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Response of Specific Stress Biomarkers in Lycopersicon Esculentum Exposed to Two Heavy Metals (Cadmium and Copper)

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KEYWORDS

Lycopersicon esculentum, cadmium, copper, oxidative stress, stress biomarkers, tolerance.

ABSTRACT:

Introduction: Certain industrial and agricultural practices are responsible for the introduction of trace metal elements into the soil. As a result, these ETMs are absorbed, translocated, and accumulated in various compartments of the plant, leading to an overproduction of ROS that induces oxidative stress

Objectives: In this context, our study aims to evaluate the response of specific stress biomarkers (total proteins, proline content, glutathione (GSH), lipid levels, malondialdehyde (MDA), and catalase (CAT) enzymatic activity) in *Lycopersicon esculentum* exposed to cadmium and copper separately.

Methods: Plants previously grown in trays were subjected to a treatment for 7 days with increasing concentrations of $CdCl_2$ (0, 50, 100, 200µM) and $CuSO_4$ (0, 200, 400, 800µM).

Results: The results show a toxic effect, characterized by a significant increase in protein levels, proline content, glutathione, MDA, and CAT activity. Conversely, lipid levels decrease significantly with increasing concentrations of both metals.

Conclusions: These results highlight the occurrence of oxidative burst following exposure to metal stress, which is counteracted by the plant's defense system, including its antioxidant system.

1. Introduction

Problems related to the discharge of heavy metals into the environment and their potential transfer to living organisms are certainly the most concerning [1]. Indeed, the accumulation of heavy metals in the soil leads, on one hand, to the alteration of its physical, chemical, and biological properties, and on the other hand, to its contamination, thus inducing deleterious effects on the entire terrestrial fauna and flora. The main sources of soil contamination are agricultural activities, urbanization, and industrial activities such as metallurgy, chemical industry, mining industry, transportation activities, as well as the combustion of petroleum products like diesel [2 - 3].

Among these heavy metals, cadmium, a highly toxic heavy metal, particularly for humans [4], represents a growing danger due to its increasing use in industry and its bioaccumulation [5 - 6]. Indeed, plants can easily absorb it, causing alterations in tissues and metabolic pathways [7 - 8]. The initial harmful effects observed in plants include growth inhibition, often accompanied by other signs of dysfunction such as foliar chlorosis, large necrotic lesions, progressive yellowing, folding or drying [9 - 10], as well as disruption of enzymatic activities necessary for chlorophyll biosynthesis [11]. Cadmium can also cause uncontrolled oxidation that disrupts cellular balance, leading to electrolyte leakage and activating biochemical responses resulting in the production of reactive oxygen species (ROS) that

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disturb plant defenses [12 - 13]. Excessive ROS production damages cell membrane permeability by producing malondialdehyde (MDA), reflecting levels of oxidative stress and membrane damage [14]. Even at low concentrations, cadmium can alter root morphology, limiting nutrient transport from the root to the shoot [15] and decreasing nutrient absorption as cadmium competes with essential nutrients such as calcium, copper, iron, manganese, and zinc [16].

Among plants, although many metals are essential for proper biological processes as trace elements, some of them can become contaminants for various forms of life when their concentration exceeds a certain threshold. For example, in plants, Copper (Cu) plays an essential role in mitochondrial respiration, electron transport chain, photosynthesis, lignin synthesis, and has a crucial function in oxidative stress response [17]. However, excessive accumulation can destabilize membrane integrity, decrease photosynthesis, and alter enzymatic activity, leading to growth inhibition and other harmful effects on plants [18]. High levels of Copper also inhibit root growth [19] and modify root morphology [20], reducing overall biomass and causing leaf chlorosis and necrosis [21].

In the face of these disturbances caused by ETMs, plants mobilize a whole range of mechanisms and employ various biochemical tactics to avoid or overcome the damages induced by ROS [21] through complex enzymatic and non-enzymatic defense mechanisms that protect plant cells against oxidative damage in order to restore cellular redox balance [22]. The cellular response involves antioxidant enzymes such as superoxide dismutase (SOD), which catalyzes the dismutation of superoxide (O2-) into hydrogen peroxide (H2O2) [23]. The latter can subsequently be eliminated by other enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) [24], and other peroxidases. Additionally, non-enzymatic mechanisms such as proline, carotenoids, ascorbate, and glutathione are also involved in the removal of excess ROS in plant tissues [25 - 26].

In recent years, intensive agricultural practices and excessive industrial waste discharge in the eastern region of Algeria have led to an increase in soil contamination by various metals, primarily Cd and Cu, and several authors have reported this excess [27 - 28]. The consequences of this pollution can be disastrous for plants and their yields.

2. Objectives

Thus, the current review addresses the adaptive potential of tomato (*Lycopersicon esculentum*), an economically important horticultural plant, in response to metal stress generated by different concentrations of copper and cadmium, through the measurement of specific stress biomarkers involved in plant defense.

3.Methods

Culture Conditions

Tomato seeds (*Lycopersicon esculentum*, var. Rio-grandé) were disinfected with a 10% (v/v) hydrogen peroxide solution, thoroughly rinsed with distilled water, and then germinated in trays filled with a mixture of soil and gravel soaked in water. Germination was carried out in darkness at a temperature of 25 ± 1°C. The seedlings were sustained by adding a basic nutrient solution every two weeks, with the pH maintained between 5.5 and 6.5. After 12 days of growth, the seedlings were treated with different concentrations of $CdCl_2$ and $CuSO_4$ for seven days. The concentration ranges were established for each metal based on the recommended field dose for copper and extrapolated relative to the tray surface area, taking into account our previous results for cadmium, as indicated in Table 1.

Table 1: Concentrations of copper sulfate and cadmium chloride used

Metal Salts	**Concentrations (µM)**			
	T	**C1**	**C2**	**C3**
Copper Sulfate ($CuSO_4$)	0	200	400	800
Cadmium Chloride ($CdCl_2$)	0	50	100	200

Analytical Techniques

Determination of Total Protein Level

Protein levels were determined using the colorimetric method described by [29]. This method involves measuring the concentration of proteins in a solution through spectroscopic analysis. To perform the assay, 0.1 g of ground plant leaf was mixed with 10 mL of

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distilled water. After filtration, 0.2 mL of the resulting supernatant was taken and mixed with 2 mL of Bradford reagent (BBC). The principle of the method is based on the binding of Comassie Blue dye to basic residues and aromatic amino acids in proteins, resulting in a blue color. The absorbance was measured at a wavelength of 595 nm using a spectrophotometer.

Determination of Proline Content

Proline content was determined using the method developed by [30], modified by [31]. Fresh material weighing 100 mg was mixed with 2 mL of 40% methanol. The mixture was then heated in a water bath at 85°C for 1 hour. Afterward, 1 mL of the extracted solution was taken and mixed with 1 mL of acetic acid, 1 mL of a mixture containing 120 mL of distilled water, 80 mL of orthophosphoric acid, and 300 mL of acetic acid, and 25 mg of ninhydrin. The mixture was placed in a water bath at 100°C for 30 minutes. Once a pink color appeared, 5 mL of toluene was added. The mixture was stirred and allowed to settle, resulting in the separation of two phases: a lower aqueous phase and an upper organic phase containing proline. The organic phase was collected and transferred to clean tubes containing anhydrous Na_2SO_4. The optical density (OD) was read at 525 nm after calibrating the instrument using a white reference.

Determination of Glutathione Level (GSH)

The enzyme extract was homogenized in a Tris/EDTA solution and subjected to deproteinization using 0.25% sulfo-salicylic acid. After centrifugation at 2000 g for 10 minutes, the resulting supernatant was used for the spectrophotometric assay with the DTNB reagent at a concentration of 0.01 M, measured at 412 nm. The concentrations of GSH were determined using the method developed by [32] and expressed in µM/mg of proteins.

Determination of Lipid Level

Total lipids were assayed using the methods described by [33]. Each sample, consisting of 0.5 g of fresh leaf material, was macerated in 10 mL of 20% TCA (trichloroacetic acid). Afterward, 1 mL of the extract was taken and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, and the pellet containing the lipids was retained. To the pellet, 1 mL of an Ether/Chloroform mixture (1:1) was added, followed by a second centrifugation at 5000 rpm for 10 minutes, resulting in the separation of two phases: a pellet and a supernatant. Next, 100 µl of the supernatant was taken and mixed with 1 mL of sulfuric acid. The mixture was placed in tubes and heated in a water bath at 100°C for 10 minutes. After cooling, 200 µl of the extract was taken and mixed with 2.5 mL of an 85% sulfo-phospho-vanillinic mixture. The spectrophotometric reading was performed at a wavelength of 530 nm.

Determination of Malondialdehyde Level (MDA)

The level of malondialdehyde (MDA), an indicator of lipid peroxidation, was estimated using the method developed by [34]. Plant tissue was homogenized in trichloroacetic acid (TCA 5%) at a ratio of 10 mL per 1 g of plant tissue. After centrifugation for 15 minutes at 12,000 g, the supernatant was mixed with an equal volume of thiobarbituric acid (TBA) at 0.5% in TCA at 20%. The mixture was then incubated for 30 minutes at 100°C.

The absorbance of the resulting supernatant, obtained after centrifugation at 10,000 g for 5 minutes, was read at a wavelength of 532 nm. The concentration of MDA was calculated using its molar extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of catalase activity (CAT)

The method developed by [35] was used to perform the spectrophotometric assay of catalase activity (CAT). The decrease in optical density (OD) was measured for 1 minute at a wavelength of 240 nm, using a linear molar extinction coefficient (ε) of 39,400 cm^{-1} M^{-1}. The reaction was carried out in a final volume of 3 mL. The reaction mixture consisted of 100 µL of the crude enzyme extract, 50 µL of hydrogen peroxide (H_2O_2) with a concentration of 0.3%, and 2.8 mL of Na K buffer (50 mM Na K, pH = 7.2). The reaction was initiated by adding hydrogen peroxide to the mixture. Catalase activity was expressed in nmol/min/mg protein.

Statistical study

The results obtained are expressed as the mean plus or minus the standard deviation (m ± sd). The means of the same series were compared pairwise using the ANOVA test with a significance threshold (P).

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4. Results

Effect of cadmium and copper on the protein content of the roots and leaves of *Lycopersicon esculentum*

Figure 1a illustrates the variation in total protein levels as a function of increasing Copper concentrations, which significantly increases ($P<0.01$) for C1, C2, and C3 compared to the control in both tomato leaves and roots. The same observations were made for the effect of cadmium (Figure 2b) on protein quantity in tomato leaves, which significantly increases ($P<0.01$) with increasing metal concentrations, reaching its maximum at C3 (102 µg/g). However, a decrease in protein levels is observed only for C1 in tomato roots compared to the control. The highest level is recorded for C3 (129.33 µg/g), representing a 65% increase.

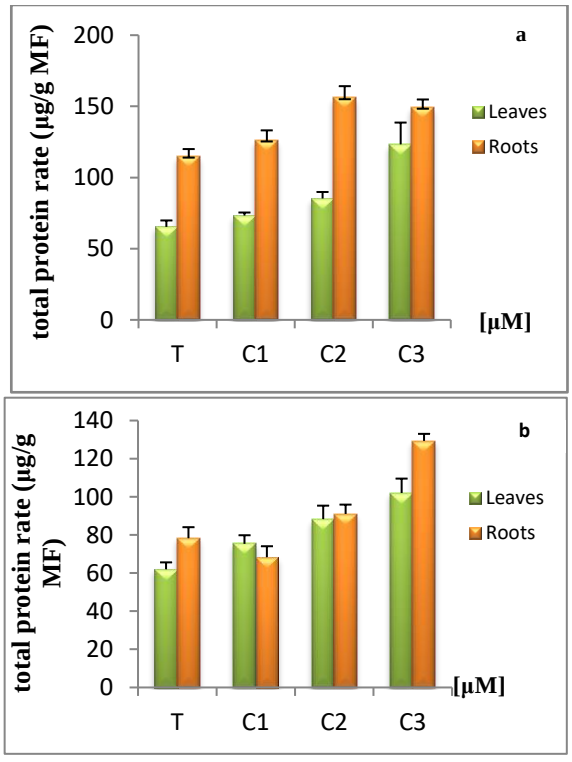


Figure 1: Effect of Cu (a) and Cd (b) on the variation in protein levels

Effect of cadmium and copper on the proline content of the roots and leaves of *Lycopersicon esculentum*

Regarding the proline content in the leaves and roots of wheat treated with copper and cadmium (Figure 2), a significant increase ($P<0.001$) in proline content is observed in a dose-dependent manner in both wheat leaves and roots compared to the controls. This increase reaches its maximum at the highest concentration (C3) in both roots and leaves, representing an increase of (89%, 184%) for copper treatment and (58%, 71%) for cadmium treatment.

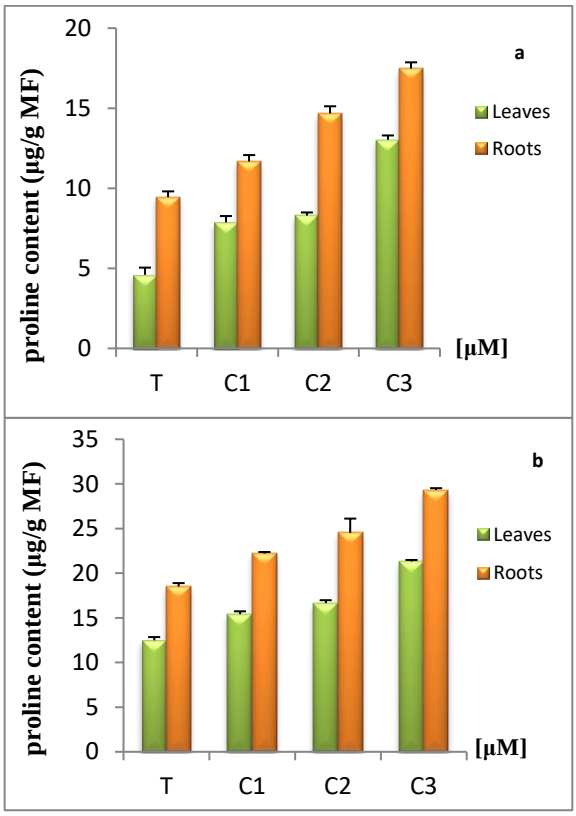
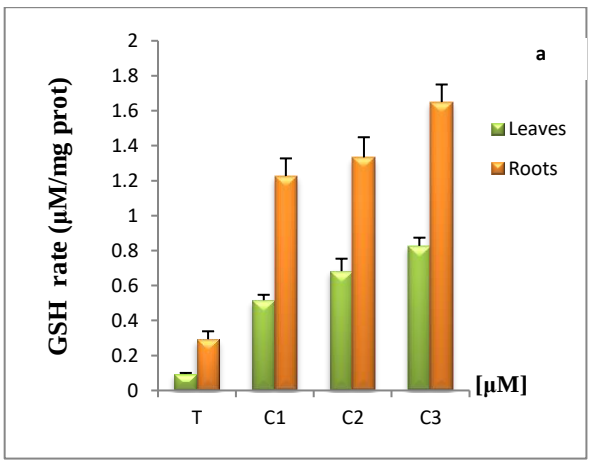


Figure 2: Effect of Cu (a) and Cd (b) on proline content

Effect of cadmium and copper on the GSH levels of the roots and leaves of *Lycopersicon esculentum*



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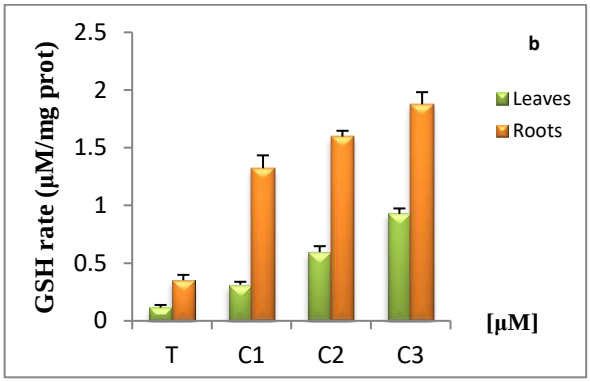
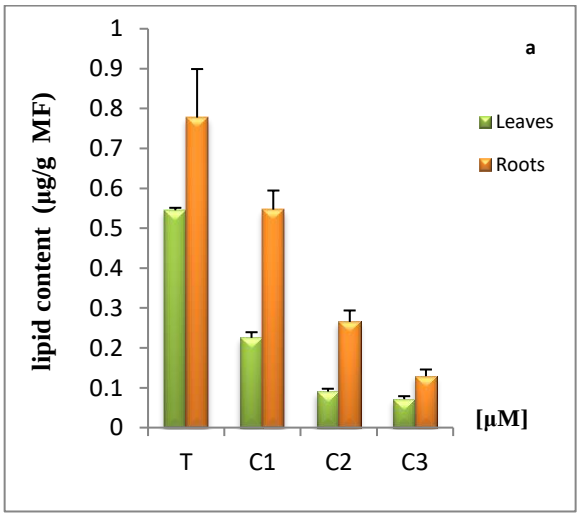


Figure 3: Effect of Cu (a) and Cd (b) on the GSH level

According to figure 3, a significant increase (P<0.001) in the GSH level was observed in the leaves and roots treated with different concentrations of copper and cadmium compared to the controls after 7 days of exposure. The levels recorded in the roots significantly exceed those observed in the leaves and reach their maximum at C3, with 1.65 µg/mg of protein in the case of copper treatment and 1.87 µg/mg of protein for cadmium treatment.

Effect of cadmium and copper on the lipid content of the roots and leaves of *Lycopersicon esculentum*



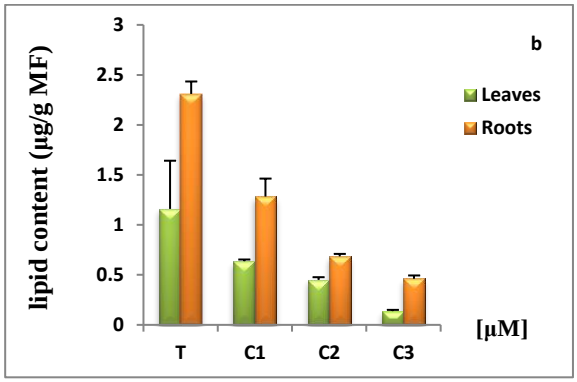
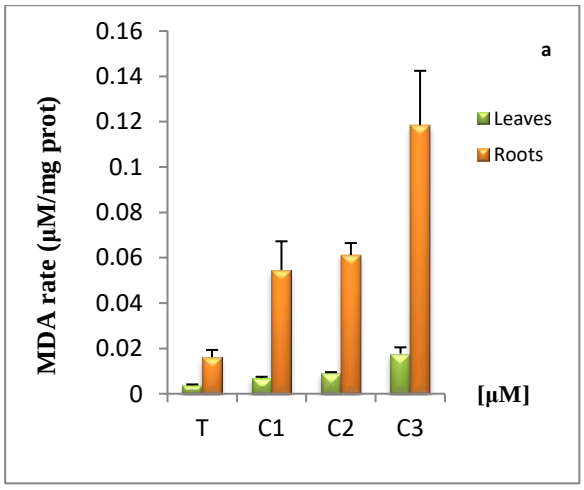


Figure 4: Effect of Cu (a) and Cd (b) on the lipid content

According to Figure 4, which represents the effect of copper and cadmium on lipid content, a significant decrease (p<0.001) in lipid content is observed in the leaves and roots of tomato plants treated with different concentrations of copper and cadmium compared to the controls. This decrease reaches its maximum for the highest concentration successively (86%; 83%) for the copper treatment and (88%; 80%) for the cadmium treatment.

Effect of cadmium and copper on the malondialdehyde level of the roots and leaves of *Lycopersicon esculentum*

As for the malondialdehyde levels measured for different treatments (Figure 5), a significant increase (p<0.01) in MDA levels was observed with increasing concentrations of copper and cadmium in both the roots and leaves compared to the controls.



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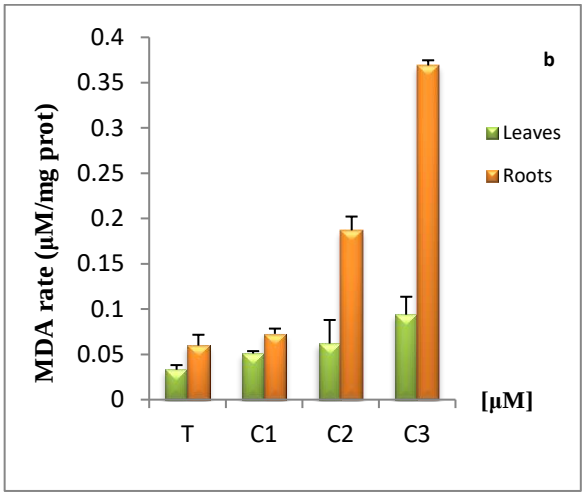
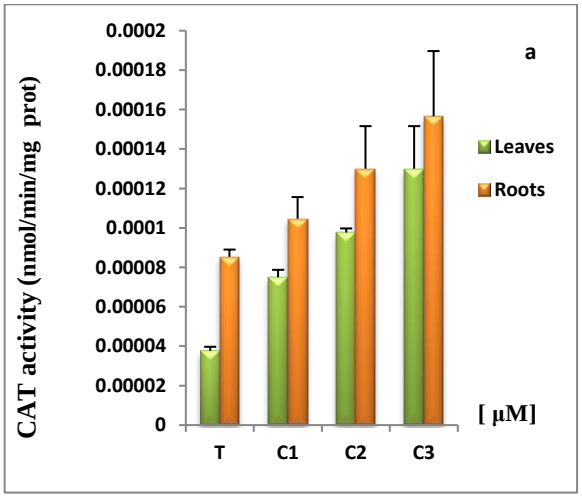


Figure 5: Effect of Cu (a) and Cd (b) on the MDA content

It should be noted that this rate is more pronounced in the roots than in the leaves, reaching a maximum at C3, namely 0.11 µg/mg of protein for the Cu treatment and 0.36 µg/mg of protein for the cadmium treatment, thus demonstrating the high toxicity of cadmium compared to copper in roots.

Effect of cadmium and copper on the Catalase activity of the roots and leaves of *Lycopersicon esculentum*



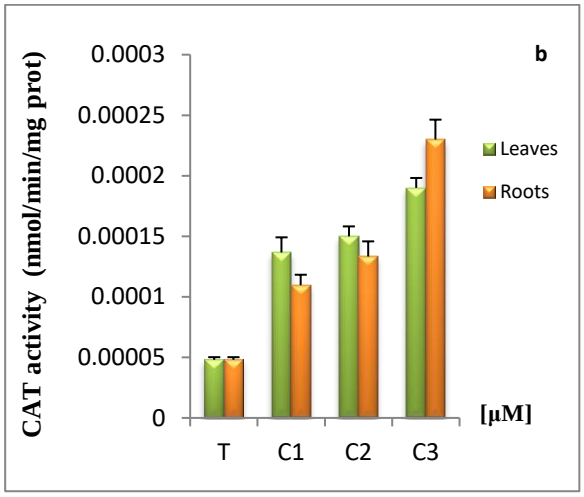


Figure 6: Effect of Cu (a) and Cd (b) on CAT activity

The results of the catalase activity monitoring are recorded in figures 6, which reveal a dose-dependent increase in CAT activity in the leaves and roots of tomato plants treated with different concentrations of copper (a) and cadmium (b) compared to the controls. It should be noted that this activity is higher in the roots than in the leaves, and higher in the cadmium treatment compared to the copper treatment. It reaches its maximum at C3 (Cd), with a five-fold increase in the roots and a four-fold increase in the leaves.

5. Discussion

Several studies have reported an increase or decrease in total protein levels, regardless of the type of stress [36]. In our study, we observed a proportional increase in protein levels in the leaves and roots of tomato plants as a result of increasing metal concentrations (Cu and Cd). This finding supports the hypothesis and underscores the crucial roles of these proteins in the plant's defense system against abiotic stress. The increase in protein levels is attributed to the activation of genes involved in the synthesis of G-type defense proteins, which are associated with membrane receptors and secondary metabolism proteins [37]. This activation is connected to the initiation of phosphorylation and the involvement of protein kinases (MAPKs) in signal transduction, leading to the induction of plant defense reactions [38]. It could also be attributed to the activation of the plant's antioxidant defense system, primarily composed of enzymes [39].

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Proline, which has been suggested to stabilize proteins and macromolecular complexes, act as a vital osmoticum, scavenge free radicals, and regulate cellular redox potential, plays a role in plants' adaptation to environmental constraints [40]. Previous studies have reported that the overaccumulation of proline is beneficial in rice, mustard, mung bean, and grass species in mitigating UV-induced stress. This is attributed not only to proline's role as an osmoprotectant but also as an antioxidant [41]. Our findings align with previous research, as we observed an increase in proline content in the roots, more so than in the leaves, in response to both metals. This increase is attributed to the continuous exposure of the roots to these metals, resulting in their initial accumulation. Subsequently, the metals are translocated to the leaves, potentially facilitated by the ZIP transporter. It is worth noting that while copper has known biological roles in the plant, the same cannot be said for cadmium.

The systemic lipid signal is one of the initial events involved in the defensive response and signal transmission mechanisms. Phospholipid signaling is a crucial component in eukaryotic signaling pathways, playing a key role in plant growth, development, and the systemic response to environmental stresses [42]. In our study, the lipid analysis revealed a significant decrease in lipid content in both tomato leaves and roots that were contaminated by copper (Cu) and cadmium (Cd). These findings contradict the existing literature, which suggests that a lipid-based molecule could serve as the mobile signal for systemic acquired resistance (SAR) [43]. When the rate of lipid content gradually decreases, it indicates the activation of systemic acquired resistance (SAR) through the observed accumulation of proteins. This activation involves various defense mechanisms against abiotic stress. One mechanism is the production of reactive oxygen species (ROS), which induces oxidative stress leading to lipid peroxidation caused by singlet oxygen or the hydroxyl radical. Additionally, other signaling pathways such as jasmonic acid (JA) and methyl jasmonate acid (MEJA) are involved. These signaling molecules are derived from fatty acids, and their biosynthesis relies on enzymes such as lipase and lipoxygenase (LOX) [11].

The elevated levels of malondialdehyde (MDA) observed in our study, particularly in the roots with high concentrations of copper (Cu) and cadmium (Cd), are likely a result of lipid oxidation triggered by oxidative stress in response to the increased levels of these metals in both tomato leaves and roots. When exposed to metals, an oxidative burst can occur, leading to an excess of reactive oxygen species (ROS) that cause direct damage, such as the oxidation of DNA, proteins, lipids, and carbohydrates. Additionally, the cytotoxic nature of the released metabolites can cause secondary damage, especially during lipid oxidation [44 - 45]. Our results support this hypothesis, as we observed significantly high levels of malondialdehyde (MDA). These elevated levels can be attributed to the excessive release of reactive oxygen species (ROS) following the addition of high concentrations of copper and cadmium to the culture medium. [46] indicated that an excess of copper in cells leads to an increase in the concentration of H2O2, which can induce oxidative stress and disrupt the homeostasis of other elements. These findings align with our study, which determined that the maximum value of malondialdehyde (MDA) reached nine times the value of the control, further demonstrating the severity of the applied stress.

Considering that any form of stress triggers an oxidative burst, leading to an imbalance in the redox state, which in turn causes oxidative damage, it is important to note that this damage can be regulated by glutathione. Glutathione plays a crucial role in reducing compounds resulting from lipid peroxidation (LOOH) or hydrogen peroxide (H2O2) [47]. Our findings align with this hypothesis, as we observed an increase in the level of glutathione in all tomato plants contaminated with both metals. This indicates that glutathione serves as a biomarker of stress and is involved in regulating the redox balance to restore cellular homeostasis. It can directly participate in this process by synthesizing phytochelatins or by conjugating with xenobiotics through the action of glutathione S-transferase (GST) for their detoxification.

In terms of monitoring catalase (CAT) activity, a stress biomarker responsible for eliminating hydrogen peroxide (H2O2) generated during stress, our results demonstrated a significant induction of CAT activity in both the leaves and roots of L. esculentum compared to the control group. These findings are consistent with the research conducted by [16] who explain this induction by the fact that there is triggering of the detoxification system which for the most part is made up of enzymes

including CAT. This leads us to postulate that CAT activity plays a crucial role in the detoxification of hydrogen peroxide (H2O2) in both the leaves and roots. According to [26], the levels of antioxidant enzymes such as APX, GPX, and CAT can determine the sensitivity of plants to lipid peroxidation. This is consistent with our study, as we observed an increase in the MDA levels following the exposure of tomato plants to the two metals, indicating the severity of the stress applied to both the roots and leaves, particularly at higher concentrations

Conclusion

In the majority of cases, the damage caused by excess ROS, if not quickly limited, leads to the death and then lysis of the cells concerned. The results of our work show an activation of protein biosynthesis, proline, GSH, MDA, and enzymatic activity (CAT) involved in the tomato antioxidant defense system as well as a drastic reduction in lipid levels. All of these results highlight the appearance of oxidative stress following exposure to metal stress, which is neutralized by the plant's defense system, in particular the antioxidant system.

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