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ARTICLE

Effect of Heavy Metals on the Morphological and Physiological Responses of the Torro Plus Variant of *Zea mays*

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ABSTRACT

This study evaluates the impact of heavy metals (zinc, copper and cadmium) on the development and metabolic responses of the maize (*Zea mays*) variety "Torro Plus". Seeds were cultivated on MS medium enriched with progressively higher concentrations of heavy metals (50, 100 and 150 µM), and plants were analyzed after 21 days. The results show a significant reduction in morphological parameters, notably an 87.28% decrease in the fresh weight of aerial parts and a 69.93% decrease in the fresh weight of roots under 150 µM of Cd. Chlorophyll a, b and total content also decreased drastically, reaching a maximum reduction of 74.31% under Cd (150 µM). In contrast, secondary metabolites such as proline and flavonoids increased, with a maximum proline accumulation of 0.71 mg/g under Cu (150 µM) and a flavonoid concentration reaching 176.33 mg/g under Cu (100 µM). These results show mechanisms of adaptation to stress, notably

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the accumulation of flavonoids and proline, while highlighting the increased toxicity of cadmium at high doses. These data are promising for applications in phytoremediation and sustainable agriculture. This study provides important data on the physiological and biochemical responses of plants to heavy metals and opens up prospects for phytoremediation applications.

Keywords: *Zea mays*; Heavy Metals; Chlorophyll; Proline; In Vitro

1. Introduction

Heavy metal pollution poses a major environmental threat, affecting crops, human health, and ecosystems [1]. These toxic elements are absorbed by plants, infiltrating the food chain and severely compromising food safety and human well-being [2, 3]. The World Health Organization (WHO) has raised significant concerns about the harm heavy metals inflict on ecosystems worldwide [4]. Currently, over 20 million hectares of soil are polluted by heavy metals [5], a situation worsened by rapid industrialization, the application of farmyard manure, and the excessive use of synthetic fertilizers [6–8].

Heavy metals are elements characterized by a high atomic density, generally ranging between 4 and 22.5 g/cm³ [9]. They can be classified into essential metals, such as zinc (Zn), copper (Cu), iron (Fe), and manganese (Mn), which play crucial roles in physiological processes, and toxic metals like cadmium (Cd), which pose significant risks even at low concentrations [10, 11].

Copper (Cu) is an essential micronutrient for plants, involved in key metabolic processes like respiration and photosynthesis [12, 13]. However, excessive Cu uptake induces phytotoxicity by generating reactive oxygen species (ROS), leading to cellular damage [14–17]. Cadmium (Cd) is regarded as one of the most toxic heavy metals, particularly in agricultural soils, due to its high mobility and toxicity. Cadmium is easily absorbed by plants, affecting their roots alongside essential nutrients. Like other heavy metals, it causes structural damage and disrupts plant processes, leading to reduced growth and productivity [18, 19]. Zinc is a vital micronutrient for plants, contributing to their growth, development, photosynthesis, and defense mechanisms [20]. However, excessive zinc levels can be detrimental, negatively affecting plant health. At optimal concentrations, zinc plays a key role in activating enzymes and transcription factors that regulate plant growth and development [21]. It is also essential for auxin regulation, a hormone crucial for cell elongation and division [22]. Conversely, at elevated concentrations, zinc becomes toxic, disrupting physiological and biochemical processes. Moreover, zinc toxicity can alter the plant's ionic balance, affecting osmotic potential and water absorption [23]. Cereals are among the crops most affected by heavy metal contamination [24].

Maize (*Zea mays* L.), domesticated approximately 9,000 years ago in the highlands of Mexico [25], is a nutritionally valuable crop, widely consumed for its richness in vitamins, proteins, starch, minerals, and bioactive compounds such as carotenoids and phenolic compounds [26–28]. In Morocco, heavy metal contamination, particularly in industrial regions, poses a serious risk to ecosystems and agriculture by accumulating in the soil, limiting plant growth, and threatening food security.

This study innovates by evaluating, for the first time in vitro, the morphological, physiological, and biochemical responses of the *Zea mays* 'Torro Plus' maize variety exposed to different concentrations of heavy metals (zinc, copper, and cadmium) to assess their impact on growth, metabolism, and adaptive mechanisms. We put forward the following hypotheses: (i) moderate concentrations of zinc stimulate growth mechanisms but become toxic at high doses, (ii) copper interferes with photosynthetic processes because of its role in redox reactions, and (iii) cadmium, because of its high toxicity, severely affects all the parameters studied.

Although studies have explored the effects of heavy metals on other crops, this research focuses on the less-studied specific variety 'Torro Plus'. It also provides information on specific adaptive responses to different heavy metals. The main objectives are, firstly, to examine the effects of different concentrations of these metals on morphological (leaf and root length, fresh and dry weight of aerial parts and roots) and physiological (chlorophyll content) parameters and, secondly, to determine their influence on secondary metabolites (proline, polyphenols, flavonoids, total sugars) involved in stress tolerance mechanisms.

2. Materials and Methods

2.1. Plant Material

The plant material used in this study consisted of seeds of the *Zea mays* variety "Torro Plus", purchased from a certified nursery.

2.2. Seed Sterilization

Seeds were immersed in an 80% ethanol solution for 1 minute for initial disinfection. After this step, the seeds were rinsed with sterile distilled water to remove the alcohol. The seeds were then immersed in a 50% bleach solution for 10 minutes, with manual agitation, to achieve complete disinfection. After incubation, the seeds were rinsed with sterile distilled water to remove all traces of bleach [29].

2.3. Treatment Preparation

Treatments were prepared from stock solutions of the heavy metals $ZnSO_4.7H_2O$ (Zn), $CuSO_4.5H_2O$ (Cu) and $CdCl_2.2.5H_2O$ (Cd) at a concentration of 1 mM for each metal. For each treatment, an appropriate amount of each stock solution was diluted with sterile distilled water to obtain the desired final concentrations of the heavy metals; the treatments applied are shown in **Table 1**.

Table 1. Treatment conditions.

Treatment	Conditions
T1	Control
T2	Seed + 50 µmol Zn
T3	Seed + 100 µmol Zn
T4	Seed + 150 µmol Zn
T5	Seed + 50 µmol Cu
T6	Seed + 100 µmol Cu
T7	Seed + 150 µmol Cu
T8	Seed + 50 µmol Cd
T9	Seed + 100 µmol Cd
T10	Seed + 150 µmol Cd

2.4. Preparation of Culture Medium

MS medium (Murashige and Skoog) was prepared, containing vitamins, sucrose (30 g/L) and agar (10 g/L). The pH of the medium was adjusted to 5.6–5.8 using drops of HCl and NaOH. The heavy metals were added to the culture medium in the desired concentrations, then the agar was incorporated. The mixture was then autoclaved at 120 °C for 20 minutes to ensure sterilization of the culture medium.

2.5. Seed Cultivation

The sterilized seeds were planted in culture tubes filled with MS medium, with or without the presence of heavy metals. These tubes were then placed in a sterilized culture chamber set at 25 ± 2 °C, with a light cycle of 16 hours of light and 8 hours of darkness, and a light intensity of 45 $\mu mol.m^{-2}.s^{-1}$ for 21 days.

2.6. Parameters Measured

2.6.1. Morphological Parameters

Leaf Length

This parameter measured using a graduated ruler and serves as an indicator of plant vigor and any potential growth inhibition caused by the treatments.

Root length was measured to evaluate the effects of heavy metals on root development. This parameter serves as an indicator of the plant's capacity to access nutrients and water, particularly under stressful conditions.

The number of leaves per plant was counted to evaluate changes in foliage development in response to heavy metal exposure. A decrease in leaf production can indicate stress or phytotoxicity.

Fresh and dry weights of aerial parts and roots were measured to assess biomass production under heavy metal stress. Fresh weight was recorded immediately after harvest, while dry weight was determined by drying samples at 40 °C for 48 hours.

2.6.2. Physiological Parameters

Physiological parameters, including chlorophyll a and b, were measured to calculate total chlorophyll content using the Arnon method [30]. Fresh leaves (1 g) were homogenized with 20 ml of 80% acetone, then incubated at 4 °C for 3 hours and centrifuged. The supernatant was adjusted to 100 ml with 80% acetone, and absorbance was measured at 645 nm and 663 nm using a spectrophotometer. Chlorophyll a, b, and total chlorophyll were calculated using specific formulas.

$$\text{Chlorophyll a (mg/g FW)} = (12{,}7 \times A663\text{nm} - 2,69 \times A645\text{nm}) \times \frac{V}{1000 \times m} \quad (1)$$

$$\text{Chlorophyll b (mg/g FW)} = (22{,}9 \times A645\text{nm} - 4,48 \times A663\text{nm}) \times \frac{V}{1000 \times m} \quad (2)$$

$$Total\ chlorophyll\ \text{(mg/g FW)} = (20{,}2 \times A645\text{nm} + 8,02 \times A663\text{nm}) \times \frac{V}{1000 \times m} \quad (3)$$

2.6.3. Biochemical Parameters

Proline

Proline concentration was measured using the method of[31]. For this, 100 mg of fresh plant tissue was homogenized in 5 ml sulfosalicylic acid (3%). After filtration, 2 ml ninhydrin acid and 2 ml glacial acetic acid were added to 1 ml of the filtered extract. The mixture was incubated at 100 °C for 1 hour, then rapidly cooled. After addition of 4 ml acetone, absorbance was measured at 520 nm. Proline concentration was determined using a calibration curve with standard proline concentrations.

Total Sugars

Quantification of total sugars was carried out according to the method of[32]. 0.5 g of plant tissue was homogenized in 10 ml distilled water, then centrifuged at 5000 rpm for 10 minutes to obtain a clear extract. To 1 ml supernatant, 1 ml 5% phenol and 5 ml concentrated sodium sulfate were added. The mixture was incubated at room temperature for 20 minutes, and absorbance was measured at 490 nm. The total sugar content was measured by constructing a calibration curve using standard glucose solutions.

Polyphenols

The method of[33] was used for polyphenol quantification. A polyphenol extract was prepared by homogenizing 0.5 g of plant tissue in 10 ml of 80% methanol. After centrifugation, 1 ml of the supernatant was mixed with 5 ml of diluted Folin-Ciocalteu reagent and 4 ml of sodium carbonate solution (7.5%). The mixture was incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 765 nm and polyphenol concentration was determined using a calibration curve based on gallic acid.

Flavonoids

Flavonoid quantification was carried out according to[34]. 0.5 g of plant tissue was homogenized in 10 ml of 80% methanol, and the mixture filtered. 1 ml of the filtered extract was added to 4 ml of 80% methanol and 0.3 ml of 2% magnesium chloride solution. After incubation at room temperature for 30 minutes, absorbance was measured at 430 nm. Flavonoid concentration was determined using a calibration curve prepared with standard quercetin.

2.7. Statistical Analysis

Statistical analyses were performed with R (version 4.2.2) and Python (version 3.9) software, R being used with the FactoMineR package for multivariate analyses, while Python enabled advanced visualizations thanks to the pandas, scipy, matplotlib, and seaborn libraries. Descriptive statistics were calculated for all measured parameters, including means and standard deviations. Analysis of variance (ANOVA) was performed to assess treatment effects. Pearson correlation analysis was used to explore linear relationships between parameters, with results presented as a correlation matrix and visualized as an annotated heatmap with significance levels ($p < 0.05$, $p < 0.01$, $p < 0.001$). PCA reduced the dimensionality of the data, making it possible to distinguish groupings according to treatments and to highlight parameters contributing to overall variability. Finally, a hierarchical clustering analysis (HCA) was performed to group samples according to their overall responses to treatments.

3. Results

3.1. Morphological Parameters

The results obtained show significant differences depending on the concentrations of heavy metals applied (**Table 2**). A notable decrease in the leaf count was observed ($p < 0.001$), leaf length was also highly significant ($p < 0.001$) as was root length ($p < 0.001$). Low concentrations of zinc, namely 50 µmol (T2) and 100 µmol (T3), did not result in any notable variations compared with the control (T1), indicating that *Zea mays* tolerates zinc well in these concentration ranges. However, at 150 µmol (T4), a slight decrease in leaf (8,96%) and root (21.07%) length was observed, although leaf number remained stable. This suggests that a high concentration of zinc begins to induce stress in the plant. Indeed, administration of 150 µmol Zn can significantly reduce root length compared to the control; conversely, it does not affect the number and length of leaves. Copper at a concentration of 50 µmol (T5) showed similar results to the control, indicating that this dose is tolerated by *Zea mays*. However, at higher concentrations, notably 100 µmol (T6), significant reductions were observed in all the parameters measured. At 150 µmol (T7), the toxic effects became even more marked, with greater reductions in leaf length (75.02%) and root length (72.34%). Indeed, administration of Cu at 100, and 150 µmol doses can significantly reduce the number and length of leaves and root length compared to the control. Cadmium showed toxic effects even at low concentrations, in fact administration of Cd at all doses can significantly reduce the number and length of leaves and root length compared to the control. At 50 µmol (T8), there was already a noticeable reduction in leaf (28.58%) and root (45.07%) length. At higher concentrations (100 µmol, T9 and 150 µmol, T10), the toxic effects are accentuated. The number of leaves fell to 1.33 for T9 (55.56% reduction) and to 0.67 for T10 (77.78% reduction). Leaf and root lengths were reduced considerably, reaching just 2.56 cm for leaves (81.81% reduction) and 3.39 cm for roots (83.71% reduction) for T10. Cadmium interferes with plant metabolic processes, inhibiting photosynthesis and disrupting nutrient uptake, even at low concentrations.

Table 2. Influence of Zn, Cu, and Cd on leaf number, leaf and root length in *Zea mays*.

Treatment	Number of Leaves	Length of Leaves (cm)	Root Length (cm)
T1 = Temoin	3 ± 0 ^{a}	14.1 ± 0.26 ^{a}	20.8 ± 0.10 ^{ab}
T2 = Zn (50)	3 ± 0 ^{a}	14.4 ± 0.45 ^{a}	21 ± 0.2 ^{a}
T3 = Zn (100)	3 ± 0 ^{a}	13.20 ± 0.70 ^{a}	19.4 ± 0.20 ^{b}
T4 = Zn (150)	3 ± 0 ^{a}	12.83 ± 0.46 ^{a}	16.43 ± 0.25 ^{c}
T5 = Cu (50)	3 ± 0 ^{a}	12.86 ± 0.55 ^{a}	14.16 ± 0.30 ^{d}
T6 = Cu (100)	2.33 ± 0.57 ^{b}	7.18 ± 0.28 ^{c}	12.1 ± 0.3 ^{e}
T7 = Cu (150)	2.33 ± 0.57 ^{b}	3.52 ± 0.33 ^{e}	5.75 ± 1.37 ^{f}
T8 = Cd (50)	1.33 ± 0.57 ^{bc}	10.06 ± 0.90 ^{b}	11.43 ± 0.45 ^{e}
T9 = Cd (100)	1.33 ± 0.57 ^{bc}	5.66 ± 0.49 ^{d}	6.37 ± 0.28 ^{f}
T10 = Cd (150)	0.66 ± 0.57 ^{bc}	2.55 ± 0.44 ^{e}	3.39 ± 0.54 ^{g}

T1 = Control, T2 = Seed + 50 µmol Zn, T3 = Seed + 100 µmol Zn, T4 = Seed + 150 µmol Zn, T5 = Seed + 50 µmol Cu, T6 = Seed + 100 µmol Cu, T7 = Seed + 150 µmol Cu, T8 = Seed + 50 µmol Cd, T9 = Seed + 100 µmol Cd, T10 = Seed + 150 µmol Cd. *(Values sharing the same letters within each category are not significantly different at a 5% significance level).*

ANOVA analyses show a significant difference between the heavy metal treatments applied (Zn, Cu and Cd) and the fresh and dry weights of aerial parts and roots (**Figure 1**). The p-values below 0.05 confirm that heavy metal stress conditions significantly influenced *Zea mays* growth. The results show that exposure to heavy metals, particularly high concentrations of Cadmium (Cd), Copper (Cu) and Zinc (Zn), leads to significant reductions in plant biomass. These effects are particularly marked for treatments with high concentrations of Cadmium (Cd) (T8, T9 and T10), where reductions in the fresh and dry weight of aerial parts and roots exceed 80%. This suggests that Cadmium (Cd) has a severe toxic effect on plant growth, disrupting several physiological processes such as water and nutrient uptake.

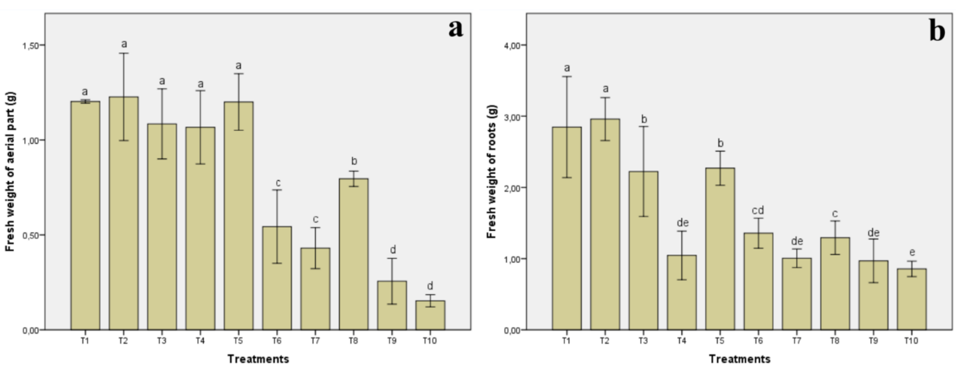

Figure 1. *Cont.*

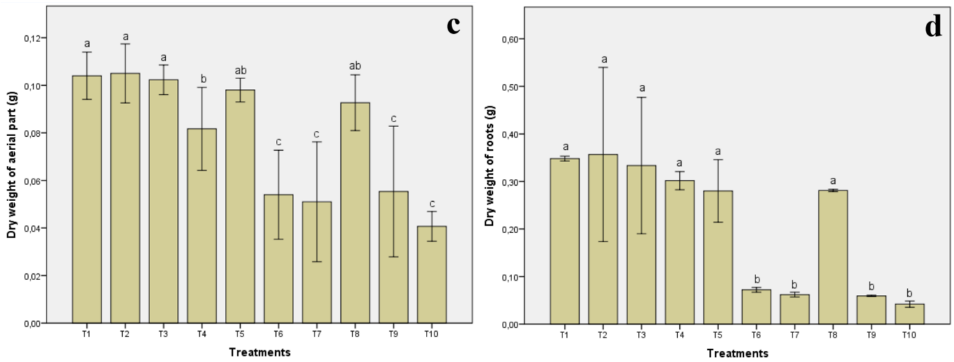

Figure 1. Impact of heavy metals (Zn, Cu and Cd) on the fresh and dry weight of *Zea mays* (T1 = Control, T2 = Seed + 50 µmol Zn, T3 = Seed + 100 µmol Zn, T4 = Seed + 150 µmol Zn, T5 = Seed + 50 µmol Cu, T6 = Seed + 100 µmol Cu, T7 = Seed + 150 µmol Cu, T8 = Seed + 50 µmol Cd, T9 = Seed + 100 µmol Cd, T10 = Seed + 150 µmol Cd). (*Values sharing the same letters within each category are not significantly different at a 5% significance level*).

3.2. Physiological Parameters

The results show that Cadmium (Cd) has the most toxic effect on chlorophyll levels, with a progressive and marked decrease in chlorophylls a, b and total as the Cd concentration increases (**Table 3**). The maximum reduction was observed in the T10 treatment (150 µmol Cd), where total chlorophyll fell by 74.31%, reflecting the high toxicity of this metal for photosynthetic processes. Copper (Cu) also has a negative effect on photosynthesis, with a significant reduction in chlorophyll levels at high concentrations (T7 treatment, 150 µmol Cu), where total chlorophyll falls by 66.19% compared to the control without stress. Zinc showed more moderate effects. Although higher concentrations (T4, 150 µmol Zn) lead to a significant reduction in chlorophyll levels, the effects are less severe compared with Cu and Cd. The reduction in total chlorophyll in the T4 treatment is 21.06%, suggesting that Zn has a lower toxicity but can still interfere with photosynthesis when present in excess.

Table 3. Effect of heavy metals on the chlorophyll a, b and total content of *Zea mays* treated with different concentrations of Zinc (Zn), Copper (Cu) and Cadmium (Cd).

Treatment	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total Chlorophyll (mg/g FW)
T1 = Temoin	1.70 ± 0.10^{a}	0.91 ± 0.01^{a}	2.61 ± 0.09^{a}
T2 = Zn (50)	1.75 ± 0.05^{a}	0.92 ± 0.01^{a}	2.67 ± 0.05^{a}
T3 = Zn (100)	1.47 ± 0.06^{b}	0.84 ± 0.02^{b}	2.31 ± 0.05^{b}
T4 = Zn (150)	1.33 ± 0.03^{b}	0.73 ± 0.03^{c}	2.06 ± 0.03^{c}
T5 = Cu (50)	1.46 ± 0.04^{b}	0.81 ± 0.06^{b}	2.27 ± 0.04^{b}
T6 = Cu (100)	1.15 ± 0.04^{c}	0.75 ± 0.02^{d}	1.90 ± 0.05^{d}
T7 = Cu (150)	0.62 ± 0.02^{e}	0.25 ± 0.04^{f}	0.88 ± 0.02^{f}
T8 = Cd (50)	1.04 ± 0.04^{cd}	0.73 ± 0.01^{d}	1.78 ± 0.04^{d}
T9 = Cd (100)	0.94 ± 0.04^{d}	0.64 ± 0.01^{e}	1.58 ± 0.04^{e}
T10 = Cd (150)	0.46 ± 0.05^{f}	0.20 ± 0.07^{f}	0.67 ± 0.04^{g}

T1 = Control, T2 = Seed + 50 µmol Zn, T3 = Seed + 100 µmol Zn, T4 = Seed + 150 µmol Zn, T5 = Seed + 50 µmol Cu, T6 = Seed + 100 µmol Cu, T7 = Seed + 150 µmol Cu, T8 = Seed + 50 µmol Cd, T9 = Seed + 100 µmol Cd, T10 = Seed + 150 µmol Cd. *(Values sharing the same letters within each category are not significantly different at a 5% significance level).*

3.3. Biochemical Parameters

The ANOVA carried out on the various parameters measured (proline, total sugars, polyphenols and flavonoids) showed significant differences ($p < 0.05$) between the heavy metal treatments applied (Zn, Cu and Cd) (**Figure 2**). The highest concentrations of heavy metals (Cu, Zn and Cd) resulted in a significant increase in proline production, which serves as a marker of oxidative stress and the plant's cellular response to metal exposure, as well as an increase in total sugar content as a compensatory mechanism for coping with stress. Polyphenols also show variations in response to heavy metal stress treatments, and their accumulation seems to be relatively less affected by Cu, Zn and Cd at low concentrations. On the other hand, flavonoids, which are also antioxidants, show a strong response to exposure to heavy metals, reflecting an increased antioxidant defence under the stress conditions induced by these elements (Cu, Zn and Cd). Overall, heavy metals had a significant impact on the synthesis of proline, total sugars, polyphenols and flavonoids, with particularly marked effects on flavonoids and total sugars. These results indicate that *Zea mays* adapt to heavy metal-induced stress by modifying their secondary metabolism, which probably contributes to defence mechanisms against oxidative damage.

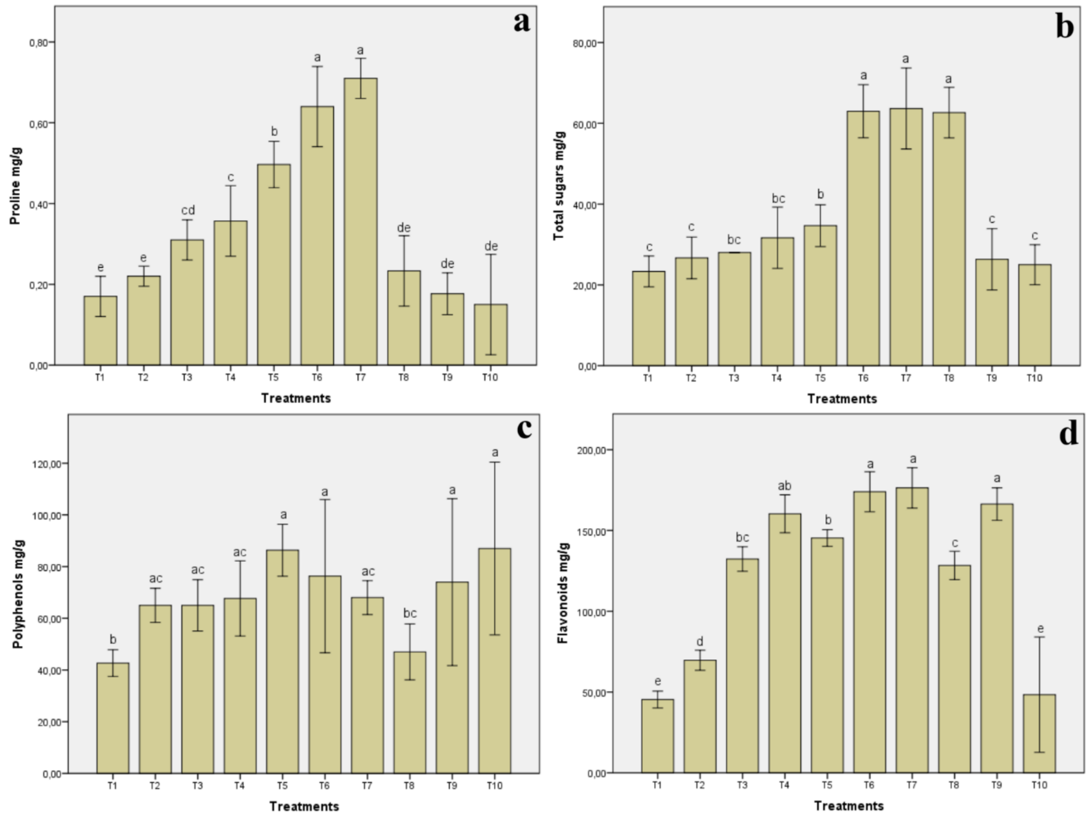

Figure 2. Effect of heavy metal treatments (Cu, Zn, Cd) on the production of proline, total sugars, polyphenols, and flavonoids in *Zea mays* (T1 = Control, T2 = Seed + 50 µmol Zn, T3 = Seed + 100 µmol Zn, T4 = Seed + 150 µmol Zn, T5 = Seed + 50 µmol Cu, T6 = Seed + 100 µmol Cu, T7 = Seed + 150 µmol Cu, T8 = Seed + 50 µmol Cd, T9 = Seed + 100 µmol Cd, T10 = Seed + 150 µmol Cd). (*Values sharing the same letters within each category are not significantly different at a 5% significance level)*.

3.4. Correlation Analysis

The correlations established between morphological, physiological and biochemical parameters in our study on stress induced by heavy metals (Cu, Zn and Cd) provide valuable information on the response mechanisms of *Zea mays* (**Figure 3**). A strong correlation was observed between the number of leaves and leaf length (r = 0.730), root length (r = 0.775) and the fresh weight of aerial parts (r = 0.781), this indicates that larger plant size is often accompanied by a higher number of leaves. Chlorophylls (a, b and total) are also positively correlated with morphological measurements, implying that better photosynthesis is associated with larger root and leaf size. On the other hand, the negative correlations between the content of secondary metabolites (polyphenols, flavonoids) and certain morphological and photosynthetic parameters indicate the redistribution of metabolic resources. Under metal stress, plants increase the production of antioxidant compounds, such as polyphenols and flavonoids, to counter the effects of reactive oxygen species generated by heavy metals. These compounds play a key role in protecting cells against oxidation, which explains their significant increase under conditions of stress. Finally, the strong correlations between proline and total sugars confirm their essential role in the stress response. These two compounds act as osmoprotectors, helping to stabilise cell structures and maintain water balance under conditions of high stress. This adaptive response is consistent with the increased toxicity of heavy metals, necessitating strengthened defence mechanisms.

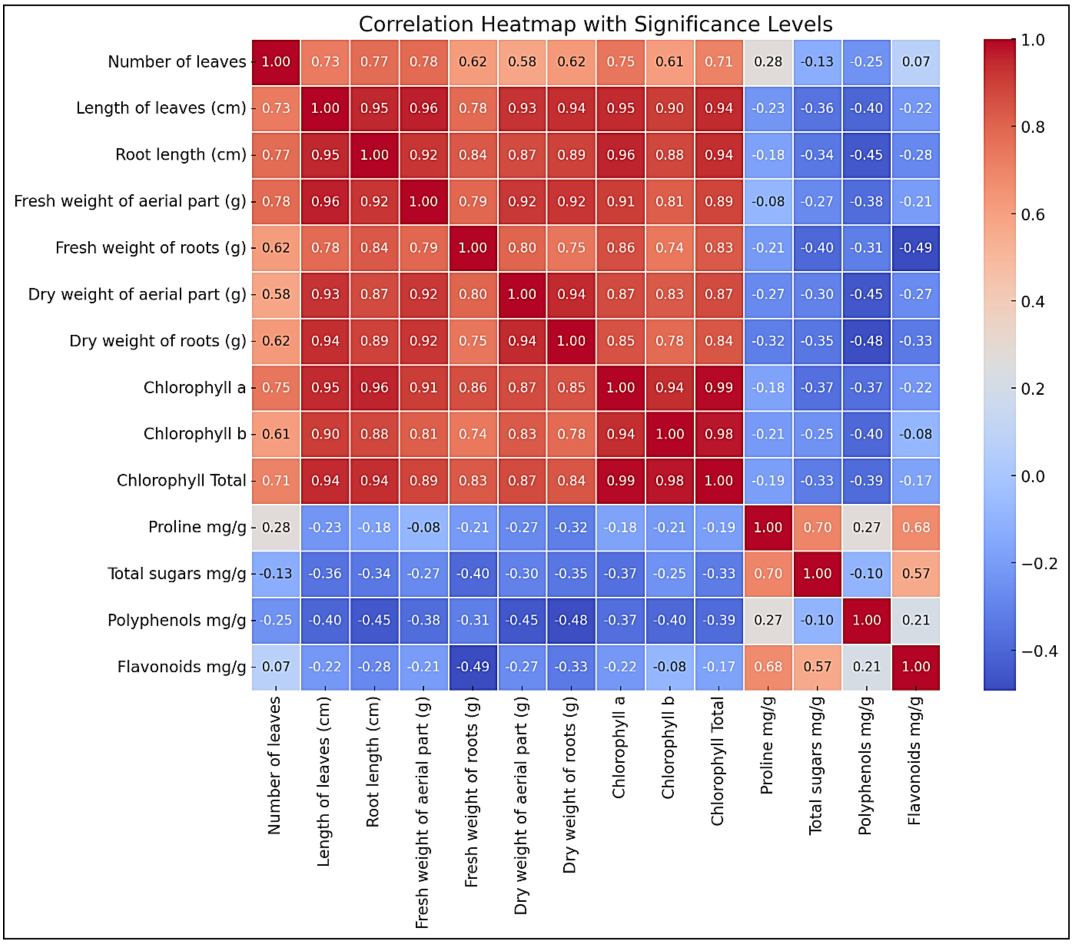

Figure 3. Correlations between the parameters studied in *Zea mays* under heavy metal stress.

3.5. Principal Component Analysis

Principal component analysis (PCA) was used to simplify and interpret the complex relationships between the parameters measured and the treatments applied to the different concentrations of heavy metals (Zn, Cu and Cd). PCA revealed that two principal components (PC1 and PC2) together explained 80.8% of the total variance (64.8% for PC1 and 16% for PC2, **Figure 4**), justifying their use to interpret the data in a meaningful way. Principal component analysis (PCA) shows that PC1 is mainly associated with morphological parameters (leaf length, root length, number of leaves, fresh and dry weights of aerial parts and roots) and chlorophyll content, and distinguishes moderate Zn concentrations (50 µmol Zn, 100 µmol Zn) from the control, promoting better plant growth. In contrast, PC2 is dominated by biochemical parameters such as proline, flavonoids and total sugars, and distinguishes high concentrations of Cd (100 µmol Cd, 150 µmol Cd) and Cu (150 µmol Cu), which are linked to metal stress response mechanisms. The biplot combines these results, showing that low Zn concentrations are associated with growth, while high Cd and Cu concentrations activate biochemical responses. In brief, PCA reveals that moderate concentrations of Zn promote morphology, while high concentrations of Cd and Cu induce biochemical stress responses.

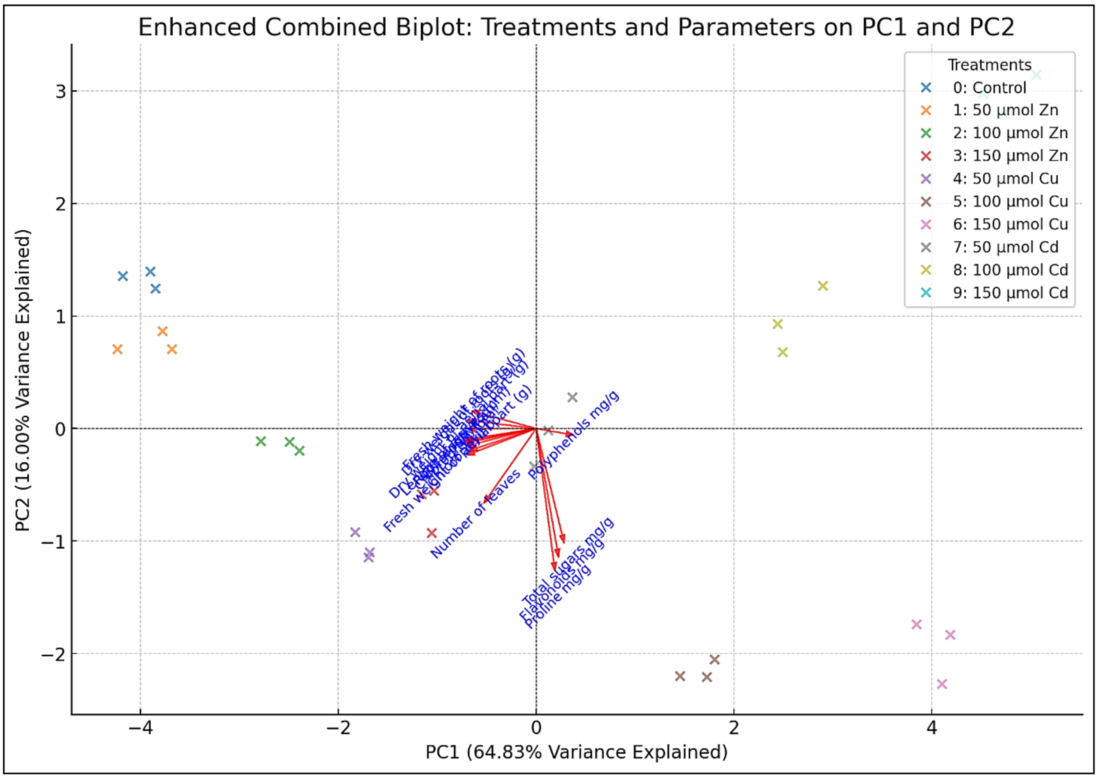

Figure 4. Enhanced combined Biplot from PCA: Associations between treatments and key parameters measured in *Zea mays* under heavy metal stress.

3.6. Hierarchical Analysis

The heatmap combined with hierarchical clustering provides a comprehensive and detailed view of the relationships between morphological, physiological and biochemical parameters and groups of samples exposed to different metal treatments (Zn, Cu and Cd). Data standardization was used to make the parameters comparable, and Euclidean distance was used as the similarity measure for clustering. The dendrograms show a consistent structure in the clustering of samples and parameters, reflecting the effects of different metal treatments (**Figure 5**). Samples exposed to high cadmium concentrations (150 µmol Cd) cluster distinctly, characterized by strongly negative normalized values for morphological parameters such as root length (−1.54) and dry weight of aerial parts (−1.87), indicating severe growth inhibition. In contrast, the control groups showed positive normalized values for these same parameters, reflecting optimal growth in the absence of metal stress (z-scores reaching 1.09 for root length and 1.17 for dry weight). Biochemical parameters such as proline secretion (1.45) and flavonoids (1.46) showed increased values in samples subjected to high concentrations of heavy metals, confirming their key role in plants' adaptive response to stress. These observations also support the idea that low concentrations of zinc (50 µmol Zn) can stimulate plant growth, with positive z-scores for leaf length (1.05) and total sugars (0.98), while higher concentrations lead to an accumulation of stress metabolites and a decrease in morphological parameters. Hierarchical clustering also reveals a clear distinction between morphological and biochemical parameters, reflecting specific responses to metal stress. Morphological parameters, such as fresh weight and root length, respond directly to metal toxicity, while metabolites, such as proline and flavonoids, act as adaptive stress indicators.

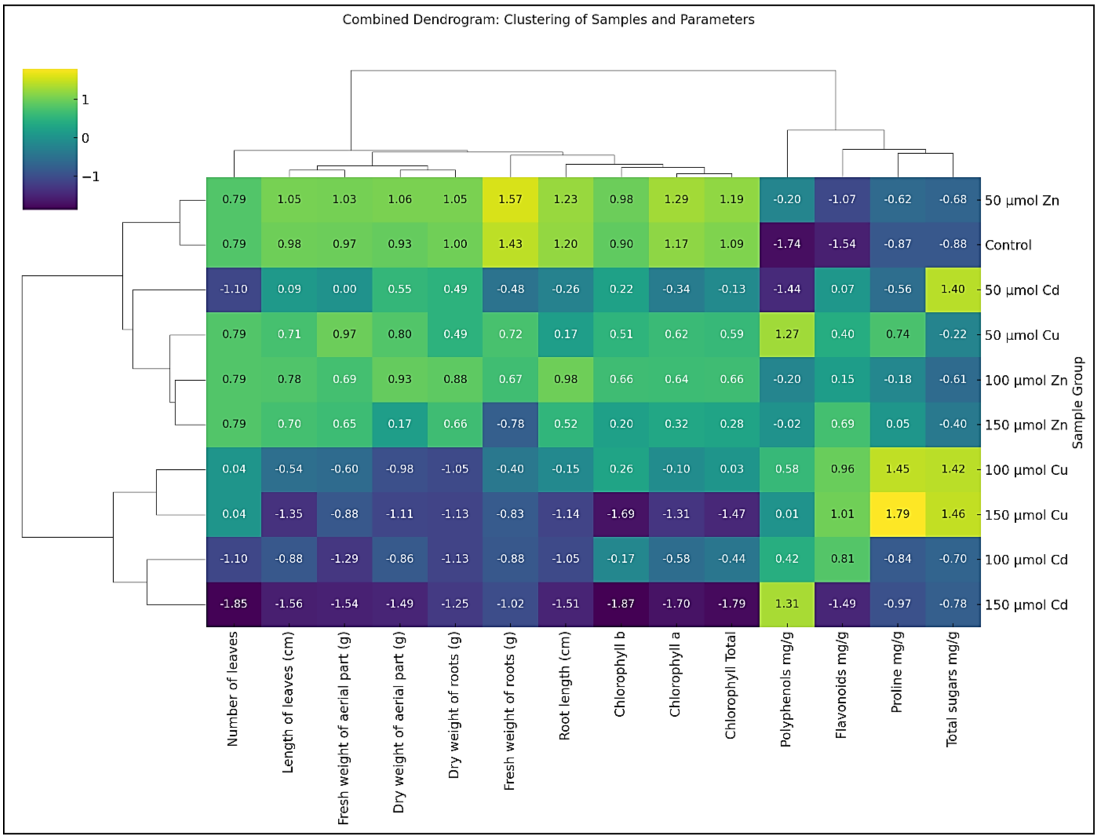

Figure 5. Hierarchical Clustering Heatmap of sample groups and parameters.

4. Discussion

Plants in their natural environment experience various stresses, such as water, salt, and metal stress, which negatively affect their yield and productivity [35–37]. Heavy metals enter the environment through natural processes like volcanic eruptions and rock weathering, as well as human activities such as industry, agriculture, mining, and waste disposal. Modern farming practices, including the use of fertilizers and pesticides, introduce heavy metals into the soil, which can be absorbed by plants through their roots [38, 39]. Factors like plant type, soil metal concentration, pH, and composition influence absorption. Heavy metals can also be deposited on plants via contaminated air, dust particles, or irrigation with polluted water. Soil disruption and erosion enhance the movement of heavy metals into plants [40–42].

4.1. Effects of Heavy Metals on Growth and Biomass

Exposure of *Zea mays* to heavy metals (Cu, Zn and Cd) has a significant impact on plant growth and biomass. The data show that morphological parameters, such as leaf length, root length, and fresh and dry weights of aerial and root parts, decrease with increasing metal concentrations. These results corroborate the work of [43], who demonstrated that Cd, in particular, inhibits cell division and disrupts essential metabolic processes in plants [43]. Zn and Cu, although necessary at low doses for enzyme function and membrane stabilization, have also shown a toxic effect at high concentrations. Similar results were reported by [29] and its team who observed a decrease in biomass and morphological parameters in Momordica cymbalaria under heavy metal stress, due to interference with nutrient uptake mechanisms and ROS generation [29]. On the other hand, zinc has a biphasic effect, with stimulation at low doses and inhibition at high doses, consistent with its essential role at low concentrations and toxicity at high concentrations [44].

4.2. Disturbance of Photosynthesis and Chlorophyll Pigments

The significant reduction in chlorophyll a, b and total levels is a key indicator of photochemical stress. This reduction results from the degradation of photosynthetic pigments by heavy metal-induced ROS. In particular, Cd disrupts chlorophyll biosynthesis by blocking key enzymes such as protochlorophyllide reductase, leading to a collapse of photosynthetic complexes [45]. In addition, Cu, at high doses, can cause photoinhibition due to excessive generation of free radicals in chloroplasts [45]. Zn, on the other hand, shows a biphasic effect: moderate stimulation of photosynthesis at low concentrations, followed by inhibition at high doses. This behavior is explained by Zn's essential role in stabilizing thylakoid membranes and its toxic effect at high doses due to excessive ROS accumulation.

4.3. Accumulation of Secondary Metabolites in Response to Stress

The increased accumulation of proline in treatments containing high doses of Cd and Cu highlights its key role as an osmoprotectant and antioxidant. Proline helps stabilize cell membranes and proteins and acts as an osmotic buffer, reducing damage caused by oxidative stress. This observation is consistent with the work of [45], who reported a significant increase in proline under salt and metal stress. Heavy metal exposure increases polyphenols and flavonoids, which, due to their antioxidant properties, help neutralize ROS and protect plant cells from oxidative damage. However, at very high doses of Cd (150 µM), a decrease in these compounds is observed, suggesting that stress exceeds the plant's capacity to produce these antioxidants. The study by [43] also reported an increase in polyphenols in plants exposed to Cd, but with a decrease at extreme doses, reflecting increased toxicity. In contrast, the increase in proline reflects its osmoprotective role, while the rise in flavonoids reflects an activation of antioxidant defense against metal stress [46–48]. The increase in total sugars observed, particularly under Cu and Zn, reflects their role in osmotic regulation and stabilization of cell structures. Sugars are also involved in the repair of stress-induced damage, as indicated in previous work [45].

These results open up several interesting perspectives for future research. Firstly, it would be relevant to explore varieties of *Zea mays* or other crops with improved tolerance to heavy metals, through genetic selection or metabolic modifications, in order to improve resistance to environmental stresses. Furthermore, the study of the phytoremediation capacities of *Zea mays* and other plants could offer a sustainable solution for the extraction of heavy metals from the soil, thus contributing to the depollution of contaminated land. In parallel, the development of innovative agronomic strategies, such as the use of biostimulants or microorganisms, could help mitigate the effects of heavy metals on plant growth and improve crop productivity [49].

4.4. Specificity of Cd in Toxicity

Cd proved to be the most toxic metal in this study. Due to its low mobility towards the aerial parts, it accumulates mainly in the roots, where it causes local toxicity by disrupting the absorption of essential nutrients such as iron and zinc. This accumulation leads to increased oxidative stress, nutritional deficiencies and growth inhibition. These results are in agreement with other studies that have reported a similar metabolic disturbance due to Cd accumulation in roots [29].

4.5. Limitations of the Study

A major limitation of this study is that the experiments were conducted exclusively under controlled *in vitro* culture conditions. While this approach minimizes environmental variables and isolates the effect of heavy metals on maize growth, it does not necessarily reflect the complex interactions that occur in real environments [50]. Agricultural soils, subject to a multitude of biotic and abiotic factors, can influence contaminant bioavailability and plant responses. Thus, further trials under field conditions would be necessary to validate the applicability of the results and refine recommendations for practical use [51, 52].

This study was carried out using a single maize variety, which limits the scope of the conclusions. To obtain generalizable results and recommendations applicable to a wider range of agricultural practices, it would be relevant to extend research to several maize varieties with different tolerances to heavy metals. Such an approach would provide a better understanding of the genetic variability of stress responses and enable us to identify the varieties best suited to polluted environments. Future research could validate these results in the field to assess the true impact of heavy metals on maize and explore molecular mechanisms of tolerance and interactions with other abiotic stresses. Finally, the study of bioremediation strategies, such as the use of plants or microorganisms, could provide sustainable solutions to improve the management of contaminated soils [53–55].

5. Conclusions

In conclusion, the results of this study confirm that exposure to heavy metals (Cu, Zn and Cd) induces significant alterations in plant growth, photosynthetic performance and secondary metabolism. Metals induce adaptive responses, such as the accumulation of proline, secondary metabolites (polyphenols and flavonoids) and sugars, which enhance plant defense mechanisms against oxidative stress. However, at high concentrations, toxic effects predominate, particularly for Cd, highlighting the limitations of *Zea mays* in the face of extreme stress conditions. Finally, long-term research into the influence of heavy metals on soil quality, biodiversity and food safety is needed to assess long-term environmental risks and propose appropriate solutions for the sustainable management of polluted soils. These results suggest that heavy metal concentration thresholds need to be defined for agricultural practices. The use of biostimulants or microorganisms could mitigate the effects of metal stress.

Author Contributions

M.O. contributed to the conceptualization, methodology, data collection, and manuscript writing. B.Y. was responsible for the experimental setup, data analysis, and manuscript review. N.B. (Noura Benlemlih) and R.Z. provided laboratory assistance and contributed to data validation. S.E.A. handled data curation and statistical analysis, while M.E.B. also contributed to statistical analysis and revision. N.A. provided resources and technical support. N.B. (Najiba Brhadda) supervised the work and edited the manuscript. S.B. contributed to the methodology. A.C. participated in conceptualization and methodology. F.Z.A. contributed to data analysis and revision, while Y.M. worked on data interpretation and manuscript revision. Finally, M.I. provided overall supervision, secured funding, and gave the final approval of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

This study does not involve human or animal subjects.

Data Availability Statement

The data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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