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Recovery of Growth in *Zinnia elegans* after Copper Stress

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Abstract. The symptoms of copper ion toxicity are well studied, but there is limited information on the mechanisms of plant recovery after stress. The paper deals with the recovery of growth in *Zinnia elegans* Jacq., treated with 50 and 100 μM Cu^{2+} for 15 days, then cultivated in normal condition for 30 days. After the recovery period the length of shoot, anatomical changes of root, hypocotyl and stem (first internode) were investigated. It was shown that the copper ions induced a decrease of plant length during stress period. After stress relief for 30 days, the length of hypocotyl and stem of Cu-treated plants increased compared to control. The diameter of root was bigger due to the increase in thickness of cortex and stele. The changes were less noticeable in shoot tissues. The diameter of hypocotyl positively correlates with the thickness of stele. Same effect of copper was observed in stem only in plants, previously treated with 100 μM. The diameter of cortex and metaxylem cells increased in all analyzed organs. No significant differences were found in cell wall thickness of metaxylem cells. The recovery of *Z. elegans* growth after cooper treatment occurred due to the expansion of cortex and metaxylem cells.

INTRODUCTION

Natural processes of rock erosion and human industrial activity, including soil contamination by heavy metal (HM) ions, in particular, copper, is known for many regions. The negative influence of HM ions on metabolic reactions, plant functions and productivity it is well known [1]. Excess of copper ions in cells catalyzes the formation of reactive oxygen species (ROS) in the Fenton and Haber–Weiss reactions, which leads to oxidative stress [1, 2]. An excess of Cu^{2+} in plants tissues causes chlorosis, induce inhibition of photosynthesis and respiration, and negatively affect morpho-anatomical parameters, cell division and growth [1]. The stress reactions on HM ions have been studied in detail in many agricultural and model plants. The information about the mechanisms of plant recovery after the stress is not so numerous.

Cell wall is a dynamic structure that plays specific role in the physical control of cell growth, cell shape and the structural integrity of the plant in response to environmental conditions and internal signals [3]. Secondary cell walls are strengthened by hydrophobic polymers: lignin in xylem, suberin and lignins in endodermis Casparian strips. As the first entry barrier for HM ions, cell walls of roots are actively involved in their absorption. Pectin and lignin contain a large number of functional groups (hydroxyl, carboxyl, methoxyl, etc.), that allow them to bind different HM ions (Cu^{2+}, Cd^{2+}, Pb^{2+}, and etc.) and reduce their entry into the cytoplasm [4]. Lignification under metallic stresses is an unspecific reaction which could lead to the thickening of the cell wall [5]. Therefore, the changes in cell walls under the influence of HM are also of great interest.

It has been shown that HM stress is able to induce modifications in the anatomy structure of root tissues, cell wall thicknesses. These changes were observed in bark, cortex and xylem cell, which may prevent further uptake and transport of the copper ions into shoot [6–8]. High copper ions concentration can induce changes not only in root, but due to the long-distance transport in hypocotyl and stem morphology and anatomy, also. The decrease of plant growth and stem length could be caused by inhibition of lateral cell expansion [9]. There is limited information about the structural modification of the tissues and cell walls in various organs of higher plants after recovery period.

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Zinnia elegans Jacq. is annual floriculture. In cell biology *Z. elegans* is one of the most used models for studying lignification especially in hypocotyls and stems [10]. The present study focuses on the structural changes of root and stem tissues and cell walls after copper stress recovery.

MATERIAL AND METHODS

Zinnia elegans Jacq. seeds were grown in pots containing perlite and vermiculite (1:1, v/v) on Knop medium at 25 °C with photoperiod of 16 h. Copper treatment was performed by watering the plants with 50 and 100 μM $CuSO_4$ in Knop solution for 15 days, then plants were grown on Knop medium for 45 days. Control plants were watered with Knop solution for the whole period of growth. The experiments were repeated three times with fifteen replicates for each treatment.

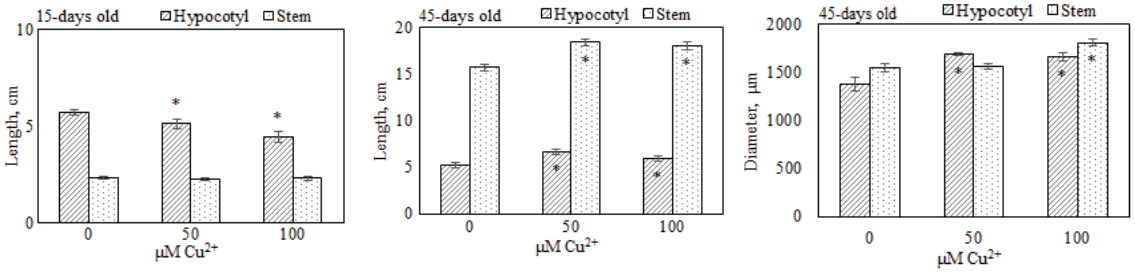
Morphological parameters were measured in 15- and 45-days old plants, including the length of hypocotyl and stem. On the 15th day, the plants had a hypocotyl and one internode, therefore, the stem length was estimated as the length of the first internode.

Root, hypocotyl and stem sections of control and stressed plants were fixed in Clarke's solution (glacial acetic acid and ethanol 1:3) at room temperature. Then samples were washed and stored in ethanol at +4 ºC. For histochemical studies, the transversal cross and longitudinal sections of root (zone of maturation), hypocotyl and the stem (first internode from hypocotyl) of 45-day-old plants were prepared with a freezing microtome M3-2 (Medpribor, Russia). The samples were stained with 1% phloroglucinol (w/v) in 12% HCl for 5 min, washed in distilled water, and stored in 50% glycerin [11]. Sections were observed with a Meiji MT 4300L light microscope ("Meiji Techno", Japan). Anatomical parameters were measured using Simagis MesoPlant software for Windows.

The results presented are the mean values ± standard error. We used fifteen replicates for each treatment for morphological parameters, at least five plants of each variant and fifteen replications for each plant for anatomical analyses. The Student's *t*-test was used for independent replicates of each sample ($p < 0.05$). Statistical analysis was completed in STATISTICA 10 for Windows, plots were constructed in Excel.

RESULTS AND DISCUSSION

The length of hypocotyl of 15-days old *Z. elegans* treated with 50 and 100 μM Cu^{2+} decreased by 10.0 and 21.7% compare to control group (Fig. 1a). After recovery period (30 days) the length of stressed plant increased compared to control ones (Fig. 1b). The length of the hypocotyl did not change in control plants from 15 to 45 days; in plants pretreated with copper ions, it increased slightly after a recovery period of 15 days. The stem length increased significantly in adult plants in comparison with young in all variants of the experiment due to the formation of new internodes and their growth. The linear size of the stem was slightly larger (by 17.2 and 14.6%) in plants previously treated with 50 and 100 μM Cu^{2+} relative to the control.



(a) (b) (c)

FIGURE 1. The length of hypocotyl and stem in 15-days old (a) and 45-day old (b) *Z. elegans* plants. The diameter of axial organs (c) in 45-days old plant. On the X axis is copper ions concentration. * indicates significant differences in comparison with the control at $p < 0.05$ (Student's *t*-test)

As plant growth recovered after the stress factor was removed, in 45-day old plants the anatomical characteristics were analyzed. In plants previously exposed to Cu^{2+} the increase of root and hypocotyl diameter was observed. The root thickness was 1180 ± 37 μm in untreated plants, 1460 ± 23 and 1420 ± 29 μm in the recovered plant, after

treatment with 50 and 100 μM Cu^{2+} or increased by 23.7 and 15.2% after recovery. The diameter of the stele was significantly higher by 12.1 and 15.5% compared to the control group (Fig. 2). The same tendency was observed in cortex thickness: the diameter increased by 27.4 and 16.0% in plants after recovery compared to the control. Ratio index stele/cortex area had tended to increase after recovery in plants, treated by 100 μM compared to control plants (Fig. 2).

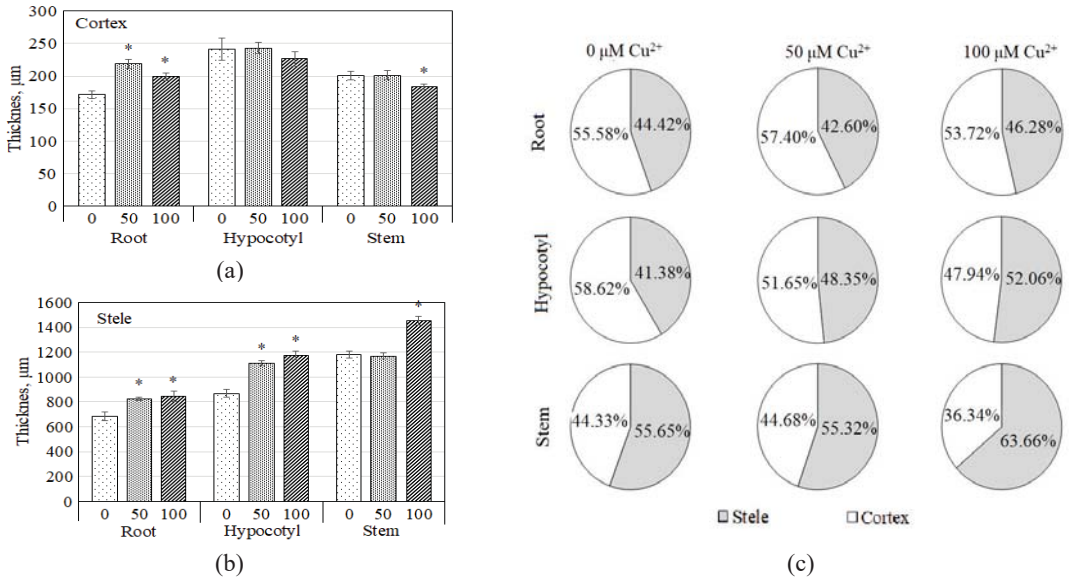


FIGURE 2. Thickness of cortex (a) and stele layer (b), the stele/cortex area index (c) in root, hypocotyl and stem in 45-day-old *Z. elegans* plants. On the X axis is copper ions concentration. *Indicates significant differences in comparison with the control at $p < 0.05$ (Student's *t*-test)

A similar effect was observed in hypocotyl. Its diameter increased by 23.3 and 20.8% (Fig. 1c). The thickness of stele was higher by 27.6 and 34.9% in plants formerly treated with 50 and 100 μM Cu^{2+} (Fig. 2). Cortex thickness had no significant changes. Ratio index stele/cortex area increased after the recovery period (Fig. 2c). The preliminary treatment with 50 μM Cu^{2+} had no impact on thickness of the stem and stela on the 45-day (Fig. 1c). On contrary, in plants, previously treated by 100 μM, the diameter of stem and stele increased by 18.5 and 23.1%, compared to control plants (Fig. 2). Index stele/cortex area did not change in stem in the case with 50 μM Cu^{2+}, compared to control, but increased in plants previously treated with 100 μM Cu^{2+} (Fig. 2c).

TABLE 1. Linear size of parenchyma cell of cortex in root, hypocotyl and stem in 45-day-old *Z. elegans* in cross and longitudinal section. *Significantly different in comparison with the control at $p < 0.05$ level (Student's *t*-test).

Parameter	Organ	Control	Cu^{2+} 50 μM	Cu^{2+} 100 μM
Diameter, μm	root	97.1 ± 2.6	123.0 ± 3.4*	133.5 ± 4.1*
	hypocotyl	79.6 ± 1.8	89.3 ± 2.3*	88.9 ± 1.8*
	stem	35.6 ± 0.7	41.8 ± 0.9*	51.8 ± 0.97*
Length, μm	root	111.8 ± 5.3	89.3 ± 2.3*	88.9 ± 1.8*
	hypocotyl	115.9 ± 2.7	138.9 ± 3.1*	159.1 ± 4.6*
	stem	114.7 ± 2.4	128.9 ± 3.0*	122.5 ± 2.2*

As the length of hypocotyl and stem was higher in copper stressed *Z. elegans*, we measured the lengths of parenchyma cells in cortex and metaxylem in root, hypocotyl and stem (Table 1, 2). The diameter of parenchyma cells was increased in plant previously treated with 50 and 100 μM Cu^{2+} (Table 1). The increase in the linear size of cells was more prominent in the root (parameter significant increased by 26.8% and 37.1% respectively, compared to the control group) and stem (raised by 45.5% under 100 μM Cu^{2+}).

We observed the decrease of cortex parenchyma cells length in root by 19.8 and 20.7% in plants formerly treated with 50 and 100 μM Cu^{2+}. Contrarily, in hypocotyl, it increased by 19.8% and 38.2% respectively. In the stem, the length of cortex parenchyma increased by 12.4 and 6.8%.in plants previously treated with 50 and 100 μM Cu^{2+} respectively.

After the recovery period, the increase of metaxylem cell diameter in axial organs of *Z. elegans* (Table 2) was shown. It was significantly higher by 15.9 and 14.4% in root, 22.2 and 37.5% in hypocotyl, 25 and 17.2% in stem in plants previously treated with 50 and 100 μM Cu^{2+} respectively compared to the control. In root and hypocotyl, the metaxylem cell length did not change in plants formerly treated with 50 μM Cu^{2+}, but significantly increased by 11.5% and 20.7% respectively at 100 μM Cu^{2+} compared to untreated plants. In the stem, the length of metaxylem cells increased by 13.6 and 8.7% in plants previously treated with 50 and 100 μM Cu^{2+} compared to untreated plants.

The metaxylem cell wall thickness had tended to decrease after stress relief. Although it is not a statistically significant difference, these changes negatively correlate with the increased diameter of cells. In the root, the metaxylem cell wall thickness significantly reduced in plants previously treated with 100 μM Cu^{2+} by 15.1% compared to untreated plants.

TABLE 2. Linear size and cell wall thicknesses of metaxylem cell in root, hypocotyl and stem in 45-day-old *Z. elegans* in cross and longitudinal section. *Significantly different in comparison with the control at $p < 0.05$ level (Student's *t*-test).

Parameter	Organ	Control	Cu^{2+} 50 μM	Cu^{2+} 100 μM
Diameter, μm	root	27.6 ± 0.8	32.0 ± 1.1*	31.6 ± 1.4*
	hypocotyl	26.1 ± 0.6	31.9 ± 0.5*	35.9 ± 0.6*
	stem	26.0 ± 0.5	29.4 ± 0.2*	30.0 ± 0.5*
Length, μm	root	196.5 ± 3.8	197.4 ± 4.3	229.8 ± 6.7*
	hypocotyl	251.0 ± 2.9	266.2 ± 8.3	303.1 ± 9.3*
	stem	300.8 ± 5.4	341.9 ± 5.6*	326.2 ± 9.2*
Metaxylem cell wall, μm	root	2.71 ± 0.18	2.51 ± 0.09	2.30 ± 0.01*
	hypocotyl	3.32 ± 0.22	3.00 ± 0.1	2.94 ± 0.14
	stem	2.88 ± 0.07	2.85 ± 0.07	2.74 ± 0.16

The hypocotyl length of young *Z. elegans* decreased under copper stress. Excess copper ions in a media lead to reduction in the length of stem that was found in *Withania somnifera* L., *Oryza sativa* L., *Belamcanda chinensis* L. [12–14]. A decrease of linear size and growth of organs are associated with the redistribution of energy for the synthesis of low-molecular-weight antioxidants and enzymes, the accumulation of ABA and ethylene [1, 2]. This redistribution of energy in stress-resistant plants leads to the induction of growth during the recovery stage [15]. According to our study, stress relief led to an increase in plant length and axial organ size.

We assume that the growth of the hypocotyl and the stem could recover due to the induction of cell elongation (auxin-dependent or acid growth). Our hypothesis is based on the fact that the removal of stress caused by copper ions leads to the normalization of auxin transport and a decrease in the pH of the apoplast to the physiological level, restoration of the activity of expansines, and, as a consequence, expansion of the cell walls [16].

The root system plays an important role in the defense mechanism in higher plants to HM stress, because it directly contacts with metal ions. It's known that almost all Cu^{2+} ions could be fixed in the root tissues in the cell walls, especially of bark and cortex [8, 7]. On the one hand, evaluation of ROS under copper stress stimulates lignification via peroxidases activities and leads to thickening of cell wall. On the other hand, if activities of peroxidases are limited, over-production of H_2O_2 will cause the breaking of the polymer links and decreases strength of cell wall [3].

The increase in diameter of root in *Z. elegans* that related to the enlargement of cortex and stele after recovery to stress. The same effect was noted for roots of Cu-treated *Oreganum vulgare* L.*:* an increase of the relative volumes of the cortex cells, diameter and volume of xylem cells [7]. Cortex cells could be the barrier against HM-transport into the vascular system in root: the accumulation of Cu^{2+} in apoplast is one of the mechanisms of plant adaptations [17].

We suggested that, the increase of the stele diameter in hypocotyl *Z. elegans* was associated with a different redistribution of Cu^{2+} and ROS as compared to the root tissues. It was reported that copper ions were predominantly localized near the vascular bundle and in cells of vascular tissues, but their amount decreased in the epidermis and endoderm [18]. Probably, these factors activated another mechanism of tissue adaptation compare to root: increase of stele diameter and volume of parenchyma cells in stele of *Z. elegans*.

The differentiation of stem tissues in *Z. elegans* started later than hypocotyl (approximately in 8-days-old plants). Stem growth took place mostly at the stage of plant recovery after stress. Because the impact of Cu^{2+} was limited during stem growth, structural changes were less pronounced. The changes in tissues were different in plants previously treated by 50 and 100 μM Cu^{2+}. The increase of stele diameter at 100 μM could be associated with the toxic impact of copper ions [12, 13].

It is known that the cell wall tensile strength is one of the factors of plant cell expansion. Probably, in our experiment, the growth of metaxylem cells by expansion increased because of pH-depended expansion of cell wall after recovery [16]. The increase of thickness of xylem cell wall due to lignification under copper stress was shown in the research [19]. In the present work, we observed the tendency of loosening cell wall, that could indicate the low resistance of *Z. elegans* to stressor. Also, the length of parenchymal cells of cortex decreased in root but increased in hypocotyl and stem. The enhancement of cell expansion could increase the stem length of copper-treated plant.

Under the impact of HM ions, the plasticity of cell walls decreases due to their binding of carboxyl groups of pectins, ectopic lignification [3, 16]. A decrease in the cross-sectional area of the vessels, a decrease in the proportion of xylem in the vascular bundles leads to a violation of the hydraulic conductivity and water regime of plants [9]. Probably, after stress relief, the normalization of physiological processes led to an increase in the activity of the cambium and contributed to an increase of the stele compared to other tissues in the stem and hypocotyl of *Z. elegans*. pH-dependent cell expansion resulted in an increase in the cross-sectional area of the xylem vessels and consequently optimal water transport to the leaves.

CONCLUSION

The anatomical changes in the root, hypocotyl and stem of copper-treated plants indicated that Cu^{2+} has a significant impact on the tissue's traits of *Z. elegans*. The increase of diameter of axial organs, stele thicknesses, diameter and length of cortex and metaxylem cells in roots and hypocotyl indicates on the recovery of growth after stress relief.

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