CADMIUM

Observed morphological changes in chickpea seedlings exposed to cadmium included changes in plant length, coloration and leaf size. Results indicated that stem color changed to a bright green-yellow. Moreover, changes were observed in leaf color (yellow) due to cadmium exposure. There was a significant reduction in shoot and root length. Shorter and less dense roots were observed in the treated samples (Table 1). The fresh and dry mass of the shoots and roots in chickpea plants were also significantly affected by cadmium with the lowest seedling mass being observed at high cadmium concentrations. Plants treated with 2 µg Cd g^{-1} perlite had a decline in leaf area which was less than half that of the control. At cadmium levels of 2 µg Cd g^{-1} perlite, the length of the first internodes increased, whereas at higher concentrations, there was a decrease, while the length of the

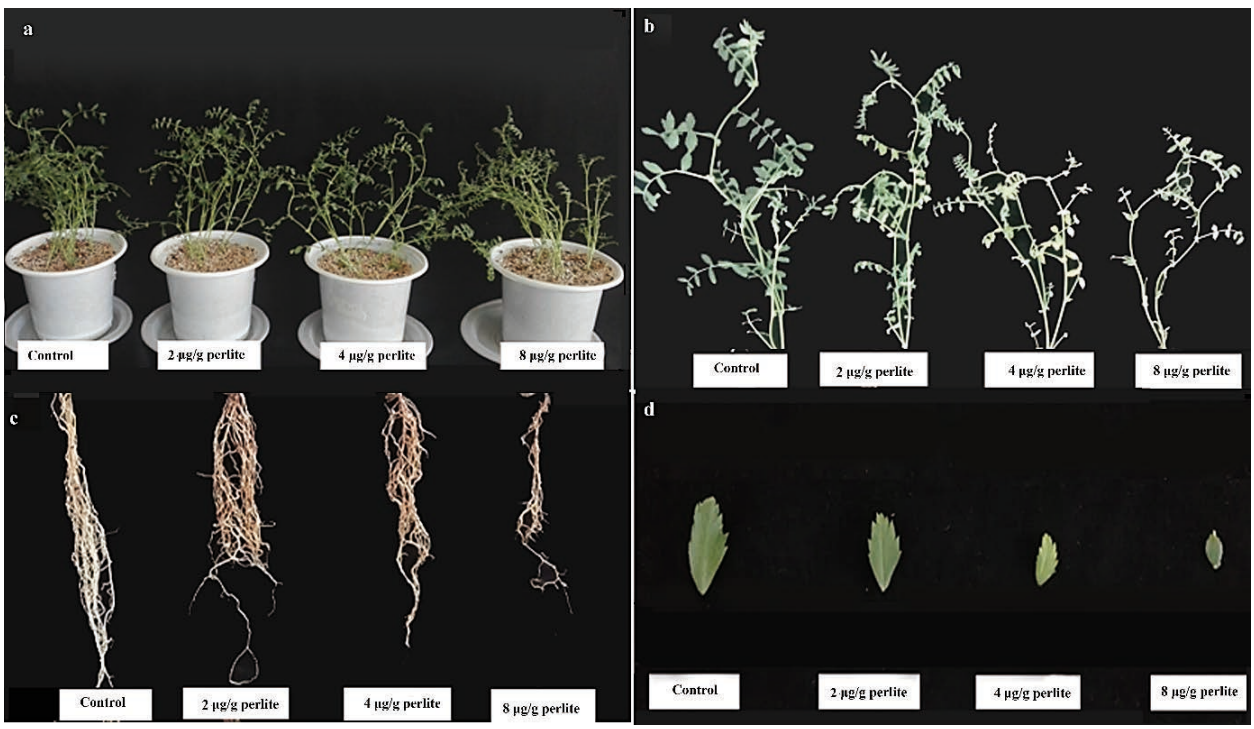

Fig. 1: Effect of cadmium on chickpea (*Cicer arietinum* L.) growth under normal and various concentrations of cadmium. a Seedlings, b Aboveground parts, c Roots, d Leaf areas (control, 2, 4 and 8 µg Cd g^{-1} perlite)

second internodes showed only a significant reduction at high concentrations of cadmium (Fig. 1, Table 1). Furthermore, with the addition of cadmium (4 µg Cd g^{-1} perlite), stomatal densities on the lower epidermis increased significantly but subsequently declined while higher concentrations of cadmium (Table1).

3.2 EFFECT OF CADMIUM ON SOD, POD AND CAT ACTIVITIES IN THE AERIAL PARTS OF CHICKPEA SEEDLINGS

Cadmium stress resulted in a significant increase in POD enzyme activity. The highest ascorbate activity was observed in cadmium treatments at 4 and 8 µg Cd g^{-1} perlite. Further increase in cadmium exposure resulted in a decline in POD activity which was however still significantly higher than that of the control and plantlets treated with 4 µg Cd g^{-1} perlite. The lowest enzyme activity was observed in the controls (Fig. 2a). SOD enzyme activity significantly increased in chickpea with the highest enzyme activity being observed in plantlets treated with 4 µg Cd g^{-1} perlite with the lowest enzyme activity being observed in the control (Fig. 2b). There was a significant increase in catalase enzyme activity. The highest catalase activity was also observed in plants treated with 4 µg Cd g^{-1} perlite with a subsequent decline when cadmium chloride concentration was increased to 8 µg Cd g^{-1} perlite. The lowest level of enzyme activity was observed in the control (Fig. 2c). Investigation of ascorbate peroxidase enzyme activity showed that this enzyme was also affected by cadmium exposure. The highest ascorbate activity was observed in cadmium treatments with 4 and 8 µg Cd g^{-1} perlite (Fig. 2d). Oxidative enzyme activity (SOD, APX or CAT) was shown to increase in the leaves of plants exposed to cadmium. Increased SOD activity is associated with an increase in the formation of superoxide, which activates gene expression by signal induction.

3.3 MEASUREMENT OF CADMIUM CONTENT AND ELEMENTAL CHANGES IN THE AERIAL PARTS OF CHICKPEA SEEDLINGS AFFECTED BY CADMIUM

The cadmium content in aerial parts of chickpea grown in different concentrations of cadmium chloride increased significantly. The highest concentrations were observed at cadmium chloride concentration of 8 µg Cd g^{-1} perlite. A doubling of cadmium accumulation was observed in the aerial parts of the plant when the cadmium content of the medium was increased from 2 to 4 µg Cd g^{-1} perlite (Fig. 3a). Moreover, elemental composition was significantly affected by cadmium levels (Fig. 3). Chickpea cultivated in cadmium-containing media showed a significant difference in the amount of manganese present in the aerial part of the plant.

With the addition of cadmium, manganese uptake increased significantly by approximately three times,

Table 1: Effect of Cd (Control, 2, 4 and 8 µg Cd g^{-1} perlite) on morphometric features in chickpea (*Cicer arietinum* L.) Values with different letters are significantly different at $p < 0.05$

Parameters	Control	2 µg Cd g^{-1} perlite	4 µg Cd g^{-1} perlite	8 µg Cd g^{-1} perlite
Plant length (cm)	62.76 ± 1.36^{a}	58 ± 0.0709^{a}	42.56 ± 1.78^{b}	37.93 ± 1.78^{b}
Shoot length (cm)	29.33 ± 0.66^{a}	25.65 ± 0.779^{b}	22.55 ± 1.35^{bc}	21.16 ± 1.092^{c}
Root length (cm)	35 ± 0.57^{a}	30.86 ± 0.69^{b}	18.56 ± 0.92^{c}	16.6 ± 0.83^{c}
Plant fresh mass (g)	4.0367 ± 0.043^{a}	3.442 ± 0.238^{b}	3.084 ± 0.169^{b}	1.715 ± 0.042^{c}
Shoot fresh mass (g)	2.291 ± 0.11^{a}	1.6317 ± 0.14^{b}	1.297 ± 0.061^{c}	0.682 ± 0.014^{d}
Root fresh mass (g)	2.24 ± 0.078^{a}	1.9 ± 0.1^{b}	1.3167 ± 0.109^{c}	0.99 ± 0.003^{d}
Shoot dry mass (g)	1.987 ± 0.01^{a}	1.4783 ± 0.11^{b}	1.0447 ± 0.029^{c}	0.606 ± 0.002^{d}
Root dry mass (g)	2.01 ± 0.04^{a}	1.696 ± 0.063^{b}	1.123 ± 0.069^{c}	0.823 ± 0.062^{d}
Leaf area (mm^2)	103.33 ± 1.76^{a}	48.33 ± 2.18^{b}	20.66 ± 0.666^{c}	18.33 ± 1.201^{c}
First internode length (cm)	1.1 ± 0.264^{b}	1.766 ± 0.0577^{a}	1.3 ± 0.3^{b}	0.833 ± 0.838^{c}
Second internode length (cm)	2.433 ± 0.513^{a}	2.266 ± 0.503^{a}	1.766 ± 0.808^{b}	1.633 ± 0.850^{b}
Stomatal densities on the upper epidermis	35 ± 0.545^{ab}	31 ± 0.564^{b}	24.33 ± 0.413^{c}	39.33 ± 0.633^{a}
Stomatal densities on the lower epidermis	29.6667 ± 0.448^{c}	39 ± 0.653^{b}	46 ± 0.765^{a}	37.33 ± 0.985^{b}

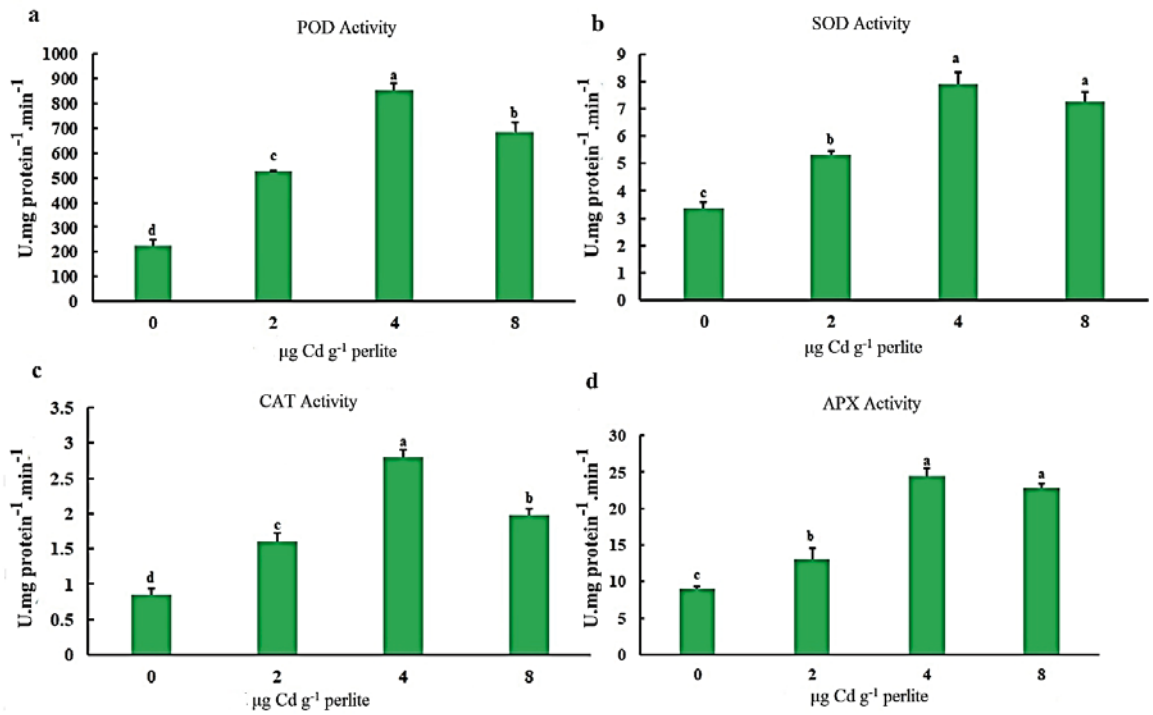

Fig. 2: The activities of a Peroxidase (POD), b Superoxide dismutase (SOD), c Catalase (CAT) and d Ascorbate peroxidase enzymes in aboveground parts of chickpea (*Cicer arietinum* L.). Values with different letters are statistically significantly different at $p < 0.05$ (One-way ANOVA, post hoc Tukey test)

while higher concentrations of cadmium reduced the amount of manganese in chickpea plants (Fig. 3b). Increase in the levels of cadmium in the culture also caused changes in the amount of zinc present in the aerial parts of pea plants. Increasing the levels of cadmium in the medium resulted in a decline in zinc (Fig. 3c). Increasing cadmium concentration, also decreased the levels of copper present in the aerial parts of chickpea seedlings. The lowest amount of copper was observed in high-cadmium seedlings (Fig. 3d). The BCF and TF values is greater than one at 8 µg Cd g^{-1} perlite (Fig. 3 e,f).

3.4 BIOINFORMATICS

In the current bioinformatics study of chickpea under cadmium stress, HMA proteins were chosen. *In silico* analysis of chickpea HMAs showed that of the 13 HMA identified, there were three proteins for each HMA3 and HMA4, two proteins for HMA5 and one protein for HMA 2, 6, 7, 8 (Table 2). The ATPase PAA2, chloroplastic, copper-transporting ATPase RAN1, and copper-transporting ATPase PAA1, chloroplastic identified in chickpea were identified as HMA6, HMA7, HMA8, in Arabidopsis, respectively. HMA7 and HMA8 all contribute to copper transport. The HMA 1, HMA 3, g HMA 2, HMA 4, HMA 5, PAA1, RAN1 and PAA2 genes are located on chromosomes 7, 1 and 7, 1, 6 and 7, 5 and 8, 6, 6, 5 respectively (Table 2). These proteins contain 130 to 1032 amino acids with 3 to 18 exons. The confidence level of predicting the three-dimensional structure of chickpea HMAs proteins is shown in Table 3. Their cellular locations are often in the nucleus and chloroplast. Using phyre2, their three-dimensional structure was determined. The protein templates and organisms used to predict the three-dimensional structure of these proteins are listed in Appendix 1. Among these templates, c3rfuC was used to predict all 13 proteins in a study related to copper-transporting PIB-type ATPase from the gram-negative bacterium *Legionella pneumophila* subsp. *pneumophila Brenner DJ,* Steigerwalt AG, McDade JE 1979. The patterns of c3j08A and c3j09A are also related to the p-type ATPase copper transporter CopA. Five (5) templates including copper-transporting proteins ATPase ATP7A, apoWLN5-6, domains 3 and 4 of human ATP7B, apo HMA domain of copper chaperone for superoxide dismutase and C2H2 type zinc finger (region 641-673) of human zinc finger protein 473 belong to humans. In total, the HMA studied in chickpeas were found to contain nine domains which are common in the 13 HMAs.

The COG4087 domain is listed as Soluble P-type ATPase and pfam00122 as E1-E2_ATPase are present

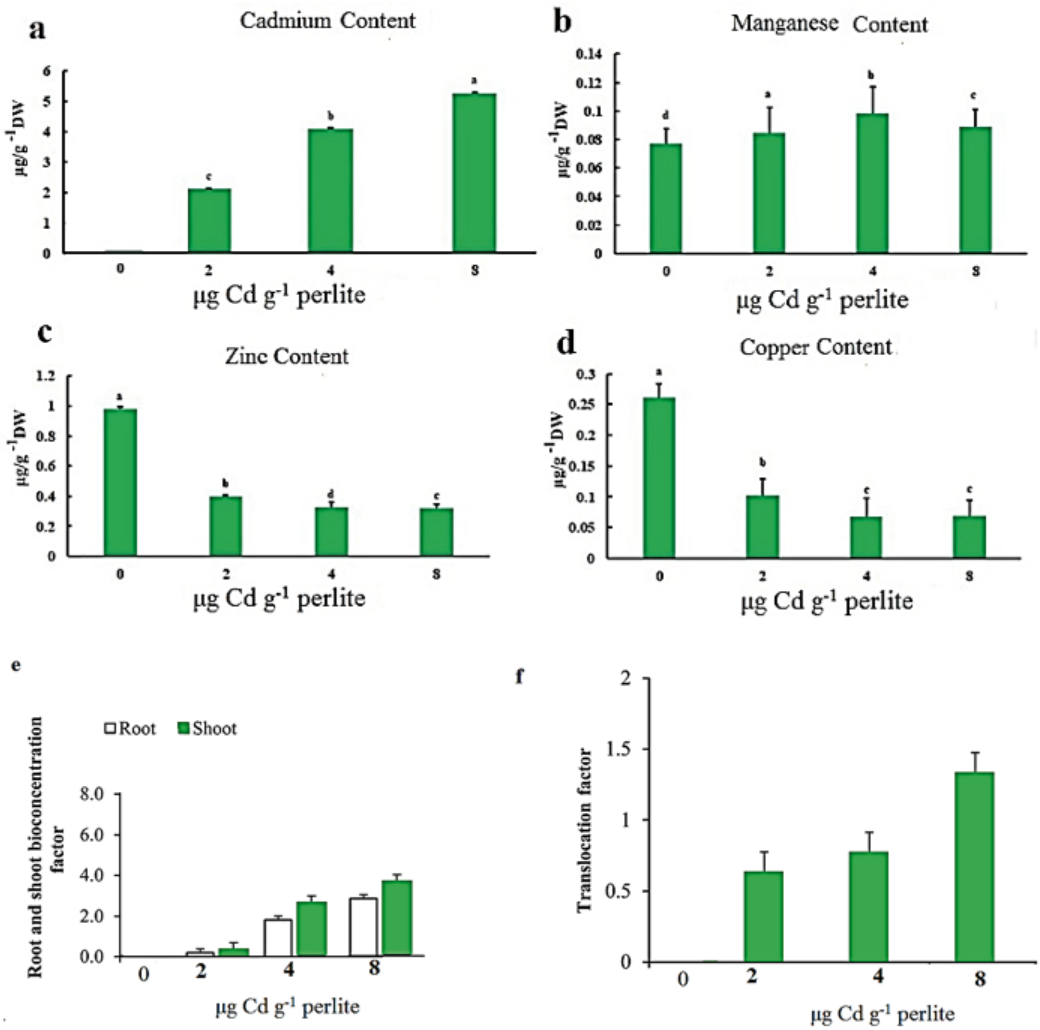

Fig. 3: Effects of different cadmium treatments on a Accumulation of cadmium, b Manganese, c Zinc, d Copper content in the aerial parts of chickpea seedlings after 10 days of cadmium treatment. Values with different letters are statistically significantly different at $p < 0.05$. (One-way ANOVA, post hoc Tukey test). Bioconcentration factor (e) and translocation factor (f). BCF values > 1 indicate that the concentration in the organism is greater than that of the medium. Translocation factor (TF) values more than one can be considered potential as Cd accumulators for phytoremediation. Mean plant tissues BCF are averages of five BCF values (n = 5) ± SEM

in ten HMAs. Following the prediction of the three-dimensional structure for chickpea HMAs, the longest tunnels for each protein and catalytic pocket predicted by CAVER Web for ion passing was determined. The longest and shortest tunnels predicted belonged to cadmium/zinc-transporting ATPase HMA3-like and cation-transporting ATPase HMA5-like, respectively. The putative inactive cadmium/zinc-transporting ATPase HMA3 was the largest HMA with 1032 amino acids and a short tunnel having a length of 41.7. No tunnel was predicted for copper-transporting ATPase PAA2, chloroplastic and copper-transporting ATPase PAA1, chloroplastic with 934 and 884 amino acids.

The three-dimensional structure with the longest predicted tunnel allowing for the passage of ions represented in color is illustrated in Fig 4. Based on the software used to analyze 8 of the 13 HMA chickpeas, the catalytic site was determined. From the proposed envelope for the HMAs the catalytic position for interaction with ions was determined. For XP_027192934, three catalytic sites with Asp residues at positions 522, 729, 733 with 40 % similarity over a specific reference of active site type and metal ion-binding site were identified. These catalytic sites can be evaluated and compared based on their pocket score. The neighboring residues of the catalytic position are also presented in the Table 3. In

Table 2: An overview of the features of chickpea HMAs proteins structure, genes loci, conserved Protein Domain Family, cellular location, Phyre2 confidence (residues modelled at > 90 % confidence), templates used for 3D prediction and longest tunnel predicted by the Caver Web for transport ions

Protein	Length	Gene	Exon count	Conserved domain	Location	Template pattern	Longest tunnel
XP_004509102.1	839	101490857 Chromosome: Ca7	13	COG4087 TIGR01512 pfam00122	Chloroplast	c3rfuC,c1mhsA,c3j08A,c5mrwF,c4umwA,c3j09A	70.1
XP_004487939	834	101492022 Chromosome: Ca1	9	COG2608 COG4087 pfam00122	Nucleus	c3rfuC, c3j08A, c4umwA, c3j09A	85.5
XP_027189340	569	101492022 chromosome: Ca1	9	cl21460 COG2608	-	c4umwA, c3rfuC, c3j08A, c3j09A	29
XP_012573401	1032	101505376 Chromosome: Ca7	11	COG2608 TIGR01512 pfam00122	Nucleus	c3rfuC,c2emcA, c3j08A,c4umwA,c3j09A	41.7
XP_004488108	832	101497233 Chromosome: Ca1	9	COG2608 COG4087 pfam00122	Nucleus	c3rfuC, c3j08A, c4umwA, c3j09A	107.3
XP_012573132	853	101504726 Chromosome: Ca6	7	COG2217 cd00371 pfam00122	-	c3rfuC, c4u9rA, c3j08A, c3j09A	43.8
XP_012574029	958	101515614 Chromosome: Ca7	10	COG2217 COG2608 COG4087 pfam00122	Nucleus	c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c3j09A	72
XP_027192934	849	101515614 Chromosome: Ca7	10	cd02094 cd00371 cl00207	Nucleus	c3rfuC, c4u9rA, c3j08A, c3j09A	95.6
XP_004500941	130	101507723 Chromosome: Ca5	3	pfam00122	-	c3rfuC, c3j08A, c3j09A, c2kijA, c2hc8A	11.3
XP_004511583	998	101498342 Chromosome: Ca8	7	COG2217 COG4087 cd00371 pfam00122	Nucleus	c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c3j09A	94.3
XP_004504792	934	101496348 Chromosome: Ca6	17	COG2217 COG4087 cd00371 pfam00122	Chloroplast	c3rfuC, c4u9rA, c3j08A, c3j09A	-
XP_004504659	995	101509532 Chromosome: Ca6	10	COG2217 COG4087 pfam00122	Nucleus	c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c2crlA, c3j09A	95.9
XP_004501429	884	101500347 Chromosome: Ca5	18	COG2217 COG4087 cd00371	Nucleus, Chloroplast	c3rfuC, c3j08A, c3j09A	-

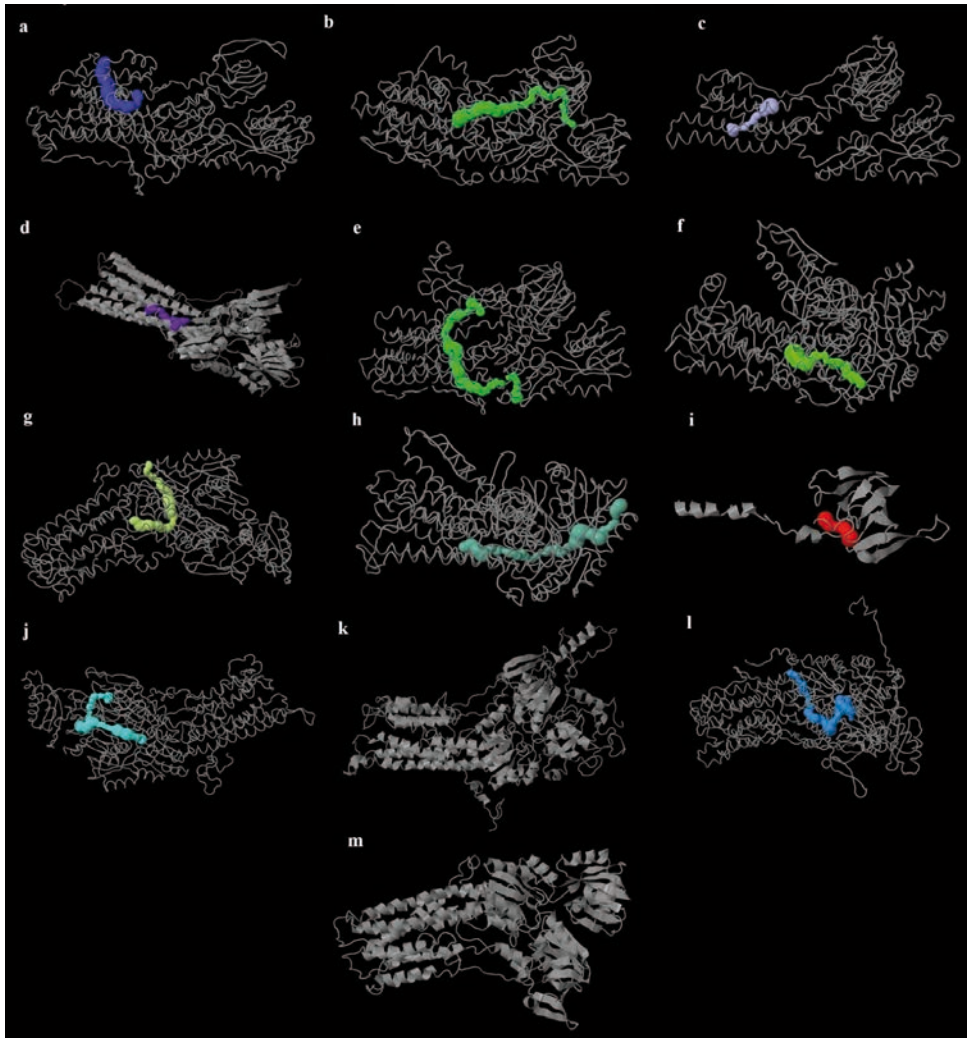

Fig. 4: An overview of the 3D model of chickpea HMAs generated by Phyre2 software. The structures were predicted using coordinate templates represented in Table 2. Colored regions in 3D structure represent the longest tunnel. a XP_004509102.1, b XP_004487939, c XP_027189340, d XP_012573401, e XP_004488108, f XP_012573132, g XP_012574029, h XP_027192934, i XP_004500941, j XP_004511583, k XP_004504792, l XP_004504659, and m XP_004501429

most cases, the amino acid Asp residue is introduced. For XP_012574029 and XP_004504659 the predicted pocket score was 100 % with XP_004504659 having an active site and three metal ion-binding sites (Table 3).

In the phylogenic tree of the HMAs (Fig. 5), comparison of the protein sequences of chickpea HMA with *Arabidopsis* revealed great similarity between these proteins in chickpea and *Arabidopsis*. HMA 2 and 4 are very similar to *Arabidopsis* and are next to HMA 3 chickpeas. HMA 3 chickpea is adjacent to HMA 3 *Arabidopsis*. HMA 1 2 3 chickpea are all involved in cadmium and zinc transfer and are in close proximity to each other in the tree. The P-type ATPases of *Arabidopsis* are very similar to the copper-transporting ATPase PAA2 chickpeas. Copper-transporting ATPase PAA1 pea is very similar to *Arabidopsis* P-type ATPases. In chickpea, copper-transporting ATPase RAN1 resembles copper-transporting ATPase HMA5, which is adjacent to copper-transporting ATPase RAN1 *Arabidopsis*. Cation-transporting ATPase HMA5-like and copper-transporting ATPase RAN1 are also in the vicinity of copper-transporting ATPase RAN1 Arabidopsis.

4 DISCUSSION

Heavy metal pollution is a significant environmental problem. Increasing our knowledge of the mechanisms by which plants are able to mitigate heavy metal stress could assist in creating new tools applicable to

Table 3: Index, residue, accession code of the reference entry, sequence identity to the reference entry, type, description, neighborhood and pocket score features of chickpea HMAs proteins structure

Protein accession number	Index	Residue	Accession code of the reference entry	Sequence identity	Type	Description	Neighborhood	Pocket score
XP_004511583	656	Asp	Q9SH30	73.8 %	active site	4-aspartylphosphate intermediate	VFDKT VFDKT	100 %
	860	Asp	Q9SH30	73.8 %	metal ion-binding	Magnesium	VGDGI VGDGI	
	864	Asp	Q9SH30	73.8 %	metal ion-binding	Magnesium	INDSP INDSP	
XP_004488108	591	Asp	P0CW78	50.2 %	metal ion-binding	Magnesium	VGDGI VGDG	33 %
XP_012573401	590	Asp	Q9SZW4	54.6 %	metal ion-binding	Magnesium	LGDGL VGDGL	28 %
XP_012574029	838	Asp	Q9SH30	56.4 %	metal ion-binding	Magnesium	VGDGI VGDGI	100 %
XP_012573132	522	Asp	Q4L970	41.3 %	active site	4-aspartylphosphate intermediate	VFDKT VFDKT	6 %
	730	Asp	Q4L970	41.3 %	metal ion-binding	Magnesium	VGDGI VGDGI	
XP_027192934	522	Asp	O32220	41.6 %	active site	4-aspartylphosphate intermediate	VFDKT VLDKT	68 %
	729	Asp	O32220	41.6 %	metal ion-binding	Magnesium	VGDGI VGDGI	
	733	Asp	O32220	41.6 %	metal ion-binding	Magnesium	INDSP INDAP	
XP_004487939	392	Asp	P0CW78	49.8 %	active site	4-aspartylphosphate intermediate	AFDKT AFDKT	13 %
	591	Asp	P0CW78	49.8 %	metal ion-binding	Magnesium	IGDGI VGDGL	
XP_004504659	649	Asp	Q9S7J8	73.4 %	active site	4-aspartylphosphate intermediate	IFDKT IFDKT	100 %
	138	Cys	Q9S7J8	73.4 %	metal ion-binding	Copper	AACVN AACVN	
	869	Asp	Q9S7J8	73.4 %	metal ion-binding	Magnesium	VGDGI VGDGI	
	873	Asp	Q9S7J8	73.4 %	metal ion-binding	Magnesium	INDSP INDSP	
XP_004509102	467	Asp	Q9M3H5	68.2 %	active site	4-aspartylphosphate intermediate	AFDKT AFDKT	25 %
	701	Asp	Q9M3H5	68.2 %	metal ion-binding	Magnesium	INDAP INDAP	

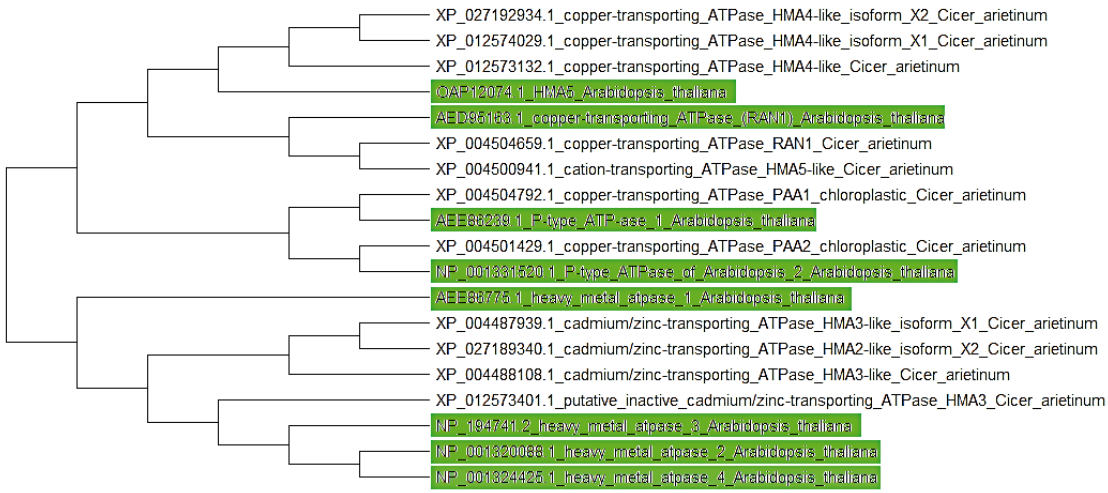

Fig. 5: Phylogenic tree of HMAs from chickpea and *Arabidopsis*. A phylogenetic tree was constructed using the neighbor-joining (NJ) method, with a bootstrap test performed using 1000 iterations in MEGA5 with the amino acid sequences of HMAs. HMAs from *Arabidopsis* are highlighted in green

phytoremediation. It is important to further research processes involved in heavy metal detoxification and signaling pathways in plants so as to identify useful targets for biotechnological applications thereby increasing plant fitness in heavy metal polluted sites (Dala-Paula et al., 2018).

Cadmium exposure reduced leaf area, shoot and root length. The effect of cadmium ion suppression on root expansion extends through its effect on cell growth (Hassan et al., 2008). Cadmium attaches to the cell wall and the middle lamella, increasing the bonding between the wall components, ultimately leading to growth inhibition and a decline in cell and organ development. Cadmium also alters water proportions in plants causing physiological dryness, which leads to metabolic dysfunction and production of ROS. These factors reduce growth and impact on plant length and mass (Zulfiqar et al. 2022). Many studies on the mechanism of cadmium blockage on cell growth have shown degradation of cell membranes by cadmium and changes in the degree of cell exchange and cellular depletion (Bücker-Neto et al., 2017). The observed changes in plants exposed to cadmium may be as a result of multiple nutritional deficiencies being experienced by the plant.

Nutrients serve an essential role in the formation, expansion, and operation of chloroplasts. Cd-phytotoxicity affects the synthesis and extensibility of cell walls (Gomes et al., 2011). Cell wall thickening in root endodermal tissue affords a greater surface area over which cadmium accumulation can occur thereby limiting its transportation to the shoot (Zulfiqar et al., 2022). Chlorosis observed in the leaves of bean plants exposed to cadmium may be due to loss of magnesium which is an integral structural feature of the porphyrin ring present in chlorophyll. Physiological changes observed in leaves are due to the associated toxic effects of cadmium including mesophyll curvature, decreased leaf thickness and a reduction in the composition of intercellular spaces of spongy parenchyma (Tuver et al., 2022). At higher doses of cadmium, the thickness of palisade and spongy tissues is reduced. A decline in the dimensions and composition of the main mid-vein bundle suggests that cadmium alters leaf expansion (Cregeen et al., 2015).

A study of the effect of heavy metals on the cell death of *Halophila stipulacea* (Forssk.) Asch leaves showed that high concentrations of metal causes necrosis of the epidermal cells and mesophyll, inhibiting surface growth of the leaves. High levels of heavy metal accumulation in plant cells inhibits the process of respiration and energy reactions, which are associated with cell growth (Ayangbenro, 2017). A decline in cell division and growth could also be a contributing factor to the observed morphological changes. Additionally, a decrease in photosynthetic rates has been observed in plants exposed to elevated levels of heavy metals. Higher concentrations of cadmium commonly result in root injury, damage to photosynthetic machinery, inhibition of plant growth, reduced nutrient and water uptake (Tuver et al., 2022). Cadmium may exert its inhibitory effect in different ways, namely binding specific groups of proteins and lipids thereby inhibiting normal function and possibly inducing free radical formation due to oxidative stress. The former may occur at transport and channel proteins of cell membranes disturbing the uptake of many other macro- and microele-

ments whereas the latter is due to the inactivation of antioxidant enzymes by cadmium (Long et al., 2017).

The results showed that oxidative enzymes activity (SOD, APX, POD and CAT) increased in the leaves of chickpea exposed to cadmium. Similar observations have been observed in CAT and POD enzymes present in cereals and squash (Ashraf, 2003). Increased activity of these enzymes is a consequence of lipid peroxidation. The effect of cadmium on growth and antioxidant enzymes in two varieties of *Brassica napus* showed that cadmium decreased the growth indices, nitrate reductase activity and leaf water potential while antioxidant enzyme activities increased. The highest level of enzyme activity was in relation to SOD enzymes, which showed more than 80 % increase in activity. The least increase in enzyme activity was observed in the catalase enzyme (Irfan et al., 2014). Increasing the absorption and accumulation of heavy metals in plants causes changes in cell metabolism, oxidative stress and cell destruction which is induced by ROS. Cadmium can induce mineral stress that reduces plant dry mass (Zhou et al., 2022). Tabarzad et al. (2017) showed that wheat seedlings grown in the presence of cadmium had changes in the level of SOD and POD activity. The observed decline in enzyme activity suggests a weakening of the oxygen and superoxide water scavenging system. Reduced activity of the other antioxidant enzymes in some tissues, is due to poor performance in oxygenate decomposition in cadmium treated tissues. ROS activity increased significantly under cadmium stress due to an increase in wall oxidation. Reduced SOD activity is justifiable as cadmium is known to be an enzyme inhibitor (Tabarzad et al., 2017).

Schutzendubel (2001) showed the inhibition of SOD, POD and total inactivation of APX in pine roots after 48 days of cadmium treatment. An increase in the activity of these enzymes under cadmium stress has been observed in other studies (Schutzendubel et al., 2001). Li et al. (2013) examined the effect of cadmium stress on growth and antioxidant enzymes and lipid oxidation in two Kenaf (*Hibiscus cannabinus* L.) species. In the study, glutathione reductase activity (GR) was greater than that of the control. The general trend was that of an increase in SOD, CAT and POD activities in the roots of cadmium-stressed plants followed by a decline. POD activity however remained relatively unchanged at all stress levels (Zhou et al., 2022). Ulusu et al. (2017) investigated the antioxidant capacity and cadmium accumulation of stressed parsley. In the study, enzyme activity increased for catalase and ascorbate peroxidase, (75 to 150 μM cadmium), while decreasing at 300 μM. The results showed that antioxidant enzymes activity was suppressed due to the accumulation of cadmium in parsley leaves and increased non-enzymatic antioxidant activity (Ulusu et al., 2017). Pereira et al. (2002) studied the activity of antioxidant enzymes in *Crotalaria juncea* L. which showed that under the influence of cadmium, catalase activity did not show any significant changes in the root. At concentrations of 2 mM cadmium, catalase activity in the leaves increased 6 fold compared to the control. Increased activity of some antioxidant enzymes exposed to metals reveal the crucial role that these enzymes play in detoxification (Pereira et al. 2002). Various antioxidant cycles under normal physiological metabolism, results in the production and scavenging of reactive oxygen species which is in a state of dynamic equilibrium (Zhou et al., 2022). Kisa (2018) studied the response of antioxidant systems to stress induced by heavy metals in the leaves and roots of tomato which showed that cadmium treatment significantly increased the activity of the APX and SOD enzymes. Antioxidant scavenging systems are connected with ROS detoxification which is a defense mechanism employed by plant tissue to combat oxidative stress (Kisa, 2018). Tomato plants exposed to cadmium showed significantly higher SOD. Catalase activity was however reduced.

The cadmium content in aerial parts of chickpea grown in different concentrations of cadmium increased significantly. Research conducted by Tang et al. (2022) revealed that cadmium concentrations in the seeds of beans from different regions and varieties is based on complex genetic factors and the environment. For different legume varieties, environmental factors such as climate, soil, agricultural and geological techniques, in comparison to genetic factors, are more important in the accumulation of heavy metals such as Cd. Compared to the genus and plant species, the accumulation of heavy metals seems to be more influenced by the genetic potential of the plant (Tang et al. 2022). The ability to absorb and distribute cadmium to the aerial regions of the plant is related to its attachment to the extracellular matrix, root flow, intracellular detoxification and transfer efficiency (Akhtar and Macfie, 2012). Cadmium is absorbed in the root of the plants subsequently accumulating in the aerial parts, which often limits the absorption and distribution of other elements (Gomes et al., 2013). Cadmium binds to the functional epidermis through direct binding to ion carriers via production of oxygen species that are associated with membrane affects (Altaf et al., 2022). Ling Liu et al. (2012) showed that legumes can increase the accumulation of cadmium in adjacent plants. Cadmium increase in plants was a direct result of planting crops in proximity to legumes. The study suggests that the system of cultivation of beans should be redesigned to prevent food contamination with cadmium (Liu et al., 2012). Vijendra et al. (2016) showed that in Moth bean (*Vigna aconitifolia* L.) cadmium concentrations in-

creased significantly in the leaves and roots. Cadmium reaches the aerial sections via the xylem of the plant (Vijendra et al., 2016). At concentrations of 0.04 to 0.32 mM, cadmium is non-polluting in soil. Knowledge about the distribution of cadmium in plant tissues is important to better understand the tolerance mechanism and accumulation of heavy metals in plants. Cadmium in plants is transferable through apoplast pathways of the stems and leaves (Benavides et al., 2005). Cadmium affects membrane potential, protein pump activity and can limit corn growth (Karcz & Kurtyka, 2007).

The result indicated that increasing cadmium concentration, also decreased the levels of copper and zinc present in the aerial parts of chickpea seedlings. Further studies also showed that zinc and copper along with cadmium have an antagonistic effect and that these minerals act in a competitive manner in relation to the transfer processes. Heavy metals, such as copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe), serve as essential micronutrients for an array of metabolic processes. These micronutrients serve as cofactors, participate in cellular redox reaction and affects protein structure (Schutzendubel & Polle, 2002). At toxic levels Cu will however interfere with physiological processes. Zn also serves as a micronutrient but can be toxic if present at high concentrations (Schutzendubel & Polle, 2002). To minimize the potential effects of excess metal contaminants, the plant utilizes various homeostasis mechanisms which include the use of specialized transport proteins which serve as carriers mediating the transfer of heavy metals across cell membranes (Lee et al., 2007). Cadmium has a negative effect on the absorption of essential nutrients. It reduces ATPase activity and decreases the exchange of ion H^+/K^+ in the plasmalema surface (Brzoska & Moniuszko-Jakoniuk, 2001). Page and Feller (2005) showed that the transfer of zinc, manganese, cobalt and cadmium in the leaves and roots of wheat were selective. When other minerals are in close proximity to cadmium, the amount of zinc in the root decreases (Page and Feller, 2005). Santos et al. (2014) showed that in the family of legumes, lead and cadmium adsorption was competitive. In this study, the concentration of zinc was eight times higher than that of cadmium, which indicates that zinc adsorption is preferable to cadmium. In plants treated with zinc and lead, lower concentrations of cadmium were observed in plant tissues in comparison to plants treated with cadmium alone. Zinc and lead along with cadmium compete for the sites of absorption and transfer (dos Santos et al., 2014). Chen et al. (2007) showed that manganese reduces the toxic effects of cadmium in corn. This suggests that manganese can be utilized to manage cadmium contamination (Chen et al., 2007). Zinc acts as a micro-element that is essential for plant growth and is part of the structure of regulatory enzymes and proteins. Zinc is very important in reducing cadmium toxicity and decreases the oxidative stress induced by cadmium. Some studies describe zinc and phosphorus interactions in plants (Marques et al., 2013). The phosphorus content in the aerial parts of plants treated with cadmium is related to the low zinc content in these sections. The negative correlation between zinc and phosphorus content in the shoots of cadmium treated plants explains the high content of phosphorus in these plants (Sarwar et al., 2010). Analysis of cadmium and manganese content in this study supports the competitive theory of absorption of these two elements. The precise mechanism for promoting growth and reducing the toxic effects of cadmium is not well known. The uptake of various cations (K^+, Ca^{2+}, $Mg2^+$, Mn^{2+}, Zn^{2+}, and Fe^{2+}) is severely affected by the presence of cadmium (Linger et al., 2005).

Different types of proteins and adsorption carriers for cadmium are known such as NRAMP family (Thomine et al. 2000), P-type ATPase (Morel et al., 2009), ABC transporter (Kim, Gustin et al., 2004), CAX family, ZIP family (Pence et al., 2000), LCT transporter and CE family (Guerinot, 2000). Researchers report that cadmium has an antagonistic and synergistic effect on the microelements and macro elements in wheat. Many studies on the effect of cadmium inhibition on cell growth suggests the destruction of cell membranes and changes in mineral levels (Rietra et al., 2017). Jibril et al. (2017), showed that the content of micronutrients and macro elements in different varieties of lettuce is significantly affected by cadmium levels. The study showed that cadmium (12 mg l^{-1}) reduced essential elements by 72, 69, 56, 61 and 52 % (nitrogen, phosphorus, potassium and calcium, respectively). Copper content was higher in the root than the shoot of cadmium treated plants. This therefore reduces the effect of cadmium toxicity. Indeed, cadmium increases the absorption of copper, but prevents it from transferring to the shoots (Jibril et al., 2017). Gomez et al. (2013) examined the effect of cadmium on nutrient distribution in *Pfaffia glomerata* (Spreng.) Pedersen. Plants were cultured with different minerals and cadmium concentration was simultaneously increased over a 20 day period. The study showed that cadmium strongly affects the distribution of microelements and macroelements in the roots and shoots. Despite the high toxicity of cadmium, the micro and macro nutrients present in plants are able to survive in contaminated environments (Gomes et al., 2013).

Present study detected that at low concentrations of cadmium, the amount of manganese increased. With an increase in cadmium concentration, the level of manganese decreased in chickpea. Manganese plays a role in many biochemical functions, such as activating

enzymes involved in respiration, redox reactions, intracellular electron transfer systems, and the Hill reaction in chloroplasts, amino acid synthesis, and regulation of hormones (He et al., 2022). Manganese concentration was higher in the shoots than the root of plants treated with cadmium. The transfer of manganese to the shoot may in fact be a tolerance mechanism that reduces the effects of cadmium toxicity on photosynthesis. Research suggests that cadmium and manganese compete for the same membrane carriers (Socha & Guerinot, 2014). Dias et al. (2013) showed that at cadmium concentrations of 5 and 10 µm there was a significant decline in the mineral content of lettuce leaves. At high concentrations of cadmium, a significant decline in manganese in the roots was observed. Cadmium appears to interfere with the transmission of macro and micro elements in the leaf (Dias et al., 2013). According to Guerinot, members of the ZIP and NRAMP or Ca channels and transporters which are responsible for the uptake of essential elements are involved in the transport of cadmium via the same route (Guerinot, 2000). Imbalance in nutrient level and growth inhibition is ultimately due to competition between nutrients and toxic metals for binding sites in the cell. Sun and Shen (2007) explained that the decrease in concentrations of Mn, Fe, Mg, S, and P in the leaves of Cd-sensitive cultivars under cadmium stress is a contributing factor to the decline in photosynthesis and the decrease of cabbage growth (Sun & Shen, 2007).

Heavy metal ATPases (HMAs), belong to the large P-type ATPase family located in the plasma membrane or tonoplast. They play an important role in the transport of metals in plants and provide resistance to the uptake and transportation of metals. The identified HMAs may contribute to the mechanisms by which chickpea plants manage, detoxify, or tolerate cadmium exposure. Understanding the structure, function, and localization of these HMAs could offer new strategies for enhancing cadmium tolerance in chickpea, a crucial crop in many parts of the world. HMAs are classified based on substrate binding with one group bound to copper and silver and the other to cadmium, lead and cobalt (Chkadua et al., 2022). HMAs 9 and 8 have been studied in rice and *Arabidopsis*, respectively. AtHMA1–4 in *A. thaliana* and OsHMA1–3 in *Oryza sativa* L. are in the first group and AtHMA5–8 and OsHMA4–9 in the second group. The expression of each of these genes is sensitive to heavy metals as indicated by mutagenesis. Typical P1B-ATPase proteins have been studied in various barley plants, *Arabidopsis* and poplar as well as in *Thlaspi caerulescens* J.Presl & C.Presl (Takahashi et al. 2012).

In poplar (*Populus trichocarpa* Torr. & A.Gray ex. Hook), seventeen HMAs are known. PtHMA1 – PtHMA4 belong to the subgroup of metals on cadmium, lead and cobalt. PtHMA5 – PtHMA8 belonging to the silver and copper groups have been identified. Most of these genes are located on chromosome 1 and 2 of poplar. On both sides of the P_{1B}-*ATPase* C and N terminals there is also a metal binding site HMA4 in poplar which produces mature RNA transcripts during alternative splicing of mRNA, containing approximately six hundred and twenty-six amino acids with an amino acid average of ninety-eight. PtHMA in poplar are all in plasma membrane except PtHMA1 and PtHMA5.1 which are located in the cytoplasm. Poplar HMAs have 5 to 16 introns, PtHMA6, 5 introns, 8 PtHMA has 16 introns and 1 PtHMA has 5 introns with the remaining possessing 10 introns (Li et al., 2015). PtHMA1 – PtHMA4 belong to the subgroup of metals consisting of cobalt and cadmium with the rest belonging to lead, silver and copper. There are 10 HMA genes related to silver and copper in poplar that are significantly higher than those in rice and *Arabidopsis*.2 OsHMA plays an important role in transmitting cadmium entry from the root to the stem and especially to rice grains (Li et al., 2015). OsHMA3 transports cadmium to root cell vacuoles. Manipulating and altering the expression of these genes is a useful tool for reducing cadmium concentration in the seeds. AtHMA1 is within the chloroplast and zinc anti-toxic while AtHMA 3 is present in the vacuolar membrane with zinc and cadmium playing a role. The motifs of poplar HMA are very similar to *Arabidopsis* and rice proteins and it seems that family members of these genes may be functionally divergent due to differences in gene organization and existing motifs (Tian et al. 2023). AtHMA 1 and 2 are in the plasma membrane and in zinc and cadmium fluxes. OsHMA 1 is involved in zinc transfer. No HMA 4 type has been reported in rice. The number of HMA genes in the soybean genome is higher than that in *Arabidopsis* and rice, probably due to duplication of the soybean genome. Phylogenetic study of these genes divides them into six groups, based on their divergent gene structure, conserved segments or protein motif patterns. Examination of the cellular location of these proteins indicates that only *GmHMA1* is involved in the secretion pathway while 1, 16, 17, 20, 20 peptides are mitochondrial targets, whereas 1, 2, 2, and 2 GmHMA2 are chloroplast peptides (Fang et al., 2016). Researchers have identified nine *typical* P_{1B}*-ATPase* in barley. HvHMA2, a P (1B)-ATPase is highly conserved among cereal crops with functionality in the transportation of zinc and cadmium. Additionally, HMA4 (Heavy Metal ATPase 4) has a key role in the translocation of cadmium in non-hyperaccumulating dicots, such as *Arabidopsis thaliana* (Mills et al., 2012).

5 CONCLUSION

Chickpea seedlings exposed to cadmium exhibited changes in their morphological features which included changes in plant length, coloration and leaf size. The results indicated that shoot and root length were significantly reduced. With the addition of cadmium (4 µg Cd g^{-1} perlite), stomatal densities on the upper epidermis decreased significantly but subsequently increased while higher concentrations of cadmium. Oxidative enzyme activities were also affected by cadmium stress. Oxidative enzyme activity (peroxidase, superoxide dismutase, catalase, ascorbate peroxidase) increased in the leaves of plants exposed to cadmium suggesting that these enzymes play an integral role in combatting heavy metal contamination. Cadmium content in aerial parts of chickpea increased significantly. The study also revealed that by increasing cadmium concentration there was a significant reduction in the amount of copper and zinc transported to the aerial regions of the plant. Moreover, at low concentrations of cadmium, the amount of manganese increased It has been suggested that there is a competitive mechanism for mineral uptake in plants. One may therefore be able to manage cadmium accumulation by varying the type of fertilizers utilized in cultivating plants. *In silico* analysis led to the identification of 13 Heavy Metal ATPases (HMAs) in chickpea. These proteins contain 130 to 1032 amino acids with 3 to 18 exons. Comparison of the protein sequences of chickpea HMA with *Arabidopsis* indicated that there was great similarity between these proteins. The presence of a variety of genes indicates the various mechanisms utilized by chickpeas to combat heavy metal stress. Genetic engineering could be utilized to create heavy metal resistant chickpea species.

6 CONFLICT OF INTEREST

There is no conflict of interest in the publication of this paper.

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