EFFECTS INDUCED BY ZINC ON SOME ANTIOXIDATIVE ENZYME ACTIVITIES AND ON SOLUBLE PROTEIN CONTENT IN YOUNG PLANTLETS OF BARLEY

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Abstract: Zinc (Zn), the second most abundant transition metal, after iron, is an essential micronutrient for plants. Generally, Zn is considered one of the least toxic heavy metals, but in excess it is toxic to plants, generally by generation of reactive oxygen species and induction of oxidative stress. For these reasons, in this study it was analyzed the impact of short-term Zn treatment on superoxide dismutase, catalase, peroxidase, and on soluble protein content in the seedlings of an autumn six-row *Hordeum vulgare* L. commercial cultivar, in the early ontogeny. Zn was added as zinc sulphate and zinc acetate at the concentrations of 10 μM, 100 μM, 250 μM, and 500 μM (Zn^{2+} content: 0.654, 6.54, 16.35, and 32.70 μg ml^{-1}, respectively). Heterogeneous enzymatic responses have been obtained under zinc stress. Soluble protein amounts lowered under Zn action, the inhibitive effect being more marked in older barley plantlets.

INTRODUCTION

Zinc, the second most abundant transition metal, after iron, is an essential micronutrient for plants (Salama and El Fouli, 2008; Jain *et al.*, 2010). It is structural stabilizing factor of cellular proteins, cell membrane and DNA-linking proteins (Salama and El Fouly, 2008) and it serves as catalyst or co-catalyst for many essential enzymes in living organisms (Päivöke, 2003), playing also a significant role in the control of gene expression and DNA transcription (Cakmak and Marschner, 1993; Päivöke, 2003). Zinc is considered one of the least toxic heavy metals (Balsberg-Pahlsson, 1989), but at high concentrations it is detrimental to plants, by inhibiting the growth and altering essential physiological processes (Reichman, 2002; Jain *et al.*, 2010). Plant species, genotype, and growth stage are the most important factors influencing the toxicity limits for zinc. The upper toxic levels range between 100 and 500 ppm (Macnicol and Beckett, 1985), whereas Paschke *et al.* (2006) sustain that EC_{50} varies between 43 and 996 mg Zn L^{-1}. Davis *et al.* (1978) reported a level of ~300 ppm zinc-inducing toxic effects in young barley. The minimum necessary amount in plant tissues is comprised between 1 and 5 μg g^{-1} depending on species (Çavusoğlu *et al.*, 2009), but in some species such as soybean, bush beans and pea, zinc requirements are high - 450, 250 and 380-500 mg Zn kg^{-1} dry weight (Macnicol and Beckett, 1985).

It is well documented that in the plants exposed to some xenobiotic stresses (chilling stress, salt stress, radiation, heavy metal stress) the formation of ROS and generation of oxidative stress are the major factors causing the cellular damage (El-Beltagi and Mohamed, 2013). In order to minimize the damaging effects of ROS, the living organisms developed non-enzymatic defence systems (ascorbic acid, reduced glutathione, tocopherols *etc*.) and enzymatic protection mechanisms (superoxide dismutase, catalase, and peroxidases). Unlike iron and copper, zinc is redox-stable under physiological conditions; it is not able to generate ROS directly through Haber–Weiss reactions, so that the formation of ROS and generation of oxidative stress in plants could be indirect consequences of zinc toxicity (Wang *et al.*, 2009a). Zinc-induced ROS determined oxidative injury in several plant organisms, consisting in adverse cellular effects such as lipid peroxidation, denaturation of proteins, and mutations in DNA (Prasad *et al.,* 1999; Chang *et al.*, 2005; Wang *et al.* 2009a; Wang *et al.*, 2009b), but in literature the data reported on the behaviour of the antioxidative enzyme activities are very different, even contrary.

In this work, the extent of the enzymatic antioxidative response was assessed in barley seedlings after short-term exposure to different concentrations of zinc, provided as zinc acetate and zinc sulphate, by analyzing the activities of superoxide dismutase, catalase, and peroxidase in two early ontogenetic moments. Also, the changes caused in the content of soluble protein were analyzed. Barley was chosen as biological material because of its multiple agricultural, economical, edible and pharmaceutical valences. It is the fourth most important crop in the world and it constitutes a main link in the food chain, so that any new data concerning zinc-induced toxicity and the extent of biochemical responses are of interest.

MATERIALS AND METHODS

Plant material and treatment conditions. Seeds of early, autumn, six-row *Hordeum vulgare* L. ('Madalin' commercial cultivar), provided by Center for Agricultural Research and Development – Secuieni (Neamt), were 3 h immersed in each of the aqueous solutions of 10 μM, 100 μM, 250 μM, 500 μM zinc sulphate - $ZnSO_4.7H_2O$ (MW = 287.54 g/mol) and zinc acetate - $Zn(CH_3COO)_2.2H_2O$ (MW = 201.483 g/mol). Zn^{2+} content in both compounds was: 0.654, 6.54, 16.35, and 32.70 μg ml^{-1}, respectively. Controls were prepared in distilled water. Then, the treated seeds were washed, placed on filter paper in Petri dishes, and maintained in dark, at 20 ºC to promote the germination. After germination, for the growth of barley seedlings it was ensured a photoperiod of 16 h light/8 h dark. The enzyme activities and the soluble protein content were determined in shoot tissues of 5-day old and 9-day old barley seedlings.

Quantification of the enzymes. For enzyme extraction, fresh plant material was weighed, cut into small pieces, homogenized and extracted in 0.2 M phosphate buffer, pH 7.0. The homogenates were centrifuged (3000 rpm, 15 min), and the resulting supernatants were used to determine enzyme activity.

Superoxide dismutase activity was measured according to Winterbourn's assay with slight modifications (Artenie *et al.,* 2008), based on enzyme ability to inhibit the photo-reduction of nitro blue tetrazolium (NBT) by the superoxide radicals generated through reoxidation of photo chemically reduced riboflavin (λ = 560 nm). One unit of superoxide dismutase activity is defined as the enzyme amount required for the inhibition of the photoreduction of NBT by 50%, under assay conditions. The activity of superoxide dismutase was expressed as units mg^{-1} protein.

Catalase 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 units mg^{-1} protein.

Peroxidase 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, at λ = 540 nm. One peroxidase unit corresponds to the enzyme amount catalyzing decomposition of 1 μM H_2O_2 min^{-1}, in optimal conditions. Enzyme activity is expressed in units mg^{-1} protein.

The soluble protein was determined according to Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard. Method principle refers to the binding of Coomassie Brilliant Blue G-250 at radicals of aromatic amino acids and measuring at λ = 595 nm. The results are expressed in mg protein g^{-1} fresh weight.

The extinctions were measured using UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer.

In order to compare the sensitivity of each parameter, changes in their values were expressed as a percentage of control value (set to 100%). The increase/decrease rates were established by the equation: $(1 – x/y) \times 100$, where *y* is the average value detected in the control and *x* is one of each treated samples.

RESULTS AND DISCUSSIONS

Activity of the antioxidative enzymes after zinc exposure. The action of some biotic or abiotic stressors such as heavy metals often results in enhanced activities of the antioxidative enzymes or in activation of some non-enzymatic scavengers in order to protect the cells against oxidative xenobiotics. Superoxide dismutase is the main enzymatic detoxifiant of superoxide radicals, by catalyzing their dismutation into H_2O_2 and molecular oxygen at a very high rate Due to the action of superoxide dismutase, the concentration of H_2O_2 is expected to increase inside the cell, but other two scavenging enzymes – catalase and peroxidase – will intervene in order to metabolize it into water and oxygen (Pastori and Foyer, 2002). The regulation of these protective enzymes is essential to keep the level of reactive compounds under permanent control (Wang *et al.* 2009b; Radić *et al.*, 2010).

In this study, the results evidenced variations in superoxide dismutase, catalase, and peroxidase activities in relation to the kind of zinc compound, concentration, and moment of enzyme determination.

In 5-day old barley seedlings, noticeable increases in superoxide dismutase activity were encountered in the variants treated with zinc sulphate at the concentrations of 10 μM (increase rate = 23.95%) and 250 μM (16.16%), while in the plant material exposed to 250 μM and 500 μM zinc acetate, superoxide dismutase registered the lowest levels as compared to the control

(inhibition rate = 31.74%, and 14.98%, respectively) (Fig. 1). These smaller amounts could indicate an over production of ROS, immediately subsequent to zinc treatment, which inactivate the protein component of the enzyme (Panda and Khan, 2004). In 9-day old seedlings, except for 250 µM zinc acetate, where a non-significant negative response was observed (decrease rate was 2.23%, as compared to the control), all the other zinc-treated variants reacted to the heavy metal stress by increases in enzyme activity, although it was not established a linear relation between zinc concentration and the extent of the phenotypic response. The enhancement is slight (2.22%) in 10 µM zinc sulphate, variant characterized by the strongest initial response, but it was significant in all the other variants (between ~58% and 103% for zinc sulphate, and from 15% to ~106%, for zinc acetate).

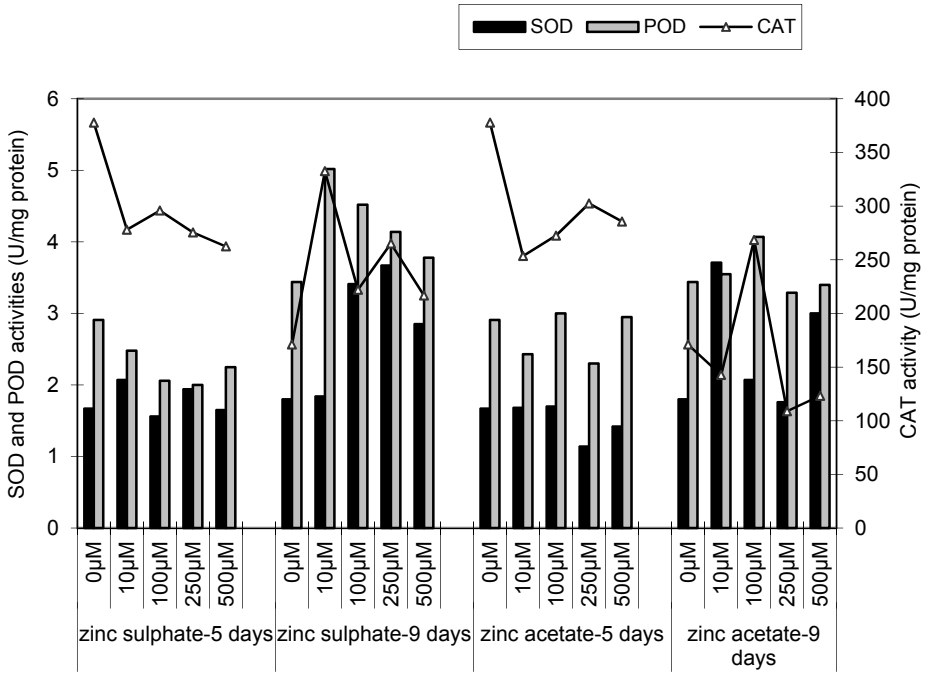

Fig. 1. The profile of superoxide dismutase, catalase, and peroxidase activities in two early ontogenetic moments of the barley seedlings, after zinc treatment

The high superoxide dismutase activity is the result of ROS overproduction caused by the disruption of cellular redox status after binding of Zn^{2+} to thiols (Dietz *et al.*, 1999) and it is generally attributed to the stimulation of the enzyme synthesis (Fatima *et al.*, 2005; Meng *et al.*, 2007). Luo *et al.* (2010) mention that the amount of the superoxide dismutase transcripts formed as response to the zinc stress is different in different tissues and at different zinc concentrations as a result of certain hierarchy of regulatory events during transcription of coding genes.

In younger barley plantlets, catalase is below the control for both zinc-containing compounds and at all tested concentrations (Fig. 1). The decrease in the catalase activity ranges between ~ 20% and ~ 30% as compared to the control, so showing the low capacity of the treated material to activate this enzymatic scavenging mechanism in the very early ontogeny. The pattern of catalase behaviour was different for the two tested compounds in 9-day old seedlings. All concentrations of zinc sulphate induced increases in catalase activity, while zinc acetate positively influenced this enzyme only at the concentration of 100 μM. Zinc-induced decline in catalase activity, reported also in other plant species (Andrade *et al.*, 2009; Radić *et al.*, 2010; Cui and Zhao, 2011), might be the result of the inhibition of enzyme biosynthesis or of the modifications in the assembling of enzyme subunits (Radić *et al.*, 2010). The enhancement in catalase level was observed in other plant species such as *Brassica juncea* (Prasad *et al.*, 1999). The lowering of catalase can be discussed in relation to the small amounts of soluble proteins detected in zinc-treated variants (Fig. 2), fact in accordance with the statement that the stress conditions inhibiting protein synthesis and reducing the rate of protein turnover cause the depletion of catalase activity (Hertwig *et al.,* 1992).

Peroxidase significantly declined in 5-day old seedlings after the exposure to zinc sulphate as well in 10 μM and 250 μM zinc acetate (Fig. 1). The concentrations of 100 μM and 500 μM zinc acetate determined no significant changes in the activity of peroxidase at this age. The most suggestive response was done by 9-day old seedlings treated with zinc sulphate, in which peroxidase activity was higher than the control in all variants and the enzyme showed a descendant trend with zinc concentration increase. Fang and Kao (2000) suggested that the stimulation of peroxidase activity by zinc may be the result of the intervention at translational level and *de novo* biosynthesis of this enzyme.

In literature, the data reported on the pattern of the antioxidative enzyme activities are very different, even inconsistent, depending on species and cultivar, age of the studied plantlets, duration of exposure to heavy metal, concentration and zinc-containing compound (Weckx and Clijsters, 1997; Panda and Khan, 2004; Salama and El Fouly, 2008; Mishra and Prakash, 2009; Jain *et al.*, 2010; Luo *et al.*, 2010; Ozdener and Aydin, 2010; Cui and Zhao, 2011; Szollosi *et al.*, 2011; Wang *et al.*, 2009a; Wang *et al.*, 2009b; Weisany *et al.*, 2012).

In this study, although zinc concentration in the two compounds is the same, the biochemical responses are different. For this, in discussion must be considered not only metal concentration, but also the intrinsic relations established between components in each chemical compound.

The rise in the activity of the antioxidative enzymes in response to certain concentrations of zinc reflects the detoxifying ability of barley seedlings by the active participation of the enzyme mechanisms to the scavenging of ROS generated under stress conditions. By contrast, the decreased levels of the antioxidative enzyme activities suggest a lower scavenging defence protection against the noxious reactive compounds formed under zinc stress, fact allowing their accumulation and the materialization of their cytotoxic and eventually genotoxic effects (Wang *et al.*, 2009b). The formation of ROS and generation of oxidative stress is one of the mechanisms by which the toxic levels of the heavy metals, including zinc, induce DNA damage and determine the occurrence of chromosome aberrations with serious repercussions on the health of the biological systems (Kawasaki *et al.*, 2013).

Effect of zinc on soluble protein content. According to Mittler (2002), the generation of the reactive oxygen intermediates under heavy metal action causes damage to proteins, nucleic acids, and lipids, and eventually leads to apoptosis and cell death. The oxidative injury of the

proteins consists in amino acid modifications, breakage of the peptide chain, aggregation of cross-linked reaction products *etc*. (Gonçalves *et al.*, 2007), while the oxidation of some amino acid residues generates the *oxo* groups which amplify the susceptibility of proteins to proteolysis (Davies, 2003). In this study, both in 5-day and 9-day old barley seedlings, all soluble protein amounts are smaller than the respective controls, but as Fig. 2 shows, the toxic effect of zinc-chromium compounds on soluble protein content was stronger in older plantlets, as compared to the levels registered in 5-day old plantlets, both in control and in the treated variants.

The soluble proteins lowered with ~15% in 9-day control as compared to 5-day control, and showed decreases ranging from ~10 % to ~35% in 9-day old barley seedlings treated with zinc-containing compounds as compared to the younger plant material. Only in 250 µM zinc acetate negligible positive values (3.03%, and 0.14%, respectively) were registered in both determinations as compared to the controls. The most deleterious effect on protein amount belongs to 10 µM zinc sulphate (inhibition rate is 21.58% in 5-day old seedlings, and 18.34% in older plantlets) and 100 µM zinc sulphate (inhibition rate is 26.61% in 9-d old plantlets), whereas zinc acetate significantly declined the protein content at the concentration of 500 µM in older plantlets (inhibition rate is 27.04%, as compared to the control).

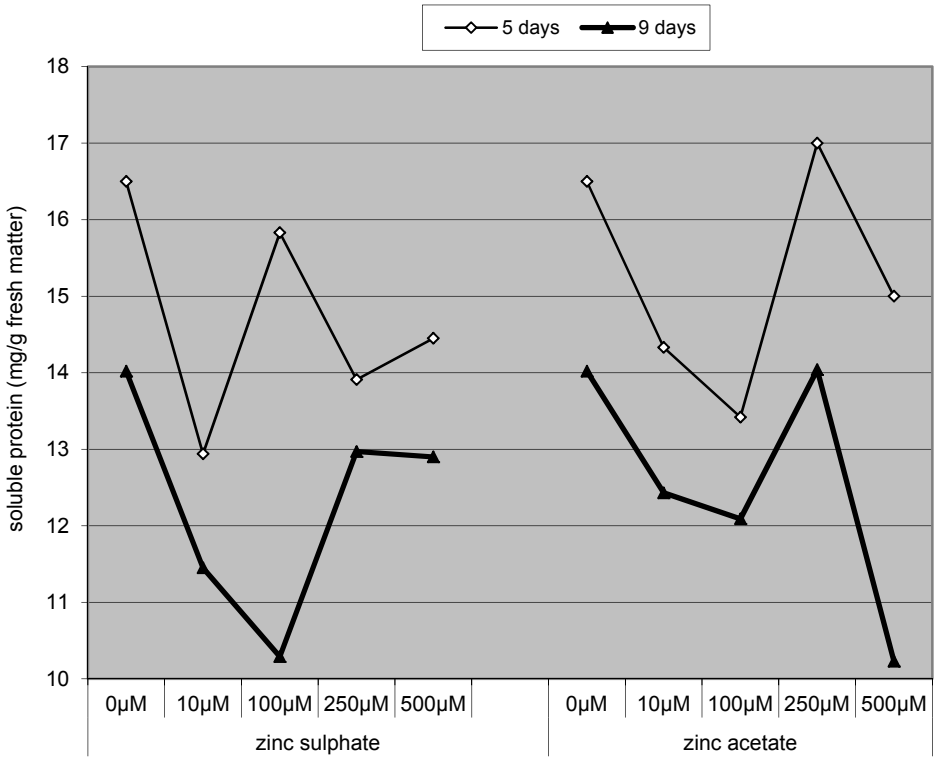

Fig. 2. Soluble protein content in early ontogeny of barley seedlings, after seed exposure to zinc sulphate and zinc acetate

The levels lower than the control reflect the toxic action of zinc on protein biosynthesis and might be due to the inhibition or the suppression of the genes responsible for protein synthesis, as result of zinc stress (Salama and El Fouli, 2008) or to the binding of heavy metal with sulfhydryl groups of proteins, with the disruption of their structure (Wu *et al.*, 1999; Manivasagaperumal *et al.*, 2011).

Also, the decline in the soluble protein amount results from the action of proteolytic enzymes or can be attributed to the loss of genetic material by certain types of chromosome aberrations, with repercussions on the synthesis of proteins encoded by the genes lost in this way.

Our results on the decrease of soluble protein content as a result of zinc treatments are in accordance with some data reported in other plant species (Khudsar *et al.*, 2004; Tripathi and Gaur, 2006; Wang *et al.*, 2009b; Radić *et al.*, 2010), but in literature the range of the results is very large; they are different – even opposite - depending on experimental conditions (Powell *et al.*, 1986; Davies *et al.*, 1991; Blinda *et al.*, 1997).

CONCLUSIONS

Although zinc is considered a less toxic heavy metal, the results of this study on the response of the antioxidative enzymes to zinc exposure as well as the negative impact of zinc on soluble protein content, together with other reported observations, inclusively on the enhancement of the rates of zinc-induced cytogenetic disturbances in plants, must constitute a signal about the risks of the increasing presence of zinc and other heavy metals in the environment.

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