Study of Oxidative Stress Indices, Morphological Response, Mineral Absorption in Chickpea (*Cicer Arietinum* L.) Under Cadmium Stress and Bioinformatics of HMA Proteins

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Abstract

Cicer arietinum L. (chickpeas) is a widely consumed legume that is impacted by heavy metal contaminants such as cadmium. Cadmium is a chemical hazard and can severely impact the morphological and physiological features of the plant. C. arietinum L. were exposed to cadmium and its impact on plant growth and antioxidant enzyme activity evaluated. Bioinformatic studies were performed to further understand the mechanism by which the plant combats heavy metal stress. Observed morphological changes included stunted growth, poor root development and yellowing of the plant. The study also revealed that increased cadmium resulted in a decline in mineral transportation to aerial regions of the plant. Antioxidative enzyme activity (peroxidase, superoxide dismutase, catalase, ascorbate peroxidase) increased in the leaves suggesting that these enzymes play an integral role in combatting heavy metal contamination. These research showed chickpea has a relatively high adsorption capacity for cadmium in aerial tissues. Special precautions should therefore be taken in the cultivation of chickpea. Increasing the levels of cadmium in the medium resulted in a decline in zinc, copper and manganese in the aerial parts of chickpea seedlings. There appears to be a competitive mechanism for mineral uptake in plants. HMAs play an important role in the transport of metals in plants and provide resistance to the uptake and transportation of metals. In silico analysis led to the identification of 13 Heavy Metal ATPases (HMAs). These proteins contain 130 to 1032 amino acids with 3 to 18 exons and assist in heavy metal detoxification.

Highlights

- Plants exposed to cadmium had stunted growth and poor root development.
- Cadmium exposure resulted in increased antioxidant enzyme activity.
- Mineral uptake is affected by cadmium exposure.
- Bioinformatics revealed the presence of 13 Heavy Metal ATPases (HMAs) in chickpeas.

Introduction

Chickpea (Cicer arietinum L.) is a major legume crop consumed globally. The legume ranks third when compared to other legumes contributing 16.4 % to the world market. In 2010, 10.9 million tons of chickpea was produced from a land mass of 12.0 million hectares. In Iran, 6.2% of total crop area is designated to chickpea cultivation. Of the total Iranian beans grown in Iran, 64.3% is in relation to chickpeas. Chickpea has high nutritional content and is an economical source of protein (12–31%). Chickpea is also a source of minerals (manganese, molybdenum, phosphorus and potassium) and vitamins. Phytoestrogens offer therapeutic benefits. Considering its economic and nutritional importance, steps need be taken to avoid contamination with heavy metals such as cadmium. Globally, environmental contamination by heavy metals is a major concern. Contamination of waterways, the soil and air are a serious threat to the health of the human population, animals, microorganisms and plant biodiversity. Consumers in developed countries could possibly suffer from nutritional deficiency due to consumption of contaminated crops (Dala-Paula, Custodio et al. 2018).

Defense mechanisms employed by the plant include redox reactions (oxidative stress and glutathione reduction), accumulation of secondary metabolites, modification in protein synthesis, transcription and gene over expression. Various mechanisms have been proposed for plants to withstand heavy metal contamination. These include competitive strategies between heavy metals and cations for absorption at the root surface, complexation of heavy metals with the sulfhydryl group (–SH) of functional proteins, exchange of essential cations from specific binding sites leading to function failure and the production of reactive oxygen species (ROS) (Zhang, Wu et al. 2019).

Environmental stresses, including heavy metals, are one of the main barriers to agricultural and horticultural production in many parts of the world. Industrialization has resulted in the release of industrial wastewater in natural ecosystems and rivers. The use of fossil fuels and accumulation of heavy metals in the soil has increased. Cadmium is readily absorbed by the root system of many plants and is considered one of the most important contaminants due to its high toxicity and high-water solubility. Important factors contributing to cadmium intake by plants include cadmium concentration, bioavailability, the presence of organic matter, soil pH, regeneration potential, temperature and the presence of other metals. Cadmium toxicity has been observed in various forms, including reduced yield, stunted plant growth, and oxidative stress. The main mechanisms of scavenging ROS in plants involves superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). SOD, APX or CAT balances the levels of superoxide and hydrogen peroxide radicals within the plant (Bowler, Van Camp et al. 1994). APX and CAT belong to two different classes of scavenging enzymes. APX adjusts the levels of ROS for signaling and CAT is responsible for removing ROS. The main pathways of scavenging ROS in plants include: SOD, found in almost all cellular components, the water cycle and ascorbate glutathione cycles in chloroplasts, cytosol, mitochondria, apoplasts, peroxisomes, glutathione peroxidase and catalase in peroxisomes. The ascorbate glutathione cycle is found in virtually all components of plant cells. APX has a high affinity for hydrogen peroxide indicating its crucial role in controlling the level of ROS. CAT is only present in the peroxisome, but is necessary to eliminate the toxic effects of ROS (Akram, Shafiq et al. 2017).

Heavy metals serve as essential micronutrients for an array of metabolic processes. These micronutrients serve as cofactors, participate in cellular redox reaction and affects protein structure. Cu is essential for plant growth serving vital roles in various biochemical reactions. At toxic levels it will however interfere with physiological processes. Zn also serves as a micronutrient but can be toxic at high concentrations (Schutzendubel and Polle 2002, Williams and Mills 2005). To minimize the potential effects of excess metal contaminants, the plant utilizes various homeostasis mechanisms such as specialized transport proteins which serve as carriers mediating the transfer of heavy metals across cell membranes (Lee, Kim et al. 2007).

P-type ATPases are present in living organisms which are involved in the translocation of ions, including H+; Na+/K+, H+/K+, and Ca2+, as well as heavy metals and possibly lipids. Ion pumps within the P-type ATPase superfamily share a common mechanistic feature. ATP hydrolysis assists in the transportation of ions across the membrane. This superfamily is divided into 5 major branches and 10 subfamilies, based on the substrate that is transported. Significant sequence similarities have been observed in the heavy-metal pumps present in bacteria, plants, and humans. They are clustered together as the P18 subfamily.
It has been hypothesized that $P_{1B}$-type heavy-metal ATPases (HMAs) are involved in the transportation of both essential and potentially toxic components across cell membranes. Through the use of phylogenetic analyses $P_{1B}$-type heavy-metal ATPases can be subdivided into 2 distinct clusters, namely the Cu cluster involved in transporting Cu and Ag, whereas the Zn cluster proteins transport Zn and other heavy metals (e.g. Co, Cd, and Pb) (Satoh-Nagasawa, Mori et al. 2012, Takahashi, Bashir et al. 2012).

In this study, the impact of cadmium on morphological features, antioxidative enzymes, mineral absorption and the cadmium content of chickpeas was evaluated. Genes involved in oxidative stress and their response to heavy metals were studied utilizing bioinformatics. HMAs are transmembrane metal-transporting proteins that play a key role in metal homeostasis. Very little is known about their function in the Fabaceae family. Utilizing bioinformatics, HMAs in chickpea were investigated. The number of genes, proteins, gene loci, cellular location, phylogenetic relationship, three-dimensional protein structure, conserved domains, similar template and catalytic site were determined.

**Materials And Method**

**Propagation and cadmium exposure**

Kabuli chickpea (*Cicer arietinum* L.) seeds were planted in sterilized Cucupite and Perlite in a greenhouse at Tabriz University, Iran utilizing an illumination cycle of 8 h light and 16 h darkness. After germination, plantlets (3) at the leaf stage were planted in pots (diameter, 12 cm and height 15 cm) under sterile conditions and irrigated with distilled water (3 days). A hydroponic nutrient solution (Hoagland solution), was then utilized for irrigating the plantlets treated with cadmium chloride in replicates of three at four different concentrations (control, 2, 4, and 8 mM) for 10 days. Plants were then harvested for further investigations.

**Morphometric parameters**

Fresh and dry weight of the roots and aerial organs were determined (mg). Plantlet height, leaf area, root length, aerial organ length and internode height were measured. Stomatal densities on the lower and upper epidermis were evaluated.

**Enzyme assays**

Enzyme extracts were prepared from fresh chickpea leaves (0.1 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 (EC 1.11.1.6) 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 enzyme extract added (0.1 mL). The absorption of the mixture was determined at 240 nm (Beers and Sizer, 1952). Peroxidase enzyme (EC 1.11.1.7) 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$_2$HPO$_4$ (0.5 M, 2.5 ml), ascorbate (0.5 mM, 0.1 ml), EDTA (0.1 mM, 0.1 ml) and H$_2$O$_2$ (1%, 0.2 ml) and the absorbance read at 290 nm. Specific enzyme activity was reported as units/g fresh weight (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).

**Elemental analysis of Cd, Zn, Cu and Mn**

Plant samples were oven dried (72 h, 60 °C) and the dry weight determined. Dried samples were ashed (550 °C, 8 h). After cooling the samples were acid digested (1N HCl, 1 ml; nitric acid, 97%, 1 ml, 1 h). The digested extract 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. Cadmium, zinc, copper and manganese nitrate in nitric acid was used as the standard.

**Bioinformatics analysis**

The NCBI gene database was searched utilizing the keyword "HMA". Gene characteristics included location, exon count and conserved domain. Protein sequences were used to predict localization 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 used 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, Dudley 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.

**Statistical analyses**

Data analyses were performed using the SPSS20.0 software package (SPSS Inc., Chicago,IL, 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. The level of significance was set at $p < 0.05$ for all tests.

**Results And Discussion**

Heavy metal pollution is a significant environmental problem. Researchers are actively seeking new cost effective and environmentally friendly technologies to be utilized in soil remediation. Increased knowledge of the mechanisms by which plants are able to mitigate heavy metal stress could assist in creating new
tools applicable to phytoremediation. Further research regarding heavy metal detoxification and signaling pathways in plants will assist in identifying useful targets for biotechnology resulting in increased plant fitness in heavy metal polluted sites (Tchounwou, Yedjou et al. 2012).

**Morphometric features in aerial 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. Stem color changed to a bright green-yellow hue. Changes were also observed in leaf size and color (yellow). There was a significant reduction in shoot and root length. Shorter and less dense roots were observed in treated samples (Table 1). The fresh and dry weight of the shoots and roots in chickpea plants were also significantly affected with lowest seedling weights being observed at high cadmium concentrations. Plants treated with 2 mM cadmium had a significant decline in leaf area (less than half that of the control). At cadmium levels of 2 mM, the length of the first internodes increased, whereas at higher concentrations, there was a decrease. The length of the second internodes showed only a significant reduction at high concentrations of cadmium (Figure 1, Table 1).

Hassan et al. (2006) investigated the effect of cadmium on chickpea and found that plant growth and development as well as carbonic anhydrase enzyme activity declined resulting in changes in plant metabolism and photosynthesis. The impact of cadmium ion suppression on root extension extends through its effect on cell growth. Cadmium attaches to the cell wall and the middle wall, increasing bonding between the wall components, ultimately leading to growth inhibition and a decline in cell and organ development. Cadmium also alters the proportion of water in plants causing physiological dryness, which leads to metabolic dysfunction and the production of reactive oxygen species (ROS). These factors reduce growth and decreases plant length and weight.

Many studies on the mechanism of cadmium blockage on cell growth have shown degradation of cell membranes, changes in the degree of cell exchange and cellular depletion (Bucker-Neto, Paiva et al. 2017). The observed changes in plants exposed to cadmium may be as a result of multiple nutritional deficiencies that are 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 (Breckle and Kahle, 1992).

**Table 1. Effect of cadmium on morphometric features of chickpea (Cicer arietinum L.) Values with different letters are significantly different at p < 0.05.**

| Treatment Parameters | Control | 2 mM Cd | 4 mM Cd | 8 mM Cd |
|----------------------|---------|---------|---------|---------|
| Plant height (cm)    | 62.76±1.36<sup>a</sup> | 58±0.0709<sup>b</sup> | 42.56±1.78<sup>b</sup> | 37.93±1.78<sup>b</sup> |
| Shoot length (cm)    | 29.33±0.66<sup>a</sup> | 25.65±0.779<sup>b</sup> | 22.55±1.38<sup>bc</sup> | 21.16±1.092<sup>c</sup> |
| Root length (cm)     | 35±0.57<sup>a</sup> | 30.86±0.69<sup>b</sup> | 18.56±0.92<sup>c</sup> | 16.6±0.83<sup>c</sup> |
| Plant fresh weight (g) | 4.0367±0.043<sup>a</sup> | 3.442±0.238<sup>b</sup> | 3.084±0.169<sup>b</sup> | 1.715±0.042<sup>c</sup> |
| Shoot fresh weight (g) | 2.291±0.11<sup>a</sup> | 1.6317±0.14<sup>b</sup> | 1.297±0.061<sup>c</sup> | 0.682±0.014<sup>d</sup> |
| Root fresh weight (g) | 2.24±0.078<sup>a</sup> | 1.9±0.1<sup>b</sup> | 1.3167±0.109<sup>c</sup> | 0.99±0.003<sup>d</sup> |
| Shoot dry weight (g) | 1.987±0.01<sup>a</sup> | 1.4783±0.11<sup>b</sup> | 1.0447±0.029<sup>c</sup> | 0.606±0.002<sup>d</sup> |
| Root dry weight (g) | 2.01±0.04<sup>a</sup> | 1.696±0.063<sup>b</sup> | 1.123±0.069<sup>c</sup> | 0.823±0.062<sup>d</sup> |
| Leaf area (mm<sup>2</sup>) | 103.33±1.76<sup>a</sup> | 48.33±2.18<sup>b</sup> | 20.66±0.666<sup>c</sup> | 18.33±1.201<sup>c</sup> |
| First internode length (cm) | 1.1±0.264<sup>b</sup> | 1.766±0.0577<sup>a</sup> | 1.3±0.3<sup>b</sup> | 0.833±0.838<sup>c</sup> |
| Second internode length (cm) | 2.433±0.513<sup>a</sup> | 2.266±0.503<sup>a</sup> | 1.7666±0.808<sup>b</sup> | 1.633±0.850<sup>b</sup> |
| Stomatal densities on the upper epidermis | 35±0.545<sup>ab</sup> | 31±0.564<sup>b</sup> | 24.333±0.413<sup>c</sup> | 39.333±0.633<sup>a</sup> |
| Stomatal densities on the lower epidermis | 29.667±0.448<sup>c</sup> | 39±0.653<sup>b</sup> | 46±0.765<sup>a</sup> | 37.333±0.985<sup>b</sup> |

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 (Gomes et al., 2011). 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 soybean 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. 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 *Halophyl astipulecea* leaves concluded 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 breathing and energy reactions, which are associated with cell growth (Ayubbheru and Babalola, 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,
Effect of cadmium on SOD, POD and CAT activities in the aerial parts of chickpea seedlings

There was a significant increase in POD enzyme activity in chickpea seedlings exposed to cadmium. Highest enzyme activity was observed at treatments of 4 mM. 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 mM cadmium. Lowest enzyme activity was observed in the controls (Figure 2A). SOD enzyme activity significantly increased with highest enzyme activity being observed in plantlets treated with 4 mM cadmium and lowest enzyme activity in the control (Figure 2B). It should be noted that the 8 mM treated plants were almost completely yellow at the same day of harvest, thus reducing all the enzymatic activities in the 8 mM plants due to possibly more cell death. There was a significant increase in CAT enzyme activity with highest levels being observed in plants treated with 4 mM cadmium. There was a subsequent decline in CAT activity when cadmium concentration was increased to 8 mM. Lowest enzyme activity was observed in the control (Figure 2C). Investigation of APX enzyme activity showed that this enzyme was also affected with highest APX activity being observed in cadmium treatments of 4 mM (Figure 2D). Antioxidative 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.

Similar observations were made in CAT and POD enzymes present in cereals, squash, and peas (Ashraf, 2003). Increased enzyme activity is as a result of lipid peroxidation. The effect of cadmium on growth and antioxidant enzymes in two varieties of Brassica showed that cadmium decreased growth indices, nitrate reductase activity and leaf water potential while antioxidant enzyme activities increased. In this study, maximum antioxidant enzyme activity was observed in plants treated with the highest concentration of cadmium. SOD enzymes had the highest levels of activity, which increased by more than 80%. The lowest increase in enzyme activity was observed in the CAT enzyme (Irfan, Ahmad et al. 2014). Increased absorption and accumulation of heavy metals in plants results in changes in cell metabolism, oxidative stress and cell destruction induced by ROS. Cadmium can induce mineral stress that reduces plant dry weight (Gill and Tuteja 2011). 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 oxygen decomposition in cadmium treated tissues. ROS activity increased significantly under cadmium stress due to an increase in wall oxidase. A reduction in SOD activity is expected as cadmium is an enzyme inhibitor (Tabarzad, Ayoubi 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 exposed to cadmium stress has been observed in other studies (Schutzendubel, Schwanz et al. 2001). Li et al. (2013) examined the effect of cadmium stress on growth, 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. SOD, CAT and POD activities increased in the roots of cadmium-stressed plants which was followed by a subsequent decline. POD activity however remained relatively unchanged at all stress levels (Schutzendubel, Schwanz et al. 2001). Ulusu et al. (2017) investigated the antioxidant capacity and cadmium accumulation in parsley. Enzyme activity increased for CAT and APX (75 to 150 µm cadmium) but decreased at 300 µm. The results showed that antioxidant enzyme activity was suppressed due to the accumulation of cadmium in parsley leaves and increased non-enzymatic antioxidant activity (Ulusu, Öztürk et al. 2017).

Studies performed by Pereira et al. (2002) on antioxidant enzymes in Crotalaria juncea exposed to cadmium, showed that there was no significant change in CAT activity in the root. At concentrations of 2 mM cadmium, CAT activity in the leaves increased 6 fold compared to the control. In the study, it was observed that of the 4 isoenzymes, two are dependent on manganese and the other two are copper-zinc-dependent. CAT activity was similar to SOD and glutathione reductase activities. Increased activity of some antioxidant enzymes exposed to metals reveal the crucial role that these enzymes play in heavy metal detoxification. Under normal physiological conditions, various antioxidant cycles lead to the production and scavenging of ROS which is in a state of dynamic equilibrium (Pereira, Molina et al. 2002). 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 involved 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 activity. There was however a decline in CAT activity.

Cadmium content in the aerial parts of chickpea seedlings

The cadmium content in aerial parts of chickpea grown at different concentrations of cadmium increased significantly. Highest levels were observed at a cadmium concentration of 8 mM. 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 mM (Figure 3C). Research conducted by Gross et al. (1987) revealed that the cadmium content in beans from different geographical 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 practices, when compared to genetic factors, played a greater role in the accumulation of heavy metals such as cadmium. Compared to the genus and plant species, the accumulation of heavy metals appeared to be more influenced by the genetic potential of the plant (Gross, Auslitz et al. 1987).

The distribution of cadmium to the aerial regions of the plant appears to be related to its attachment to the extracellular matrix, root flow, intracellular detoxification and transfer efficiency (Di Cagno, Guidi et al. 1999, 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 minerals (Gomes, Marques 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 (Hernandez, Carpena-Ruiz et al. 1996, Campbell, Brand et al. 1999).
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 close proximity to legumes. The study suggests that the system of cultivation of beans should be redesigned to prevent food contamination with cadmium (Liu, Zhang et al. 2012). Vijendra et al. (2016) showed that in moth bean (Vigna aconitifolia L.) cadmium concentrations increased significantly in the leaves and roots. Cadmium reaches the aerial sections of the plant via the xylem (Vijendra, Huchappa 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 from apoplastic pathways of the stems and leaves (Benavides, Gallego et al. 2005). Cadmium affects membrane potential, protein pump activity and can limit corn growth (Karcz and Kurtyka 2007).

Changes in the mineral composition of aerial regions of chickpea seedlings

Mineral composition was significantly affected by cadmium (Figure 3). Cadmium accumulation in plant species with varying tolerance to other heavy metals determines the effect of cadmium on the absorption of these other minerals in plants. Chickpeas cultivated in cadmium-containing media showed a significant difference in the levels of manganese in the aerial part of the plant. With the addition of cadmium (2 mM), manganese uptake significantly increased, while higher concentrations of cadmium reduced the levels of manganese in chickpea plants (Figure 3B). Increase in the levels of cadmium in the culture also caused changes in the levels of zinc present in the aerial parts of pea plants. Increasing the levels of cadmium in the medium resulted in a decline in zinc (Figure 3A). Increasing cadmium concentration decreased the levels of copper present in the aerial parts of chickpea seedlings. The lowest amount of copper was observed in high-cadmium seedlings (Figure 3D). 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. Cadmium has a negative impact on the absorption of essential minerals. It reduces ATPase activity and decreases the exchange of $\text{H}^+ / \text{K}^+$ ions in the plasmalemma surface (Brzoska and 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 were in close proximity to cadmium, the amount of zinc in the root decreased (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, Schmidt 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, Lu 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, Moreira et al. 2013). The phosphorus content in the aerial parts of plants treated with cadmium is related to low zinc content. The negative correlation between zinc and phosphorus in the shoots of cadmium treated plants explains the high content of phosphorus in these plants (Sarwar, Malhi et al. 2010). Analysis of cadmium and manganese content in this study supports the competitive theory of the 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 ($\text{K}^+$, $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, $\text{Mn}^{2+}$, $\text{Zn}^{2+}$, and $\text{Fe}^{2+}$) is severely affected by the presence of cadmium (Linger, Ostwald et al. 2005).

Different types of proteins and adsorption carriers for cadmium are known such as the NRAMP family (Thomine, Wang et al. 2000), P-type ATPase (Morel, Crouzet et al. 2009), ABC transporter (Kim, Gustin et al. 2004), CAX family (Hirschi, Zhen et al. 1996), ZIP family (Pence, Larsen et al. 2000), LCT transporter and CE family (Guerinot 2000). Researchers report that cadmium has an antagonistic and synergistic effect on the micro-elements 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, Heinen 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) reduced essential elements by 72, 69, 56, 61 and 52 % ($\text{N}$, $\text{P}$, $\text{K}$, $\text{Cu}$ and $\text{Zn}$, respectively). Copper content was higher in the root than the shoot of cadmium treated plants. This therefore reduces the effect of cadmium toxicity (Jibril, Hassan et al. 2017). Based on studies by Obata & Umeya (1997), cadmium increases copper absorption, but prevents its transfer to the shoots (Obata and Umeya 1997).

Gomez et al. (2013) examined the effect of cadmium on nutrient distribution in Pfaffia glomerata. Plants were cultured with different minerals and cadmium concentration simultaneously increased over a 20 day period. The study showed that cadmium strongly affects the distribution of micro and macro elements in the roots and shoots. Despite the high toxicity of cadmium, micro and macro nutrients are able to survive in contaminated environments (Gomes, Marques et al. 2013).

Manganese plays a role in many biochemical functions, such as enzyme activation in respiration, redox reactions, intracellular electron transfer systems, the Hill reaction in chloroplasts, amino acid synthesis, and hormone regulation. Manganese concentration was higher in the shoots than the roots of plants treated with cadmium. 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 and 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, Monteiro 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 $\text{Mn}$, $\text{Fe}$, $\text{Mg}$, $\text{S}$, and $\text{P}$ in the leaves of Cd-sensitive cultivars under cadmium stress is a contributing factor to the decline in photosynthesis and decrease of cabbage growth (Sun and Shen 2007).
Some researchers show that increasing zinc concentration along with cadmium reduces cadmium toxicity. There appears to be a common competitive mechanism for absorption of these elements. In our study the concentration of zinc was kept constant while the concentration of cadmium was increased. By increasing cadmium, a significant reduction was observed in the levels of copper and zinc in the aerial sections of chickpea seedlings. Lowest levels of copper and zinc were observed at high concentrations of cadmium. At low concentrations of cadmium, the amount of manganese increased. With an increase in cadmium, the level of manganese decreased. In the current study, high concentrations of cadmium did not significantly affect mineral concentration. At high concentrations there is saturation within channels or adsorption receptors preventing mineral absorption.

**Bioinformatics**

Heavy metal ATPases (HMAs), belong to the large P-type ATPase family. They play an important role in the transport of metals in plants and provide resistance to the uptake and transportation of metals. For the current bioinformatics study HMA proteins were selected. *In silico* analysis of chickpea HMAs identified 13 HMA. There were three proteins each for HMA3 and HMA4, two for HMA5 and one for HMA 2, 6, 7, 8 (Table 2). 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. 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*. 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. The HMAs in chickpeas contain nine domains which are common in all 13 HMAs. The characteristics of these domains are listed in the Appendix.

The COG4087 domain which is listed as Soluble P-type ATPase and pfam00122 as E1-E2_ATPase are present in ten HMAs. The three-dimensional structure for chickpea HMAs, the longest tunnels for each protein and catalytic pocket utilizing CAVER Web for ion passing was determined. The longest and shortest tunnels 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 which had 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.

**Table 2**: An overview of chickpea HMAs protein structure, gene 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 Coach Software for transport ions. 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 HMA3-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].
| Protein     | Protein length | Gene        | Exon count | Conserved Protein Domain Family | Localizer | Phyre2 confidence | Template                                      |
|-------------|----------------|-------------|------------|--------------------------------|-----------|-------------------|-----------------------------------------------|
| XP_004509102.1 | 839            | 101490857   | 13         | COG4087, TIGR01512            | Chloroplast | 81%               | c3rfuC, c1mhsA, c3j08A, c5mnwF, c4umwA, c3   |
| XP_004487939  | 834            | 101492022   | 9          | COG2608, COG4087              | Nucleus    | 83%               | c3rfuC, c3j08A, c4umwA, c3j09A              |
| XP_027189340  | 569            | 101492022   | 9          | c21460, COG2608               | -          | 99%               | c4umwA, c3rfuC, c3j08A, c3j09A              |
| XP_012573401  | 1032           | 101505376   | 11         | COG2608, COG4087              | Nucleus    | 72%               | c3rfuC, c2emcA, c3j08A, c4umwA, c3j09A      |
| XP_004488108  | 832            | 101497233   | 9          | COG2608, COG4087              | Nucleus    | 83%               | c3rfuC, c3j08A, c4umwA, c3j09A              |
| XP_012573132  | 853            | 101504726   | 7          | COG2217, COG4087              | -          | 99%               | c3rfuC, c4u9rA, c3j08A, c3j09A              |
| XP_012574029  | 958            | 101515614   | 10         | COG2217, COG2608              | Nucleus    | 96%               | c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c3j09A |
| XP_012574029  | 958            | 101515614   | 10         | cd02094, cd00371, cd00207     | Nucleus    | 99%               | c3rfuC, c4u9rA, c3j08A, c3j09A              |
| XP_004500941  | 130            | 101507723   | 3          | pfam00122                      | -          | 100%              | c3rfuC, c3j08A, c3j09A, c2kijA, c2hc8A      |
| XP_004511583  | 998            | 101498342   | 7          | COG2217, COG4087              | Nucleus    | 92%               | c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c3j09A |
| XP_004504792  | 934            | 101496348   | 17         | COG2217, COG4087              | Chloroplast | 84%               | c3rfuC, c4u9rA, c3j08A, c3j09A              |
| XP_004504659  | 995            | 101509532   | 10         | COG2217, COG4087              | Nucleus    | 88%               | c2ew9A, c3rfuC, c2rmlA, c2ropA, c3j08A, c3j09A |
| XP_004501429  | 884            | 101500347   | 18         | COG2217                        | Nucleus    | 86%               | c3rfuC, c3j08A, c3j09A                      |
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 protein structure.

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

The three-dimensional structure with the longest predicted tunnel allowing for passage of ions is illustrated in colour in Figure 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 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 phylogenetic tree of the HMAs, 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 in Arabidopsis and are next to HMA 3 chickpea. HMA 3 Chickpea is adjacent to HMA 3 Arabidopsis. HMA 1, 2, 3 chickpea are 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.

HMAs are classified based on substrate binding with one group bound to copper and silver and the other to cadmium, lead and cobalt. HMAs 9 and 8 have been studied in rice and Arabidopsis, respectively. AtHMA1–4 in A. thaliana and OsHMA1–3 in Oryza sativa 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-ATPases proteins have been studied in various barley plants, Arabidopsis and poplar as well as Thlaspi caerulescens (Takahashi, Bashir et al. 2012). In poplar (Populus trichocarpa), seventeen HMAs are known. PTHMA1–P1HMA4 belong to the subgroup of metals on cadmium, lead and cobalt. PTHMA5–P1HMA8 belonging to the silver and copper groups have been identified. Most of these genes are located on chromosome 1 and 2 of poplar. Typical P1B ATPase have 1 to 3 tracheal helix and one domain bound to a soluble nucleotide binding domain and phosphorylation domain and a stoator that plays a key role in the interaction of these domains. On both sides of the C and N terminals there is also a metal binding site HM44 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 plasmalaspe except PTHMA1 and P1HMA5.1 which are located in the cytoplasm. Poppal HMAs have 5 to 16 introns, PTHMA6, 5 introns, 8. PTHMA, 16 introns and 1 P1HMA, 5 introns with the remaining possessing 10 introns. PTHMA1–P1HMA4 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 from the root to the stem especially in rice grains (Li, Xu 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 vascular 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. AtHMA1 and 2 are in the plasma membrane and in zinc and cadmium fluxes. OsHMA1 is involved in zinc transfer. No HMA II 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 divided 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, Wang et al. 2016). Researchers have identified nine typical P1B ATPase in barley. HvHMA2, a P1B-ATPase is highly conserved among cereal crops with functionality in the transportation of zinc and cadmium. Additionally, HMA4 (Heavy Metal ATPase 4) plays a key role in the translocation of cadmium in non-hyperaccumulating dicots, such as Arabidopsis thaliana (Mills, Peaston et al. 2012).

Conclusion

Chickpea seedlings exposed to cadmium exhibited changes in their morphological features. These included changes in plant length, coloration and leaf size. There was a significant reduction in shoot and root length. Antioxidative enzyme activities were also affected by cadmium stress. A significant increase in POD, SOD, CAT APX enzyme activity was observed at 4 mM cadmium with a subsequent decline when concentrations were increased to 8 mM. Plants treated with 8 mM cadmium were discolored (almost completely yellow) and had a reduction in enzymatic activities possibly due to cell death. Cadmium content 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. AtHMA1 and 2 are in the plasma membrane and in zinc and cadmium fluxes. OsHMA1 is involved in zinc transfer. No HMA II 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 divided 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, Wang et al. 2016). Researchers have identified nine typical P1B ATPase in barley. HvHMA2, a P1B-ATPase is highly conserved among cereal crops with functionality in the transportation of zinc and cadmium. Additionally, HMA4 (Heavy Metal ATPase 4) plays a key role in the translocation of cadmium in non-hyperaccumulating dicots, such as Arabidopsis thaliana (Mills, Peaston et al. 2012).

Declarations

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Ethical Approval
Research not involving living human participants.

Consent to Participate
Not applicable.

Consent to Publish
Not applicable.

Authors Contributions
Maryam Kolahi and Elham Mohajel Kazemi were the supervisors and designed the study. Milad Yazdi carried out the field studies. Andrea Goldson-Barnaby carried out the statistic studies. All authors read and approved the final manuscript.

Competing Interests
The authors declare that they have no conflict of interest.

Availability of data and materials
Not applicable

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Figures

**Figure 1**

Effect of cadmium on chickpea (Cicer arietinum L.) growth under normal and various concentrations of cadmium. A: Chickpea seedlings, B: Aerial part of seedlings, C: Roots D: Leaf area (control, 2, 4 and 8 mM cadmium).
Figure 2

The activities of A) Peroxidase (POD), B) Superoxide dismutase (SOD), C) Catalase (CAT) and D) Ascorbate peroxidase (APX) enzymes in aerial organs of chickpea (C. arietinum L.). Values with different letters are statistically significantly different at p < 0.05.

Figure 3

Effects of different cadmium treatments on A) Accumulation of Zinc, B) Manganese, C) Cadmium, D) Copper content in the aerial parts of chickpea seedlings after 10 days of cadmium treatment. Values with different letters are statistically different at p < 0.05.
Figure 4

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.

Supplementary Files

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- FigS1.jpg