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Evaluation of the distribution of cadmium and its toxic effects on the biological responses of *Nicotiana tabacum* L.

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Evaluation of the distribution of cadmium and its toxic effects on the biological responses of *Nicotiana tabacum* L.

Abstract: The environmental pollutant cadmium (Cd) contributes to cell destruction in plants by elevated ROS. This study investigated the effects of Cd on tobacco plants (*Nicotiana tabacum* L.) to understand their response to oxidative stress induced by Cd, with a focus on morphological and biochemical characteristics. The finding revealed that the increased concentration of Cd reduced the values of PLSTI, PDSTI, and RWC compared with the control. In addition, the functions of CAT, POD, and APX enzymes were increased, while SOD enzyme activity decreased. Cd significantly increased proline, antioxidant capacity, MDA, and H_2O_2 levels. As the concentration of Cd in the environment elevates, its accumulation in roots and shoots increases. However, the amount of Cd accumulated in the roots was greater than in the shoots. Furthermore, the accumulation of Cd in the roots reduced amount of elements such as zinc, calcium, manganese, copper, and iron in the tissues of plants. The high potential of tobacco to absorb heavy metals makes it a suitable Cd accumulator, and its non-edible nature allows its use in phytoremediation. This study helps to better understand the interaction of different antioxidant pathways with Cd toxicity and the biochemical changes resulting from oxidative stress pathways in tobacco plants.

Key words: antioxidant enzymes, cadmium, oxidative stress, tobacco

Ovrednotenje razporeditve kadmija in njegovi toksični učinki na biološki odziv v tobaku (*Nicotiana tabacum* L.)

Izvleček: Okoljsko onesnaženje s kadmijem (Cd) prispeva k uničenju celic in povečanju reaktivnih zvrsti kisika v rastlinah (ROS). V raziskavi so bili preučevani učinki Cd na rastline tobaka (*Nicotiana tabacum* L.) za razumevanje njihovega odziva na oksidacijski stres, ki ga vzpodbudi Cd s poudarkom na morfoloških in biokemičnih lastnostih. Izsledki so pokazali, da je povečana koncentracija Cd zmanjšala vrednosti PLSTI, PDSTI in RWC v primerjavi s kontrolo. Dodatno se je povečalo delovanje encimov kot so CAT, POD in APX med tem, ko se je aktivnost encima SOD zmanjšala. Povečenaje Cd je značilno povečalo vsebnosti prolina, MDA, H_2O_2 in antioksidacijsko aktivnost. S povečevanjem koncentracije Cd v okolju se povečuje njegovo kopičenje v koreninah in poganjkih. Povečevanje vsebnosti Cd je večje v koreninah kot v poganjkih. Kopičenje Cd v koreninah je zmanjšalo vsebnosti elementov v rastlinskih tkivih kot so zink, kalcij, mangan, baker in železo. Zaradi velikega potenciala tobaka za absorbcijo težkih kovin bi lahko bil ta primeren akumulator Cd in ker ni užiten bi bil primeren tudi za fitoremediacijo. Raziskava prispeva tudi k boljšemu razumevanju interakcij različnih antioksidacijskih mehanizmov s strupenostjo Cd in biokemijskih sprememb, ki nastanejo zaradi oksidacijskega stresa v rastlinah tobaka.

Ključne besede: antioksidacijski encimi, kadmij, oksidacijski stres, tobak

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1 INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is an herbaceous species belonging to the Solanaceae family. Tobacco is produced commercially as a major ingredient in cigarettes and as a nicotine extract for medicinal purposes on a large scale. Nicotine, which is the specific and major alkaloid of the tobacco plant (95 % of total alkaloid content), is used to improve symptoms of Parkinson's and Alzheimer's diseases also manufactures e-cigarettes, nicotine patches, and gum. In addition, nicotine derivatives, including N-acyl nornicotine, have potent insecticidal properties (Barreto et al., 2015). This plant is used as a template organism in botanical research such as plastid studies, tissue culture, genetic engineering, genetic transformation, and the production of secondary metabolites with therapeutic value and transgenic proteins (Weathers et al., 2010).

The persistence and high tendency to accumulate heavy metals (HM) cause damage to the cellular structure of plants. Among HMs, plant uptake of Cd has been the subject of various research due to its excellent water solvability, high toxicity, and mobility (Pal and Maiti, 2019). The threshold level of Cd in agricultural soils (approximately 100 mg kg^{-1}) is increasing due to human activities, including the application of phosphate fertilizers and sewage sludge for soil amendment (Zulfiqar et al., 2022). Since the beginning of the 21st century, approximately 30,000 tons of Cd are produced annually, with around 13,000 tons resulting from human activities. Levels exceeding 3 mg kg^{-1} in soil can cause toxicity in plants (Asgher et al., 2015). Other reasons for soil contamination with Cd include forest fires and industrial activities, including refining, ore mining, leaks of contaminated water (warehouses and waste), pesticides, and tire wear (Zulfiqar et al., 2022). Cd competes for uptake by plants due to its similarity in physicochemical properties to micronutrients, utilizing similar transporters. Cd ions are transported across membranes by a special type of metal carrier, so roots are the first organs that are directly in contact with toxic metal ions and will also have more metal content than shoots (Chen et al., 2018). Cd toxicity leads to reduced plant growth due to disruptions in cell division, chlorosis, leaf necrosis, and accelerated leaf senescence. These symptoms are attributed to alterations in the biochemical components of the plant, including the induction of lipid peroxidation, deficiencies in nutrient uptake, disruptions in water absorption, and the plant's hydrological relationships. These modifications are often the result of oxidative stress due to an increase in cellular reactive oxygen species (ROS) (Burzyn´ski and Zurek, 2007). In ordinary condition, ROS is produced at some point of cellular metabolism, and numerous approaches, which includes the mitochondrial electron delivery chain, using the reaction of semiquinone with oxygen, fatty acid β-oxidation, and enzymatic reactions such as peroxidase, NADPH oxidase, and xanthine oxidase. At the same time, ROS is elevated in abiotic stress situations, including HM, due to an imbalance between ROS production and elimination (Zhang et al., 2007). Cd can elevate ROS production, including superoxide radicals, at complex III of the mitochondrial electron delivery chain. Furthermore, plasma membrane NADPH oxidase (NOX) may be involved in Cd toxicity-induced ROS production after short-term exposure to HM (Garnier et al., 2006). Plant tolerance to Cd is supported by different mechanisms such as Cd chelation by cell wall components, vacuolar compartmentalization, and Cd chelation by cytosolic organic acids or peptides (Rizwan et al., 2016).

Here, we investigat the impact of cadmium chloride ($CdCl_2$) toxicity on tobacco plant growth, biochemical parameters, oxidative damage, and antioxidant enzyme activity during stress periods, as well as how Cd interacts with iron (Fe), zinc (Zn), calcium (Ca), copper (Cu), and manganese (Mn). Those results offer data on the defense mechanisms of tobacco, Cd accumulation, and the tolerance of tobacco, which may be grown as crops and medicinal plants on Cd-infected soils.

2 MATERIALS AND METHODS

2.1 PLANT CULTURE AND CD TREATMENT

Tobacco seeds (*Nicotiana tabacum* 'Samsun') were obtained from the Institute of Plant Molecular Biology, University of Strasbourg, France. Seeds were surface-sterilized with ethanol and sodium hypochlorite solution. Seeds were grown in sterilized coco peat and perlite (in a 1:2 ratio) in a greenhouse (25-30 °C, 16/8 h light/dark, light intensity 100 mmol m^{-2} s^{-1}, relative humidity 60–75 %). After germination, the seedlings were irrigated with Hoagland 25 %, 50 %, and 100 % solution (pH 5.8–6) consisting of micronutrients: KCl, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_2MoO_4, and macronutrients: KNO_3, Ca $(NO_3)_2.4H_2O$, $NH_4H_2PO_4$, $MgSO_4$, and iron solution: $FeSO_4.H_2O$, EDTA once a week, and distilled water every 3 days. At the start of the trifoliate phase, $CdCl_2$ was irrigated in three replicates at five different concentrations (0, 1, 1.5, 2, and 2.5 mM) once a week for 21 days (to determine the above concentrations, a concentration determination was conducted, which indicated that tobacco plants do not survive at $CdCl_2$ con-

centrations of 4 mM and above). After the treatment, the plants were harvested for the relevant experiments.

2.2 TOLERANCE INDICES (%)

Stress tolerance indices were calculated by the following formula Amin et al. (2014):

PLSTI: (Plantlet length (shoot + root) for stressed plant/ plantlet length (shoot + root) for control plant) × 100

PDSTI: (Plantlet dry mass (shoot + root) for stressed plant/plantlet dry mass (shoot + root) for control plant) × 100

2.3 LEAF RELATIVE WATER CONTENT (RWC)

Relative water content (RWC) was analyzed according to the formula (Ritchie et al., 1990):
RWC (%) = (FM-DM)/(TM-DM) .

Leaf samples from each treatment were weighed to measure fresh mass (*FM*), placed in distilled water for 24 h, and weighed to determine the turgid mass (TM). Then the samples were dried at 65 ˚C for 24 h, to measure dry mass (*DM*).

2.4 ENZYME ASSAYS

Activities of antioxidative enzymes were estimated according to the protocols described by Hajiboland et al. (2010). The activity of the enzyme superoxide dismutase (SOD) was performed using a monoformazan formation at 560 nm. Ascorbate peroxidase (APX) activity was assayed by measuring the oxidation of ascorbic acid at 290 nm. Catalase (CAT) activity was determined by investigating H_2O_2 reduction at 240 nm. Peroxidase (POD) activity was investigated using the guaiacol at 470 nm.

2.5 MEASUREMENT OF OXIDATIVE STRESS INDICATORS

Proline was investigated via the method of Bates et al. (1973). Lipid peroxidation was determined utilizing thiobarbituric acid at 532 nm. The H_2O_2 concentration was estimated utilizing potassium iodide at 390 nm (Hajiboland et al., 2010). Radical scavenging ability was assayed by the DPPH method at 517 nm (Miliauskas et al., 2004).

2.6 DETERMINATION OF CD, CA, CU, FE, MN, AND ZN CONTENT AND CD DISTRIBUTION

The Cd content of the samples and other mineral elements was measured by the method of Dániel et al. (1997). For this purpose, the roots were immersed for 10 minutes in EDTA-Na_2 solution (0.1 M) to facilitate the removal of Cd from the root surfaces, followed by rinsing with distilled water. The shoot and root portions of the plants were dried at 25 °C and then ground into a powder. To 0.5 g of the dried samples, 10 ml of nitric acid (65 %) were added for acid digestion, and the samples were placed under a fume hood (24 h). Subsequently, the samples were heated to 90 °C to facilitate the evaporation of acidic vapors from the solution. After cooling, 1 ml of H_2O_2 (30 %) was added to the digested samples and heated until the solution became clear. The digested extract was then diluted with distilled water to a final volume of 25 ml. The Cd content and other mineral elements in the samples were measured using atomic absorption spectrometry and reported as mg g^{-1} dry mass. The translocation factor (TF), bioaccumulation factor (BF), and transfer coefficient (TC) were determined by the following formula (Eid and Shaltout, 2016).

$$TF = Cd_{shoot}/Cd_{root}, BF = Cd_{root}/Cd_{medium}, TC = Cd_{shoot}/Cd_{medium}$$

2.7 STATISTICAL ANALYSES

Data analysis was enforced with the SPSS 24.0 software package. Experimental data was released as the mean ± SD. One-way ANOVA was employed to define differences between means. Duncan's test and the significance level at $p \leq 0.05$ were used.

3 RESULTS AND DISCUSSION

3.1 TOLERANCE INDICES OF TOBACCO PLANTS UNDER CADMIUM STRESS

The PDSTI (plantlet dry mass stress tolerance index) and PLSTI (plantlet length stress tolerance index) tolerance indices decreased significantly with increasing $CdCl_2$ concentration. PLSTI values of 42.50 % and 19.51 % were the highest and lowest when $CdCl_2$ was applied at 1 and 2.5 mM, respectively. In addition, similar results were obtained for PDSTI. Treatments 1 and 2.5 mM had the highest and lowest rates of PDSTI, 30.79 %, and 9.20 %, respectively ($p \leq 0.05$) (Figure 1, Figure 2A-B).

Plant growth characteristics are considered sensitive

parameters to measure their resistance to metal toxicity (Imtiaz et al., 2015). Our experimental data showed that Cd stress inhibited the growth of tobacco plants. Similarly, a reduction in DWSTI and PHSTI indices was reported in *Cicer arietinum* L. under Cd stress (Mohajel Kazemi et al., 2020). Additionally, some researchers have reported a decrease in the dry mass of both roots and shoots of plants with increased exposure to Cd (Yazdi et al., 2019). In the present study, it is presumed that the inadequate absorption and transfer of essential minerals, including calcium, phosphate, potassium, and iron, along with the inhibition of cell division and abnormal mitosis -which are direct outcomes of root metabolism disruption -are likely factors contributing to the reduction in plant height and dry mass (Muradoglu et al., 2015; Kolahi et al., 2020). In addition, by disrupting water absorption, Cd reduces cell water potential and cell wall elasticity, reduces hydraulic conductivity through aquaporin, which causes cells to remain small, reduces intercellular space, and inhibits plant length growth (Karcz and Kurtyka, 2007; Ehlert et al., 2009). A similar scenario was observed by Monteiro et al. (2012).

Figure 1: Effects of different concentrations CdCl2 (0, 1, 1.5, 2 and 2.5 mM) on morphology and growth of *Nicotiana tabacum* for 21 days.

They attributed the greatest reduction in plant growth under Cd stress to oxidative damage. The production of ROS due to the presence of heavy metals leads to the degradation of cell biomolecules and organelles and membrane lipids, resulting in decreased plant growth and ultimately plant mortality (Monteiro et al., 2012: Yazdi et al., 2019).

3.2 LEAF RELATIVE WATER CONTENT (RWC) OF TOBACCO PLANTS UNDER CADMIUM STRESS

The changes of water content in plants under $CdCl_2$ stress showed RWC decreased significantly with an increase in the concentration of $CdCl_2$ (Figure 3). Similar result was obtained in potato due to Cd-influenced water imbalance (Li et al., 2019). The change in root structure caused by Cd stress (such as increased root suberization and lignification and loss of endoderm integrity) disrupts the root-soil relationship, ultimately reducing water uptake (Barcelo and Poschenriedr, 1990). Elevated Cd concentrations in plant tissues cause membrane damage, electrolyte leakage, and cytoplasmic thickening. Therefore, the presence of Cd reduces the ability to absorb water and inhibits short-range migration in the apoplast and symplast pathways, ultimately reducing its availability to physiological processes. Cd reduces root hydraulic conductivity and turgor pressure by interfering with aquaporin function and altering gene expression. Furthermore, Cd disrupts stomatal conductivity and water balance in plant cells by reducing stomata, leaf transpiration rate and RWC, and limiting water availability to plants for cell expansion (Gall et al., 2015).

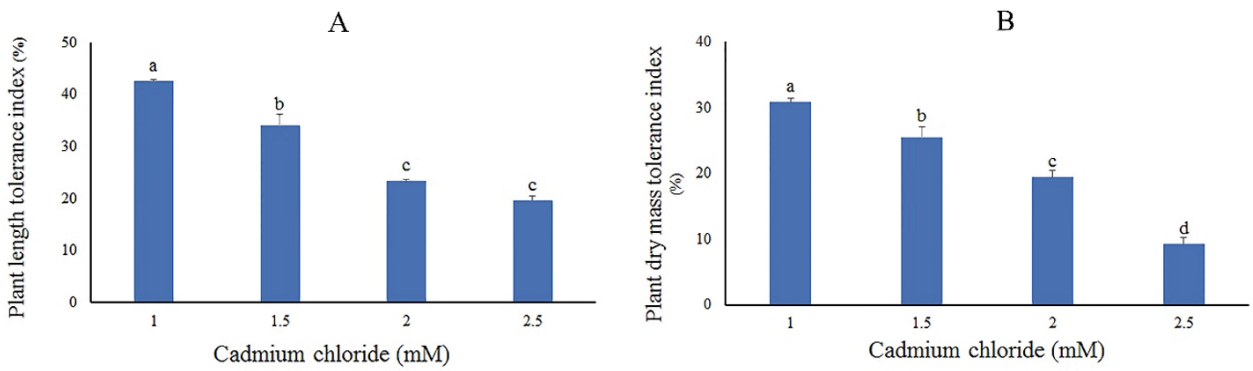


Figure 2: Effects of different concentrations CdCl2 on A) Plant length tolerance index (PLSTI), B) Plant dry mass tolerance index (PDSTI) for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

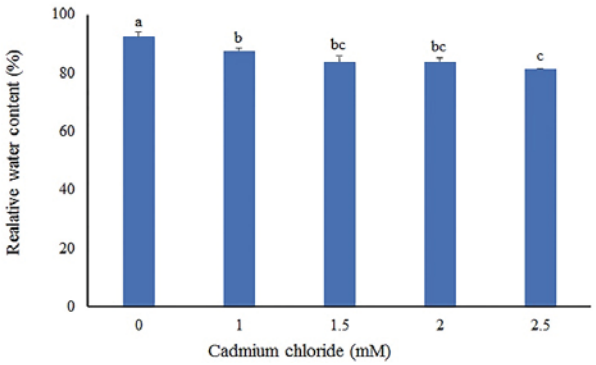


Figure 3: Effects of different concentrations CdCl2 on Relative water content (RWC) for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

3.3 CHANGES IN ANTIOXIDANT ENZYMES ACTIVITIES OF TOBACCO PLANTS UNDER CADMIUM STRESS

The activity of SOD enzyme considerably decreased in the plants under stress. The smallest enzyme activity (64.88 % decrease compared to control) was obtained by 1 mM $CdCl_2$ (equivalent to 9.15 U mg protein^{-1}) (Figure 4A). With the increase in $CdCl_2$ concentration, the activity of the ascorbate peroxidase enzyme increased, and the highest activity of this enzyme was in the treatment of 2.5 mM $CdCl_2$ with a value of 9.41 U mg^{-1} protein. An increase in APX enzyme activity was observed with increasing $CdCl_2$ and its peak activity was shown at 2.5 mM $CdCl_2$ reaching 69.54 % of controls (Figure 4B). A considerable change in CAT enzyme activity was also shown. Plants treated with 2 mM $CdCl_2$ showed the highest CAT enzyme activity (0.4226 U mg^{-1} protein) (Figure 4C). POD enzyme activity showed a considerable difference in all treatments except the 1 mM $CdCl_2$ treatment. The highest level of enzyme activity was observed (87.96 %) in 2 mM $CdCl_2$ (13.21 U mg^{-1} protein) ($p \leq 0.05$) (Figure 4D).

The binding of HM to the sulfhydryl group of enzymes inhibits their activity and disrupts their structure in plants under Cd stress. Moreover, other reports have revealed that protein denaturation and inactivation are vital in the plant reaction to HM toxicity (Emamverdian et al., 2015). In addition, the plant's resistance to Cd-related stress is closely related to the ability of the antioxidant system to eliminate ROS; also, the synergistic antioxidant enzymes in response to Cd treatment, which actively eliminates oxidative conditions. In this research, increased activity of CAT, POD, and APX enzymes was observed under Cd stress, although decreased SOD enzyme activity in tobacco leaves suggested the involvement of other protective factors. Overall, this condition may be a direct result of the enhanced production of ROS compounds and, ultimately the stimulation of antioxidant enzyme defense systems to neutralize these compounds. The results showed that the activities of POD, APX, and CAT enzymes were improved in contrast to the decrease of SOD enzyme activity, which could confirm that the H_2O_2 removal was favorable. Similar results were obtained for the antioxidant enzymes activity of *Dittrichia viscosa* (L.) Greuter affected by Cd (Fernandez et al., 2013).

APX enzymes remove excess H_2O_2 using ascorbate; so can act as ROS modulation for signal transmission. Alternatively, H_2O_2 can be degraded to water and oxygen by the POD enzyme in the cell wall and cytoplasm or by the CAT enzyme in peroxisomes and mitochondria (Chen et al., 2003). POD enzymes have heme groups in their structure; they preferentially oxidize aromatic electron donors such as guaiacol and pyragallol with the help of H_2O_2. Peroxidase enzymes are associated with some vital cellular processes, such as growth, differentiation, and resistance to various abiotic and biotic stresses. Due to the role of this enzyme in lignin biosynthesis, it can create a physical barrier against HMs. Different stress states, including heavy metals, herbicides, ozone, and polycyclic aromatic hydrocarbon, changed the function of the GPX enzyme (Bhaduri and Fulekar, 2012). In recent research, Cd led to the continuous production of H_2O_2 and increased POD activity; resulting in the breakdown of excess H_2O_2 at the cytoplasmic level. Similar results are consistent with our study; antioxidant enzymes activity (*e.g.*, POD, APX, and CAT) was increased in sugarcane affected by Cd (Yousefi et al., 2018). In this study, the induction of CAT, APX, and POD enzymes under Cd stress may indicate a role for these three enzymes in enhancing tobacco defense mechanisms against oxidative damage.

3.4 OXIDATIVE STRESS INDICES OF TOBACCO PLANTS UNDER CADMIUM STRESS

3.4.1 Evaluation of proline content

A significant increase in proline was shown in the treated tobacco compared to the control, which had the highest levels at 2 and 2.5 mM $CdCl_2$ and equal to 1.21 and 1.20 mg g^{-1} FM, respectively ($p \leq 0.05$) (Figure 5A).

Under Cd stress, proline reduces the destructive effect in plants by cheating Cd, creating a non-toxic

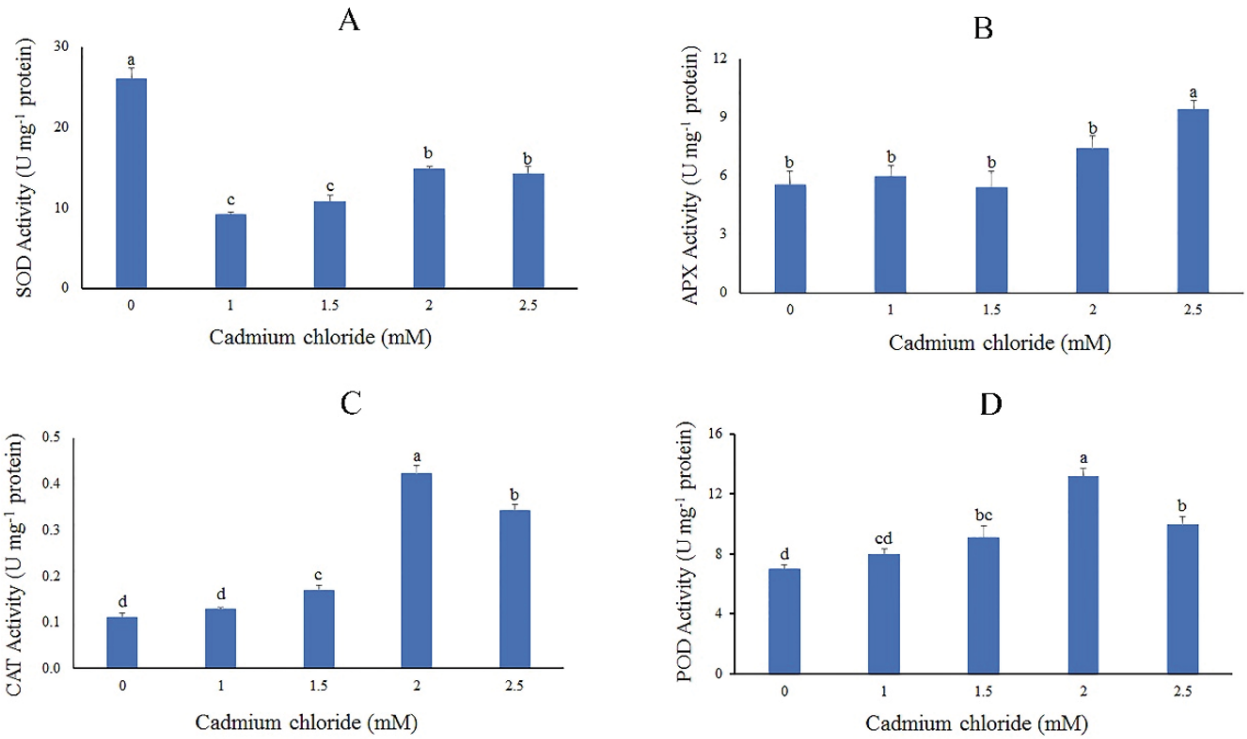


Figure 4: Effects of different concentrations CdCl2 on the activities of A) SOD (Superoxide dismutase), B) APX (Ascorbate peroxidase), C) CAT (Catalase), D) POD (Peroxidase) for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

proline-Cd complex, stabilizing macromolecules and organelles, stabilizing protein synthesis, and preventing enzyme denaturation. In addition, proline, either free or bound to polypeptides, can react directly to H_2O_2, hydroxide ions, dioxygen, and inhibit free radicals induced by Cd toxicity (Rejeb et al., 2014). It has been suggested that the increase of proline in Cd-treated plants is not directly associated with high HM concentrations but rather reduced water capacity and disturbances in water balance in the cells. Therefore, proline accumulation may be directly related to plant water balance (Clemens, 2006). According to the present study, tobacco plants under $CdCl_2$ stress accumulate more proline, which is attributed to ROS detoxification and elevated resistance to Cd. Comparable findings have additionally been noted in some Cd-affected crops, such as *Arabidopsis thaliana* (L.) Heynh. (Xiao et al., 2020).

3.4.2 Estimation of malondialdehyde (MDA)

The MDA amount in tobaccos increased significantly, getting the top level at 2.5 mM of $CdCl_2$ with a value of 3.63 µmol g^{-1} FM ($p \leq 0.05$) (Figure 5B). The significant ion in MDA amount (lipid peroxidation) causes damage to membrane nature, increased cell permeability, dysfunction of enzymes, ion leakage, and cell death. An increase in the amount of MDA can be an ideal detector for assessing metal toxicity and determining Cd tolerance in tobacco (Nazar et al., 2012). HM stress stimulates free radical production, including superoxide radicals, by increasing lipoxygenase enzymatic activity, followed by lipid peroxidation and elevated MDA levels (Ahmad et al., 2009). Indeed, due to the increase in free radicals under stress, the antioxidant system cannot remove them effectively, ultimately damaging the cellular composition of the plant. Various studies are consistent with the present research. High amounts of inner membrane peroxidation influenced by Cd were observed in sassafras (Zhao et al., 2021). Furthermore, an increase in ROS and MDA in Cd treatment has blended in crops such as cotton, confirming the present study's finding (Khan et al., 2013). In a recent study, Cd accumulation in tobacco plants also elevated the production of free radicals such as H_2O_2, increased MDA, and induced oxidative stress.

3.4.3 Evaluation of H_2O_2 content

The H_2O_2 content increased significantly with the rising $CdCl_2$ concentration except for the treatment of 1

mM $CdCl_2$. This increase was more noticeable (90 %) in 2.5 mM $CdCl_2$ treatment with 0.38 µmol g^{-1} FM of H_2O_2 ($p \leq 0.05$) (Figure 5C).

HM, directly and indirectly, leads to ROS production and subsequent oxidative stress in plant tissues. Cd induces ROS production indirectly by disrupting the structure of leaf chloroplasts. Furthermore, the inhibition of electron transport by Cd toxicity leads to the photoinactivation of photosystem II (Farooq et al., 2016). It has been suggested that during plant treatment, which includes rice and peas, Cd increases the production of ROS such as H_2O_2 by indirectly activating NADPH oxidase enzyme bound to the membranes of peroxisomes and leading to an oxidative burst (Tran and Popova, 2013). Also, an elevated H_2O_2 has been stated in *Vaccinium corymbosum* L. under the influence of Cd (Manquián- Cerda et al., 2016).

Furthermore, Cd toxicity causes a rise in ROS in the mitochondrial electron transport chain, and ROS overproduction leads to ATP depletion and decreased respiration (Singh et al., 2016). An increase in H_2O_2, after disrupting the harmony between its production and removal, induces senescence, lipid peroxidation, and disruption of integrated membranes in plants (Shiyu et al., 2020). It has been advised that to protect plant cells against H_2O_2 accumulation caused by various environmental stresses, different proteins and compounds act as ROS scavengers, i.e. antioxidant defense mechanisms (Shahid et al., 2014).

3.4.4 Evaluation of the antioxidant content by the DPPH assay

The antioxidant content of plants revealed a considerable enhancement in tobacco plants under $CdCl_2$ treatment. The maximum antioxidant capacity was stated in 2 mM $CdCl_2$ with a value of 99.35 % ($p \leq 0.05$) (Figure 5D).

The DPPH test defines phenolic compounds' free radical stabilizing capacity (Müller et al., 2011). With the increase of phenolic acids under the influence of Cd, the antioxidant capacity of the plant also increased, as well as the ability to resist oxidative damage caused by HM (Shan, 2022). Research has shown that phenolic compounds such as flavonoids, phenolic acids, and flavonolignans act as antioxidants in plants. High concentrations of polyphenols induce hydrogen from the hydroxyl groups of the aromatic circle to ROS (Sharififar

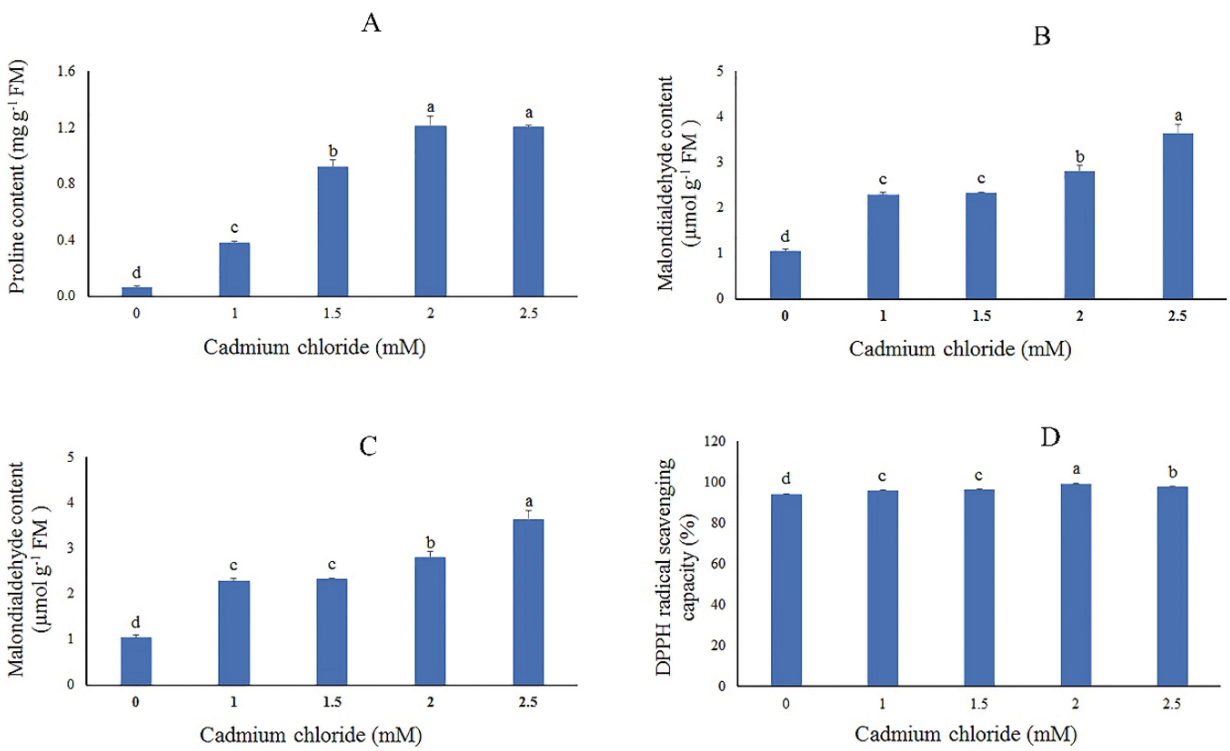


Figure 5: Effects of different concentrations CdCl2 on A) Proline, B) Malondialdehyde (MDA), C) Hydrogen peroxide (H_2O_2), D) DPPH radical scavenging capacity for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

et al., 2009). Under Cd stress, tobacco plants stimulate phenolic pathways, enhancing the plant's antioxidant capacity to fight oxidative harm. These effects are similar to data on *Vaccinium corymbosum* L., treated with HM, confirming an increase in antioxidant activity after exposure to HM (Manquián-Cerda et al., 2018). Antioxidant capacity was increased in *Gynura procumbens* (Lour.) Merr. and *Ocimum basilicum* L. under HM stress (*e.g.*, Cd, Cu, and Al), confirming a positive correlation between DPPH activity and the amount of metabolic compounds, such as phenols and flavonoids (Ibrahim et al., 2017).

3.5 CD ACCUMULATION, TRANSLOCATION FACTOR (TF), BIOACCUMULATION FACTOR (BF), AND TRANSLOCATION COEFFICIENT (TC) OF TOBACCO PLANTS UNDER CADMIUM STRESS

Based on our data, the amount of Cd in the roots and stems of tobacco plants changed significantly under the influence of different Cd treatments. As shown in Figure 6, Cd accumulation in roots and stems elevated steadily with the addition of Cd concentration in the medium. The concentration of Cd in the roots was remarkably higher than in the shoots. Compared with the control, the lowest and highest Cd concentrations in

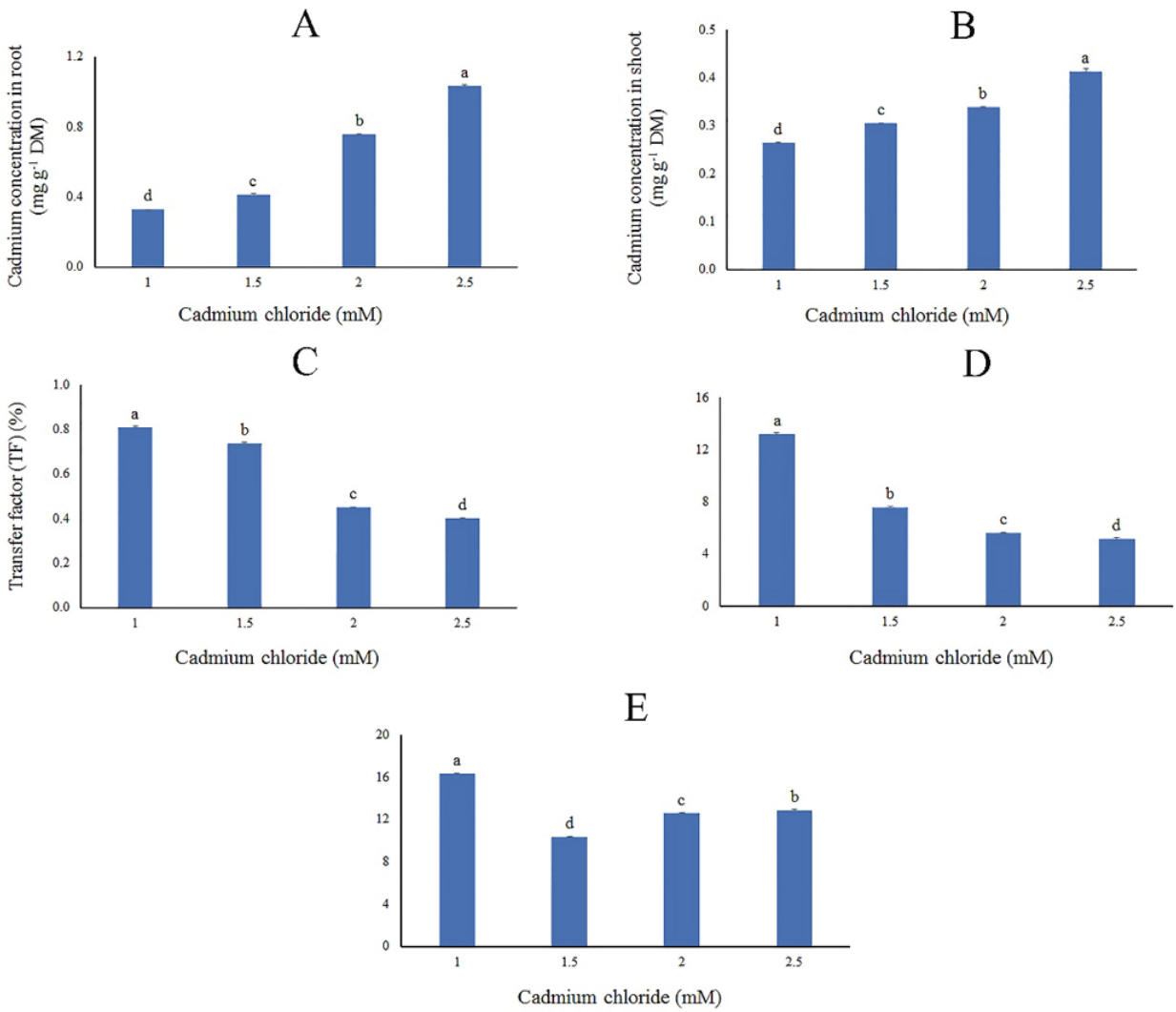


Figure 6: Effects of different concentrations $CdCl_2$ on accumulation of Cd in A) roots and B) shoots. Study of C) Transfer factor (TF), D) Transfer coefficient (TC) and E) Bioaccumulation factor (BF). Values with different letters are statistically significantly different at $p \leq 0.05$.

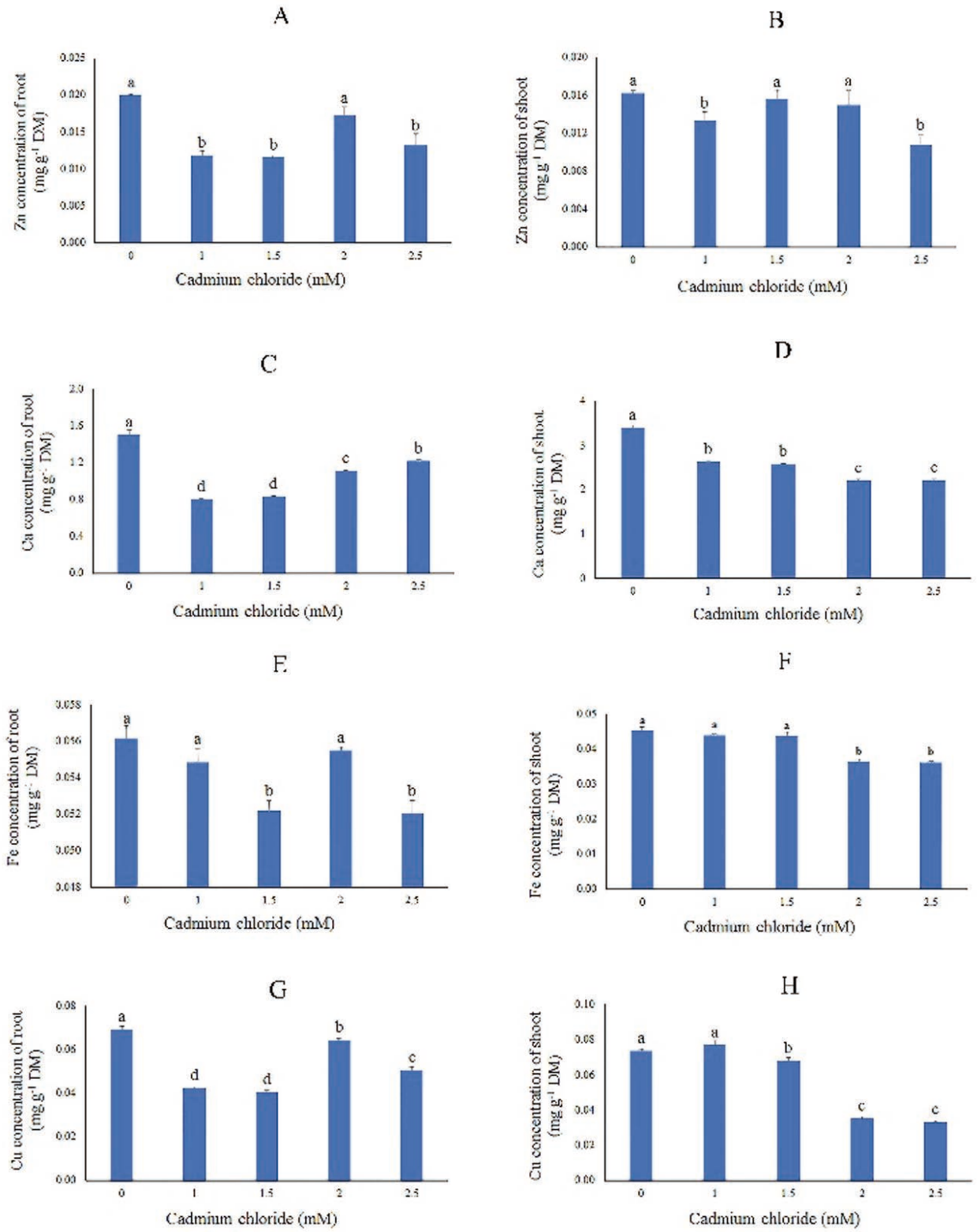
the roots and the shoots were observed in the 1 and 2.5 mM $CdCl_2$ treatments, respectively (Figure 6A-B). In contrast, the TF index continuously decreased with increasing $CdCl_2$ concentration, indicating a decrease in Cd transfer from the roots to the shoots of the tobacco plant (Figure 6C). BF and TC indices also showed a significant decrease with increasing $CdCl_2$ concentration (Figure 6 D-E) ($p \leq 0.05$).

Because Cd contacts the roots and prevents their transfer to the shoots, the roots contain a larger amount of Cd. In addition, Cd accumulates in the underground parts relative to the aerial organs by entering the root apoplastic pathway, where it penetrates and cumulates in root tissue. Furthermore, Cd accumulation in vacuoles of root cells prevents the xylem loading, thus reducing the toxicity symptoms and protecting the plant from stress (Lux et al., 2011). Akhter (2012) reported the reduction of root-to-shoot Cd transfer at high Cd levels was associated with elevated biosynthesis of phytochelatins (PCs) and relative compounds (Cys, Glu, and γ-Glu-Cys) that bind to Cd. The PC gene expression increased in the roots of *Saccharum officinarum* L. during the duration of Cd treatment, resulting in higher PC levels in roots compared with the shoots (Yousefi et al., 2018). The plant's ability to decrease in TF and TC indices has also been reported in plants such as *Saccharum officinarum,* and *Satureja hortensis* L. as Cd concentrations increase (Yousefi et al., 2018; Azizollahi et al., 2019). The capacity of plants to compile metals in shoots and transfer them from root to stem was determined by TF and TC indices, respectively. A TF index higher than 1 indicates the greatest efficiency of the seedling in transferring metals from the root to the aerial organs (Eid and Shaltout, 2016). In the recent search, the TF amount was less than 1 due to Cd concentration in tobacco roots contrasted to stems and leaves. The TF and TC values decreased with increasing Cd concentration, indicating an improved resistance barrier. Root endurance prevents the transfer of Cd to tobacco shoots. Niu et al. (2007) showed a reduction in BF in plants likewise alfalfa, castor bean, and mustard with increasing Cd concentrations; this index's value varied depending on the concentration, nature of HM, and environmental conditions. The value of the BF index evaluates the capacity of plant roots to uptake Cd from the ground; this index is higher than 1 in HM-accumulating plants and less than 1 in HM-remover plants (Yanqun et al., 2005). In recent research, the value of the BF index is greater than 1, which indicates a higher Cd absorption capacity of tobacco roots; therefore, tobacco is considered a Cd accumulator. Equal findings have also been stated in chickpeas (Mohajel Kazemi et al., 2020).

3.6 ZN, CA, FE, CU, AND MN CONCENTRATIONS OF TOBACCO PLANTS UNDER CADMIUM STRESS

Zn concentration was notably decreased by treatment of tobacco roots with $CdCl_2$, except for the 2 mM treatment. Zn concentration in the shoots decreased remarkably at 1 and 2.5 mM while increasing the $CdCl_2$ levels in the medium (Figure 7A-B). In addition, increasing $CdCl_2$ levels in the treatments resulted in decreased Ca^{2+} concentrations in tobacco tissues compared with controls (Figure 7C-D). Also, Fe concentrations reported a remarkable induction in tobacco roots at 1.5 and 2.5 mM treatments. As the $CdCl_2$ concentration increased, the iron concentration decreased in the tobacco shoots in treatments 2 and 2.5 mM (Fig. 7E-F). Cu concentrations decreased at all tested $CdCl_2$ concentrations except 1 mM (Fig. 7G-H). Furthermore, the levels of Mn in the aerial parts also decreased significantly in all treatments except 1.5 mM treatment ($p \leq 0.05$) (Figure 7I-J).

By disrupting membrane transport proteins, Cd causes membrane permeability changes and affects nutrient absorption and accumulation, thereby causing nutritional deficiencies and disorders (Yao et al., 2009). Possible evidence for tobacco essential element reduction is lipid peroxidation due to Cd toxicity, which can alter cell membrane function and ultimately disrupt the plant's nutritional balance; however chemically similar to Cd, the concentration of each element decreases for other specific reasons under the influence of Cd. For example, Cu, the electron transporter in photosynthetic organisms, is adversely affected by Cd because it serves as a cofactor for enzyme structure and occupies the active site of the enzyme cofactor. Cd tends to react with compounds containing the -SH functional group, thus competing with copper for this position and reducing the copper concentration (Qian et al., 2009). As another micronutrient, Mn is required for important metabolic processes such as the photolysis of H_2O by photosystem II and the uptake of NO_2^- in chloroplasts (Wu et al., 2003). The amount of Mn decreases because Cd and Mn compete for a membrane transporter (Ramachandran and D'Souza, 2002). The presence of Cd in the medium can affect the nutritional processes of leaves and roots by preventing the loading of ions into the branches of the plant and affecting PC production. Fe transport is dependent on PC production, and Cd pollution affects Fe transport to the shoots by increasing PC production and occupying Fe transporters such as IRT1 (iron-regulated-transporter^{-1}, belongs to the ZIP family of transporters) as well as Nramp (natural-resistance-associated-macrophage protein) family transporters (Takahashi et al., 2011). The decrease in Fe concentration caused by Cd is always associated with


A
Zn concentration of root (mg g-1 DM)
Cadmium chloride (mM)
B
Zn concentration of shoot (mg g-1 DM)
Cadmium chloride (mM)
C
Ca concentration of root (mg g-1 DM)
Cadmium chloride (mM)
D
Ca concentration of shoot (mg g-1 DM)
Cadmium chloride (mM)
E
Fe concentration of root (mg g-1 DM)
Cadmium chloride (mM)
F
Fe concentration of shoot (mg g-1 DM)
Cadmium chloride (mM)
G
Cu concentration of root (mg g-1 DM)
Cadmium chloride (mM)
H
Cu concentration of shoot (mg g-1 DM)
Cadmium chloride (mM)

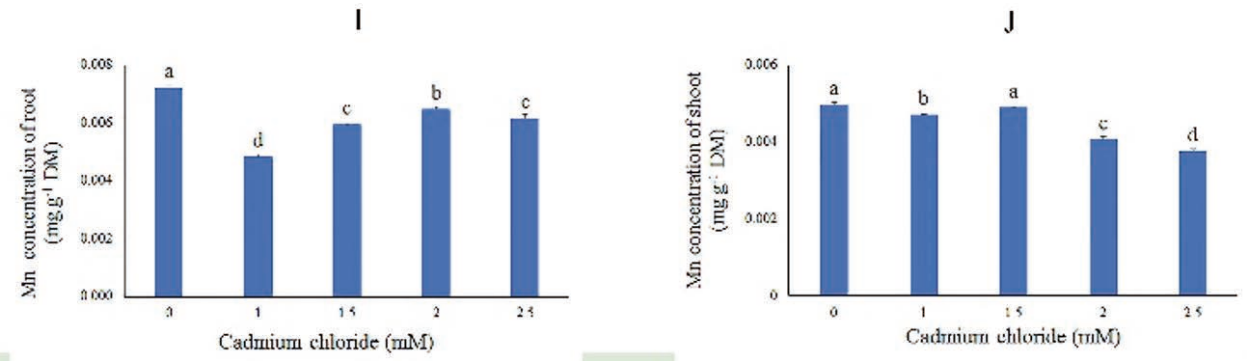


Figure 7: Effects of different concentrations $CdCl_2$ on A) Zn root, B) Zn shoot, C) Ca root, D) Ca shoot, E) Fe root, F) Fe shoot, G) Cu root, H) Cu shoot, I) Mn root, J) Mn shoot concentrations. Values with different letters are statistically significantly different at $p \leq 0.05$.

the antagonistic effects of these two elements. In addition, Fe and Zn play a role in the formation of protective enzymes, likewise CAT and SOD enzymes. SOD is present in the cell in three different forms, FeSOD (chloroplasts), MnSOD (mitochondrial and peroxisomes), and Cu/ZnSOD (chloroplasts and cytoplasm). Cd can replace Fe and Zn in various macromolecules and inactivate this form of the enzyme. On the other hand, the release of free Fe due to the presence of Cd can lead to redox reactions and induce oxidative stress (Cuypers et al., 2010). Many studies have reported a reduction of Zn absorption in the Cd treatment, suggesting an opposite relationship in the transportation of Cd and Zn. In *Gynura pseudochina* (L.) DC., Cd uptake by the roots decreased as Zn concentration increased. Therefore, Cd entry is attributed to Zn transporters, which tend to occupy more Zn than Cd (Panitlertumpai et al., 2013). In plants, both Ca and Cd compete for the same Ca channel. Cd can enter the plasmalemma of root tissues via Ca channels, and depolarization of the membrane potential can also inhibit Ca uptake during Cd processing. (Li et al., 2012). One of the reasons for limiting Ca movement into the aerial parts of the plant during Cd treatment could be the presence of calcium oxalate crystals in the plant's vessels (Barcelo' et al., 1988). Increasing the Ca concentration in the growth medium remarkably decreased Cd harmful in *Mesembryanthemum crystallinum* L., sea purslane, and *Arabidopsis*, which is consistent with the idea of an opposition between two cations all through uptake (Suzuki et al., 2005). With Ca application, Cd tolerance was also increased in *Nicotiana tabacum* due to the formation and elimination of Cd and Ca-containing crystals through the trichome head cells. In addition, adding the amount of Ca in the culture medium increased the expression of the LCT1 gene in tobacco, which is a non-selective transmembrane transporter for K, Na, and Ca and blocks Cd absorption to reducing its toxicity (Antosiewicz and Hennig, 2004).

4 CONCLUSION

The present study investigated growth indices, oxidative damage, antioxidant mechanisms, and Cd accumulation in tobacco plants under Cd stress. Tobacco plants' growth and biochemical responses under Cd stress reveal their high resistance to HM. Raising the level of H_2O_2 (as a signaling molecule) increased MDA production; therefore, oxidative stress is triggered in tobacco seedlings under Cd treatment. Also the CAT, POD, and APX enzymes are enhanced to deal with ROS toxicity. Enzymatic and non-enzymatic antioxidant systems appeared to be initiated in tobacco reacting to Cd toxicity, with different roles in these responses. In reaction to toxicity due to Cd accumulation, tobacco plants enhanced proline, decreased water potential, and disrupted water balance.

Furthermore, adding Cd to the culture medium resulted in Cd penetration into the roots and shoots of tobacco plants. Accumulation of Cd in roots and lipid peroxidation disturbs the plant nutrient balance and reduces mineral nutrients such as Zn, Ca, Mn, Cu, and Fe in roots and shoots. Finally, the reduction in tobacco plant growth properties may be due to the chemical similarity of some ions with Cd and their antagonistic relationships and competition for inhibit absorption and transfer. The reduction of these nutrients by Cd stress can lead to decreased growth rate and dysfunction of several enzymes involved in growth and defense responses. A BF value greater than 1 indicates that tobacco roots can uptake Cd from the environment, so tobacco as an inedible and Cd-accumulating plant can be used in Cd-contaminated soil.

Changes in oxidative stress indices and activation of antioxidant pathways are valuable for understanding cellular mechanisms and processes implicated in plant cell reaction to Cd in cell biological stress and the development of environmental targets. Tobacco provides a valuable model for assessing plants' metabolic and cytoprotective responses under Cd.

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