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COPPER-INDUCED CHANGES IN ANTIOXIDATIVE RESPONSE AND SOLUBLE PROTEIN LEVEL IN *TRITICUM AESTIVUM* CV. BETI SEEDLINGS

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ABSTRACT

The changes in superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities, and in soluble protein level were analysed in *Triticum aestivum* cv Beti seedlings, after seed exposure to copper, provided as copper acetate and copper citrate, at four concentrations (10, 25, 50, and 100 µM) containing 0.64, 1.59, 3.18, 6.35 µg ml^{-1} Cu^{2+}, and 1.91, 4.77, 9.53, 19.06 µg ml^{-1} Cu^{2+}, respectively. SOD and POD showed similar patterns, with relatively small fluctuations compared to control. Considerable rise of activity was registered only in 25 µM copper citrate (increase rate of +152.90% for SOD, and +70.51% for POD). CAT activity was lower than control in all variants, the smallest level being in 25 µM copper citrate-treated variant (decrease rate of -65.41%). Generally, copper had negative repercussions on soluble protein level. Higher SOD and POD activities and CAT decline in all copper-treated variants indicate that SOD and POD play a more important role than CAT in preventing copper-induced oxidative stress, in the studied wheat cultivar.

Key words: copper, pollution, antioxidative enzymes, oxidative stress, soluble protein, wheat.

INTRODUCTION

Heavy metal pollution has become a serious environmental problem in the last decades. The most toxic metals both for higher plants and microorganisms are Hg, Cu, Ni, Pb, Co, Cd, and possibly Ag, Be, and Sn (Kabata-Pendias and Pendias, 2001). Copper is among the most abundant metals in agricultural soils, one of the sources for its high amount being the largely use of copper compounds as fungicides, algaecides, and bactericides. The concentration can reach 500 mg kg^{-1} in vineyard soils, even 5800 mg kg^{-1} in the vicinity of copper-nickel smelters (Souguir et al., 2008). As an essential micronutrient required in trace amount to plants, copper is involved in protein and carbohydrate metabolism, cell wall lignification, photosynthesis, respiration, seed germination or disease resistance (Kabata-Pendias and Pendias, 2001; Meng et al., 2007; Sudo et al., 2008), but excess copper has negative repercussions on photosynthesis, electron transport, respiration, growth, membrane integrity, and plant vigour. In range of 20-100 ppm/dry weight, copper is considered to be excessive or toxic for crops (Kabata-Pendias and Pendias, 2001). In Romania, in 2004, 0.9 million hectares were affected by chemical pollution, 0.2 from them being excessively polluted with heavy metals, acid rains and other noxious agents etc. In severely polluted areas, some heavy metals exceeded the maximum allowable limits: 3-30 - fold for Pb, 2-32 - fold for Cd, 2-3 - fold for Zn, 2-4 - fold for Cu, and excessive contents of these metals were detected in sugar beet, forage beet, maize, potatoes and winter wheat (Romanian Sanitation Country Profile, 2004).

Copper is a redox active heavy metal and its auto oxidation results in formation of superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) *via* Fenton-type reactions, with subsequent oxidative injury of proteins, membrane lipids, nucleic acids (Schützendübel and Polle, 2002;

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Qi et al., 2006; Sudo et al., 2008; Singh et al., 2010). To protect the cells against oxidative injury, the living organisms developed complex antioxidative systems, both non-enzymatic (low molecular mass antioxidants such as ascorbate, glutathione, tocopherols, phenolic compounds) and enzymatic scavengers of activated oxygen (SOD, E.C. 1.15.1.1; POD, E.C. 1.11.1.7; CAT, E.C. 1.11.1.6) (Boojar and Farahi, 2011).

Plants are valuable systems in monitoring of cytotoxic effects induced by environmental stressors, but sometimes the reports are contradictory or even conflicting, inclusively concerning the copper action on the plant defence systems against free radicals, due to different growth conditions, copper concentration, types of copper compounds, plant parts taken into consideration, different threshold levels of susceptibility to copper stress (Navari-Izzo et al., 1998; Souguir et al., 2008).

Wheat is one of the world's major crops. About 1/3 of the world's population uses it for nourishment and it is also important as livestock feed. In Romania, wheat represents ~ 25% of arable surface and 40% of cereal areas, winter cultivars occupying 99% of wheat cultivated surface. Out of edible value, wheat grain antioxidants are known to lower the carcinogenic risk by reducing the level of free radicals in human body - lignans showed antitumor activity against colon cancer SW480 cells (Qu et al., 2005).

For these reasons, the overall objective of this work was to evaluate the amplitude of antioxidative response, by quantification of protective enzyme (SOD, CAT, POD) activities and of soluble protein level in seedlings of *Triticum aestivum* L. cv. Beti, after exposure to different concentrations of copper (Cu^{2+}) provided as acetate and citrate.

MATERIAL AND METHODS

Seeds of *Triticum aestivum* L. cv. Beti were used. Beti is a commercial cultivar of winter common wheat, obtained at Agricultural Research and Development Station of Podu-Iloaiei, Romania, with a three years average yield = 5600 kg/ha, and 1000-grain weight = 42-46 g (Ioan and Ciolpan, 2004). Seeds were surface-sterilized with freshly prepared 5% sodium hypochloride, then rinsed and 4 h treated with 10 μM, 25 μM, 50 μM, 100 μM copper acetate monohydrate (MW = 199.63 g mol^{-1}) and copper citrate (MW = 568.84 g mol^{-1}). Copper concentrations in solutions were the following: 0.64, 1.59, 3.18, 6.35 μg ml^{-1} Cu^{2+} for copper acetate, and 1.91, 4.77, 9.53, 19.06 μg ml^{-1} Cu^{2+} for copper citrate. Control was set up by seed immersion in distilled water. After treatment, the seeds were washed with running tap water, and placed in Petri dishes for germination. Antioxidative enzyme activity and soluble protein amount were evaluated in 7-days old wheat seedlings.

To prepare enzyme extracts, the fresh samples were weighed, cut into small pieces, homogenized with a mortar and pestle, and collected in 0.2 M phosphate buffer, pH 7.0. After centrifugation (3000 rpm/min, 15 min) of homogenates, the supernatant fractions were used to determine enzyme activity.

SOD activity was measured according to Winterbourn's assay with slight modifications (Artenie et al., 2008), based on SOD ability to inhibit the reduction of nitro blue tetrazolium (NBT) by the superoxide radicals generated through reoxidation of photochemically reduced riboflavin. Absorbance was recorded at λ = 560 nm using UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer. One SOD unit is defined as the enzyme amount producing 50% inhibition of NBT reduction under assay conditions.

CAT activity was assayed by Sinha's procedure with minor adaptations (Artenie et al., 2008), based on determination of chromium acid, obtained by reduction of $K_2Cr_2O_7$, in acid medium, in the presence of non decomposed H_2O_2, at λ = 570 nm. Enzyme activity was expressed as CAT units per mg protein.

POD activity was established by Gudkova and Degtiari method, with minor adaptations (Artenie et al., 2008), based on the measurement of the colour intensity of product of o-dianisidine oxidation with H_2O_2, in the presence of peroxidase. Colour intensity was measured at λ = 540 nm. One POD unit

corresponds to the enzyme amount catalysing decomposition of 1 μM H_2O_2 min^{-1}, in optimal conditions. Enzyme activity is expressed in POD units per mg protein.

The soluble protein content was determined according to Bradford method (Bradford, 1976), by using bovine serum albumin as standard. Method principle refers to the binding of Coomassie Brilliant Blue G-250 at aromatic amino acid radicals and measuring of extinction at λ = 595 nm. The results are expressed in mg protein per g fresh weight. In order to compare the sensitivity of each parameter, changes in their values were calculated as a percentage of control value (set to 100%). The increase/decrease rates were established by the equation: $(1 - x/y)$ 100, where y is the average value detected in the control and x is one of each treated samples. Data are given as means ± standard error of the means ($\bar{x}$ ±SE). To calculate and to graphically represent the statistical parameters, the Microsoft Office Excel 2003 software of Windows XP operating system was used.

RESULTS

In our study, heterogeneous responses were obtained from antioxidative enzymes determined in 7-days old wheat seedlings, after copper treatment (Table 1, Figure 1). By catalysing dismutation reaction ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) and neutralizing the reactive $O_2^{\bullet-}$ radicals, SOD represents the first line of cell defence against ROS generated by heavy metal exposure.

Table 1. SOD, CAT, and POD activities ($\bar{x}$ ±SE) and increase/decrease rates of antioxidative enzyme activity in 7-days old wheat seedlings, after copper treatment

Variant		SOD activity		CAT activity		POD activity	
		U/mg protein	-/+ rate (%)	U/mg protein	-/+ rate (%)	U/mg protein	-/+ rate (%)
Copper acetate	Control	4.31±0.13	0.00	393.82±14.33	0.00	6.24±0.28	0.00
	10 μM	5.04±0.40	+16.93	349.43±21.10	-11.28	6.50±0.55	+4.16
	25 μM	5.20±0.17	+20.64	337.27±23.23	-14.36	6.21±0.74	-0.49
	50 μM	4.26±0.62	-1.17	305.43±17.05	-22.45	5.93±0.44	-4.97
	100 μM	5.23±0.26	+21.34	240.65±15.12	-8.90	7.28±0.69	+16.66
Copper citrate	Control	4.31±0.13	0.00	393.82±14.33	0.00	6.24±0.28	0.00
	10 μM	4.83±0.34	+12.06	186.09±8.67	-52.75	5.98±0.31	-4.17
	25 μM	10.90±0.76	+152.90	136.26±11.42	-65.41	10.64±0.97	+70.51
	50 μM	5.10±0.38	+18.32	345.59±20.43	-12.25	6.66±0.64	+6.73
	100 μM	4.04±0.23	-6.27	334.33±17.28	-15.11	5.89±0.80	-5.61

- decrease rate; + increase rate

In this work, except for 50 μM copper acetate and 100 μM copper citrate, in which small decreases of SOD activity were noticed, all the other variants exceeded the control with ~17-21%, but the treatment resulted in a considerable rise of SOD activity (stimulation rate is 152.9%) in 25 μM copper citrate. This fact indicates the detoxification by neutralization of $O_2^{\bullet-}$ radicals generated at this concentration. At 25 μM, copper citrate also determined significant changes in CAT and POD activity, and in soluble protein level.

CAT, a major ROS-scavenging enzyme in all aerobic organisms, converts the toxic H_2O_2 resulted from SOD reaction to H_2O and O_2, in peroxisomes. In copper acetate treatments, CAT response registered a descendant trend with copper concentration increase (inhibition rate increased from 11% to 38%).

Also, all copper citrate concentrations showed CAT activity smaller than control, but the pattern was different (Figure 1). So, at 10 µM and 25 µM copper citrate, inhibition rate of CAT activity was high (-52%, and -65%, respectively), whereas 50 µM and 100 µM concentrations reduced the CAT activity with 12-15% only, compared to control. The smallest CAT activity was in 25 µM copper citrate - treated variant.

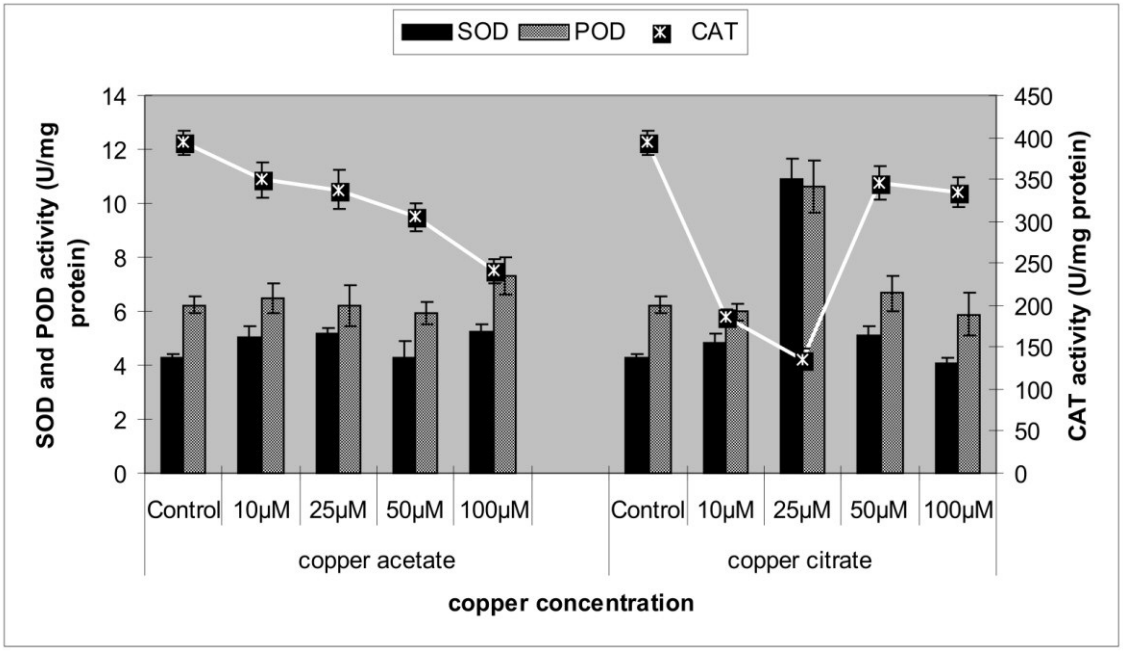

Figure 1. Comparative behaviour of SOD, CAT and POD in 7-days old wheat seedlings, after copper exposure (bars represent standard errors of the means)

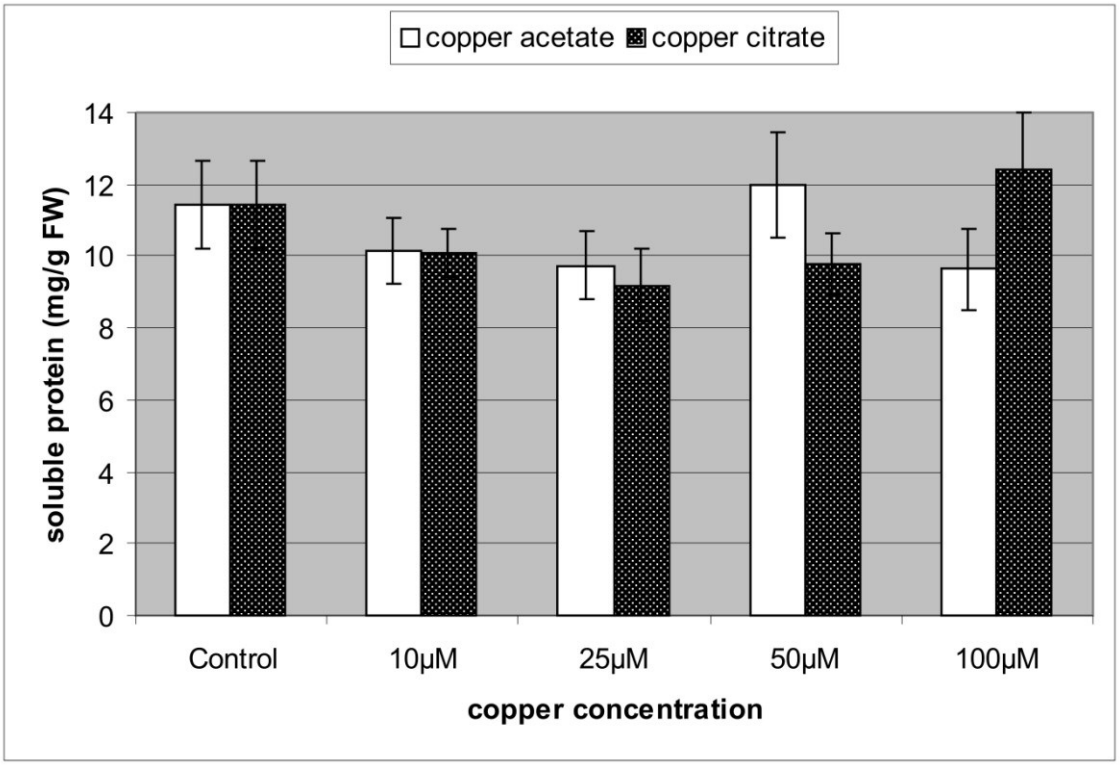

Figure 2. Mean values of soluble protein level in 7-days old wheat seedlings, after copper exposure (bars represent standard errors of the means)

POD is involved in the oxidation of many substrata, in the presence of H_2O_2, with H_2O production: $AH_2 + H_2O_2 \rightarrow A + 2\ H_2O$. This enzyme is stimulated by H_2O_2 accumulation, being able to scavenge this toxic compound. Compared to CAT, POD possesses a higher affinity towards H_2O_2, but has lower processing rate. Our research evidenced small increases of POD activity in 10 µM and 100 µM copper acetate, and 50 µM copper citrate. In 25 µM and 50 µM copper acetate, 10 µM and 100 µM copper citrate, the scavenging function of POD was impaired and lower levels of enzyme activity were noticed. In variant exposed to 25 µM copper citrate, POD reached the maximum activity (stimulation rate over 70%), CAT activity is ~3-fold smaller, but POD is 1.7-fold greater than control. As Figure 1 shows, SOD and POD profiles were very similar for both copper compounds and in all concentrations. Significant differences were not evidenced compared to control, except for 25 µM copper citrate only, with enhanced POD and SOD activities. A similar situation, but at a smaller scale of values, was present in the variant exposed to 100 µM copper acetate, in which SOD and POD were higher than control, and CAT was 30% under control. Except CAT evolution in copper acetate treated variants, in which decline of enzyme activity was in direct relation to the increase of copper concentration, other evident linear relationships between enzyme activities and heavy metal concentration were not observed.

No direct relationship between copper concentration and protein level was evidenced (Figure 2). Inhibition rates of protein synthesis ranged between 11 and 19%, except for 50 µM copper acetate and 100 µM copper citrate where small increases were noticed. The most important decline of soluble protein level was also registered in 25 µM copper citrate.

DISCUSSION

For a long time, ROS have been considered only as dangerous molecules, whose levels need to be kept as low as possible. Now it has been realized that they play important roles in the defence against pathogens, in programmed cell death, in plant development and in regulation of gene expression (Schützendübel and Polle, 2002). Therefore, it is necessary for cells to control the level of ROS tightly, but not to eliminate them completely (Pitzschke et al., 2006). In the study of García et al. (1999), as in 25 µM copper citrate in our study, a lower copper concentration was found to be toxic for sunflower plants causing oxidative stress and SOD and POD increase. In garlic, SOD and POD increases were generally induced by higher Cu^{2+} concentrations and exposures, and CAT was strongly inhibited at greater levels of the heavy metal (Meng et al., 2007). Increment of POD and SOD has been evidenced in rice, and no changes were found in CAT activity (Chen et al., 2000). It seems that 25 µM concentration of copper citrate constitutes the "stress point" for copper toxicity in the studied wheat cultivar, because the physiological state of the cell was changed and this change was reflected in the increase of the enzymes responsible for antioxidative protection, and in the decrease of soluble protein amount. CAT decreases observed in all copper-treated variants indicate a limited ability of this enzyme to eliminate ROS and to minimize the oxidative state. POD appears as alternate mechanism in H_2O_2 removal, so compensating for reduced CAT activity in respective variant. Possibly, CAT is a less efficient H_2O_2-scavenger than POD because of its low substrate affinity. Numerous studies evidenced a considerable variation of antioxidative enzyme responses to copper stress, depending on species, analysed organ and even tissue type, plant age, treatment duration (Meng et al., 2007; Gao et al., 2008; Zhao et al., 2010; Boojar and Farahi, 2011). For example, SOD increases have been reported in pea, soybean, and oat as response to excess copper (Luna et al., 1994; Rabinowitch and Fridovich, 1983), whereas in pearl millet and in rice Lidon and Henriques, 1993; Reddy and Venkaiah, 1988) an inhibition of SOD activity has been noticed. High levels of POD in copper exposures have been reported in other plants such as common

duckweed and brown mustard (Teisseire and Guy, 2000; Singh et al., 2010). CAT enhancement was reported in *Arabidopsis* and *Lycopersicon* plants, after copper treatment (Mediouni et al., 2008), but the metal had an adverse effect on CAT activity in cabbage (Chatterjee and Chatterjee, 2000). Despite of numerous studies, the mechanisms by which the enzymes ensure the antioxidative defence are not entirely clear. Like other heavy metals, copper can induce the overexpression of genes encoding for antioxidative enzymes or can determine changes at transcriptional level (Sudo et al., 2007). High SOD activity results from copper-induced accumulation of $O_2^{\bullet-}$ radicals which trigger the genes encoding for SOD and from *de novo* enzyme synthesis (Meng et al., 2007), whereas a decrease in SOD activity under copper treatment might indicate production of oxygen radicals at levels inactivating SOD protein. CAT decline may arise from copper-induced modifications in the assembly of enzyme subunits, as it happens with other heavy metals (Verma and Dubey, 2003). For POD, enzyme augmentation is possible by releasing of cell wall-located enzyme as response to the stress to which the plants are subjected.

The multigene determinism of the studied antioxidative enzymes offers possible explanations for some contradictory results relative to variable trends of SOD, CAT and POD. Plants have multiple enzyme isoforms encoded by more than one gene because they developed more complex antioxidant defence strategies. Plant CAT gene family is constituted by many genes. Ten POD genes were identified in *Tamarix hispida* (Gao et al., 2010), while in *Eucalyptus grandis* L., 36 gene clusters encoding for antioxidant enzymes have been identified, 6 from these for ascorbate-POD isozymes, 3 for CAT proteins, and 12 for SOD isozymes (Teixeira et al., 2005). In wheat, three SOD isoforms have been detected, both in roots and leaves, and 16 POD isoforms (Song et al., 2007). Another factor influencing the antioxidative enzyme patterns is the different degree of plant tolerance or sensitivity to the heavy metals: alfalfa and barley are highly tolerant, rice and potato are less tolerant to copper stress, and wheat is very sensitive to copper deficiency (Sudo et al., 2008). Schützendübel and Polle (2002) sustained the assumption that the ability of plants to ensure the antioxidative protection against heavy metal induced stress is rather limited, since many studies showed that exposure to elevated concentrations of redox reactive metals resulted in decreased and not in increased activities of antioxidative enzymes. Low values of antioxidative enzyme activities are equivalent with a reduced protection against ROS, fact promoting accumulation of these radicals and allowing the materialization of cytotoxic and eventually genotoxic effect. The rise of enzyme activity as response to heavy metal exposure is a proof of detoxifying ability of wheat seedlings by removal of ROS generated in stressed organisms. Higher values of SOD and POD, more or less noticeable, and the CAT decline in all copper-treated variants indicate that SOD and POD play a more important role in preventing heavy metal-induced oxidative stress.

Protein oxidative injury induced by ROS generated under heavy metal stress refers to amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, changes in electrical charge. In some cases, oxidation of susceptible residues such as cysteine and histidine leads to the production of *oxo* groups which “mark” the protein macromolecules for an increased susceptibility to proteolysis (Davies, 2003). In literature, the reported data are numerous and greatly variable. For example, copper excess lowered protein level in cabbage and barley (Chatterjee and Chatterjee, 2000; Guo et al., 2007), but copper-related increases have been evidenced in maize and *Jatropha curcas* (Gao et al., 2010; Qi et al., 2006). Possible explanations for protein increase could be the formation of carbonyl derivatives in presence of copper, or *de novo* synthesis of some stress proteins as result of exposure to exogenous factor (Verma and Dubey, 2003). The decline in soluble protein amount results from action of proteolytic enzymes or can be attributed to the loss of genetic material by chromosome fragmentation, micronuclei and lagging

chromosomes (Truta et al., unpublished), with repercussions on synthesis of proteins encoded by the genes lost in this way. Heterogeneous responses showing large interspecific and intraspecific variability are signalled in literature for many other heavy metals. Probably, the oxidative stress induced by heavy metals is a general phenomenon, but the antioxidative responses are specific and depend on genetic potential of each cultivar or species. We presume that these observations could contribute to the knowledge basis on complex antioxidative responses of plants to the stress exerted by numerous xenobiotic environmental factors, some of them with toxic potential.

CONCLUSIONS

Two of the measured enzymes - SOD and POD - exhibited similar profiles, with relatively small fluctuations compared to control, but with considerable rise of activity in 25 μM copper citrate. In copper acetate treatments, slight increases of SOD and POD were evidenced comparatively to control, whereas CAT registered a progressive decline with concentration increase. CAT activity was lower than control in all variants, the smallest level being in 25 μM copper citrate-treated variant. Generally, copper had negative repercussions on soluble protein level. Higher SOD and POD activities and lower CAT activity in all copper-treated variants indicate that SOD and POD play a more important role than CAT in preventing copper-induced oxidative stress, in the studied wheat cultivar. Except CAT evolution in copper acetate treated variants, in which decline of enzyme activity was in direct relation to the increase of copper concentration, other evident linear relationship between enzyme activities and heavy metal concentration was not observed.

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