Physiological and Elemental analysis of *Triticum aestivum* for HMAs gene family under Zinc and Copper metal stress

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A thesis submitted to the National University of Sciences and Technology, Islamabad in partial fulfillment of the requirements for the degree of

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II

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Warda Jabbar

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V

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"My dissertation is dedicated to my Family and Friends"

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LIST OF ABBREVIATIONS

CEC	Cation Exchange Capacity
Cu	Copper
GFP	Green fluorescent protein
HMAs	Heavy Metal ATPases
HMs	Heavy Metals
MBD	Metal Binding Domains
SAR	Systemic Acquired Resistance
WEEE	Waste electrical and electronic equipment
NR	Nitrate Reductase
Zn	Zinc

Abstract

Agriculture is the main occupation of around 80% of people living in rural areas. Hence playing a primary role in improving their livelihoods, income, and food. The industrialization has accelerated in tandem with global economic growth. One of the negative consequences of rapid industrialization is heavy metal pollution. Its contamination has the potential to harm human health via food chains. In this study morphological characteristics of *Triticum aestivum* were observed to be affected by the uptake and accumulation of various amounts of zinc and copper metal under controlled glasshouse conditions using the hydroponic technique. Dharabi-11 variety (moderately sensitive and drought tolerant) of wheat was selected for this research. The concentration of 50 µM and 100 µM Zinc chloride ($ZnCl_2$) and Copper chloride ($CuCl_2$) were added for moderate and toxic treatment respectively. One-way ANOVA analysis of phenotypic data obtained under different duration of treatments showed non-significant effects as compared to the control groups. Heavy metal ATPases (HMA) are integral membrane proteins that help in the translocation of heavy metals in plants. Previously 9 HMA genes (HMA1 to HMA9) were reported. The current study has just focused on HMA1 gene.

Chapter 1

1.Introduction

Agriculture has been playing a vital role in improving livelihoods, source of income for people, and providing food. It is the main occupation for 80% of people living in rural areas. Wheat provides basic proteins and calories for 85% and 82% of the worldwide population (Jasrotia, Kashyap, Bhardwaj, Kumar, & Singh, 2018). Wheat production worldwide is 600 million tons per year. It is the third-largest crop (Barlow, Christy, O'leary, Riffkin, & Nuttall, 2015) and 200 million to 218 million hectares of the wheat yield globally covers 21% of the global population in which 80 million farmers depend on wheat (Giraldo, Benavente, Manzano-Agugliaro, & Gimenez, 2019).Wheat as a whole grain has 13% of protein content and source of minerals, fats, micronutrients, vitamins, and dietary fibers than other cereals. Even with unfavorable climate conditions in different countries, consumption of wheat is still increasing globally as it is important source of feed for animals as well. Wheat provides balance between proteins and carbohydrates, therefore, it has nutritional importance in human life (Jeber & Khaeim, 2019).

1.1. Origin of wheat

Wheat originated when evidence from archeological excavations was found from 12000BC to 6500BC, wheat cultivation was first done in Mesopotamian Fertile Crescent almost 6000 years ago then after spreading in Asia, the Middle East, North Africa, and then toward Europe. In 1500AD it was spread to South Africa and the Americas and then in 1970 to Australia (Tiwari & Shoran, 2010). Wheat quality and its yield depends on some important characteristics of wheat grain such as texture, color, plumpness, and size. The correlation between kernel shape and size with grain weight is too strong that has a role in yield potential (Gao et al., 2021).

1.2.Morphology of wheat

Morphology of wheat is related to the grass family (rhizomatous) its shoot has leafy clumps also called tillers which are hollow and cylindrical with solid nodes and the diameter of clumps starts to reduce towards the internode having spikes (peduncle) and the length of plant is genetically determined (Tiwari & Shoran, 2010).

Wheat plants have long, thin leaves and stems, as well as numerous blooms borne in a spikelet. Wheat may be produced in a range of temperatures and soils, but it thrives in

temperate areas with rainfall ranging from 12 to 36 inches (30 to 90 cm) (Evans & Wardlaw, 2017). Wheat is a crop that is farmed extensively, and its seed, which is a cereal grain, is used as a primary food source all over the world. The prominent types of wheat are common wheat (*Triticum aestivum*), durum wheat (*T. Durum*), and club wheat (*Triticum aestivum*) (*T. compactum*) (Shewry, 2009).

1.3.Nutritional influence on wheat

For the growth of the wheat crop, its nutrition is important such as micronutrients which include B, Zn, Fe, Mo, Mn, and Cu. These are the major ones that required help in increasing the growth of a wheat plant. Because of their role in increasing plant growth and nutrition of plant they have huge importance (Zain et al., 2015). Wheat contains carbohydrates. It provides around 13% of the vegetable protein in meals for humans (Rosenfelder, Eklund, & Mosenthin, 2013). When ingested as a whole grain, wheat is a valuable source of nutrients and dietary fiber. Wheat contains a protein called gluten, which can cause Celiac disease in people who have it. Celiac disease is a genetically prone immune disease. When someone with celiac disease eats gluten, their small intestine is damaged, which can lead to health problems. Dermatitis herpetiformis, gluten sensitivity, and gluten ataxia are among the conditions that can be caused by gluten (Shewry, Halford, & Lafiandra, 2003).

1.4.Factors affecting wheat productivity

Many biotic and abiotic factors play role in wheat productivity, use of improper fertilizers, their inconsistent use, and lack of information on wheat variants. Soil's condition is significant in the wheat productivity and the use of fertilizers, and their requirement is decided, humidity and moisture must be insufficient amount for the plants that are under fertilization. Therefore, absorption of nutrients, water, and their stress response to plants differ from species to species and genotypes (Pandey, Shrestha, Subedi, & Shah, 2020). Foliar application is the technology that reduces the increasing amount of chemical fertilizers and losses, amino and organic fertilizers have also benefited plants by improving the yield and growth by activating internal immunity and plant synthesis efficiency (Jeber & Khaeim, 2019).

A proper number of micronutrients in wheat is important for human health, less or more amount leads to serious health issues, number of micronutrients is becoming less day by day with the increase in yield and due to extreme use of synthetic fertilizers and, cropping intensity has also been increased. Due to this Zn deficiency was reported in World Health Report and it became the fifth major cause of death and disease in human beings this can be overcome with the help of the foliar application by the process of bio-fortification (Zain et al., 2015).

1.5.Heavy metals tolerance

As wheat is our major food so how much we intake heavy metals depends on the amount present in wheat. Through natural processes and anthropogenic activities, heavy metals are found in soil, the normal concentration of heavy allowed by international and national agencies for Pb, Zn, and Cd in wheat is 0.5-10, 10-150, 0.05-2 mg/kg. Tolerance to heavy metals varies in different crops. Different origins of heavy metals are atmospheric sources, agricultural, industrial, domestic and natural effluents (EL-Gharbawy, 2015). The combination of organic matter with heavy metal ions forms complexes due to which heavy metals mobility and activity are reduced in this way their amount becomes limited as micronutrients (S. Wang, Wu, Liu, Liao, & Hu, 2017). Heavy metal contamination of soil as a result of mining, industrial, agricultural, or military activities (Satoh-Nagasawa et al., 2012). Tolerance to heavy metals depends on the genetic variations or genotypes of the wheat plant, to achieve the genetic improvement of tolerant bread wheat and high yield depends on the genetic variability between different genotypes of wheat by evaluating the variation from performance.

1.6.Development of wheat varieties

Crop productivity in Pakistan is threatened by both biotic and abiotic stresses. Two major diseases of wheat are yellow rust (*Puccinia striiformis*) and brown rust (*Puccinia recondita*). Due to the rusts' vulnerability, most wheat types have been swapped with new cultivars (Rattu, Akhtar, Fayyaz, & Bashir, 2007). To achieve a superior quality of wheat with improved yield potential, Pakistan is now working on developing new varieties of wheat so they can increase wheat yield per hectare. A wide climatic range is required for the growth of wheat varieties (Ikhtiar & Alam, 2007).

Several types of varieties of wheat are present and every variety has its specific property some are drought tolerant, and some give a high yield. These varieties are Bakhtawar-92, Tatara-96, Chakwal-50, Dharabi-2011, and BARSAT-10. Dharabi-2011, tolerant genotypes that were identified as MRV, ADM, AAS, EM13, NRC, TTR, VLN, GRG, WC11, PIR, and WC13. Dharabi-11 is drought- and disease-resistant in addition to having a high potential yield. If this variety is grown in the country, not only will it prevent the risk of Ug99 (stem rust), but it will also help the country grow more wheat (Tariq et al., 2013). Genotypes with intermediate performance were PIR, TTR, and NRC, sensitive genotypes were D86, BKH and EB14. Others that were the same sensitive included DRB, EB12, CKW, EB10, and D93, however, KRN, EB13, EB10, CKW, and DRB were more tolerant due to their better chemical profiles by Principles Component pk Analyses (PCA) and Hierarchical Agglomerative Cluster Analysis (HACA) (Ali, Razi-ul-Hasnain, Quraishi, & Malik, 2018).

1.7.Heavy Metal ATPases (HMAs)

Recent research has shown that HMAs are transmembrane proteins for transporting metal playing an important part in the translocation and absorption of heavy metals (Colangelo & Guerinot, 2006). P1B-type ATPases which transport heavy metals using ATP which is why these are also called heavy metal ATPases (HMAs). P-type ATPases control the active transport of a variety of cations through cell membranes. There are more than 50 P-type ATPases. The energy released during the hydrolysis of ATP's terminal phosphate bond is used to move cations against their electrochemical potential gradient (J. M. Argüello, E. Eren, & M. González-Guerrero, 2007). P-type ATPases come in five different subtypes, P_{1B}-type ATPases translocate heavy metal ions, P2A and P2B groups play their role in calcium ion transportation, and all eukaryotes have P4- and P5-ATPases, while prokaryotes lack themP4-ATPases maintain lipid bilayer equilibrium and vesicle-mediated transport. P5-ATPases' function is still unidentified (X. C. Zhang & Zhang, 2019).

According to numerous studies, HMAs are split into subclasses based on their heavy metal sensitivity. The normal concentration of heavy metals is the basic requirement for the normal growth of plants. Unnecessarily increased concentrations of heavy metals

become harmful to plant growth (Takahashi, Bashir, Ishimaru, Nishizawa, & Nakanishi, 2012). HMAs have two functions. They provide heavy metals needed for the normal growth of the plant. They also exude toxic heavy metals over the plasma membrane (high concentrations of Zn, Cu, Cd, Pb, Co, etc. are toxic to plants) (Palmgren & Nissen, 2011). Zn and Cd are transported out of the cell and their movement via the xylem, from roots to shoots, with the assistance of *AtHMA2* and *AtHMA4* transporters. *HMA3* proteins, like *OsHMA3*, *TcHMA3*, and *AtHMA3* which are found in tonoplasts help in pumping Cd and/or Zn into vacuoles. A lot of evidence shows that HMAs play a substantial role in the trafficking of heavy metals via transmembrane (Qiao, Gong, Tian, Wang, & Chai, 2018).

1.8.Aims and Objectives

- To identify the genes that help in the translocation of Cu and Zn heavy metals
- To identify heavy metal uptake after every 7 days interval through elemental analysis
- To check the gene expression through qRT PCR
- Analyze the data
- Compare the data with replicates

Chapter 2

2. Review of Literature

2.1. Processing and utilization of wheat

Wheat used for food must be processed, which involves cleaning and breaking up the grain using water. Wheat is split and pushed through rollers to form smaller particles during milling. Most of the wheat flour is utilized in the production of bread. Cereal, cookies, crackers, flour, pastries, pasta, and muffins are all made with wheat. Wheat can also be used to make alcohol, malt, dextrose, gluten, starch, and a variety of other goods (Rosenfelder et al., 2013).

2.2. Worldwide production of wheat

Wheat is grown as a primary crop because it has a maximum yield relative to the amount of land it occupies. It does best in a climate that is relatively mild and has a relatively brief growing season. The United States Department of Agriculture (USDA) forecasts world wheat output for 2021/2022 at 778.52 million metric tons, up 2.10 million tons over the previous month's forecast. Last year, 776,000,000 tons of wheat were produced. The predicted 778.52 million tons of wheat produced this year could indicate a 2.52-million-ton gain, or 0.32 percent, above last year's total.

Wheat is Pakistan's most popular food grain and a cornerstone of Pakistani cuisine. It is an extremely important part of the process of developing agrarian programs. It adds 14.4 percent to the value that agriculture already has, and it adds 3.1% of the country's GDP (GDP). Wheat productivity in the United States was 24,231,000 tons in 2012–2013, up 3.2 percent from 23,473,000 tons in 2011–2012. The amount of wheat that was produced on a global scale in the 2019/2020 marketing year was approximately 765 million metric tons. This was a 30-million-ton increase from the previous year (Shahbandeh, 2022).

2.3. Genome of wheat

Wheat was first cultivated around the time called the "Neolithic Revolution," which occurred around 10,000 years ago and marked the transition from a foraging and hunting lifestyle to a more established agricultural system (Dubcovsky & Dvorak, 2007). The fact that the early cultivated wheat types were diploid (genome AA) (*einkorn*) and tetraploid (genome AABB) (*emmer*), respectively (Kilian, Martin, & Salamini, 2010).

Nearly nine thousand years ago, hexaploidy wheat bread was first spotted, and people in the Near East began to dabble with crop cultivation for the first time. Wheat's genomes have three sub genomes: A, B, and D so it is more difficult to study than the genomes of other crops. It is believed that the domesticated tetraploid wheat (*T. turgidum, ssp dicoccum*) that was used to cultivate common wheat was crossed with the goat grass (*Aegilops tauschii*). It is believed that *T. urartu* (AA) is the source of the genome A, whereas the origin of the genome B is unknown (Guan et al., 2020). The hexaploidy of the wheat genome needs to exist for common wheat to be a superior crop to its ancestors. This is because interactions among the wheat's sub genomes lead to heterogeneity in its gene expression, which may explain its increased tolerance to a variety of environmental factors (Brenchley et al., 2012).

2.4. Issues in wheat productivity

There are three key issues in crop productivity: maximizing and protecting yield potential, and improving supply consumption for long-term sustainability (Iftikhar, Ghori, Ali, Sheikh, & Gul, 2019). These issues are intertwined, and there is conclusive proof that increasing average yield often leads to higher yields, especially under stressful situations. Wheat genetic improvement has gotten a lot of attention in recent decades, intending to increase grain output while reducing crop yields owing to adverse climatic conditions and harm from numerous viruses and pests (Agrios, 2005). Yield reduction, caused by biotic and abiotic pressures, damages not just the farmer but also the entire country's economy.

Because crop yields remain constant, the profit expected from crop harvest rises exponentially. Pests cause a 34.5 % loss of cereals every year. This loss varies depending on abiotic stresses. The goals of gene engineering shifted in the early 1960s to reduce yield variability induced by biotic and abiotic stressors and to improve resource usage efficiency. Following that, due to changes in global food strategy over the last several years, biotechnology initiatives provided a viable solution, initially by lowering farm-level production costs by offering plants that are considerate to various biotic and abiotic stresses and, more importantly, by refining product quality (Iftikhar et al., 2019).

2.5. Metal Toxicity

Metals are typically present in soil. They can be beneficial or damaging to plants. Metals are required in small numbers for crop growth. But when metals detected in enormous quantities in the soil, they hinder crop growth. When these ions and harmful metals build up in the cellular structure, the primary issue arises. The number of metals accumulated in plant cells is determined by the plant species, their capacity of nutrients absorption, and the number of metals in the soil. Industrialization is to blame for the rise in toxicity in the soil (Rizvi et al., 2020). Metal poisoning can be caused by both natural and artificial resources. The qualities of soil are affected by metal toxicity. Metal poisoning is caused by extreme absorptions of metals such as zinc, cobalt, aluminum, lead, mercury, and copper.

Heavy Metals (HMs) with concentrations greater than $5gcm^{-3}$ can pollute the soil (Kamitani, Oba, & Kaneko, 2006). As a result, the world's cultivable land is diminishing due to soil degradation, which has become a severe concern in many countries. (Tchounwou, Yedjou, Patlolla, & Sutton, 2012). Other sources of HMs contamination in agricultural soils comprise volcanic emissions, metal-containing dust dissemination, and the degradation product of HM-enriched rocks (Algreen et al., 2012). Apart from these natural sources, anthropogenic operations involving fast-developing enterprises, mines, and smelters, can also add a lot of HMs to cultivable soils (Gautam, Gautam, Banerjee, Chattopadhyaya, & Pandey, 2016).

2.5.1. Heavy Metals Uptake and Accumulation

Plant uptake of HMs is reliant on the heavy metal activity in the soil. Additionally, it is extremely change the pH of the soil as well as the quantity of organic matter that is already in soil (Maiz, Esnaola, & Millan, 1997). The pH of the soil can influence the complex metal cations formation as well as the rate of ion exchange. In addition, because a high pH soil is electronegative, it can increase both the adsorption and the precipitation of metal cations , hence impacting the metal uptake by plants (Adachi & Tainosho, 2004). Generally, organic material in soil tend to mix with ions of heavy metal to produce organic complexes. These complexes can decrease the accessibility and transportation of HMs (McGrath & Cegarra, 1992).

Research has discovered that the activity of several HMs directly proportional to the amount of soil organic matter increases. Because of this, the subject of whether soil organic matter might assist in the uptake of HMs by plants continues to be disputed (Smith, 2009). In addition to the redox and chemical form of HMs, several factors affect the circulation and allocation of HMs in soil-plant systems, including the contents of soil clay, iron and manganese oxides contents, cation exchange capacity (CEC), plants specie, climatical situations, and irrigation with polluted water (Sindhu, Saharma, & Pooja, 2015).

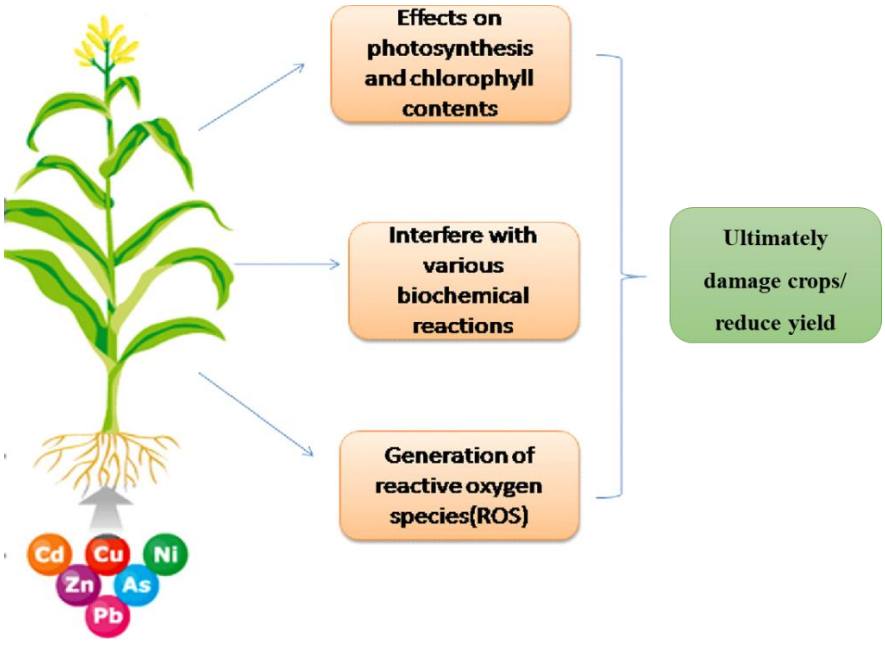

Figure 1: Heavy metals accumulation in plants

Accumulation of HMs is one of the primary agricultural issues that has gravely impacted food safety, among several others (Ghani et al., 2015). Overly high HMs concentration in agrarian commodities caused by heavy metal pollution (HMP) in the soil has gotten a lot of attention in recent years. Due to various problems, soil biologists and agronomists have recently expressed worry about heavy metal pollution, which is wreaking havoc on agro-ecosystems and agricultural output. Toxic HMs, once accumulated beyond a certain acceptable limit, harm the density, composition, and physiological activities of microbiota, as well as the dynamics and fertility of the soil. this results in a decrease in agricultural output and, via the food supply, human and animal health (Kumar & Chopra, 2015).

2.5.2. Zinc

Zn and magnesium (Mg) are chemically identical, with identical sizes and oxidation states of + 2. Zn^{2+} ions have strong binding affinities for nitrogen, sulfur, or oxygen. The typical range of zinc concentration that is necessary for many crops is between 30 and 200 g Zn g^{-1} dry weight (DW) (Leuci et al., 2020).

Additionally, tryptophan a precursor to auxin requires zinc (Tsonev, Cebola Lidon, & Agriculture, 2012). Zn is essential for more than 300 enzymes, including phospholipase, superoxide dismutase, alcohol dehydrogenase, alkaline phosphatases, carboxypeptidases, aldolases, carbonic anhydrase, etc. (Gupta, Ram, Kumar, & Bio/Technology, 2016). Zn metalloenzymes include RNA polymerases and reverse transcriptase, which synthesize DNA and RNA. Additionally, zinc aids plant defense mechanisms; for instance, Zn finger WRKY transcription factors are responsible for the regulation of systemic acquired resistance (SAR). In the salicylate defense signaling pathway, the Zn-binding protein *HPP3* is necessary (Choi, Liu, & Pan, 2018).

The combustion of fossil fuels, phosphate herbicides and pesticides (50–1450 µg Zn g^{-1}), hummus (15–250 µg Zn g^{-1}), sandstone (10–450 µg Zn g^{-1}), rubber mulches, particles from Zn-coated surfaces, and carbonaceous modifications (10–450 µg Zn g^{-1}) are additional man-made sources of zinc that are introduced into soils. It has been reported that waste electrical and electronic equipment (WEEE) has an average zinc content of about 4,000 mg kg^{-1}DS, that sewage sludge contains about 1,000 mg kg^{-1}DS,

that municipal solid waste (MSW) contains between 3,000 and 4,000 mg kg-^{1}DS, and that recovered waste wood contains between 400 and 600 mgkg^{-1}DS.Increased Zn levels in soils are toxic to plants, causing a variety of structural and functional problems that degrade plant productivity. These responses can vary extensively depending on the species of plant and the stage it is in its development (Chen, Gaillardet, Louvat, & technology, 2008).

2.5.2.1 Zinc Toxicity

The most visible physiological reactions of plants to Zn toxicity are a decrease in germination activity and biomass, which subsequently leads to a loss in productivity and product quality (Rout & Das, 2009). Excess Zn reduces nitrate (NO_{3-}) absorption and translocation along with total nitrogen content so that disrupting Brassica species' nitrogen metabolism. Zn stress causes chloroplast instability and restricts NO_{3-} at the nitrate reductase (NR) site that inhibiting its action. A decrease in NR activity decreases the number of free amino acids (such as asparagine and glutamate), which impedes photosynthesis in plants. Metals in the soil are taken up by more than 500 different species (Nagajyoti, Lee, & Sreekanth, 2010).

A decrease in NR activity decreases the number of free amino acids (such as glutamate and asparagine), which impedes photosynthesis in plants. Wheat, like so many plants, is heavy metal susceptible. HMs stress plants, affecting germination, growth, metabolic reactions, and reducing yield in wheat (X. Zhang et al., 2011). The utilization of cadmium by plant roots altered the sustainability and yield of wheat. In a growth chamber of the University of Agriculture Faisalabad, ZnSO4 was examined on two types of wheat growing in soils with sandy loam. The lethal amount of Zn harmed wheat morphology, physiological traits, and yield (Dong et al., 2011). The highest Zn level was found in roadside soil. In soil-wheat systems near roadways, researchers discovered that heavy metal concentrations steadily drop with distance from the roadbed (S. Wang, Wu, Liu, Liao, & Hu, 2017).

2.5.2.2. Mechanism of Zn Uptake and Accumulation

It's important to remember that roots aren't just fixed organs when it comes to nutrient absorption. In the rhizosphere, plant roots release a variety of, amino acids,

protons, carbohydrates, organic acids, and even some mineral ions (Kaur & Garg, 2021). These help them operate and grow properly. Roots acquire zinc as the divalent metal ion Zn^{2+} via flow rate and diffusion processes. In the process of passive zinc uptake by these channels, water molecules, which act as a solvent, are involved, as change in the zinc concentrations that exist across the root cell-plasma membrane (RCPM). The signaling cascade of RCPM, is aided by the functioning of the RCPM H+-ATPase system. This is the fundamental driving force (cation uptake) (Lin, Aarts, & sciences, 2012).

The RCPM H^{+}-ATPase system aggressively pumps H+ ions out of the cell. As it hydrolysis ATP. On the one hand, The RCPM becomes more hyperpolarized because of the release of H^{+} ions in the rhizosphere, while on the other hand, it lowers the pH of the soil, which ultimately leads to a higher cation uptake rate (Gupta et al., 2016). Charged zinc ions, in contrast to water, cannot freely move through cell membranes. These divalent cations must therefore be carried by proteins that are intended for the job. These proteins are not linked to ATP hydrolysis, indicating that Zn absorption is passive rather than active. In addition, through non-selective cation channels connected to the passive flow of many cations, Zn^{2+} can be taken up. It is believed that the low cytoplasmic activity of many metal cations, which is caused by metal absorption and binding to intracellular sites, is a driving force in the absorption of these metals (Loneragan & Webb, 1993).

Zinc accumulation differs dramatically between flowering and post-flowering periods. At the post-flowering stage of *Solanum nigrum*, greater Zn was found in the root cortical parenchyma, according to a recent study. After blooming, the plant was able to tolerate larger amounts of zinc due to the strong constitutive synthesis of organic acids in root tissue. There is limited transfer of zinc to aerial sections. The amount of zinc in leaves varies depending on where they are on the stem. The amount of Zn accumulated by older leaves is lower than that of younger leaves. Zn concentrations change from the top of the plant's stem to the bottom of the stem. There were also found to be seasonal changes in the distribution of zinc in *Phragmites australis*, with the amounts of zinc gradually falling from spring through winter. Furthermore, metal concentrations in

deciduous trees, shrubs, and grasses are highest in the autumn and lowest in the spring (Lin et al., 2012).

The meristematic cells of *T. aestivum* roots were affected by Zn^{2+} poisoning, which resulted in a rise in the number of vacuoles, thickening of cell walls, and the granular material building up inside the vacuoles. These modifications show that in the presence of surplus Zn, the synthesis of cell wall components is accelerated. These also allowing the apoplast to sequester the overloaded metal in less damaging locations. An increase in vacuoles filled with granular deposits shows that plants store additional zinc to protect themselves from metals (Broadley, White, Hammond, Zelko, & Lux, 2007).

2.5.3. Copper

Copper (Cu) is a trace element found in plants, soil, rocks, and animals. Agricultural soils absorb significant hazardous amounts of Cu due to anthropological activities (Adhikari, Kundu, Biswas, Tarafdar, & Rao, 2012). Cu has been deposited in the top layer of agricultural soils as a direct Copper-based insecticide is widely used, including insecticides (e.g., vineyards), fungicides, and bactericides (Mackie, Müller, & Kandeler, 2012). Bordeaux combination ($Ca(OH)_2$ + $CuSO_4$) will be sprayed as a preventative measure against vine downy mildew. Because of this, the level of copper pollution in European vineyard soils has increased. A specific implementation of the Bordeaux combination brought in 3–5 kg Cu ha^{-1} in Champagne (France) (Legros et al., 2010).

Copper concentrations in uncontaminated soil range from three to one hundred mg kg^{-1}. However, crustal rocks include 55 mg kg^{-1} of this element. Depending on the kind of soil, the concentration of copper in cultivated fields might range anywhere from 5 to 30 mg kg^{--1}. Vineyard soils have copper levels ranging from 200 to 500 mg kg^{-1} on average. The concentration of copper in the soil solution is often relatively low in unpolluted soils. The average copper concentration in sandy soil is 11 M, whereas the average concentration of copper in limestone soil is 0.8 M (Q.-Y. Wang, Liu, Wang, & Yu, 2015).

The impacts of relationships between Chromium and Copper and temperature on wheat seedlings were investigated in a study. High doses of Cr and Cu at 40°C reduced

root and shoot length and dried weight. While 30 μM Cr at 40°C reduced total chlorophyll content (Mench, 1990). Higher concentrations of Cr and Cu, on the other hand, boosted carotenoid and proline levels while decreasing soluble protein in comparison to control plants. When contrasted to Cu, Cr had the most damaging impact on the development and metabolic parameters among the metals. The location with the maximum soil Cu concentration was one where wastewater irrigation had been used for a long time (C. Wu, Luo, & Zhang, 2010).

2.5.3.1 Copper Poisoning

Copper poisoning is a considerable issue in crops. Higher Cu concentrations are poisonous to plants, generating considerable damaging consequences at all stages of plant life, spanning from phenotypical to molecular and cellular levels (Feigl et al., 2013). Copper-induced morphological modifications in various crop plants have been studied. Higher concentrations of Cu in plants modify the morphological characteristics. Length of root and shoot in various plants gradually decreases to inordinate Cu concentration. In comparison to the control plants, maize plants exposed to nutritional solutions containing higher concentrations of copper (103 mM) had shorter shoots, roots, and leaf lengths. Extreme Cu dosages also resulted in a reduction in maize plant height (Barbosa et al., 2013).

Furthermore, in maize, a 15.7 μM Cu treatment decrease root length by 90% when compared to the control. Maize shoot length decreased by 23% when treated with 10 μM Cu (Shahbaz et al., 2010). Similarly, the root and shoot length of wheat was reduced by 72 and 31 percent, respectively, when exposed to a concentration of copper in nutritional media of 50 parts per million (ppm) over six days. At 25 ppm copper in the nutrient solution, mung bean root length is inhibited (Gajewska & SkŁodowska, 2010).

Furthermore, the toxicity of high Cu concentrations in cucumber (*Cucumis sativus L.*) and tomato (*Solanum Lycopersicum L.*) roots was studied after 7 days of treatment. At the same Cu treatment, the roots of cucumbers were more susceptible to damage from Cu than tomato roots were. Cucumber roots were more toxic to copper than tomato roots under the same conditions. Furthermore, rice plants exposed to 5 μM Cu had their root length reduced by 55% (compared to control). Rice plant height was shown to be lowered

by up to 48.4 percent when exposed to a soil Cu level of 1000 mg/kg, in comparison to control that had 75.4 mg/kg of soil.

Durum wheat was used in the study, and it was cultivated under varied concentrations of Cu stress for eight days. The results showed that, similar to earlier research, 2420nM Cu reduced root length and prevented the growth of lateral roots (Sánchez-Pardo, Fernández-Pascual, & Zornoza, 2014). In a similar vein, if there is an excessive amount of copper in the culture medium, it will inhibit the growth of the durum wheat roots, causing them to become brown and thick (Feigl et al., 2015).

Copper poisoning affects the number of leaves, elongation, and stem size. For example, at greater Cu concentrations (100 μg/ha) in maize plants diameter, leaf area, and length are reduced. Indian mustard (*Brassica juncea L.*) and rapeseed both experienced a reduction in stem size because of Cu stress (*Brassica napus L.*). The leaf area of cucumber plants was reduced comparably when Cu (10μg/g) was applied. When cucumber cells are exposed to too much Cu (20 mg/kg of sand) for 20 days, their leaf area and stomatal conductance drop by 37 and 52 percent (Yotsova, Dobrikova, Stefanov, Kouzmanova, & Apostolova, 2018). Similarly, a high concentration of copper (1.55 ppm or higher) caused a reduction in the number of leaflets and leaf area in cucumber plants for ten weeks. In comparison to the control plants, the leaves of the soybeans that were given 192 μM of copper for 35 days resulted in a significant narrowing of the leaf blades, and both the adaxial epidermal cells and the lamina parenchyma cells were reduced in size. The ultrastructure of soybean chloroplasts was also altered as a result of Cu stress (D. Singh, Nath, & Sharma, 2007).

The wheat plant's overall leaf area and height decreased as soil Cu content increased. Many plants' leaves show varied visual toxicity symptoms when exposed to too much copper (L. Liu et al., 2022; Luo, Li, & Zhou, 2008). Chlorosis of the leaves was caused by copper stress, for instance, and was observed in rapeseed plants and Indian mustard. Similarly, chlorosis was observed in maize plants that had an external Cu concentration of 78.7 M (Yotsova et al., 2018). The damaging effects of copper on plant biomass vary depending on the species studied; for instance, wheat and sorghum (*Sorghum bicolor L.*) plants were found to be more subtle to copper stress when

paralleled to maize plants, and they also showed a decreasing trend as excess Cu accumulated (Wodala, Eitel, Gyula, Ördög, & Horváth, 2012).

In numerous studies, Cu has been shown to interfere with variability of metabolic pathways that are essential for plant development and growth. Phytotoxicity (the ability of a plant to be harmful to Cu) differs significantly within species and between species. The growth of roots is more vulnerable to copper poisoning than the growth of shoots. Cu toxicity can be decreased by using the right soil and plant species/cultivars (Dresler, Hanaka, Bednarek, & Maksymiec, 2014).

2.5.3.2. Copper Uptake Mechanism

Cellular Cu concentration is controlled by a sophisticated homoeostatic mechanism in plants, and it depends on a series of stages like a) uptake of Cu HM from root, b) translocation of Cu from root to shoot, c) compartmentation in vacuole d) and distribution and/ or redistribution of Cu to various plant organs (I. J. B. J. o. P. P. Yruela, 2005). Different families of Cu heavy metal transporters have been identified. These transporters are P- Type ATPases copper transporters, COPT (CTR-like high-affinity Cu transporter), and copper chaperones (Burnstock, Williams, & Therapeutics, 2000; Markossian & Kurganov, 2003; I. J. F. P. B. Yruela, 2009). Plants mainly uptake Cu in reduced form (i.e., Cu^{+}) by highly selective transporters COPT proteins like *AtCOPT1* and *OsCOPT/Ctr* (Huang et al., 2011; Jouvin et al., 2012; Sancenón et al., 2004). Free Cu^{+} stimulates the production of ROS in the cytosol which opens non-selective cationic channels. This allows entry of Ca^{+} ions and induces the root growth. Plant's root growth may further be modulated by Cu if it interacts with auxin efflux carrier pin formed 1 (PIN_1) and with the low phosphate response multi-copper oxidases ($LPR_{1/2}$) (Yuan, Xu, Liu, Lu, & Physiology, 2013).

In case of Cu toxicity, immense generation of ROS takes place which activates the K^{+} efflux via non-selective cationic channels. This causes activation of programmed cell death. ROS generation can be prevented by chelating Cu^{+} through specific chaperones or intracellular metallothionein (RODRIGO- MORENO et al., 2013). Several

$P1_B$-ATPases members have been identified which are involved in Cu homeostasis in monocot model plant *Arabidopsis* and dicot model plant *O. sativa*. For instant, *AtHMA5* participates in removal of Cu in plant roots and/ or performs function of Cu translocation from root-shoot via xylem (Andrés- Colás et al., 2006; Kobayashi et al., 2008). *AtHMA6/PAA1* is involved in transport of Cu across the chloroplast envelope.

Similarly, *AtHMA6/PAA1* and *AtHMA8/PAA2* both are involved in transportation of Cu into the chloroplast (Abdel-Ghany, Müller-Moulé, Niyogi, Pilon, & Shikanai, 2005; Shikanai, Müller-Moulé, Munekage, Niyogi, & Pilon, 2003).Function of *AtHMA7/RAN1* was identified during highlighting the role of Cu transportation to the ethylene signaling pathway where, it is required for the formation of functional ethylene receptors (Hirayama, Alonso, & physiology, 2000; Rodrıguez et al., 1999).

In *Oryza sativa*, *OsHMA4* and *OsHMA5* has been identified which are involved in transportation and loading of Cu from root to shoot and detoxification in roots (F. Deng, Yamaji, Xia, & Ma, 2013). Similarly, study on heritable changes in gene expression analysis of rice elucidated significant upregulation of *OsHMA2 and OsHMA5* and transcriptional activation of *OsHMA6* and *OsHMA7* under Cu stress (Cong et al., 2019).

2.6. P-type ATPases

The P-type ATPases are a vast protein family that results in the transportation of ions and lipids via cytomembrane. Various subfamilies of pumps, each with a distinct transport specialty, are required for the survival of almost all life forms. For instance, metabolic energy is converted to electrochemical energy using pumps. That can be used for the uptake of cellular mechanisms, facilitate cellular signaling, supply metalloenzymes, and initiate vesicle budding, which serves as the foundation for eukaryotic organelles.

2.6.1. Domains of P-type ATPases

The five major and unique domains on basis of structure of a P-type ATPase. Each domain is composed of three cytoplasmic domains.

1. Actuator (A) domain
2. Nucleotide-binding (N) domain

3. Phosphorylation (P) domain

There are two membrane-fixed domains.

1. Transport (T) domain
2. Class-specific support (S) domain
3. The regulatory (R) domain is the name given to the fourth cytoplasmic domain if it is present. It is always located at the N or C terminal, and both have it. Asp residues present at P-domain is phosphorylated, occurs during each catalytic cycle, which is followed by P-domain dephosphorylation by the N and A domains. This means that the P-domain, A-domain, and N-domain all work as protein kinases. While the P-domain works as a substrate for both.

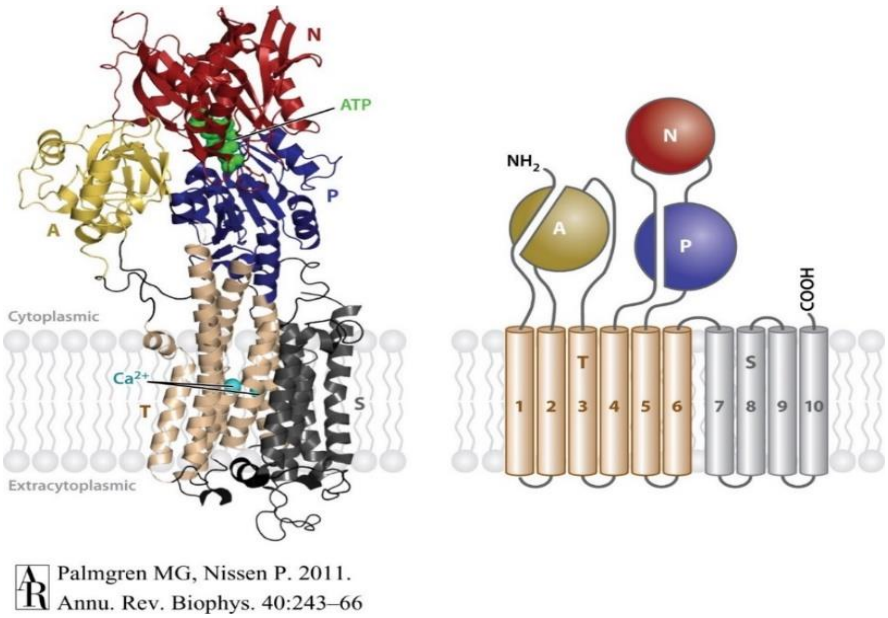

Figure 2: Structure of P-type ATPases

2.6.2. Subgroups of P-type ATPases

The P-type ATPases essential structures are unique. In genomes, various P-type ATPase sequences can be identified due to the presence of conserved sequences and signature motifs. The superfamily of P-type ATPase may be categorized into five separate subfamilies P1, P2, P3, and P5. Each further subdivided into subgroups (A, B, and so on). The following are the most significant organizations

i. P1A-ATPases are essential to the transport of bacterial potassium.
ii. P1B-ATPases are significant for the transport of metal.
iii. P2A and P2B ATPases are responsible for Ca^{2+} pumps.
iv. P2C and P2D ATPases subtypes are presumed Na+/K+ and H+/K+ pumps of mammals. P2D ATPases are presumed Na+ pumps of fungi.
v. P3A ATPases have presumed plasma membrane H+ pumps.
vi. P4-ATPases are presumed lipid lipases.
vii. P5-ATPase pumps have not any specificity yet.

Table 1: Plant P-Type ATPases and their functions

Plant P-type	**Characteristic Domain**	**Transporting Ion**	**Functions**
P1B	Heavy metal-binding domain	Cu^{+}, Cu^{2+}, Cd2+, Zn^{2+}, Pb^{2+}, Co^{2+}, Ag^{+}	Phytoremediation of metal ions, Homeostasis
P2A(ECAs)	Ca^{+2} binding domains (M5, M6, and M8)	Ca^{2+}, Mn^{2+}, Zn^{+2}	Cell signaling, Trace element homeostasis
P2B(ACAs)	N-terminal autoinhibitory domain (CaM binding)	Ca^{2+}	Calcium ions are transported in the plasma membrane, Signaling
P3A	C-terminal autoinhibitory domain (14-3-3 protein binding)	H+	Involved in Plasma membrane potential, pH homeostasis
P4	Autophosphorylation at a conserved aspartate residue	Phospholipids	Lipid transport and lipid bi-layer asymmetry
P5A	Ma and Mb catalytic domains	Unknown	Fertilization and pollen development

2.6.3. Heavy metal ATPases (P1B-ATPases)

P1B-ATPases present in every living body, common in bacteria and archaea (Chan et al., 2010). Based on their evolutionary relationship, the monovalent and divalent metal pumps make up the P1B subfamily's respective classifications. There are two distinct functions of P1B-ATPases (Mills, Krijger, Baccarini, Hall, & Williams, 2003). They offer HMs (most often Cu and Zn) for the production of metalloenzymes, which are found in the periplasmic space of prokaryotes and the lumen of secretory vesicles in eukaryotes, respectively (Mills et al., 2008). Hazardous HMs are extruded over the plasma membrane during this procedure (Cu and Zn are harmful at high concentrations)(Satoh-Nagasawa et al., 2013). The N-domain of P1B-ATPases very slightly resembles other pumps, as compared to the bacterial P1A-ATPase, (Mills et al., 2008).

P1B ATPase's A-domain exhibits a startling contrast between primary and tertiary structural conservation despite showing little sequence homology to other pumps(Mou et al., 2020). The unique terminal extensions of P1B-ATPases, which are densely packed with Cyst and, in some cases, His residues, serve as a distinctive feature (F. Deng et al., 2013). Due to their tight coordination, binding metals have more affinity for P1B ATPases' terminal domains (Chan et al., 2010).

Transduction of metal ions involves the P1B subfamily of P-type ATPases including zinc, copper, cobalt, lead, and cadmium thereby maintaining a homeostatic environment in the cell which is possible due to its metal-binding domain in C- or N-terminal along with a conserved sequence of CPx/SPC located in 6^{th} out of 8 membrane-spanning domain and *Arabidopsis* plant contains eight types of HMAs having distinguishing characteristics (Andrés- Colás et al., 2006). HP locus is conserved only in

plant HMAs and absent in two divalent cation transporters of algae i.e., *CrHMA1* and *CmHMA2*, which along with a nearby glutamate help in nucleotide coordination and also in catalytic activity as suggested by an experiment done on *E. coli* Znta (*EcZntA*) mutants (Williams & Mills, 2005).

Arabidopsis HMA1 localized in green tissues (chloroplast envelope) for the transport of copper ions and analyzed by experiment on the basis of deletion mutants of *hma1* that absence of N-terminal histidine-domain somewhat affects this transport and these mutants are also characterized to have lower Cu^{+2} ion concentration and complete blockage of superoxide dismutase activity in the chloroplast (Mills et al., 2003). *HMA6/PAA1* also has the same catalytic activity as that of *HMA1* and mediates the import of copper ions in the chloroplast envelope which are essential micronutrients as well as activate cell-damaging free radicals having functional contribution in the Cu^{+2} homeostasis which is diverse pathway and *paa1* mutants recovered their phenotypes when provided with copper supplements (Okkeri, Bencomo, Pietilä, & Haltia, 2002).

HMA2 maintains the heavy metal content in plants mainly Zn^{+2} translocation and metal-binding domain (MBD) located at the C-terminal of *HMA2* rich in cysteine-histidine residues when modified after removing 244 amino acids lowered its enzymatic activity by 43% and diethylpyrocarbonate activity disrupted zinc binding to histidine residues showing a crucial part of His-residues in metal binding (Seigneurin-Berny et al., 2006). *HMA4* was involved in maintaining Zn and Cu concentration, when investigated for heavy metal homeostasis grouped in HMAs (transporting Cu/Zn/Cd/Pb/Co) for the evolutionary development and its cDNA sequencing also showed the same conserved motifs that are the characteristic of this sub-class. Yeast expression system also represented Cd resistance in the cells having *HMA4* ATPase while increased levels of Manganese and Zinc in roots elevated the expression of *AtHMA4* with the highest expression in roots as compared to other organs.

OsHMA3 (*Oryza sativa* HMA3) was found to control Cd transport from root to shoot analyzed by positional cloning and localized in the vacuolar membrane by green fluorescent protein (GFP) while *OsHMA3mc* is a defective allele that hyper accumulates Cd for translocating it to xylem cells because of this shortcoming Cd is transported to

shoot instead of its sequestration in the vacuole. Micronutrients for plant growth and development are also heavy metals such as Cu, Zn, Ni, Mn, Co, and Fe are essential but an excess of these elements along with non-essential elements like Cd, Hg, Ag, Se and Pb can cause phytotoxicity which can be the consequence of blockage or displacement of crucial biological molecules or enzymes that regulate the metabolic pathways, explained in figure 3(Chaudhary, Jan, & Khan, 2016; Park, Ahn, & science, 2014).

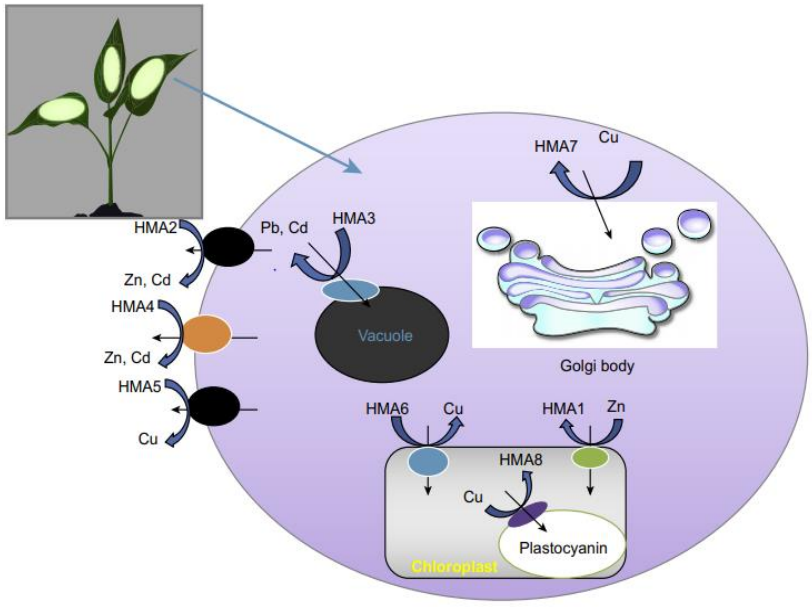

Figure 3: Hyperaccumulation of heavy metals by plant HMAs (Chaudhary et al., 2016)

HMA2 gene of *Triticum aestivum* (*TaHMA2*) when overexpressed in rice plants, showed an improved level of translocation of Cd and Zn. Zea mays cultivar also hyper accumulates chromium from the industrial wastewater into roots and young leaves when toxicity tests were conducted for tannery leftover water (Boutigny et al., 2014). *AtHMA5* has been identified as a transporter of Cu that delivers it in the secretory pathway with the help of ATX1-like metallochaperone and its mutants after T-DNA insertion copper responds well unlike other metals, defining their regulatory role in copper accumulation in roots and this regulation is due to binding of chaperons with strictly conserved amino acid residue (Chaudhary et al., 2016).*OsHMA9* is involved in zinc transport as a metal

ion efflux. ATPase and can be utilized to increase levels of essential micronutrients such as Zn and Fe as a medium for biofortification in wheat and rice by QTL (Quantitative Trait Loci) mapping and high throughput genotyping and in this way candidate genes can be identified, and allelic variations can be studied(J. M. Argüello, E. Eren, & M. J. B. González-Guerrero, 2007).

2.7. Metal Binding Domains

At the C terminus (in eukaryotes, humans, and other animals) (MBDs) and N terminus in (prokaryotes, plants, and fungi) metal-binding domains or P1BATPases can transport HMs in the absence of their MBDs, contrasting against the notion that these domains are required for metal transport (Yu, Dolgova, & Dmitriev, 2017). MBDs often contain many metals binding sites, making them particularly well suited for use as metal radars. Half-life of Yeast's Pca1p Cd pump is less than 5 minutes, making it one of the most transient proteins known (Mandal & Argüello, 2003). A part of the MBD domain located at the proteasome's N-terminus serves as a breakdown signal for the degradation of ubiquitin-mediated proteins. (C.-C. Wu, Rice, & Stokes, 2008). Because of Pca1's more constrained folding and ability to tolerate degradation, Cd binding to its N-terminal MBDs appears to mask this signal (T. Liu, Reyes-Caballero, Li, Scott, & Giedroc, 2007). The pump can break free after being bound by the MBDs and be transported to the cell membrane, where it performs as a reliable and long-lasting pump (Drees, Beyer, Lenders- Lomscher, & Lübben, 2015).

Increasing the concentration of Cu in the cell activates P1B-ATPases in humans which does not appear to need enzymatic cleavage of the protein (T. Liu et al., 2007). MBD metal sensors may alter pump protein at different cellular sites in response to heavy-metal concentrations, allowing the protein to switch from metalloprotein production to heavy-metal detoxification. Heterologous systems have a limited turnover of P1B-ATPase (Malecki, Hsu, Truong, & Sanchez, 2002). Bacterial CopA and human Cu-ATPase activity in yeast are both two times lower than SERCA pumps. The heterologous systems utilized to examine these pumps lack an auxiliary component, metallochaperone, or particular lipids (Wijekoon et al., 2017). A list of the gene involved

in Co, Pb, Cu, and Cd uptake, transport, and metabolism in wheat is given in the below

Name	**Remarks**	**Metals**	**References**
TaABCC2	Metal detoxification transporters in response to metals	Cd/Co/Zn	(Shafiq et al., 2019)
TaABCC3	Metal detoxification transporters in response to metals	Cd/Co/Zn	(Shafiq et al., 2019)
TaABCC4	Metal detoxification transporters in response to metals	Cd/Co/Zn	(Shafiq et al., 2019)
TaHMA2	Plasma membrane located transporter, Export the	Cd/Zn	(Tan et al., 2013)

table.

Table 2: Reported HMA genes in Wheat

	Zn/Cd toward the apoplast		
TaCT1	Localized to the Golgi apparatus, promotes copper uptake, and keeps copper homeostasis	Cu	(Li et al., 2014)
TpNRAMP5	Localized at the plasma membrane, involved in Cd/Co transportation	Cd/Co/Zn	(Fan et al., 2018)
TaCNR2	Transport heavy metal ions and maintain ions balance in plants.	Cd/Zn/Mn	(Qiao et al., 2019)

Chapter 3

3.Methodology

3.1.Wheat Planting in Hydroponic System

3.1.1. Seedlings Preparation

Dharabi-2011 (DRB), a moderately sensitive wheat cultivar, got from the National Agriculture Research Council in Islamabad. Healthy seeds were collected, and surface sterilized for 30 minutes with 1% sodium hypochlorite (Honeywell FlukaTM) before being thoroughly washed with autoclaved distilled water for 10 minutes. Inoculation of seeds were done in 125 mm autoclaved glass Petri plates using forceps and moist autoclaved filter paper (Whatman No 40). Petri plates were kept in the dark for 48 hours at 25°C±1. The plates were then moved to a growth chamber for 24 hours at 25°C, with a diurnal cycle of 16 hours of daylight and 8 hours in night. Throughout the experiment, autoclaved water was employed to ensure the filter paper wet. The seedlings of the same size were chosen for transplantation on the fourth day.

3.1.2. Setting-up Hydroponic System

The hydroponic system was made up of nine black boxes that were 6 inches long, 12 inches wide, and 6 inches deep. Each box had 32 lid holes measuring one inch in diameter and holding 3.5 L of solution each. 12 feet of 1-inch diameter PCV pipe were connected to an air pump with an output capacity of 100 L/min and a voltage range of 220-240 volts (PA:100 Jebao®). Small tubes with a diameter of 0.1 inches that were 2 feet long were used to connect this PVC pipe. A knob was used to adjust the air pressure, and the tubes were connected at a distance of 10 inches from one another. One of the 32 holes in the box was intended to insert an air tube for the uniform circulation of air and nutrients, while the remaining 31 holes were used for the transplantation of seedlings.

3.1.3. Solution Media Preparation for Hydroponic System

The hydroponic solution media were made using the previously published Lombnaes and Singh recipe (2003) (Lombn s, Singh, & Science, 2003). All glassware was dried after being cleaned with distilled water. The final concentration in the medium was adjusted following the information provided in I Tables 1 and 2, respectively, for each nutrient's stock solution. After adding macro- and micronutrients, pH was set to 6.0 with 1M HCL and 5M NaOH before adding 1.0 mM/L 2-(N-morpholino) ethane sulfonic acid (MES). A media solution volume of 3.5 liters was placed within each box.

Table 3: Concentration of macronutrients in Lombnaes media

Stock solutions (500ml each)	Final concentration in media	Final volume per liter in media
10 mM $MnCl_2.4H_2O$	0.6 µM	60µl
MES	1Mm	195.2mg
1 M Ca $(NO_3)_2.4H_20$	2 Mm	1ml
1 M KNO_3	1 Mm	1ml
80 mM KH_2PO_4	80 µM	1ml
0.5 M $MgSO_4.7H_2O$	0.5 mM	1ml
0.9 M NaOH	0.9 mM	1ml
75mM$Fe(NO3)_3.9H_2O$	75 µM	1ml

Table 4: Final concentration of micronutrients in Lombnaes media.

Micronutrient stock solution (250 ml each)	1000X Stock solution Concentration	Final concentration required in 1x diluted stock solution	Final volume per liter
80 mM $ZnCl_2$	8000 µM	8 µM	1ml
0.5M$CuCl_2.2H_2O$	2000 µM	2.0 µM	1ml
0.5 M $NiCl_2.6H_2O$	100 µM	0.1 µM	1ml
0.5 M $Na_2MoO_4.2H_2O$	100 µM	0.1 µM	1ml
0.5 M H_3BO_3	10000 µM	10 µM	1ml

3.2. Transplantation and Heavy Metal Treatments

Glass Petri plate lids were taken off on the fourth day of seedling germination, and distilled water was added to the base (which included filter paper and wheat seedlings). Petri plates were kept under a white lamp to get erect seedlings. For transplantation, seedlings that were upright, healthy, and of the same size were chosen after 20 minutes of light exposure. A cotton plug was wrapped around the seedling's crow region and moistened with the prepared Lombnaes media solution. Batches of 279 seedlings of the same size were planted in 9 boxes filled with media solution. The boxes were kept in a growth chamber for 28 days, with 12 hours of light and 12 hours of darkness. Two heavy metals Zn, and Cu were chosen for this study. Each metal had three different treatments: Control (Lombnaes media without stress), Moderate (Lombnaes media together with a moderate dosage of heavy metal (50 µl/L), and Toxic (Lombnaes media along with a toxic dose of heavy metal (100 µl/L). There were three replicates of each treatment. The heavy metal stress doses and sources employed in the current study were drawn from previously published investigations. As a source of Zn, and Cu for stress treatments, Zinc chloride ($ZnCl_2$) (Shafiq et al., 2019) and Copper chloride ($CuCl_2$) (Lwalaba et al., 2019).Plants were cultivated exclusively on Lombnaes medium solution for the first seven days. On days 7, 14, and 21 following sampling, the media were then changed with freshly made Lombnaes media and heavy metal salt, apart from the control, which received no replacement.

3.3. Sampling and Data Collection

Samples were taken on days 7, 14, 21, and 28 after the transplantation. Total length in cm, root length in cm, shoot length in cm, total fresh weight in gm, shoot weight in gm, and root weight in gm were all recorded. For RNA extraction and elemental analysis, each plant's shoot and root were cut in half and stored right away at -80 in 2ml microcentrifuge tubes and 15ml falcon tubes.

3.4. Statistical Analysis

The Kolmogorov-Smirnov’s and Levene's tests were performed for the normality of residuals and the homogeneity of variance, respectively. One-way ANOVA was performed using the F- Test followed by an independent t-test post-hoc analysis. This

was used to compare moderate (set I) and toxic doses (set II) of heavy metals with the control treatment. The obtained P- value using F- Test post-hoc analysis was used to indicate whether there were any differences among the treatments or not. ANOVA and t-test was performed using ggplots, dplyr, and Nagpur R-packages.

ANOVA model:

$$X_{ij} = \mu + \tau_j + \varepsilon_{ij}$$

Where X_{ij}= each observation for i^{th} replication and j^{th} treatment, μ= total mean, τ_j= main effect of j^{th} treatment, ε_{ij}= error of each observation for i^{th} replication and j^{th} treatment.

3.5. Elemental Analysis:

For sample preparation, wet oxidizing acids, such as HNO3-HClO4 (di-acid digestion), were used to get heavy metals out of plant tissues (Estefan, Sommer, Ryan, & region, 2013). First, samples were dried in an air dryer (FD130-1340, ARSHiA®) until they were completely dry, and then they were crushed in an autoclaved pestle motor. For pre-digestion, 500 milligrams of dried, ground plant material were put in a conical flask and weighed. Then, in the fume hood, 5ml of sulfuric acid added in flask in concentrated form (H_2SO_4) then carefully stirred. A glass funnel was put on the conical flask, and it was left for about 6 to 8 hours. Mixing concentrated HNO_3 and $HClO_4$ in a 9:4 ratio and letting the mixture cool made a di-acid mixture. After the first step of pre-digestion, the di-acid mixture was added carefully and stirred. Then, on a hot plate a conical flask was put, and temperature was slowly raised to 180-200 °C. The flask was heated until dense white fumes came out and the white liquid inside became clear. Whatman No. 1 filter paper was used to filter out the remaining content, and distilled water was used to bring volume to 50 ml. Atomic Absorption Spectrophotometer was used to find the heavy metals Zn, and Cu.

3.6. RNA extraction

In this study, samples of roots and shoots were used to get total genomic RNA with TRIzol reagent (Simms, Cizdziel, & Chomczynski, 1993). In an autoclaved pestle and motor, liquid nitrogen was used to make 200 mg of fresh sample (leaf and root separately) into a fine powder and then put into a micro-centrifuge tube of 1.5 ml, and 1

ml TRIzol (Ambion®, USA) was added to each tube. For 15 seconds tubes were then shaken vigorously by hand, and they were put in an incubator at 4°C for 10 minutes. Sample tubes were put into a centrifuge (5810R, Eppendorf) 14000rpm 10 minutes and 4°C. Supernatant was then put in other1.5 ml micro-centrifuge tube. For chloroform phase separation, 200 µl of chloroform (Sigma ALDRICH®, Germany) was added to the supernatant, which was then manually mixed for 15 seconds and left 2-3 minutes at 4°C. The tubes were centrifuged 15 minutes 4°C at 14000rpm. Top aqueous phase was put into a new 1.5 ml micro-centrifuge tube. For the precipitation of the RNA, 100% isopropanol (Sigma ALDRICH®, Germany) was added and kept for incubation for 10 minutes at 4°C. RNA was extracted by spinning the sample at 14000 rpm 15 minutes at a temperature of 10°C. RNA pellet washed and centrifuged 4°C for 5 minutes at 7500rpm with 75% ethanol (Merch KGaA, Germany). The pallet was then suspended in 30 µl RNase-free water (Ambion®, USA).

3.7. Quantitative Analysis

The concentration of extracted RNA sample was determined on Nanophotometer at 260 nm and 280 nm. The quality was further checked by the 230/260 ratio.

3.8. cDNA Synthesis

To eliminate the possibility of genomic contamination, the isolated total genomic RNA of roots and shoots was processed with DNase enzyme (Thermo Scientific, Lithuania). DNase enzyme-treated RNA sample was utilized for complementary DNA (cDNA) synthesis using the Revert Aid First strand cDNA synthesis kit from Thermo scientific in Lithuania. One µg of extracted total RNA was added to a 200 µL PCR tube. Then, 1 L of oligo (dT)18 primer (0.5 g/l), 4 L of 5x reaction buffer, 2 L of riboLock RNase inhibitor enzyme (20 u/L), 2 L of the dNTPs mix (10 mM), and 1 L of reverted reverse transcriptase enzyme (200 u/L) were added. RNase-free water was used to make the final volume of 20L. Tubes were quick spined in minicentrifuge and were incubated at 42 °C for 60 minutes, then at 70 °C for 5 minutes, and finally at 4 °C for ∞ in swift ™ Max Pro Thermal Cycler (ESCO® Micro Pte. Ltd). After the cycle was finished, the cDNA tubes were kept at -80 °C till the expression analysis. All reagents and tubes used in the cDNA synthesis process were kept on ice during the whole process.

3.9. Primer Designing

To design primers for qRT-PCR CDS sequences of all three genomes of *TaHMA1-27* were retrieved from the EnsemblPlants database. Accession numbers of *TaHMA1-27* CDS sequences are given in Annexure III. Primer-BLAST option of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to get the suitable primers for TaHMA1-27 expression analysis (Ye et al., 2012).

3.10. Primer Optimization

Optimization of primers HMA1.1, HMA1.2 and HMA1.3 were done by using gradient PCR. For each temperature three annealing temperatures were used 58 ˚C, 60 ˚C and 62 ˚C. Forward and reverse primers were also changed according to protocol. Protocol that followed for the optimization of primers is given in Table 6:

Table 5: Setting up of PCR reaction using Thermo Scientific

Reagents	**Volume**
10X Taq Buffer	2.5 µl
Taq DNA polymerase	0.5 µl
$MgCl_2$	2.5 µl
2.5mM d	2 µl
Primer forward	0.5 µl
Primer Reverse	0.5 µl
Template DNA	2 µl
H_2O	4.9 µl
Total	**15 µl**

3.11. qPCR Plate Design

The reference gene i.e., housekeeping gene Actin is used for expression analysis. Three biological replicates and technical replicates were loaded with *Actin* and *HMA1.1*, *HMA1.2* and *HMA1.3.* Roots and shoots samples of day 21 was used for expression analysis.

3.12. qRT-PCR

For qRT-PCR, EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) was used to find out the expression of selected HMA1 in hydroponically grown wheat tissue samples. 12 PCR 1.5ul PCR strips were used for the loading of the samples. In each PCR strip tubes 8 samples were loaded and was sealed with clear caps (BIO Genetics) PCR strip tubes were vortexed to ensure mixing of the reagents. The reaction was run on qPCR (AB Quant Gene) using the Quant Gene program. Concentrations of the reagents and PCR conditions used are given in (Table 7 and Table 8).

Table 6: List of PCR reagents for qRT-PCR

Reagents	**Volume**
Template DNA	1 µl
Primer Forward	0.3 µl
Primer Reverse	0.3 µl
5x HOT FIREPOL EvaGreen qPCR Mix Plus	4 µl
H_2O PCR grade	14.4 µl
Total	**20 µl**

Table 7: Cycling conditions for different PCR reactions.

Steps	Temperatures and Duration		
Initial denaturation	98 °C, 30 sec	94 °C, 2 min	95 °C, 1 min
Denaturation	98 °C, 10 sec	94 °C, 30 sec	95 °C, 50 s
Annealing	55-60°C, 30 sec	55 °C, 1 min	65 °C, 50 s
Extension	72 °C, 30 sec	72 °C, 1 min	68 °C, 4 min
Repeat	72 °C, 30 sec	35 cycles	40 cycles
Final Extension	72 °C, 30 sec	72 °C, 3 min	69 °C, 7 min

Chapter 4

4.Results

4.1. Statistical analysis on Physiological data

4.1.1. Plant length under Zinc Stress

The data were analyzed one-way ANOVA with t-test independent post-hoc analysis for total length, shoot length and root length obtained during the different duration of treatment. Analysis of root length data by one-way ANOVA showed a significant effect of treatment [F (2,6) = 25.48, add actual F values here)] at day 21. T-test post-hoc analysis revealed that the root length was significantly increased in set II ($p<0.05$) when compared with the control group. Statistical analysis showed a significant effect of treatment on total length, shoot length, and root length of set II at day 28 only when compared with the control. Significant effect of treatment on shoot length only of set I was found at day 28 as when compared to control. Post-hoc analysis by t-test showed that total length($p<0.05$), and root length ($p<0.01$) in set II were significantly increased by the treatment on day 28 as compared to that of control group. Whereas shoot length was also significantly decreased ($p<0.05$) by the treatment on day 28 in set I and set II when compared with the control (Figure 4). Table of statistical analysis are shown in Annexure III.

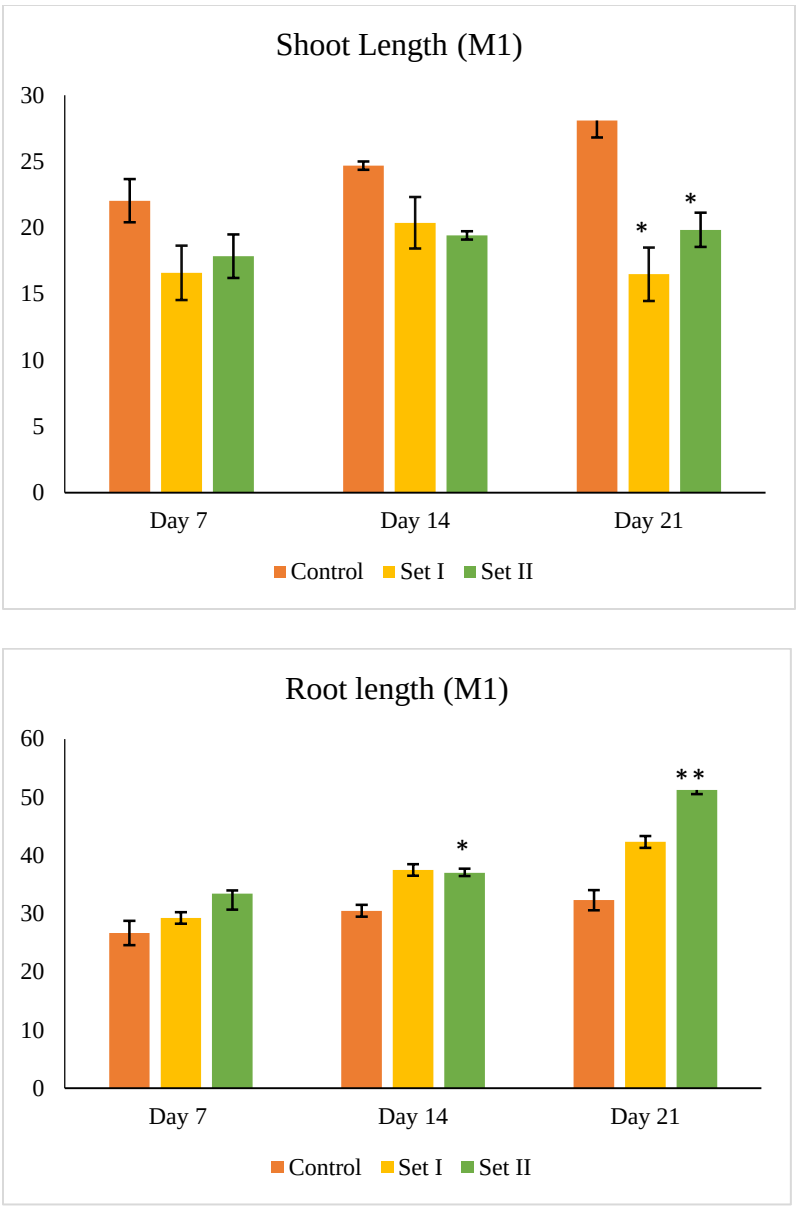

Figure 4: Length data of shoot and root was analyzed by one-way ANOVA with t-test post-hoc test. * is showing P<0.05, ** is showing P<0.01 as compared to the control group. Whereas each treatment group had three biological replicates collected on day 7, day 14, and day 21.

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4.1.2. Plant weight under Zinc Stress

Analysis of plant weight, root weight and shoot weight data by one-way ANOVA revealed nonsignificant treatment effects. T-test post-hoc analysis did not reveal any significant results of treatments on plant weight, shoot weight and root weight during different intervals of treatment in set I and II in contrast to the group that served as the control. (Figure.5) (Annexure III)

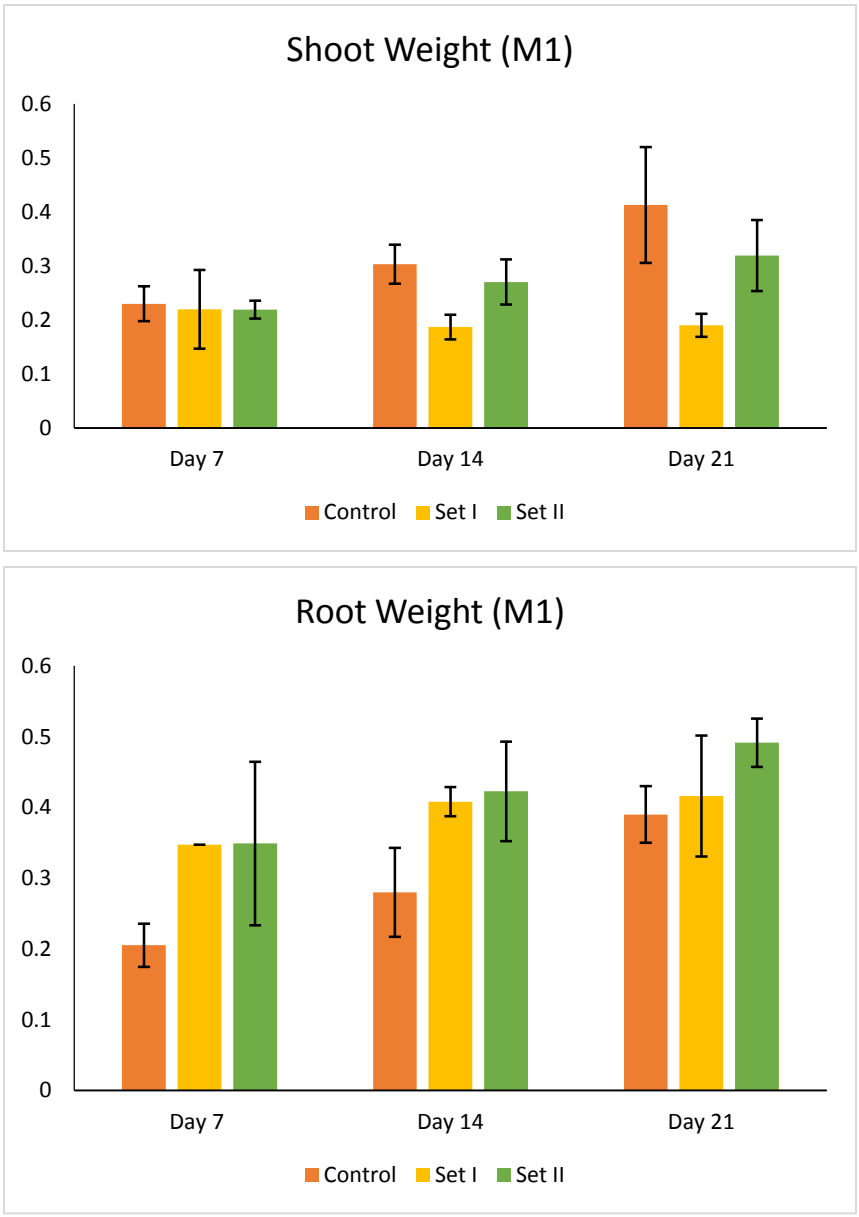
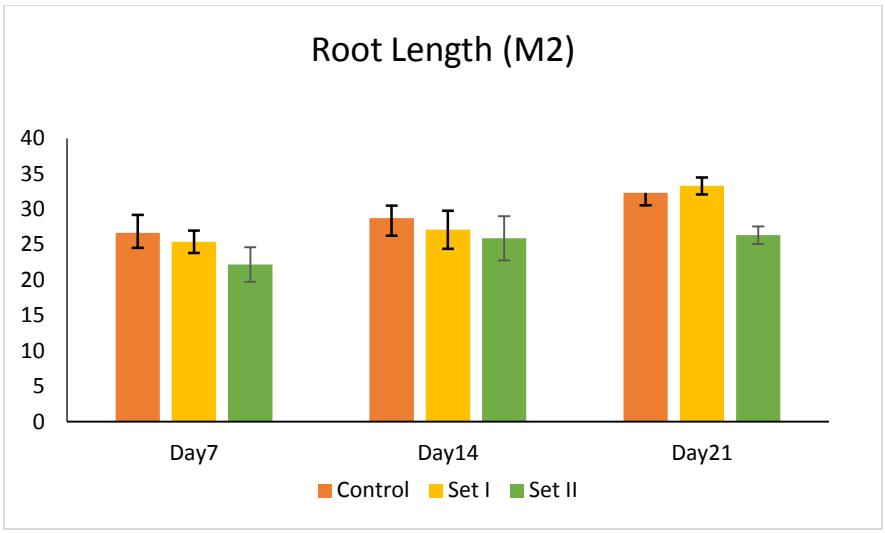


Figure 5: Weight data of shoot and root was subjected to a one-way analysis of variance followed by a t-test. No significant change was observed after post-hoc analysis as compared to control. Whereas each treatment group had three biological replicates collected on day 7, day 14, and day 21 for phenotypic study.

4.1.3. Plant Length Under Copper Stress

One-way ANOVA Analysis of phenotypic data obtained under the different duration of treatments showed non-significant effects of treatments compared to the control groups. Furthermore, t-test post hoc analysis didn't show any significant result for treatments of total plant length, shoot length, root length, total plant weight, shoot weight, and root weight. Plants treated under copper stress root length data and treatment effects were not statistically significant using a one-way ANOVA. The total length of copper-treated plants also showed non-significant from the control to toxic group as shown in figure 6 and Annexure III.



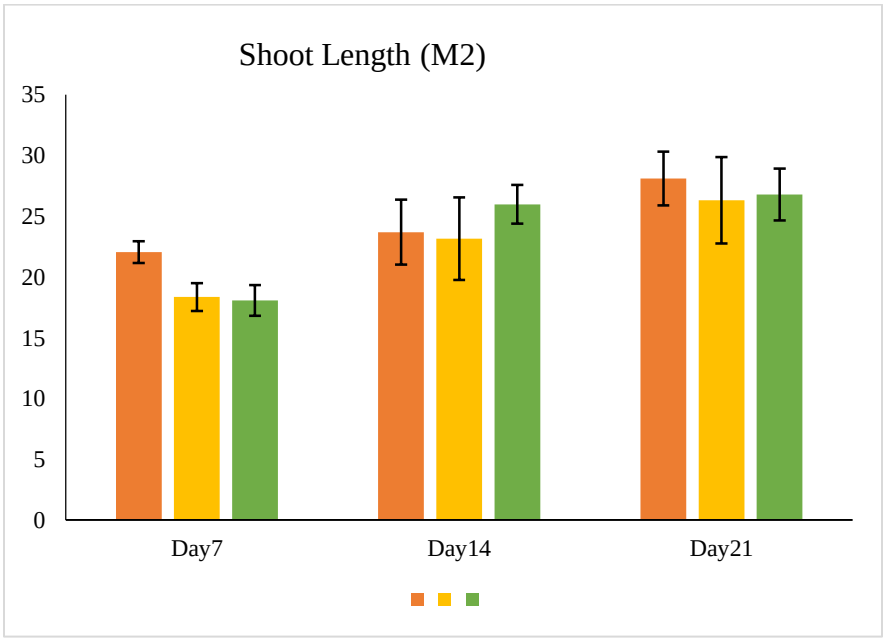


Figure 6: The lengths of the shoots and roots were measured and evaluated using one-way ANOVA followed by a t-test for comparison. No significant change was observed after post-hoc analysis as compared to control. Whereas three biological replicates were harvested from each treatment group on day 7, day 14, and day 21 for phenotypic study.

4.1.4. Plant Weight Under Copper Stress

One-way ANOVA Analysis of phenotypic data obtained under different duration of treatments showed non-significant effects of treatments compared to the control groups. Furthermore, t- test post hoc analysis didn’t show any significant result for treatments of shoot weight and root weight. Plants treated under copper stress root weight data by Treatment effects were not statistically significant using a one-way ANOVA. The shoot length of copper-treated plants also showed non-significant from control to toxic groups shown I Figure 7.

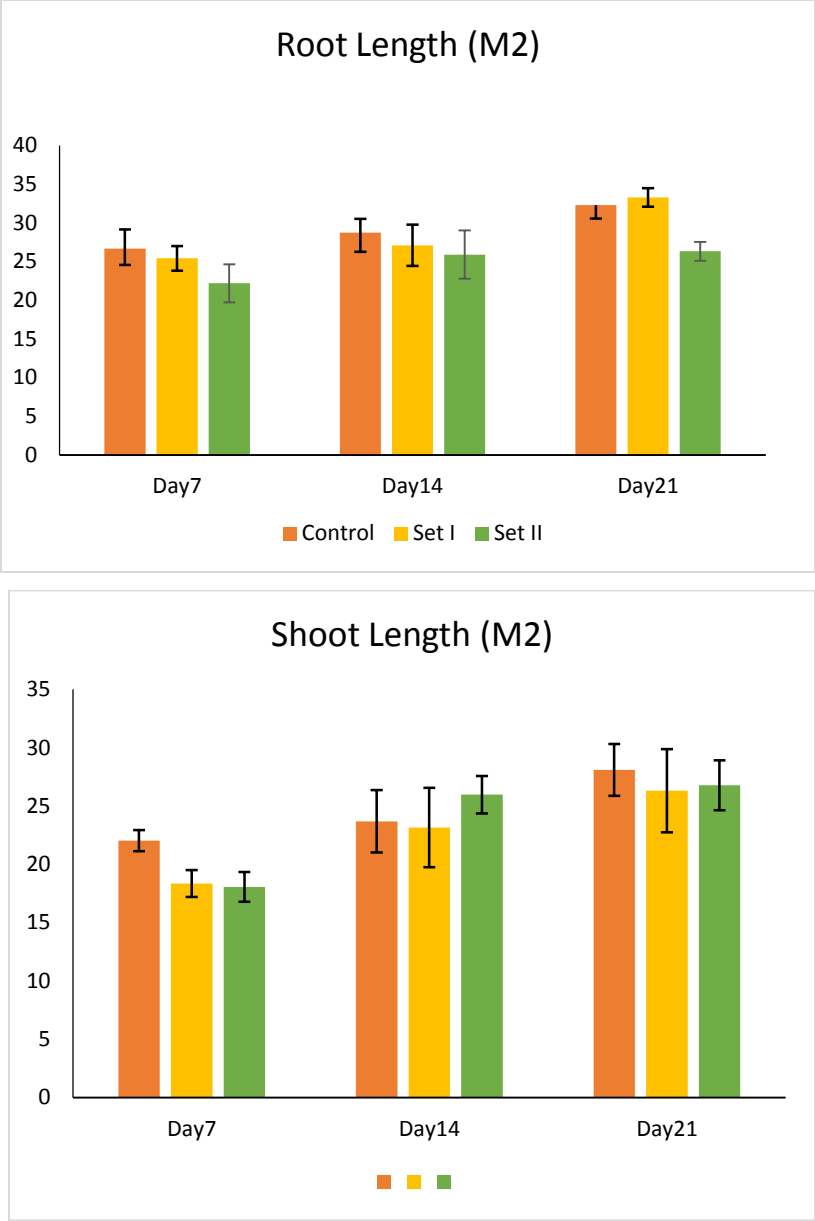
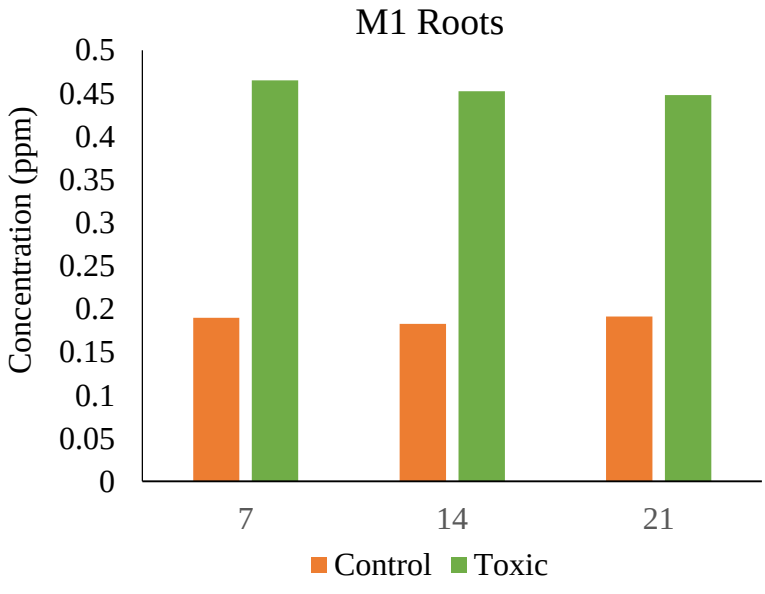


Figure 7: Weight data of shoot and root was subjected to a one-way analysis of variance followed by a t-test. No significant change was observed after post-hoc analysis as compared to control. While each treatment group gathered three biological replicates on day 7, day 14, and day 21 for phenotypic study.

4.2. Elemental Analysis

4.2.1 Zinc Metal analysis

The accumulation of Zinc in roots and shoots at day 7, 14, 21 was higher in toxic (set Ⅱ) in contrast to the group that served as the control. The higher accumulation of zinc in roots is at day 7 in toxic with the concentration of 0.464983082 ppm in contrast to the group that served as the control. Whereases in shoot the higher concentration of 0.459848907 ppm of zinc was obtained at day 14 as compared to control group. (Figure 8)



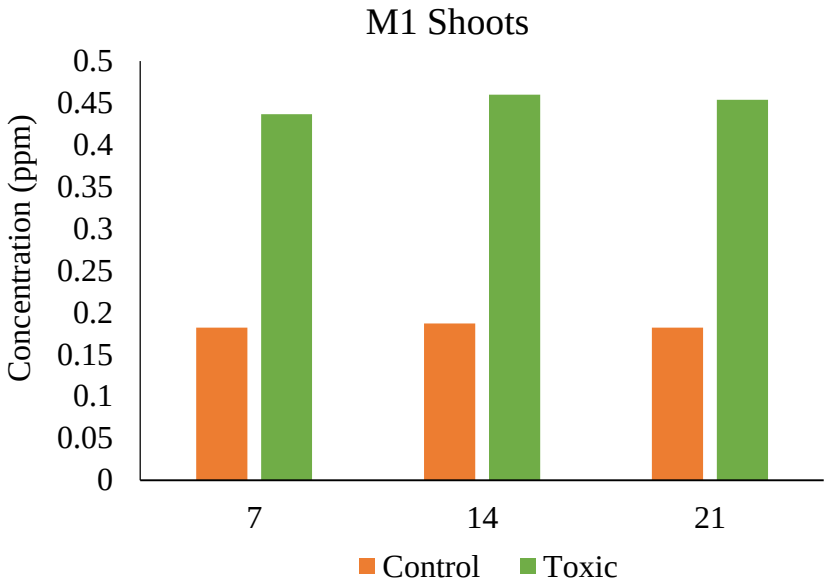


Figure 8: Graphical presentation of Zinc metal in ppm accumulated in shoots and roots of toxic group of day 7, 14, and 21 using atomic absorption spectrophotometer at 425nm wavelength.

4.2.2. Copper metal analysis

The accumulation of copper in roots at day 7, 14, and 21 was significantly higher in toxic (set Ⅱ) in contrast to the group that served as the control. In shoots, copper accumulation in control group on day 7 was lower as compared to the toxic group. The higher concentration of copper in roots is at day 14 in toxic with the concentration of 0.248655734 ppm as compared to control group. Whereases in shoot the higher concentration of 0.176576382 ppm of copper was obtained on day 21 as compared to control group.

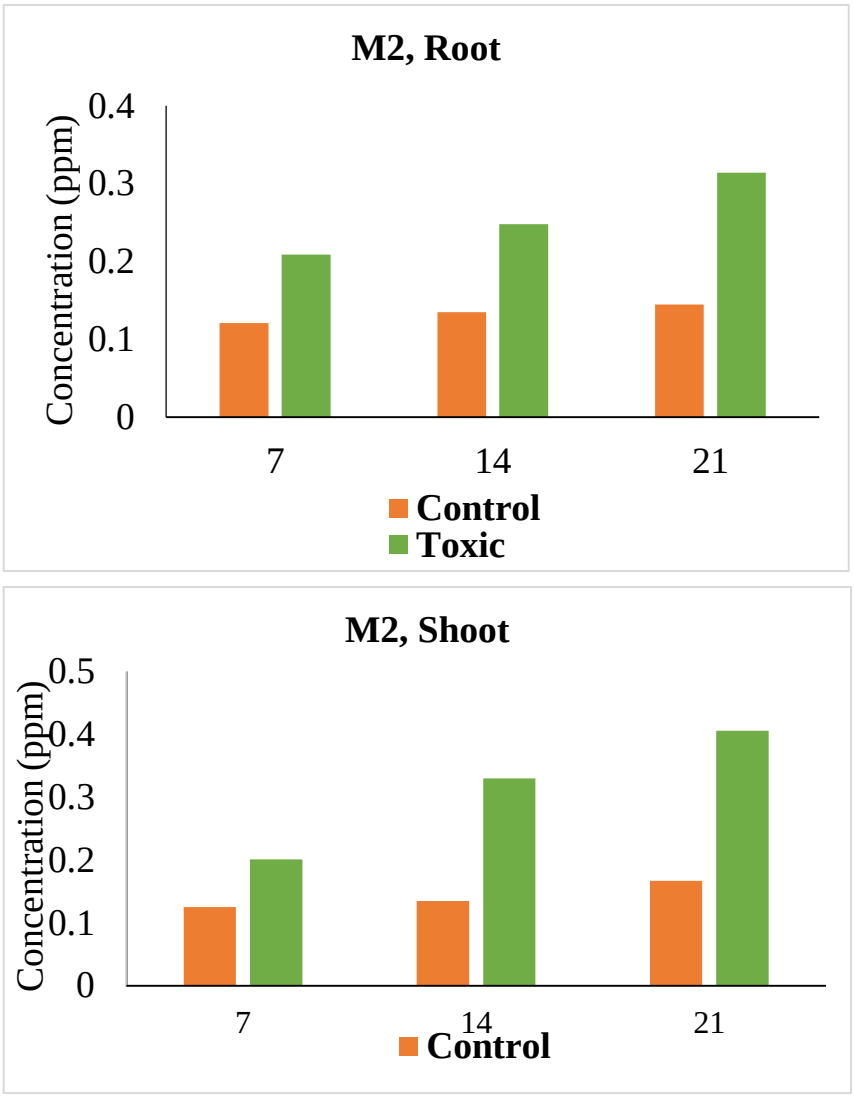


Figure 9: Graphical presentation of copper metal in ppm accumulated in shoots and roots of toxic group of day 7, 14, and 21 using atomic absorption spectrophotometer

4.3. Designed Primer

The primers that design for HMA 1 gene of ABD genome of wheat named as *HMA1.1, HMA1.2, HMA 1.3*.

Table 8: List of designed primers with sequence ID

Gene name	Sequence ID Strand	Primer Sequence	
HMA1.1	TraesCS7A02G439100.1	F	AGCTGAGCAATCTGAGTAGGAG
		R	CCCAACATGGTCTTGGGCAG
HMA1.2	TraesCS7B02G337700.1	F	AACCCTCCGACATGGTCTTG
		R	TGAGCAATCTGAGGAGGAGC
HMA1.3	TraesCS7D02G428700.1	F	GCTGAGCAATCTGAGGAGCTA
		R	AACTCAATACGGGCTCTCAACC

4.4. Expression profiling of HMA1 under Zinc Metal Stress

The expression profiling of *HMA1* under zinc stress has shown an abundance of *HMA1* in wheat shoots and roots (Figure 10 ang Figure 11). In *HMA1.1* toxic roots showed downregulation as compared to control but there is no expression of *HMA1.1* in shoots in both control as well as toxic. Similarly, *HMA1.2* also showed 3.8 relative expression fold in toxic roots as compared to control. *HMA1.3* showed the highest expression as compared to *HMA1.1* and *HMA1.2* in both toxic roots and shoots. However, *HMA1* was found to be expressed more in wheat roots as compared to shoots.

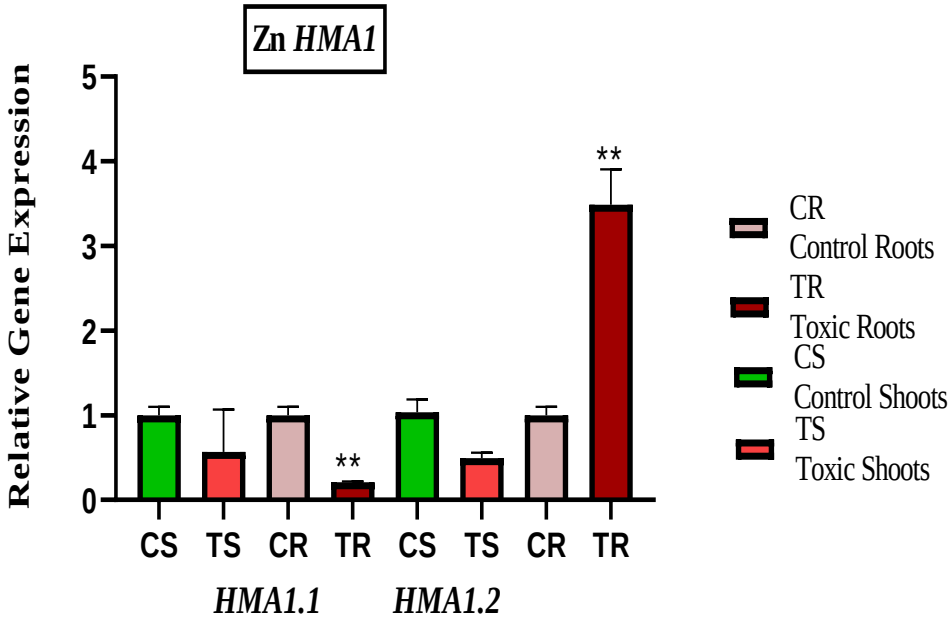


Figure 9: qRT-PCR analysis shows that both *HMA1.1* and *HMA1.2* are expressed in the shoots and roots of *T. aestivum* when the plant is subjected to zinc poisoning. The significant differences in expression from control values are indicated by * where, P<0.05.

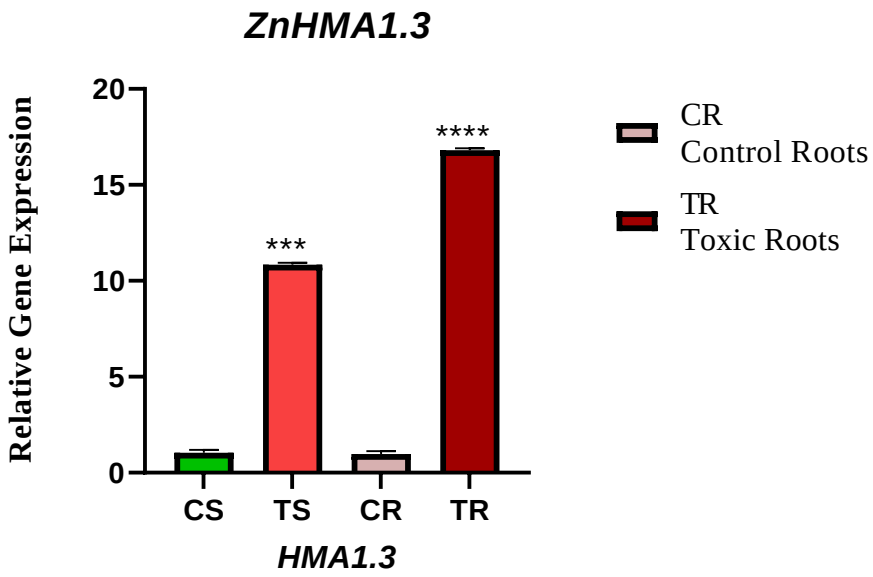


Figure 10: qRT-PCR data indicating the expression of *HMA1.3* in *T. aestivum* shoots and roots under zinc toxicity. The significant differences in expression from control values are indicated by * where, P<0.05.

4.5. Expression profiling of HMA1 under copper metal stress

The expression profiling of *HMA1* under copper stress has shown an abundance of *HMA1* in wheat shoots and roots (Figure 12). In *HMA1.1* toxic shoots showed upregulation as compared to control but there is no expression of *HMA1.1* in roots in both control as well as toxic. Similarly, *HMA1.2* also showed 4.5 relative expression fold in toxic roots as compared to control. *HMA1.3* showed the highest expression in toxic roots as compared to *HMA1.1* and *HMA1.2* roots. while *HMA1.3* is also expressed in toxic shoots. However, *HMA1* was found to be expressed more in wheat roots as compared to shoots.

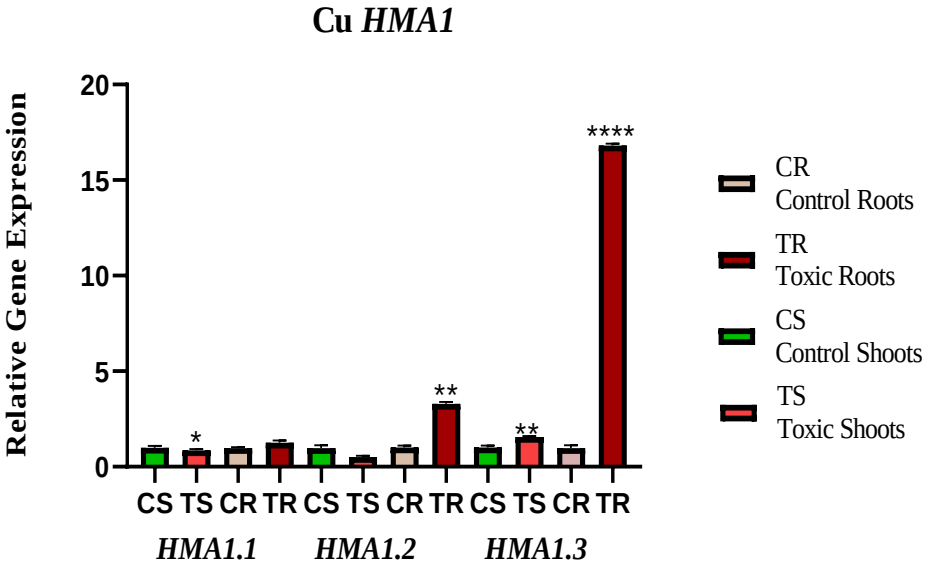


Figure 11: qRT-PCR data indicating the expression of *HMA1.3* in *T. aestivum* shoots and roots under copper toxicity. The significant differences in expression from control values are indicated by * where, P<0.05.

Chapter 5

5.Discussion

5. Discussion

Around 4.5 billion people worldwide rely on wheat, a major cereal crop for their nutritional requirements (Arzani, Ashraf, & Safety, 2017). Large cultivated area of wheat is called the "king of grains" (Mathur, Raikalal, & Jajoo, 2019). Individual grain, grain yield, and total biomass production are the metrics most frequently used to assess wheat yield. A difficult agronomic feature is wheat yield. The term "biological yield" is most frequently used to describe total biomass yield, whereas "economic yield" is used to describe grain output. The grains are the main product of wheat, hence economic yield would seem to be the most important yield factor. Shoot and root biomass are included in the standard definition of biological yield, but as the latter is typically not recoverable, the term is typically applied to shoot biomass.

Heavy metals (HMs) are metals that have densities several times higher than those of water. The earth's crust contains ores and minerals that contain heavy metals. On the other hand, the release of HMs into the environment can be attributed not just to human activities but also natural processes (R. Singh, Gautam, Mishra, & Gupta, 2011). This pollution has a substantial impact on the productivity, growth, reproduction, and physiological functioning of virtually all living organisms, including plants (Chaudhary, Agarwal, & Khan, 2018). One of the most significant of these impacts is on yield. However, plants have a variety of physiochemical and biochemical defenses against the stress caused by HM (Rensing, Ghosh, & Rosen, 1999).

The toxicity of heavy metals poses a global risk to human health, as well as to the environment and plants. They don't decompose or volatilize easily, so accumulate in the soil (Lin et al., 2012). Lead and cadmium are the two most hazardous heavy metals because of their ability to migrate through the soil and enter the food chain, where they pose a harm to both humans and animals (Cobbett, Hussain, & Haydon, 2003).

It has been discovered that both eukaryotes and prokaryotes, such as plants, insects, mammals, and yeasts include P1B heavy metal ATPases. These carriers' metal substrates in prokaryotes include the ions Ag, Cu, Co, Cd, Pb, and Zn. Most of the time, each transporter acts as an efflux pump to impart flexibility with regard to the metal ion substrate (Petris et al., 2002).

However, in bacteria, P1B ATPases have been linked to maintenance of internal equilibrium and metal absorption. Cu transporters are the only type P1B ATPases found in non-plant eukaryotes yet. These include the proteins known as ATP7A (Menkes) and ATP7B (Wilsons) found in humans and CCC2p in yeast (Nagajyoti et al., 2010). Type P1B ATPases are typically divided into two further types based on sequence comparisons: Cu/Ag transporters and Cd/Pb/Zn/Co transporters (Solioz & Vulpe, 1996).

Previous studies revealed that *TaHMA2* was necessary for the movement of the heavy metals zinc and cadmium and played a crucial role in this process; however, little is known about TaHMA2's metal-binding domains. N-MBD transgenic Arabidopsis was employed in this investigation to evaluate the role of MBDs in Zn2+/Cd2+ tolerance and accumulation. In this work, the N-terminal and C-terminal were removed, and the CPC and CCxxE motif was mutated. The plasma membrane was linked to the *TaHMA2*, *TaHMA2-C*, *TaHMA2C24*, *TaHMA2C25*, *TaHMA2E28*, and TaHMA2C357S-EGFP fusion proteins, however, TaHMA2-N-EGFP was found in the cytosol. It was suggested that *TaHMA2* has crucial structural and functional locations in its N-terminal region and that *TaHMA2* transports metal cations through the plasma membrane. The accumulation and tolerance of lead, zinc, cobalt, and cadmium were significantly enhanced in A. thaliana by overexpressing *AtHMA3* (Andrés- Colás et al., 2006).

The genetic transformation system of Arabidopsis halleri is an excellent model for the hyperaccumulation of zinc and cadmium. This makes it possible to investigate the physiological and molecular processes that contribute to the hyperaccumulation of heavy metals in this species. By using RNA interference (RNAi) to suppress *AhHMA4* expression in *A. halleri* transgenic plants, it was possible to find out what the major role of *AhHMA4* is in the root-to-shoot transport of zinc in A. halleri. Strong Cd hyperaccumulators such as *N. caerulescens, S. alfredii*, and *S. plumbizincicola* have greater Cd concentrations in their shoots compared to A. halleri, which collects more Cd in its roots. A. halleri also accumulates more Cd in its leaves. In contrast to an *AtHMA3*-knockout mutant, when *AtHMA3* overexpresses in A. thaliana it increases tolerance and accumulation of Cd, Zn, Pb, and Co (Saavedra-Mella, Liu, Southam, & Huang, 2019).

Current study shows the highest relative fold expression oh HMA1 gene in roots as compared to the control.

Foundries, mining, combustion, smelters, and agriculture are just a few of the sources where heavy metals including iron (Fe), cobalt (Co), molybdenum (Mo), zinc (Zn), copper (Cu), nickel (Ni), manganese (Mn) and enter the soil (Wuana & Okieimen, 2011). To control how these metals get into the whole plant, plant genomes code for different transporters with different substrate specificities, cellular localizations, and expression patterns (Wuana & Okieimen, 2011). Concentration gradients and selective absorption allow the plant to absorb manganese, zinc, iron and copper from the soil. For instance,(L. Deng, Wang, Li, Zhao, & Shangguan, 2016) claimed in their study that the Pb, Cr, Cu, Cd, and Zn accumulate in Baoji, China, smelting and peri-urban plants due to air deposition. (Tang et al., 2020). Zinc levels are rising primarily because steel processing, coal mining and garbage combustion are anthropogenic. Electroplating procedures and waste pesticides damage the environment with fungicides, bactericides, insecticides, Cr and Cu used to manage plant diseases and pests. This causes copper to accumulate in topsoil (Tang et al., 2020).

Components of beneficial metal nutrients such as cobalt, iron, manganese, copper, molybdenum, nickel, and zinc are required for appropriate plant growth and development. The soil contains trace amounts of these essential metal nutrients, which are then transported to the plant in a mutually beneficial way by symbiotic metal transporters (C. Zhang et al., 2016).

According to (Grotz & Guerinot, 2006), a class of transporters called the ZIP family, including ZRT, mediates Zn and Fe ions uptake (Zinc regulated transporters). *HMA2* and *HMA4* are crucial in Zn homeostasis were disclosed by (D. Hussain et al., 2004) in their article "P-Type ATPase Heavy Metal Transporters with Roles in Essential Zinc Homeostasis in Arabidopsis" 2004. CTR and COPT1 transport copper in plants (Sancenón, Puig, Mira, Thiele, & Peñarrubia, 2003). Cu is transported in plants through RAN1 (Responsive-to-Antagonist) and P-type HMA ATPases.

Current studies showed, for copper HMA1 gene is highly expressed in Treated roots and shoots as compared to the control group.Many times, plants are sensitive to

heavy metal ions accessibility as vital micronutrients, both at low and high levels. Useful heavy metals in large concentrations can disrupt the soil ecosystem, reducing soil fertility and stunting plant growth. When there is an overabundance of the element copper (Cu), plant development is slowed down and cellular processes, such electron transport during photosynthesis, are compromised. Cu do damage to plants in a cytotoxic manner at high level this stunts plant growth and led to chlorosis. Copper toxicity has a harmful effect on the development, dry matter, yield Vigna radiata as well as the oxidative mechanism and growth of tea plants, claim (Dey, Mazumder, & Paul, 2015; Manivasagaperumal, Balamurugan, Thiyagarajan, & Sekar, 2011).

According to Warne et al. (2008), an increase in soil Zn concentration impairs plant metabolism, resulting in senescence and retarded development. Zn has a deleterious effect on plant development and metabolism at higher concentrations. At 7.5 mM, zinc disrupts cortical cells, dilutes the nuclear membrane, and significantly changes the nucleoli of the root tips cells (Warne et al., 2008). According to Neumann and zur Nieden (2001), different metals are deposited in changed forms. For example, Minuaria verna's cell wall contained Zn silicate (Neumann & Zur Nieden, 2001).

According to the recent research, a single employment of copper and cobalt in a growth medium increased the concentration of metal found in the tissue, with copper being far less effective than cobalt. According to research conducted by Gondar et al., the increased exchange capacity of Cu may be related to its limited mobility (2006). According to Zhou et al. (2007), Cu adsorption was thought to be quite tight, retaining a significant proportion of the metal cell barriers at the root, decreasing its mobility (Liang & Zhou, 2007).

According to the translocation factor, Cu in the growth medium impeded Co absorption and translocation from roots to shoots. Comparing the matching single therapy to the tissue Cu concentration, there was no change ($p = 0.05$). Divalent ions have been demonstrated to interact with one another in soil and other plant-growing media. Based on their findings, they concluded that even a single dose of Co or Cu applied to a growth medium was extremely hazardous to barley plants, since it not only induced oxidative stress and lipid peroxidation but also slowed down photosynthesis. By reducing Co

uptake and storage in plant tissues, combining these two metals reduces their damaging effects. Ea52 was more disrupted than Yan66 by the effect, which varied depending on the genotype. However, because the substrate influences how metals behave, the findings of this study demand further investigation in a solid medium like soil.

Numerous transporters have shown that certain heavy metals are segregated in root vacuoles. For instance, the sequestration of Cd, Zn, Co, and Pb in vacuoles is facilitated by both Arabidopsis *AtHMA3* and *AtMTP3*. Recent research has shown that the P1B-type ATPases found in cucumbers, designated *CsHMA5.1* and *CsHMA5.2*, are responsible for transporting copper into yeast vacuoles. Zn poisoning also causes the buildup of additional heavy metals including copper and manganese in shoot and root tissue (Nagajyoti et al., 2010). Additionally, phosphorus (P) insufficiency can result from Zn toxicity and show up as the purple-red color of the leaf indicator. In addition to having roots with limited cell division and lengthening, they have a bloated, blunt appearance. *P. vulgaris*' ATP synthesis is harmed because of Zn poisoning because of the suppression of photosynthetic photosystems I and II. These outcomes are reversible, but prolonged stress can have irreversible consequences. Zn inhibits the carbon-fixing enzyme RuBisCO (ribulose-1,5-bisphosphate-carboxylase/oxygenase) by dislodging Mg2+ ions. RuBisCO is a divalent ion. Studying poplar leaves, Todeschini, and colleagues (2011) observed that Zn was found in the cell walls of xylem and parenchyma cells. As a result of their research, they concluded that leaves exposed to zinc toxicity had an increased amount of calcium oxalate crystals, which suggests that zinc poisoning enhances the amount of free calcium in plants. Copper excess negatively impacts the germination, size of seedlings, and quantity of roots in Solanum melongena seeds, according to Neelima and Reddy's study from 2006. The plant will suffer severe harm from all of these impacts (Kantam, Neelima, Reddy, & Neeraja, 2006).

According to the study by Brian J. Alloway in 2013, All soils contain the majority of the heavy metals listed in the periodic table, however the amounts vary widely, and some may not be detectable by analytical methods. Concentrations of elements in soils can be categorized as "total" and "available," where "available" refers to the portion of this total content that may be accessible to plants. All forms of the element that are

present in soil have been accounted for in the total concentrations. This includes soluble organic and inorganic complexes in the soil solution, free ions, ions that are bound in the crystal structure of primary and secondary minerals, ions that are adsorbed on the surfaces of secondary minerals like clays, oxides, and carbonates, and those that are bound in solid-state organic matter (Alloway, 2013).

Most of the time, a large portion of an element's total content won't be immediately available for uptake by plants. Exudates released by the roots of plants can have a substantial impact on the availability of metals. Although there are many more potential sources of heavy metals in an urban setting, they are also probably dispersed very differently. Lead, which frequently coexists with Cu and Zn in urban soils, is the most common heavy metal contaminant.(Alloway, 2013).

Metals are inherently a part of the crust of the earth, but today Heavy metal pollution of soil is a problem that affects the environment on a worldwide scale that is becoming more significant as industrialization grows. Heavy metals and other released toxins are present in large volumes of wastewater produced by industrial processes including mining, electroplating, and the production of necessary goods. Because heavy metals cannot be broken down by biological processes, they are unable to disappear from the environment and can accumulate to harmful amounts in soil, water, and the food chain (S. Wang, Wu, Liu, Liao, Hu, et al., 2017). Due to wastewater application over an extended period, metals can potentially accumulate in the soil at dangerous amounts. Metals regularly infiltrate the water supply that is used by humans and animals, besides the discharges from various industrial regions, harming their growth and health. Many times, local farmers use these discharges to irrigate their crops, exposing the crops to these toxins. The chemical makeup of plants is frequently altered without obvious damage, and Plants that are cultivated in soils that are polluted have greater amounts of metals than plants that are grown in soils that are not affected (Pruvot, Douay, Hervé, Waterlot, & sediments, 2006).

Pakistan is a developing country with a large population. However, Pakistan's soils aren't naturally fertile enough to support commercial crop cultivation. Due to the high cost and lack of availability of chemical fertilizers, impoverished farmers in

Pakistan make extensive use of land disposal of agricultural, municipal, and industrial wastes as a primary and cost-effective source of nutrients and organic matter for the production of cereal crops. This practice is widespread throughout the country. With 16.2 TG produced in 1995, Pakistan's garbage production increased by 120 percent between 1980 and 1996. Cu, Pb, and Zn are the three most commonly reported heavy metals in waste-amended agricultural soils (Mahmood, Islam, & Muhammad, 2007).

Due to industrialization, the majority of Pakistan's agricultural land is polluted with heavy metals. In a 2015 study by Noor-ul-Amin and Tauseef Ahmad, atomic absorption spectrometry was used to measure the Seven heavy metals build up and get concentrated in vegetables grown in an area near Hayatabad, Peshawar, Pakistan, where industrial wastewater is used to water crops. In the edible sections of various vegetables growing in the area, concentrations of copper, cobalt, iron, lead, chromium, manganese, zinc, and nickel ranged from 0.044 to 0.504, 0.009 to 0.085, 0.243 to 0.496, 0.005-0.033, 0.019 to 2.019, 0.045 to 0.703, and 0.017 to 0.108 ppm, respectively. All veggies had the highest concentration of iron (Fe). The metals were often highly polluted throughout the root, demonstrating a noticeably larger concentration than in other areas. Health concerns were raised since a sizeable portion of the metals absorbed by the veggies was transferred to the edible sections. Without specific treatment, growing vegetables in the examined area with such industrial effluent is not advised (Amin & Ahmad, 2015).After the rice harvest, the wheat crop is the one that is most widely used as a food source worldwide. It is the most significant and useful crop in a nation like Pakistan since it boosts GDP by 1.9 percent and wheat crop production annually stayed at 25.75 million tonnes, up 0.5 percent from the previous year's output. Since wheat is a staple meal for Pakistanis, there is a need to improve wheat crop production to keep up with the country's rapidly growing population. A high yielding, disease- and drought-tolerant wheat cultivar named Dharabi-11 has been authorized for widespread cultivation (I. Hussain, Khakwani, & Khan, 2018).

Metals are widely believed to be the primary factor in environmental contamination caused by both natural and human activities. In nature, you can find trace levels of metals including cadmium (Cd), copper (Cu), and zinc (Zn). Plants require Zn and Cu, two micronutrients found in the growth media, for growth as well as several

biochemical and physiological processes. Sensitive plants are harmful to some elements at high concentrations. Damage to the root system, chlorosis, necrosis, and plasma membrane permeability are all results of metal concentration toxicity (Al Khateeb, Al-Qwasemeh, & Plants, 2014). Zinc is a mineral essential for plants' healthy development and growth (Zn). It's vitally important in several metabolic processes in plants. Harmful concentrations of Zn in the terrestrial environment can impair mineral feeding, growth, and photosynthesis. Due to our dependence on cereal grains, especially wheat-based diets, Zn deficiency is a global health issue in micronutrient malnutrition. Research on the accumulation of heavy metals in soil-plant systems is the basis for risk assessments of the threats to human health, which have attracted international attention due to the widespread nature of the health concerns connected with heavy metal pollution (HMP) in agricultural soils.

According to the study of (S. Wang, Wu, Liu, Liao, Hu, et al., 2017), compared to corn, Heavy metal contamination was more likely in wheat. According to research of the seven heavy metals, corn and wheat both had high BCFs of Zn, Cd, and Cu during the eight periods, but both also had relatively low BCFs of Hg, As, Cr, and Pb. This suggests that Zn, Cd, and Cu have higher potentials to accumulate than the other four heavy metals.

It is commonly known that Zn, Cd, and Cu may very easily accumulate in other plants. High amounts of Cu have been documented to have harmful effects on wheat crops and to have negative effects on protein synthesis, photosynthetic activities, enzyme activity, and plasma membrane permeability. Plant growth and development may also be stunted by an excess of Zn because of the potential for it to interfere with metabolic activities, the antioxidant defence system, and mineral distribution and absorption (Al Khateeb et al., 2014).

Germination and early development of wheat, rice, and barley seedlings were considerably stunted as the concentration of heavy metals increased at higher and higher levels. The factors that had the most significant impact on seed germination were the seedlings' root length, root to shoot ratio, and shoot height. According to the tolerance index, the overall inhibitive effects of metals were more evident on wheat and rice

seedlings than they were on barley seedlings. This was the case for all three types of grains. Copper had a more significant inhibitive effect on the seedlings of grain crops when compared to lead and zinc. (Mahmood et al., 2007).

Metals are bioaccumulated by wheat plants, which is a significant factor. The idea of Phyto-toxicity, according to which metals enter the food chain through plants, is supported by this fact. Crops in some locations contain high levels of metals, indicating their geological origin, even though production and transportation are not usually well-established. Because wheat plants have a predisposition to accumulate heavy metals in its aerial parts and because wheat is such a key component of the food chain, it is imperative that wheat not be grown on farms or in areas that contain heavy metal contamination (Al-Othman, Ali, Al-Othman, Ali, & Habila, 2016).

Conclusively, according to the study of (Vinod, Awasthi, Chauchan, & Biochemistry, 2012), High amounts of Cu and Zn were shown to promote growth, chlorophyll content, protein and DNA content, carbohydrate content, proline, total phenol, and hydrogen peroxide content. Additionally, it was claimed that while low concentrations of Although copper and zinc help wheat grow, an excess of either is toxic, stunting the plant's development while also causing structural damage and disrupting the plant's physiological and biochemical processes and its ability to function.

In a research work by (Wei et al., 2022), looked at how two types of wheat reacted morphologically and physiologically to exposure to zinc. Zn produced hormetic-like biphasic dosage responses. Treatment with low Zn (10–100 μM) boosted accumulation and enhanced tolerance. Via boosting photosynthetic capacity and stimulating the production enzymes that function as antioxidants. On the other hand, high Zn treatment. So, the current study also shows the significantly increase of root and shoot length at both sets as compared to control group. Zinc stress is also one of the reasons of increase root hairs. Our findings indicate that Zn has a favorable impact at a negative effect at high doses and a low dose.

A study was conducted on tomato plant, in which 50 and 100 mg kg^{-1} of zinc amount is beneficial for the proper growth of tomato plants (Vijayarengan & Mahalakshmi, 2013). The current study on wheat shows that at the two treatments of 50

µM/L and 100 µM/L of Zn is advantageous for the plant growth and development as compared to control group which has 8 µM/L zinc concentration. Statistical analysis showed both zinc treatment has no significant effect on root shoot weight when compared with control group.

Previously study showed 50 µM Zn exhibited no deleterious effects on *T. aestivum* growth, but 300 µM Zn did. 50 µM Zn wheat absorbed High metal, no ion effects morphological and cytological differences were noted. The effective adaptive alteration of expanded root cell metal compartmentation vacuoles (Glińska, Gapińska, Michlewska, Skiba, & Kubicki, 2016). Current study showed the parallel results according to this previously conducted studies. As Zinc metal accumulated at day 7, 14 and 21 of toxic stress (set II) as compared to the respective control.

The previous findings demonstrated that while Fakhar-e-root sarhad's length decreased in comparison to control, Pirsabak 2004's root length did not respond to Cd and Zn stresses (Shafiq et al., 2019). While the present study infer that Dharabi-11 wheat variety showed significant increase in root length in set I and set II (moderate and toxic treatment) of zinc. Dharabi-11 wheat variety is the moderately sensitive and drought tolerant variety while Pirsabak 2004 and Fakhar-e-root sarhad's are zinc resistance and sensitive respectively.

In a study of rice, *OsHMA1* show expression and highly upregulated by Zn deficiency in shoot tissue (Takahashi, Bashir, Ishimaru, Nishizawa, Nakanishi, et al., 2012). This current study reveals the *HMA1* shows highest expression in treated roots as compared to control in wheat plant. *HMA1.1* and *HMA1.2* shows highest expression in toxic roots, while *HMA1.3* shows highest relative fold change in toxic roots and shoots as compared to control group.

Chapter 6

6. Conclusion

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In this study heavy metal stress was given to wheat plants under controlled glass house environment using hydroponics to notice their morphological deviation under metal toxicity. For this purpose, samples were taken on different days intervals (7, 14, 21, and 28) after the transplantation. Total length, root length, shoot length in cm, total fresh weight, shoot and root weight in gm were recorded. After that RNA extraction and elemental analysis was performed. The Kolmogorov-Smirnov's and Levene's tests were performed to confirm the normality of residuals and the homogeneity of variance, respectively. Furthermore, one-way ANOVA was performed using the F- Test followed by an independent t-test post-hoc analysis. The results indicated that under zinc stress shoots and roots length increase as compared to control without no effect on the weight of plants. furthermore, Metal accumulation and upregulated gene expression of HMA1 were found in wheat var. Dharabi-11. No significant changes in length and weight of root and shoot were found when plants were exposed to copper metal stress. However, elemental analysis of copper shows the accumulation of metal in treated plants when compared to control. Further RT analysis showed thatHMA1including three homeologs (HMA1.1, HMA1.2, and HMA1.3) gene were highly expressed in tested plants. This research will be help for the identification of microRNA targets which will be valuable for selecting and breeding low-Cd accumulation wheat lines.

References

Abdel-Ghany, S. E., Müller-Moulé, P., Niyogi, K. K., Pilon, M., & Shikanai, T. J. T. P. C. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. *17*(4), 1233-1251.

Adachi, K., & Tainosho, Y. (2004). Characterization of heavy metal particles embedded in tire dust. *Environment international, 30*(8), 1009-1017.

Adhikari, T., Kundu, S., Biswas, A. K., Tarafdar, J. C., & Rao, A. S. (2012). Effect of copper oxide nano particle on seed germination of selected crops. *Journal of Agricultural Science and Technology. A, 2*(6A), 815.

Agrios, G. N. (2005). *Plant pathology*: Elsevier.

Al-Othman, Z. A., Ali, R., Al-Othman, A. M., Ali, J., & Habila, M. A. J. A. J. o. C. (2016). Assessment of toxic metals in wheat crops grown on selected soils, irrigated by different water sources. *9*, S1555-S1562.

Al Khateeb, W., Al-Qwasemeh, H. J. P., & Plants, M. B. o. (2014). Cadmium, copper and zinc toxicity effects on growth, proline content and genetic stability of Solanum nigrum L., a crop wild relative for tomato; comparative study. *20*(1), 31-39.

Algreen, M., Rein, A., Legind, C. N., Amundsen, C. E., Karlson, U. G., & Trapp, S. (2012). Test of tree core sampling for screening of toxic elements in soils from a Norwegian site. *International journal of phytoremediation, 14*(4), 305-319.

Ali, Z., Razi-ul-Hasnain, R., Quraishi, U. M., & Malik, R. N. (2018). Heavy metal tolerance and biochemical attributes of selected wheat genotypes on irrigation with industrial wastewaters. *Pakistan Journal of Agricultural Sciences, 55*(4).

Alloway, B. J. (2013). Sources of heavy metals and metalloids in soils. In *Heavy metals in soils* (pp. 11-50): Springer.

Amin, N.-u., & Ahmad, T. J. R. a. (2015). Contamination of soil with heavy metals from industrial effluent and their translocation in green vegetables of Peshawar, Pakistan. *5*(19), 14322-14329.

Andrés- Colás, N., Sancenón, V., Rodríguez- Navarro, S., Mayo, S., Thiele, D. J., Ecker, J. R., . . . Peñarrubia, L. J. T. P. J. (2006). The Arabidopsis heavy metal P- type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. *45*(2), 225-236.

Argüello, J. M., Eren, E., & González-Guerrero, M. (2007). The structure and function of heavy metal transport P 1B-ATPases. *Biometals, 20*(3-4), 233.

Argüello, J. M., Eren, E., & González-Guerrero, M. J. B. (2007). The structure and function of heavy metal transport P1B-ATPases. *20*(3), 233-248.

Arzani, A., Ashraf, M. J. C. R. i. F. S., & Safety, F. (2017). Cultivated ancient wheats (Triticum spp.): A potential source of health- beneficial food products. *16*(3), 477-488.

Barbosa, R. H., Tabaldi, L. A., Miyazaki, F. R., Pilecco, M., Kassab, S. O., & Bigaton, D. (2013). Foliar copper uptake by maize plants: effects on growth and yield. *Ciência Rural, 43*(9), 1561-1568.

Barlow, K., Christy, B., O'leary, G., Riffkin, P., & Nuttall, J. (2015). Simulating the impact of extreme heat and frost events on wheat crop production: A review. *Field Crops Research, 171*, 109-119.

Boutigny, S., Sautron, E., Finazzi, G., Rivasseau, C., Frelet-Barrand, A., Pilon, M., . . . Seigneurin-Berny, D. J. J. o. E. B. (2014). HMA1 and PAA1, two chloroplast-envelope PIB-ATPases, play distinct roles in chloroplast copper homeostasis. *65*(6), 1529-1540.

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L., D'Amore, R., Allen, A. M., . . . Bolser, D. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature, 491*(7426), 705-710.

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., & Lux, A. J. N. p. (2007). Zinc in plants. *173*(4), 677-702.

Burnstock, G., Williams, M. J. J. o. P., & Therapeutics, E. (2000). P2 purinergic receptors: modulation of cell function and therapeutic potential. *295*(3), 862-869.

Chan, H., Babayan, V., Blyumin, E., Gandhi, C., Hak, K., Harake, D., . . . Liu, H. Y. J. M. P. (2010). The p-type ATPase superfamily. *19*(1-2), 5-104.

Chaudhary, K., Agarwal, S., & Khan, S. (2018). Role of phytochelatins (PCs), metallothioneins (MTs), and heavy metal ATPase (HMA) genes in heavy metal tolerance. In *Mycoremediation and environmental sustainability* (pp. 39-60): Springer.

Chaudhary, K., Jan, S., & Khan, S. J. P. M. I. (2016). Heavy metal ATPase (HMA2, HMA3, and HMA4) genes in hyperaccumulation mechanism of heavy metals. 545-556.

Chen, J., Gaillardet, J., Louvat, P. J. E. s., & technology. (2008). Zinc isotopes in the Seine River waters, France: a probe of anthropogenic contamination. *42*(17), 6494-6501.

Choi, S., Liu, X., & Pan, Z. J. A. P. S. (2018). Zinc deficiency and cellular oxidative stress: prognostic implications in cardiovascular diseases. *39*(7), 1120-1132.

Cobbett, C. S., Hussain, D., & Haydon, M. J. J. N. P. (2003). Structural and functional relationships between type 1B heavy metal- transporting P- type ATPases in Arabidopsis. *159*(2), 315-321.

Colangelo, E. P., & Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Current opinion in plant biology, 9*(3), 322-330.

Cong, W., Miao, Y., Xu, L., Zhang, Y., Yuan, C., Wang, J., . . . Wang, N. J. B. p. b. (2019). Transgenerational memory of gene expression changes induced by heavy metal stress in rice (Oryza sativa L.). *19*(1), 1-14.

Deng, F., Yamaji, N., Xia, J., & Ma, J. F. J. P. p. (2013). A member of the heavy metal P-type ATPase OsHMA5 is involved in xylem loading of copper in rice. *163*(3), 1353-1362.

Deng, L., Wang, K., Li, J., Zhao, G., & Shangguan, Z. J. E. E. (2016). Effect of soil moisture and atmospheric humidity on both plant productivity and diversity of native grasslands across the Loess Plateau, China. *94*, 525-531.

Dey, S., Mazumder, P. á., & Paul, S. á. J. A. J. o. B. (2015). Copper-induced changes in growth and antioxidative mechanisms of tea plant (Camellia sinensis (L.) O. Kuntze). *14*(7), 582-592.

Dong, J., Yang, Q.-w., Sun, L.-n., Zeng, Q., Liu, S.-j., Pan, J., & Liu, X.-l. (2011). Assessing the concentration and potential dietary risk of heavy metals in vegetables at a Pb/Zn mine site, China. *Environmental Earth Sciences, 64*(5), 1317-1321.

Drees, S. L., Beyer, D. F., Lenders- Lomscher, C., & Lübben, M. J. M. m. (2015). Distinct functions of serial metal- binding domains in the E scherichia coli P 1 B- ATP ase CopA. *97*(3), 423-438.

Dresler, S., Hanaka, A., Bednarek, W., & Maksymiec, W. (2014). Accumulation of low-molecular-weight organic acids in roots and leaf segments of Zea mays plants treated with cadmium and copper. *Acta Physiologiae Plantarum, 36*(6), 1565-1575.

Dubcovsky, J., & Dvorak, J. (2007). Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science, 316*(5833), 1862-1866.

EL-Gharbawy, S. S. (2015). Wheat breeding for tolerance to heavy metals pollution. *Agron Department, Faculty of Agriculture, Zagazig University, Egypt*.

Estefan, G., Sommer, R., Ryan, J. J. A. m. f. t. W. A., & region, N. A. (2013). Methods of soil, plant, and water analysis. *3*, 65-119.

Evans, L. T., & Wardlaw, I. F. (2017). Wheat. In *Photoassimilate distribution in plants and crops* (pp. 501-518): Routledge.

Fan, P., Wang, C., Zhu, J., Zeng, J., Kang, H., Fan, X., . . . Wang, Y. J. P. (2018). Expression of TpNRAMP5, a metal transporter from Polish wheat (Triticum polonicum L.), enhances the accumulation of Cd, Co and Mn in transgenic Arabidopsis plants. *247*(6), 1395-1406.

Feigl, G., Kumar, D., Lehotai, N., Pető, A., Molnár, Á., Rácz, É., . . . Laskay, G. (2015). Comparing the effects of excess copper in the leaves of Brassica juncea (L. Czern) and Brassica napus (L.) seedlings: growth inhibition, oxidative stress and photosynthetic damage. *Acta Biologica Hungarica, 66*(2), 205-221.

Feigl, G., Kumar, D., Lehotai, N., Tugyi, N., Molnár, Á., Ördög, A., . . . Erdei, L. (2013). Physiological and morphological responses of the root system of Indian mustard (Brassica juncea L. Czern.) and rapeseed (Brassica napus L.) to copper stress. *Ecotoxicology and environmental safety, 94*, 179-189.

Gajewska, E., & SkŁodowska, M. (2010). Differential effect of equal copper, cadmium and nickel concentration on biochemical reactions in wheat seedlings. *Ecotoxicology and environmental safety, 73*(5), 996-1003.

Gao, L., Yang, J., Song, S.-j., Xu, K., Liu, H.-d., Zhang, S.-h., . . . Zhao, Y. (2021). Genome–wide association study of grain morphology in wheat. *Euphytica, 217*(8), 1-12.

Gautam, P. K., Gautam, R. K., Banerjee, S., Chattopadhyaya, M., & Pandey, J. (2016). Heavy metals in the environment: fate, transport, toxicity and remediation technologies. *Nova Sci Publishers, 60*, 101-130.

Ghani, A., Khan, I., Umer, S., Ahmed, I., Mustafa, I., & Mohammad, N. (2015). Response of wheat (Triticum aestivum) to exogenously applied chromium: effect

on growth, chlorophyll and mineral composition. *J. Environ. Anal. Toxicol, 5*(3), 2161-0525.1000273.
Giraldo, P., Benavente, E., Manzano-Agugliaro, F., & Gimenez, E. (2019). Worldwide research trends on wheat and barley: A bibliometric comparative analysis. *Agronomy, 9*(7), 352.
Glińska, S., Gapińska, M., Michlewska, S., Skiba, E., & Kubicki, J. J. P. (2016). Analysis of Triticum aestivum seedling response to the excess of zinc. *253*(2), 367-377.
Grotz, N., & Guerinot, M. L. J. B. e. B. A.-M. C. R. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *1763*(7), 595-608.
Guan, J., Garcia, D. F., Zhou, Y., Appels, R., Li, A., & Mao, L. (2020). The battle to sequence the bread wheat genome: a tale of the three kingdoms. *Genomics, Proteomics & Bioinformatics, 18*(3), 221-229.
Gupta, N., Ram, H., Kumar, B. J. R. i. E. S., & Bio/Technology. (2016). Mechanism of Zinc absorption in plants: uptake, transport, translocation and accumulation. *15*(1), 89-109.
Hirayama, T., Alonso, J. M. J. P., & physiology, c. (2000). Ethylene captures a metal! Metal ions are involved in ethylene perception and signal transduction. *41*(5), 548-555.
Huang, H., Yuan, X., Zeng, G., Zhu, H., Li, H., Liu, Z., . . . Bi, W. J. B. T. (2011). Quantitative evaluation of heavy metals' pollution hazards in liquefaction residues of sewage sludge. *102*(22), 10346-10351.
Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., . . . Cobbett, C. S. J. T. p. c. (2004). P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *16*(5), 1327-1339.
Hussain, I., Khakwani, A. A., & Khan, A. A. J. G. U. J. o. R. (2018). THE EVALUATION OF DIFFERENT REGIMES WHEAT CULTIVARS UNDER AGRO CLIMATE OF DERA ISMAIL KHAN. *34*(1), 32-37.
Iftikhar, H., Ghori, Z., Ali, S. H., Sheikh, S., & Gul, A. (2019). Wheat responses to stress and biotechnological approaches for improvement. In *Wheat production in changing environments* (pp. 343-392): Springer.
Ikhtiar, K., & Alam, Z. (2007). Nutritional composition of Pakistani wheat varieties. *Journal of Zhejiang University Science B, 8*(8), 555-559.
Jasrotia, P., Kashyap, P. L., Bhardwaj, A. K., Kumar, S., & Singh, G. (2018). Nanotechnology scope and applications for wheat production: A review of recent advances.
Jeber, B. A., & Khaeim, H. M. (2019). Effect of foliar application of amino acids, organic acids, and naphthalene acetic acid on growth and yield traits of wheat. *Plant archives, 19*(2), 824-826.
Jouvin, D., Weiss, D., Mason, T., Bravin, M., Louvat, P., Zhao, F., . . . technology. (2012). Stable isotopes of Cu and Zn in higher plants: evidence for Cu reduction at the root surface and two conceptual models for isotopic fractionation processes. *46*(5), 2652-2660.
Kamitani, T., Oba, H., & Kaneko, N. (2006). Microbial biomass and tolerance of microbial community on an aged heavy metal polluted floodplain in Japan. *Water, air, and soil pollution, 172*(1), 185-200.

Kantam, M. L., Neelima, B., Reddy, C. V., & Neeraja, V. J. J. o. M. C. A. C. (2006). N-Arylation of imidazoles, imides, amines, amides and sulfonamides with boronic acids using a recyclable Cu (OAc) 2· H2O/[bmim][BF4] system. *249*(1-2), 201-206.

Kaur, H., & Garg, N. J. P. (2021). Zinc toxicity in plants: a review. *253*(6), 1-28.

Kilian, B., Martin, W., & Salamini, F. (2010). Genetic diversity, evolution and domestication of wheat and barley in the Fertile Crescent. *Evolution in action*, 137-166.

Kobayashi, Y., Kuroda, K., Kimura, K., Southron-Francis, J. L., Furuzawa, A., Kimura, K., . . . Koyama, H. J. P. p. (2008). Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in Arabidopsis. *148*(2), 969-980.

Kumar, V., & Chopra, A. (2015). Toxicity of chromium in agricultural crops with respect to its chemical speciation-a review. *World Appl Sci J, 33*(6), 944-969.

Legros, S., Chaurand, P., Rose, J., Masion, A., Briois, V., Ferrasse, J.-H., . . . Doelsch, E. (2010). Investigation of copper speciation in pig slurry by a multitechnique approach. *Environmental science & technology, 44*(18), 6926-6932.

Leuci, R., Brunetti, L., Laghezza, A., Loiodice, F., Tortorella, P., & Piemontese, L. J. A. S. (2020). Importance of biometals as targets in medicinal chemistry: An overview about the role of Zinc (II) chelating agents. *10*(12), 4118.

Li, H., Fan, R., Li, L., Wei, B., Li, G., Gu, L., . . . environment. (2014). Identification and characterization of a novel copper transporter gene family TaCT 1 in common wheat. *37*(7), 1561-1573.

Liang, Q., & Zhou, B. J. M. b. o. t. c. (2007). Copper and manganese induce yeast apoptosis via different pathways. *18*(12), 4741-4749.

Lin, Y.-F., Aarts, M. G. J. C., & sciences, m. l. (2012). The molecular mechanism of zinc and cadmium stress response in plants. *69*(19), 3187-3206.

Liu, L., Song, Z., Li, Q., Ellam, R. M., Tang, J., Wang, Y., . . . Wang, H. (2022). Accumulation and partitioning of toxic trace metal (loid) s in phytoliths of wheat grown in a multi-element contaminated soil. *Environmental Pollution, 294*, 118645.

Liu, T., Reyes-Caballero, H., Li, C., Scott, R. A., & Giedroc, D. P. J. B. (2007). Multiple metal binding domains enhance the Zn (II) selectivity of the divalent metal ion transporter AztA. *46*(39), 11057-11068.

Lombn s, P., Singh, B. R. J. J. o. P. N., & Science, S. (2003). Varietal tolerance to zinc deficiency in wheat and barley grown in chelatorbuffered nutrient solution and its effect on uptake of Cu, Fe, and Mn. *166*(1), 76-83.

Loneragan, J. F., & Webb, M. J. (1993). Interactions between zinc and other nutrients affecting the growth of plants. In *Zinc in soils and plants* (pp. 119-134): Springer.

Luo, X.-S., Li, L.-Z., & Zhou, D.-M. (2008). Effect of cations on copper toxicity to wheat root: Implications for the biotic ligand model. *Chemosphere, 73*(3), 401-406.

Lwalaba, J. L. W., Louis, L. T., Zvobgo, G., Fu, L., Mwamba, T. M., Mundende, R. P. M., . . . safety, e. (2019). Copper alleviates cobalt toxicity in barley by antagonistic interaction of the two metals. *180*, 234-241.

Mackie, K., Müller, T., & Kandeler, E. (2012). Remediation of copper in vineyards–a mini review. *Environmental Pollution, 167*, 16-26.

Mahmood, T., Islam, K., & Muhammad, S. J. P. J. o. B. (2007). Toxic effects of heavy metals on early growth and tolerance of cereal crops. *39*(2), 451.
Maiz, I., Esnaola, M. V., & Millan, E. (1997). Evaluation of heavy metal availability in contaminated soils by a short sequential extraction procedure. *Science of the Total Environment, 206*(2-3), 107-115.
Malecki, M., Hsu, A., Truong, L., & Sanchez, S. J. P. o. t. N. A. o. S. (2002). Molecular immunolabeling with recombinant single-chain variable fragment (scFv) antibodies designed with metal-binding domains. *99*(1), 213-218.
Mandal, A. K., & Argüello, J. M. J. B. (2003). Functional roles of metal binding domains of the Archaeoglobus fulgidus Cu+-ATPase CopA. *42*(37), 11040-11047.
Manivasagaperumal, R., Balamurugan, S., Thiyagarajan, G., & Sekar, J. J. C. B. (2011). Effect of zinc on germination, seedling growth and biochemical content of cluster bean (Cyamopsis tetragonoloba (L.) Taub). *2*(5), 11-15.
Markossian, K., & Kurganov, B. J. B. (2003). Copper chaperones, intracellular copper trafficking proteins. Function, structure, and mechanism of action. *68*(8), 827-837.
Mathur, S., Raikalal, P., & Jajoo, A. (2019). Physiological responses of wheat to environmental stresses. In *Wheat Production in Changing Environments* (pp. 31-61): Springer.
McGrath, S., & Cegarra, J. (1992). Chemical extractability of heavy metals during and after long- term applications of sewage sludge to soil. *Journal of Soil Science, 43*(2), 313-321.
Mench, M. (1990). Transfert des oligo-éléments du sol à la racine et absorption. *Comptes Rendus de l'Académie d'Agriculture de France, 76*(2), 17-30.
Mills, R. F., Doherty, M. L., López-Marqués, R. L., Weimar, T., Dupree, P., Palmgren, M. G., . . . Williams, L. E. J. P. p. (2008). ECA3, a Golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in Arabidopsis. *146*(1), 116-128.
Mills, R. F., Krijger, G. C., Baccarini, P. J., Hall, J., & Williams, L. E. J. T. P. J. (2003). Functional expression of AtHMA4, a P1B- type ATPase of the Zn/Co/Cd/Pb subclass. *35*(2), 164-176.
Mou, Y.-N., Gao, B.-J., Ren, K., Tong, S.-M., Ying, S.-H., & Feng, M.-G. J. V. (2020). P-type Na+/K+ ATPases essential and nonessential for cellular homeostasis and insect pathogenicity of Beauveria bassiana. *11*(1), 1415-1431.
Nagajyoti, P. C., Lee, K. D., & Sreekanth, T. J. E. c. l. (2010). Heavy metals, occurrence and toxicity for plants: a review. *8*(3), 199-216.
Neumann, D., & Zur Nieden, U. J. P. (2001). Silicon and heavy metal tolerance of higher plants. *56*(7), 685-692.
Okkeri, J., Bencomo, E., Pietilä, M., & Haltia, T. J. E. j. o. b. (2002). Introducing Wilson disease mutations into the zinc- transporting P- type ATPase of Escherichia coli: The mutation P634L in the 'hinge'motif (GDGXNDXP) perturbs the formation of the E2P state. *269*(5), 1579-1586.
Palmgren, M. G., & Nissen, P. (2011). P-type ATPases. *Annual review of biophysics, 40*, 243-266.
Pandey, M., Shrestha, J., Subedi, S., & Shah, K. K. (2020). Role of nutrients in wheat: a review. *Tropical Agrobiodiversity, 1*(1), 18-23.

Park, W., Ahn, S. J. J. J. o. p. n., & science, s. (2014). How do heavy metal ATPases contribute to hyperaccumulation? , *177*(2), 121-127.
Petris, M. J., Voskoboinik, I., Cater, M., Smith, K., Kim, B.-E., Llanos, R. M., . . . Mercer, J. F. J. J. o. b. c. (2002). Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate. *277*(48), 46736-46742.
Pruvot, C., Douay, F., Hervé, F., Waterlot, C. J. J. o. s., & sediments. (2006). Heavy metals in soil, crops and grass as a source of human exposure in the former mining areas (6 pp). *6*(4), 215-220.
Qiao, K., Gong, L., Tian, Y., Wang, H., & Chai, T. (2018). The metal-binding domain of wheat heavy metal ATPase 2 (TaHMA2) is involved in zinc/cadmium tolerance and translocation in Arabidopsis. *Plant cell reports, 37*(9), 1343-1352.
Qiao, K., Wang, F., Liang, S., Wang, H., Hu, Z., & Chai, T. J. S. R. (2019). Improved Cd, Zn and Mn tolerance and reduced Cd accumulation in grains with wheat-based cell number regulator TaCNR2. *9*(1), 1-10.
Rattu, A., Akhtar, M., Fayyaz, M., & Bashir, M. (2007). Screening of wheat against yellow and leaf rusts under NUWYT and NWDSN and wheat rust situation in Pakistan during 200-07. *Crop Diseases Research Programme, NARC, Islamabad*.
Rensing, C., Ghosh, M., & Rosen, B. P. J. J. o. b. (1999). Families of soft-metal-ion-transporting ATPases. *181*(19), 5891-5897.
Rizvi, A., Zaidi, A., Ameen, F., Ahmed, B., AlKahtani, M. D., & Khan, M. S. (2020). Heavy metal induced stress on wheat: phytotoxicity and microbiological management. *RSC Advances, 10*(63), 38379-38403.
RODRIGO- MORENO, A., ANDRÉS- COLÁS, N., Poschenrieder, C., Gunse, B., Penarrubia, L., Shabala, S. J. P., Cell, & Environment. (2013). Calcium- and potassium- permeable plasma membrane transporters are activated by copper in Arabidopsis root tips: linking copper transport with cytosolic hydroxyl radical production. *36*(4), 844-855.
Rodrıguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E., & Bleecker, A. B. J. S. (1999). A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. *283*(5404), 996-998.
Rosenfelder, P., Eklund, M., & Mosenthin, R. (2013). Nutritive value of wheat and wheat by-products in pig nutrition: A review. *Animal Feed Science and Technology, 185*(3-4), 107-125.
Rout, G. R., & Das, P. (2009). Effect of metal toxicity on plant growth and metabolism: I. Zinc. In *Sustainable agriculture* (pp. 873-884): Springer.
Saavedra-Mella, F., Liu, Y., Southam, G., & Huang, L. J. E. P. (2019). Phosphate treatment alleviated acute phytotoxicity of heavy metals in sulfidic Pb-Zn mine tailings. *250*, 676-685.
Sancenón, V., Puig, S., Mateu-Andrés, I., Dorcey, E., Thiele, D. J., & Peñarrubia, L. J. J. o. B. C. (2004). The Arabidopsis copper transporter COPT1 functions in root elongation and pollen development. *279*(15), 15348-15355.
Sancenón, V., Puig, S., Mira, H., Thiele, D. J., & Peñarrubia, L. J. P. m. b. (2003). Identification of a copper transporter family in Arabidopsis thaliana. *51*(4), 577-587.

Sánchez-Pardo, B., Fernández-Pascual, M., & Zornoza, P. (2014). Copper microlocalisation and changes in leaf morphology, chloroplast ultrastructure and antioxidative response in white lupin and soybean grown in copper excess. *Journal of plant research, 127*(1), 119-129.

Satoh-Nagasawa, N., Mori, M., Nakazawa, N., Kawamoto, T., Nagato, Y., Sakurai, K., . . . Physiology, C. (2012). Mutations in rice (Oryza sativa) heavy metal ATPase 2 (OsHMA2) restrict the translocation of zinc and cadmium. *53*(1), 213-224.

Satoh-Nagasawa, N., Mori, M., Sakurai, K., Takahashi, H., Watanabe, A., & Akagi, H. J. P. B. (2013). Functional relationship heavy metal P-type ATPases (OsHMA 2 and OsHMA3) of rice (Oryza sativa) using RNAi. 13.0616 a.

Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., . . . Joyard, J. J. J. o. B. C. (2006). HMA1, a new Cu-atpase of the chloro plast envelope, is essential for growth under adverse light conditions. *281*(5), 2882-2892.

Shafiq, S., Zeb, Q., Ali, A., Sajjad, Y., Nazir, R., Widemann, E., & Liu, L. J. I. j. o. m. s. (2019). Lead, cadmium and zinc phytotoxicity alter DNA methylation levels to confer heavy metal tolerance in wheat. *20*(19), 4676.

Shahbandeh, M. (2022). Wheat: production volume worldwide 2011/2012-2021/22. *website*.

Shahbaz, M., Tseng, M. H., Stuiver, C. E. E., Koralewska, A., Posthumus, F. S., Venema, J. H., . . . De Kok, L. J. (2010). Copper exposure interferes with the regulation of the uptake, distribution and metabolism of sulfate in Chinese cabbage. *Journal of Plant Physiology, 167*(6), 438-446.

Shewry, P. R. (2009). Wheat. *Journal of experimental botany, 60*(6), 1537-1553.

Shewry, P. R., Halford, N. G., & Lafiandra, D. (2003). Genetics of wheat gluten proteins. *Advances in genetics, 49*, 111-184.

Shikanai, T., Müller-Moulé, P., Munekage, Y., Niyogi, K. K., & Pilon, M. J. T. P. C. (2003). PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. *15*(6), 1333-1346.

Simms, D., Cizdziel, P. E., & Chomczynski, P. J. F. (1993). TRIzol: A new reagent for optimal single-step isolation of RNA. *15*(4), 532-535.

Sindhu, P., Saharma, A., & Pooja, P. (2015). Total chlorophyll and total protein content in wheat (Triticum aestivum) grown under arsenic stress. *Int. J. Recent Sci. Res, 6*, 5072-5075.

Singh, D., Nath, K., & Sharma, Y. K. (2007). Response of wheat seed germination and seedling growth under copper stress. *Journal of Environmental Biology, 28*(2), 409.

Singh, R., Gautam, N., Mishra, A., & Gupta, R. J. I. j. o. p. (2011). Heavy metals and living systems: An overview. *43*(3), 246.

Smith, S. R. (2009). A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. *Environment international, 35*(1), 142-156.

Solioz, M., & Vulpe, C. J. T. i. b. s. (1996). CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *21*(7), 237-241.

Takahashi, R., Bashir, K., Ishimaru, Y., Nishizawa, N. K., & Nakanishi, H. (2012). The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. *Plant signaling & behavior, 7*(12), 1605-1607.

Takahashi, R., Bashir, K., Ishimaru, Y., Nishizawa, N. K., Nakanishi, H. J. P. s., & behavior. (2012). The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. *7*(12), 1605-1607.

Tan, J., Wang, J., Chai, T., Zhang, Y., Feng, S., Li, Y., . . . Chai, X. J. P. B. J. (2013). Functional analyses of T a HMA 2, a P 1B- type ATP ase in wheat. *11*(4), 420-431.

Tang, J., Zhang, L., Zhang, J., Ren, L., Zhou, Y., Zheng, Y., . . . Chen, A. J. S. o. t. T. E. (2020). Physicochemical features, metal availability and enzyme activity in heavy metal-polluted soil remediated by biochar and compost. *701*, 134751.

Tariq, M., Mahmood, A., Mian, M. A., Cheema, N. M., Sabar, M., & Ihsan, M. (2013). Dharabi-11: a new high yielding drought and disease tolerant wheat variety. *International Journal of Agriculture and Biology*, *15*(4).

Tchounwou, P., Yedjou, C., Patlolla, A., & Sutton, D. (2012). Heavy metal toxicity and the environment. InMolecular, clinical and environmental toxicology 2012 (pp. 133-164). In: Springer, Basel.

Tiwari, V., & Shoran, J. (2010). Growth and production of wheat. *Verheye, HW Soils, Plant Growth and Crop Production. Eolss publishers. Co. Ltd. United Kingdom, 1*, 298.

Tsonev, T., Cebola Lidon, F. J. J. E. J. o. F., & Agriculture. (2012). Zinc in plants-an overview. *24*(4).

Vijayarengan, P., & Mahalakshmi, G. J. W. A. S. J. (2013). Zinc toxicity in tomato plants. *24*(5), 649-653.

Vinod, K., Awasthi, G., Chauchan, P. J. J. o. S. P., & Biochemistry. (2012). Cu and Zn tolerance and responses of the biochemical and physiochemical system of wheat. *8*(3), 203-213.

Wang, Q.-Y., Liu, J.-S., Wang, Y., & Yu, H.-W. (2015). Accumulations of copper in apple orchard soils: distribution and availability in soil aggregate fractions. *Journal of Soils and Sediments, 15*(5), 1075-1082.

Wang, S., Wu, W., Liu, F., Liao, R., & Hu, Y. (2017). Accumulation of heavy metals in soil-crop systems: a review for wheat and corn. *Environmental Science and Pollution Research, 24*(18), 15209-15225.

Wang, S., Wu, W., Liu, F., Liao, R., Hu, Y. J. E. S., & Research, P. (2017). Accumulation of heavy metals in soil-crop systems: a review for wheat and corn. *24*(18), 15209-15225.

Warne, M. S. J., Heemsbergen, D., Stevens, D., McLaughlin, M., Cozens, G., Whatmuff, M., . . . Journal, C. A. I. (2008). Modeling the toxicity of copper and zinc salts to wheat in 14 soils. *27*(4), 786-792.

Wei, C., Jiao, Q., Agathokleous, E., Liu, H., Li, G., Zhang, J., . . . Jiang, Y. J. S. o. T. T. E. (2022). Hormetic effects of zinc on growth and antioxidant defense system of wheat plants. *807*, 150992.

Wijekoon, C. J., Udagedara, S. R., Knorr, R. L., Dimova, R., Wedd, A. G., & Xiao, Z. J. J. o. t. A. C. S. (2017). Copper ATPase CopA from Escherichia coli: quantitative

correlation between ATPase activity and vectorial copper transport. *139*(12), 4266-4269.
Williams, L. E., & Mills, R. F. J. T. i. p. s. (2005). P1B-ATPases–an ancient family of transition metal pumps with diverse functions in plants. *10*(10), 491-502.
Wodala, B., Eitel, G., Gyula, T., Ördög, A., & Horváth, F. (2012). Monitoring moderate Cu and Cd toxicity by chlorophyll fluorescence and P700 absorbance in pea leaves. *Photosynthetica, 50*(3), 380-386.
Wu, C.-C., Rice, W. J., & Stokes, D. L. J. S. (2008). Structure of a copper pump suggests a regulatory role for its metal-binding domain. *16*(6), 976-985.
Wu, C., Luo, Y., & Zhang, L. (2010). Variability of copper availability in paddy fields in relation to selected soil properties in southeast China. *Geoderma, 156*(3-4), 200-206.
Wuana, R. A., & Okieimen, F. E. J. I. S. R. N. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *2011*.
Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. J. B. b. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *13*(1), 1-11.
Yotsova, E. K., Dobrikova, A. G., Stefanov, M. A., Kouzmanova, M., & Apostolova, E. L. (2018). Improvement of the rice photosynthetic apparatus defence under cadmium stress modulated by salicylic acid supply to roots. *Theoretical and Experimental Plant Physiology, 30*(1), 57-70.
Yruela, I. J. B. J. o. P. P. (2005). Copper in plants. *17*, 145-156.
Yruela, I. J. F. P. B. (2009). Copper in plants: acquisition, transport and interactions. *36*(5), 409-430.
Yu, C. H., Dolgova, N. V., & Dmitriev, O. Y. J. I. l. (2017). Dynamics of the metal binding domains and regulation of the human copper transporters ATP7B and ATP7A. *69*(4), 226-235.
Yuan, H.-M., Xu, H.-H., Liu, W.-C., Lu, Y.-T. J. P., & Physiology, C. (2013). Copper regulates primary root elongation through PIN1-mediated auxin redistribution. *54*(5), 766-778.
Zain, M., Khan, I., Qadri, R. W. K., Ashraf, U., Hussain, S., Minhas, S., . . . Bashir, M. (2015). Foliar application of micronutrients enhances wheat growth, yield and related attributes. *American Journal of Plant Sciences, 6*(07), 864.
Zhang, C., Nie, S., Liang, J., Zeng, G., Wu, H., Hua, S., . . . Deng, L. J. S. o. t. T. E. (2016). Effects of heavy metals and soil physicochemical properties on wetland soil microbial biomass and bacterial community structure. *557*, 785-790.
Zhang, X., Zhang, G., Guo, L., Wang, H., Zeng, D., Dong, G., . . . Xue, D. (2011). Identification of quantitative trait loci for Cd and Zn concentrations of brown rice grown in Cd-polluted soils. *Euphytica, 180*(2), 173-179.
Zhang, X. C., & Zhang, H. (2019). P-type ATPases use a domain-association mechanism to couple ATP hydrolysis to conformational change. *Biophysics Reports, 5*(4), 167-175.

ANNEXURES

Annexure I: Composition of stock solutions for Lombnaes media.

Table 1: Concentration of macronutrients in Lombnaes media.

Stock solutions (500ml each)	**Final concentration in media**	**Final volume per liter in media**
1 mM MES 10 mM $MnCl_2.4H_2O$	1mM 0.6 µM	195.2mg 60µl
1 M Ca $(NO_3)_2.4H_20$	2 mM	1ml
1 M KNO_3	1 mM	1ml
80 mM KH_2PO_4	80 µM	1ml
0.5 M $MgSO_4.7H_2O$	0.5 mM	1ml
0.9 M NaOH	0.9 mM	1ml
75mM$Fe(NO3)_3.9H_2O$	75 µM	1ml

Table 2: Final concentration of micronutrients in Lombnaes media.

Micro nutrient stock solution (250 ml each)	**1000X Stock solution concentration**	**Final concentration required in 1x diluted stock solution**	**Final volume per liter**
80 mM $ZnCl_2$	8000 µM	8 µM	1ml
0.5M$CuCl_2.2H_2O$	2000 µM	2.0 µM	1ml
0.5 M $NiCl_2.6H_2O$	100 µM	0.1 µM	1ml
0.5 M $Na_2MoO_4.2H_2O$	100 µM	0.1 µM	1ml
0.5 M H_3BO_3	10000 µM	10 µM	1ml

Annexure II: Statistical analysis of phenotypic data

COMB	Day	Dose	Total Length	Shoot Length	Root Length	Fresh Weight	Shoot Weight	Root Weight
M1	7	Control	34.72 ± 0.72	15.98 ± 0.47	18.74 ± 0.33	0.3166 ± 0.0168	0.1442 ± 0.0188	0.1326 ± 0.0138
		Moderate	36.87 ± 1.86**(ns)**	14.97 ± 0.35(ns)	21.90 ± 2.22(ns)	0.3756 ± 0.0606(ns)	0.1343 ± 0.0149(ns)	0.1634 ± 0.0345(ns)
		Toxic	35.14 ± 2.35**(ns)**	13.92 ± 0.11(*)	21.77 ± 2.45(ns)	0.317 ± 0.0375(ns)	0.2247 ± 0.1131(ns)	0.1460 ± 0.0286(ns)
	14	Control	48.69 ± 1.22	22.03 ± 0.9	26.66 ± 2.1	0.4722 ± 0.0698	0.2302 ± 0.0323	0.2051 ± 0.0305
		Moderate	45.82 ± 2.33**(ns)**	16.58 ± 2.05(ns)	29.24 ± 2.31(ns)	0.6133- ± 0.1876(ns)	0.2200 ± 0.0729(ns)	0.3475 ± 0.1213(ns)
		Toxic	51.26 ± 1.66**(ns)**	17.85- ± 1.64(ns)	33.41 ± 2.76(ns)	0.6093- ± 0.1236(ns)	0.2193 ± 0.0164(ns)	0.3490 ± 0.1155(ns)
	21	Control	55.06 ± 2.55	24.68 ± 1.67	30.46 ± 1.02	0.6313- ± 0.0578	0.3032 ± 0.036	0.2799- ± 0.063
		Moderate	57.84 ± 2.96**(ns)**	20.36 ± 1.95(ns)	37.49 ± 3.1(ns)	0.9014 ± 0.1675(ns)	0.1873 ± 0.0228(ns)	0.4080 ± 0.0206(ns)
		Toxic	56.39 ± 0.82**(ns)**	19.41 ± 0.32(ns)	36.98 ± 0.56(*)	0.7642 ± 0.1093(ns)	0.2705 ± 0.042(ns)	0.4227 ± 0.0703(ns)
	28	Control	60.38 ± 2.88	28.09 ± 2.21	32.30 ± 1.75	0.8359 ± 0.1268	0.4131 ± 0.1073	0.3900 ± 0.0402
		Moderate	58.79 ± 5.28**(ns)**	16.48 ± 2.02(*)	42.31 ± 4.2(ns)	0.6449- ± 0.1271(ns)	0.1902 ± 0.0213(ns)	0.4159 ± 0.0854(ns)
		Toxic	71.09 ± 1.78**(*)**	19.83 ± 1.29(*)	51.26 ± 0.73(**)	0.9991 ± 0.1952(ns)	0.3194 ± 0.0658(ns)	0.4914 ± 0.0341(ns)
M2	7	Control	34.72 ± 0.72	15.98 ± 0.47	18.74 ± 0.33	0.3166 ± 0.0168	0.1442 ± 0.0188	0.1326 ± 0.0138
		Moderate	27.14 ± 2.22	13.28 ± 1.35	18.23 ± 2.62	0.2163 ± 0.0355	0.1185 ± 0.0069	0.1092 ± 0.0099
		Toxic	27.80 ± 1.87	11.73 ± 1.36	16.07 ± 0.51	0.2216 ± 0.0146	0.0876 ± 0.0084	0.1252 ± 0.0566
	14	Control	48.69 ± 1.22	22.03 ± 0.9	26.66 ± 2.1	0.4722 ± 0.0698	0.2302- ± 0.0323	0.2051 ± 0.0305
		Moderate	43.74 ± 2.51	18.34 ± 1.15	25.41 ± 1.59	0.3596 ± 0.0492	0.2381- ± 0.0849	0.1734 ± 0.0424
		Toxic	40.23 ± 3.36	18.06 ± 1.27	22.19 ± 2.45	0.405 ± 0.0804	0.1719- ± 0.0317	0.1960 ± 0.0538
	21	Control	52.34 ± 5.21	23.68 ± 2.67	28.75 ± 2.51	0.5584 ± 0.0254	0.2823 ± 0.0465	0.2311 ± 0.0144
		Moderate	49.41 ± 4.39	23.15 ± 3.4	27.10 ± 2.68	0.4585 ± 0.0337	0.1994- ± 0.0334	0.2106 ± 0.0074
		Toxic	51.70 ± 3.29	25.97 ± 1.6	25.90 ± 3.13	0.4986 ± 0.0523	0.2765- ± 0.0373	0.2202 ± 0.0325
	28	Control	60.38 ± 2.88	28.09 ± 2.21	32.30 ± 1.75	0.8359 ± 0.1268	0.4131 ± 0.1073	0.3900 ± 0.0402
		Moderate	59.58 ± 4	26.30 ± 3.56	33.28 ± 1.2	0.7835 ± 0.0446	0.3321 ± 0.0271	0.4048 ± 0.0649

		Toxic	52.16 ± 1.46	26.78 ± 2.14	26.33 ± 1.23	1.1583 ± 0.0291	0.5438 ± 0.0443	0.5851 ± 0.0655

Here M1= Metal 1 (Zinc), M2= Metal 2 (Copper) ns= non-significant, * means $p<0.05$, ** means $p<0.01$.

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