BEHAVIOUR OF ANTIOXIDATIVE ENZYMES AND OF SOLUBLE PROTEIN IN WHEAT SEEDLINGS AFTER LEAD-INDUCED STRESS

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Abstract. The amplitude of antioxidative enzymatic response was investigated in *Triticum aestivum* cv. *Maruca*. Pb^{2+} was provided as solutions of lead acetate [$Pb(C_2H_3O_2)_2.3H_2O$] and lead nitrate [$Pb(NO_3)_2$], at four concentrations (10, 25, 50, 100 μM) containing 2.07, 5.18, 10.36, respectively 20.72 μg ml^{-1} Pb^{2+}. The results support idea that mainly superoxide dismutase and peroxidase are involved in the defence mechanism of wheat seedlings against Pb^{2+} toxicity, by scavenging reactive oxygen species. All Pb^{2+} concentrations enhanced SOD activity (the increase rates range between 24.59%-65.19%, for Pb^{2+} acetate, and between 20.88%-175.40%, for Pb^{2+} nitrate treated variants, comparatively to control). Pb^{2+} induced the decline of soluble protein level in all variants, indifferently of compound type and lead concentration.

INTRODUCTION

The problem of heavy metal toxicity acquired new dimensions in the industrial era. Besides the beneficial component, the progress of human society had destructive effects on environment, with disastrous repercussions on biological systems. Increment of civilization degree has meant the irrational exploitation of the nature, the increase of non-biodegradable waste, amplification of physical (radioactive, thermal, noise), chemical (heavy metals and other noxious agents) and biological (pathogen agents such as viruses, bacteria, fungi) pollution, increasing of greenhouse effect (global warming) by depletion of the earth’s stratospheric ozone layer etc. Heavy metals come from natural (volcanoes and continental dusts) and anthropogenic activities (mining operations, combustion of fossil fuels, metalworking industries, domestic garbage dumps, utilization of fertilizers etc.) resulting in their emission and accumulation in ecosystems. Such metals are released in the biosphere through air, water and soil and ultimately affect the plant, animal and human systems.

Lead is the most common heavy metal contaminant in aquatic and terrestrial ecosystems having various natural and anthropogenic sources (Sharma *et al*., 2005; Liu *et al*., 2009 a, b). It is naturally found in small amounts in the earth crust and is largely used in the production of containers of foods, stills, batteries, paints, and leathers. Human activities like burning of fossil fuels, mining, and manufacturing are lead sources. Its use as tetraethyl and tetra methyl additives in gasoline to increase octane rating has transformed lead into one of the metals of high toxic risk. In 1965 – 1990 lead consumption increased in the world to 5.6 x 10^6 tones (OECD, 1993), its concentration in biosphere being 1,000 – 100,000 times higher than the natural level (WHO, 1995). Since the half-life in biological systems is one of the longest among metals (150 – 5000 years), the consequences of lead pollution can be devastating.

Although lead has no known biological function, numerous investigations show that plants can accumulate lead via root and shoot. Important alterations have been reported in structure, biochemistry and physiology of plant cells in lead excess. In *Helianthus annuus* L., Pb^{2+} showed the highest phytotoxicity comparatively with Al, Cd, Cu, Ni, Pb and Zn (Chakravarty and Srivastava, 1992). This metal alters the transcriptional process, denatures the proteins (Rathore et al., 2007) and disturbs photosynthesis (Akinci *et al*., 2010). It causes changes in lipid composition of thylakoid membranes and modifies membrane permeability (Stefanov et al., 1995). Root elongation, plant growth, seed germination, transpiration, photosynthesis, mineral nutrition, plant water status and enzymatic activities can be also negatively influenced by lead treatment (Jiang and Liu, 2010; Kaznina *et al.*, 2005; Pinero *et al*., 2002).

An important feature of lead toxicity is the generation of reactive oxygen species (ROS) and free radicals, such as superoxide anion radical (O_2^-), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) which cause oxidative stress to plants. Lead and other heavy metals promote oxidative damage not only by direct increasing of the cellular concentration of reactive oxygen species but also by the diminution of the cellular antioxidant capacity (Pinto et al., 2003). 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 plant development and in regulation of gene expression. Therefore, it is necessary for cells to control the level of ROS tightly, but not to eliminate them completely (Pitzschke *et al*., 2006). To minimize the damaging effects of ROS, aerobic organisms evolved non-enzymatic defence systems (ascorbic acid, reduced glutathione, carotenoids, tocopherols, flavonoids, alkaloids) and enzymatic protection mechanisms (superoxide dismutase, SOD, E.C. 1.15.1.1; peroxidases, POD, E.C. 1.11.1.7; catalase, CAT, E.C. 1.11.1.6).

Wheat is a plant of a worldwide economic importance, a main link in trophic chain and a pathway of pollutant ingestion for animals and humans. The main objectives of the present investigation are to evaluate the antioxidative response in *Triticum aestivum* L. cv. *Maruca* seedlings, by analyzing the activity patterns of antioxidative enzymes and the protein level after lead treatment, provided as lead acetate and lead nitrate. We tested two lead compounds, with important industrial uses, at different concentrations, to establish if the chemical structure in which lead is included induces significant differences in the studied parameters. *Lead acetate trihydrate*, the common form of lead acetate, is called *sugar of lead* and it is used as a mordant in dyeing and as a drier in certain paints. *Lead nitrate* is used in heat stabilization of nylon and polyesters, in coatings of photothermographic paper, in gold cyanidation, as oxidizer in the dye industry, as a metal stain for ultra-thin sections and as medical astringent.

MATERIAL AND METHODS

Plant material and treatment conditions. Biological material is represented by wheat seeds (*Triticum aestivum* L. cv. *Maruca*), Agricultural Research Station, Podu Iloaie, Romania). The seeds were 4 h treated with four concentrations (10 µM, 25 µM, 50 µM, 100 µM) for each lead compound. The lead concentrations (µg ml^{-1}) in each solution are presented in Table 1. In control, distilled water was used.

Table 1 Lead concentration in tested solutions.

variant	molar concentration of salt solution	lead concentration (µg ml^{-1})
Control – distilled water		
Lead acetate trihydrate, $Pb(C_2H_3O_2)_2.3H_2O$, mol. weight=379.33 g/mol	10 µM	2.07
	25 µM	5.18
	50 µM	10.36
	100 µM	20.72
Lead nitrate, $Pb(NO_3)_2$, mol. weight=331.20 g/mol	10 µM	2.07
	25 µM	5.18
	50 µM	10.36
	100 µM	20.72

Determination of antioxidative enzyme activities. The activity of antioxidative enzymes and soluble protein content were evaluated in 7 days old wheat seedlings.

Superoxide dismutase (SOD) activity was measured according to spectrophotometric assay (Winterbourn *et a*l., 1975) with slight modifications (Artenie et al., 2008), based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radicals generated upon reoxidation of photochemically reduced riboflavin. Absorbance was recorded at λ=560 nm using UV-VIS 1700 Shimadzu PharmaSpec spectrophotometer (Kyoto, Japan). One unit of SOD is defined as the enzyme amount producing 50% inhibition of NBT reduction in the standard conditions.

Catalase (CAT) activity was assayed by Sinha's procedure with minor adaptations (Artenie *et al.,* 2008). The method principle is based on spectrophotometrical 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, using UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer.

Peroxidase (POD) activity was established by method of Gudkova and Degtiari (1968), 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 is measured at UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer (λ=540 nm). The calculus of results uses the coefficient of micromolecular extinction (0.0128). One peroxidase unit corresponds to the enzyme amount catalyzing the decomposition of 1 µM H_2O_2 min^{-1}, in optimal conditions.

Determination of soluble protein content. Soluble protein content in enzyme extracts was established by Bradford method (Bradford, 1976). Method is based on binding of Coomassie Brilliant Blue G-250 solution to the amino acids radicals and recording of absorbance at λ = 595 nm. Protein content was established with a standard curve constructed with bovine serum albumin.

In order to compare the sensitivity of each parameter (enzyme activities, protein content), changes in these values were calculated as a percentage of their control value (set to 100%).

RESULTS AND DISCUSSIONS

Effects induced by Pb^{2+} on the activity of antioxidative enzymes in 7 days old wheat seedlings. Unlike iron, Pb^{2+} has no redox capacity. Therefore, lead-induced oxidative stress in treated plants seems to be an indirect effect of its toxicity leading to the production of ROS which enhance pro-oxidant status of cell by reducing the pool of reduced glutathione (GSH), activating Ca-dependent systems and influencing iron-mediated processes (Pinto *et al*., 2003). Increase of endogenous ROS levels and activation of antioxidant enzymes represent the most rapid indicators of oxidative stress resulted from the imbalance between production and elimination of ROS generated after lead treatment. Our investigations showed alterations in the activities of the three antioxidant enzymes in relation to lead exposure (Table 2).

Table 2 Increase/decrease rates of antioxidative enzyme activities and soluble protein levels in 7-days old wheat seedlings, after lead treatment.

Variant		SOD activity			CAT activity			POD activity		
		units mg^{-1} protein	%	–/+ rate (%)*	units mg^{-1} protein	%	–/+ rate (%)*	units mg^{-1} protein	%	–/+ rate (%)*
Lead acetate	control	4.31	100.00	0.00	393.82	100.00	0.00	6.24	100.00	0.00
	10µM	6.40	148.49	+48.49	284.30	72.19	-27.91	4.99	79.96	-20.04
	25 µM	6.75	156.61	+56.61	353.97	89.88	-10.12	7.78	124.67	+24.67
	50 µM	5.37	124.59	+24.59	446.32	113.33	+13.33	6.63	106.25	+6.25
	100µM	7.12	165.19	+65.19	350.84	89.08	-10.92	7.78	124.67	+24.67
Lead nitrate	control	4.31	100.00	0.00	393.82	100.00	0.00	6.24	100.00	0.00
	10µM	11.87	275.40	+175.40	559.73	142.12	+42.12	11.64	186.53	+86.53
	25 µM	5.58	129.46	+29.46	373.56	94.85	+5.15	6.94	111.21	+11.21
	50 µM	6.57	152.43	+52.43	317.67	80.66	-19.34	6.01	96.31	-3.69
	100µM	5.21	120.88	+20.88	355.06	90.31	-9.69	6.67	106.89	+6.89

*– decrease rate; + increase rate

Superoxide dismutase, the main scavenger of superoxide radicals, is a strong antioxidant which converts the toxic superoxide (O_2^-) to hydrogen peroxide and oxygen, by so called *dismutation reaction*: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. This enzyme represents the first line of cell defence against ROS generated by lead exposure, so preventing the tissue damage. Concerning SOD activity under lead stress (Table 2; Fig. 1), the treatment resulted in a considerable rise of enzyme activity in all variants, exposed either to lead acetate or lead nitrate, fact proving activation of wheat detoxification mechanisms. SOD increases are the result of the formation of superoxide radicals in lead exposed seedlings. This increment indicates the superoxide as being the central component of the signal transduction which triggers the genes responsible for antioxidant enzymes including SOD (Liu *et al.*, 2009b). SOD increased values were accompanied by significant lowering of protein level.

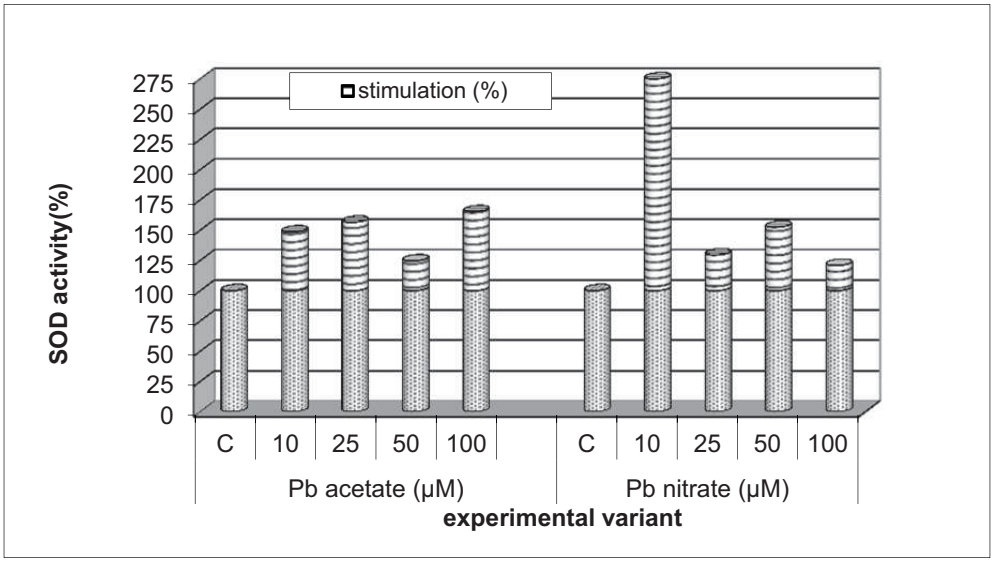

Fig. 1. Graphic representation of SOD activity in wheat seedlings, after lead treatment.

It was not observed a dose – SOD response relationship, but the differences from the controls are important. In 10 µM lead nitrate, SOD activity is 2.75 times higher than that of control (an increase of 175% in terms of percentage value) being an indicative of oxidative stress generated by superoxide radical production in the presence of Pb^{2+}. This concentration seems to be critical for wheat seedlings because this variant also showed the most increased activities of CAT and POD. CAT surpasses the control with more than 42%, while POD is almost 2 times higher than control. Soluble protein level has also one of the lowered values in 10 µM lead nitrate treated variant - 1.6 times smaller than control. The decrease both in peroxidase and catalase activity in 10µM lead acetate treated variant suggests a greater accumulation of H_2O_2 in the context of an amplified SOD activity. In literature, different trends of SOD activity (Dey *et al*., 2007) or significant increases of this enzyme (Pang *et al.*, 2001) have been noted in wheat seedlings exposed to lead stress.

SOD increase has been reported also in other plant species under lead stress, such as *Oryza sativa* (Verma and Dubey, 2003), *Medicago sativa* (Olteanu *et al*., 2008), *Sesbania drummondii* (Ruley *et al*., 2004), *Cassia angustifolia* (Qureshi *et al*., 2007), *Jatropha curcas* (Gao *et al*., 2009), and *Luffa cylindrica* (Jiang *et al*., 2010). SOD augmentation can be the result of two main factors: increase of amount of superoxide radicals and *de novo* enzyme synthesis which in turn can be associated with induction of SOD gene expression by superoxide mediated signalling transduction (Slooten *et al.,* 1995; Fatima *et al*., 2005).

Although lead was administrated as two different compounds, the effective level of metal was the same in the corresponding concentration variants (Table 1). The differences evidenced between correspondent variants of concentration can be due to the types of lead bindings to the other components in salt molecules.

Catalase is a major ROS-scavenging enzyme in all aerobic organisms, catalyzing the conversion of toxic H_2O_2 resulted in SOD dismutation reaction to H_2O and O_2, in peroxisomes.

In these conditions, SOD and CAT are complementary in their action to diminish the effects of oxidative stress. Depending on H_2O_2 concentration, catalase exerts a dual function (Scandalios, 2005). At low H_2O_2 concentrations (<1 μM) and in presence of increased levels of other substrata (ethanol, ascorbic acid etc.), catalase acts like a peroxidase: $RH_2 + H_2O_2 \rightarrow R + 2H_2O$. At high H_2O_2 concentrations, catalase degrades extremely rapid the hydrogen peroxide, by specific catalasic reaction: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$.

In the presence of Pb^{2+}, high levels of catalase activity have been reported in *Pteris vittata* L. (Fayiga *et al*., 2004) or *Sesbania drummondii* (Ruley *et al*., 2004), but in our experiments the mechanism of protection by catalase action is rather inefficient in wheat seedlings because, except the variant treated with 10 μM lead nitrate which shows a significant positive reaction and with 50 μM lead acetate which exceeds control with 13.33%, the others variants registered a more or less marked decline comparatively with control (Fig. 2). Lead-induced accumulation of H_2O_2 can result in the inactivation of catalase followed by decrease of its activity (Qureshi *et al*., 2007). CAT is more sensitive to Pb^{2+} than SOD and POD, as shows the decline in its activity. Decrease of CAT activity indicates a limited ability of this enzyme to eliminate the formed ROS and to decrease the oxidative state. Possibly, CAT is a less efficient H_2O_2 scavenger than POD because of its low substrate affinity.

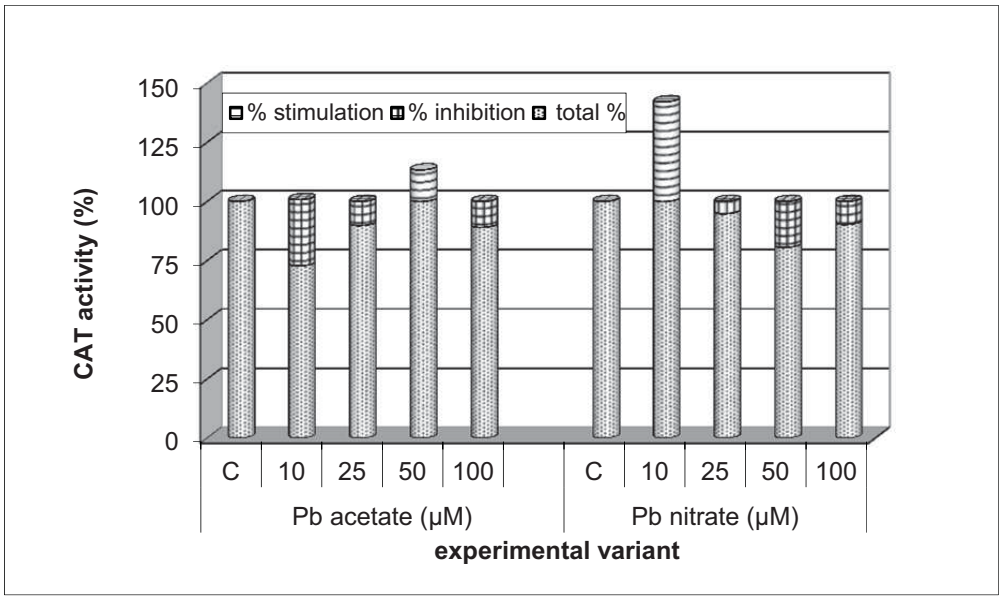

Fig. 2. Graphic representation of CAT activity in wheat seedlings, after lead treatment.

Data from literature differ on CAT behaviour in wheat seedlings exposed to lead treatment. Lead action determined either CAT decline (Dey *et al*., 2007) either moderate increases of this enzyme (Pang *et al*., 2001). The decline of CAT activity in Pb^{2+}-stressed plants can result not only from decrease of its synthesis but also from some changes in the assembly of enzyme subunits. Verma and Dubey (2003) observed lower intensity of two isozymic forms of catalase in

shoots of Pb^{2+}-stressed rice seedlings, consistent with decreased activity of the enzyme under Pb^{2+} treatment.

Peroxidase catalyzes the oxidation of many substrata (phenols, aromatic amines, ascorbic acid, glutathione, nitrites) in the presence of H_2O_2, with H_2O production: $AH_2 + H_2O_2 \rightarrow A + 2H_2O$. Peroxidase activity is considered as a potential biomarker of sublethal toxicity of heavy metals in plants, its role as a stress enzyme being widely accepted (Zhang *et al*., 2007). Peroxidase is stimulated by the accumulation of H_2O_2 in plant and it is able to scavenge this toxic compound. Compared to CAT, peroxidases possess a higher affinity towards H_2O_2, but have lower processing rate.

The pattern of peroxidase behaviour is different from that of catalase in our experiments (Fig. 3). Except 10 µM lead acetate treated variant, which shows an important POD decrease, and 50 µM lead nitrate, with small POD decrease, the other variants engaged this antioxidative defence system and POD levels registered more or less important increases. Since in contrast to CAT, POD activity increased, this enzyme seems to play a more significant role than CAT in detoxifying of the produced H_2O_2. POD augmentation can be also the result of release of those enzymes located in cell wall as response to the stress to which the plants are subjected (Gaspar *et al*., 1976).

Lead has been reported to induce peroxidase activity in soybean (Lee *et al*., 1976), rice (Verma and Dubey, 2003), *Sesbania drummondii* (Ruley *et al*., 2004), *Vicia faba* (Wang *et al.,* 2008) and *Luffa cylindrica* (Jiang *et al.*, 2010). In *Cicer arietinum* L. (cv. Radhey), successive increases and decreases of peroxidase activities were noted, depending on Pb^{2+} concentration (Reddy *et al*., 2005). In *Sonchus oleraceus* L., with increasing amounts of Pb^{2+}, the POD activity generally increased (Xiong, 1997), but in *Triticum aestivum* cv. *Maruca* it was not established a direct relation between increase of Pb^{2+} concentration and peroxidase activity.

In plants, multigene families encode the major antioxidant enzymes. This fact confers a great adaptive advantage by allowing a differential regulation of each gene family member in response to different endogenous and exogenous stimuli. Unlike most other organisms that have only one of each type of SOD in the various cell compartments, plants have multiple forms of each type encoded by more than one gene because they evolved more complex antioxidant defence strategies. As in the case of SOD, plant CATs are encoded by a small gene family constituted by three genes, as previously was described in maize, tobacco, cottonseed, *Arabidopsis*, and rice, whereas animals exhibit one CAT form. In *Eucalyptus grandis* L., 36 clusters as encoding antioxidant enzymes have been identified, 6 from these encoding POD isozymes, 3 encoding CAT proteins and 12 of them encoding SODs (Teixeira *et al*., 2005). Both *cat* and *sod* genes respond in a differential manner to various stresses known to generate ROS. This fact makes more difficult the explanation of some contradictory results relative to variable trends of the studied enzymes. Various behaviours of certain enzymes can be also due to the different degrees of tolerance or sensitivity of the plants to the heavy metal (Sharma and Dubey, 2005) or depend on tested organs (Jiang *et al*., 2010).

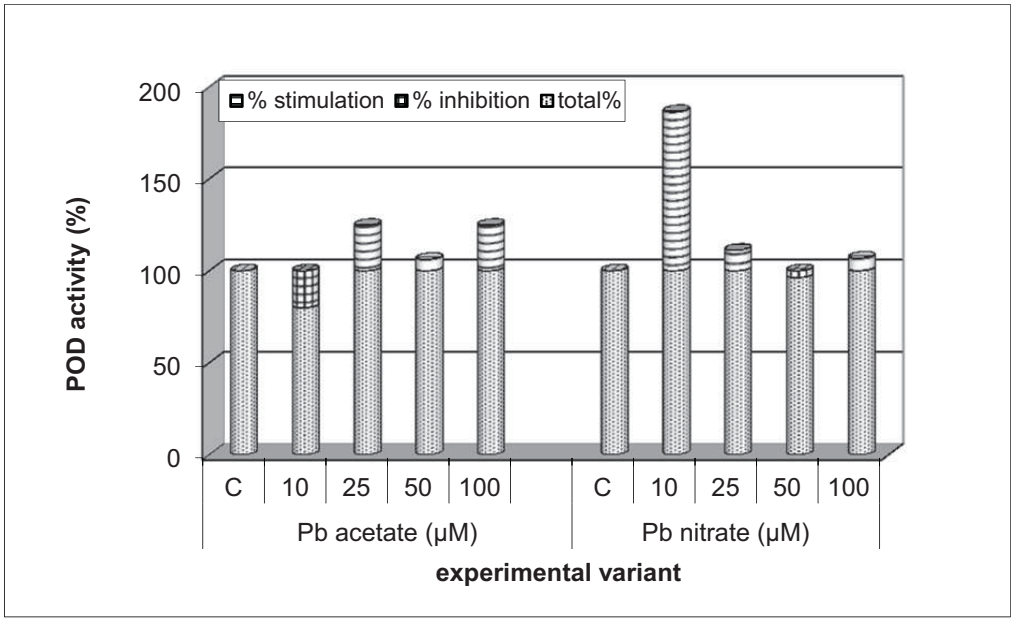

Fig. 3. Graphic representation of POD activity in wheat seedlings, after lead treatment.

Our results generally are in accordance with those published for wheat or other plant species, with some differences in enzyme behaviour. Lowering values of antioxidative enzyme activities are equivalent with a reduced protection against ROS, fact promoting accumulation of these and allowing the materialization of cytotoxic and eventually of genotoxic effect. The rise of enzyme activity as response to lead exposure is a proof of detoxifying ability of wheat seedlings by removal of ROS generated in stressed organisms. So, plants are able to overcome metal stress using an effective antioxidant defence mechanism in order to maintain the balance between ROS generation and their elimination. Therefore, in our experiments, for all tested concentrations of lead acetate and lead nitrate a stimulation of SOD activity was produced, but a distinct trend of CAT and POD activities in relation to Pb^{2+} concentration or compound type was not found. Various lead concentrations resulted either in inhibition or in increase activity of these enzymes. This situation is also signalled in literature for other heavy metals and plant species (Parmar *et al.*, 2002). The results published until now reveal a large diversity of antioxidative responses to heavy metal stress not only plant species but also a large intraspecific variability. Probably, the oxidative stress induced by heavy metals is a general phenomenon in plant species, but the antioxidative response is specific and depends on genetic potential of each cultivar or species.

Effects induced by Pb^{2+} on soluble protein level in 7 days old wheat seedlings. Oxidative damage of ROS on proteins refers to site-specific amino acid modifications such as formation of carbonyl derivatives on lateral chains of some amino acids (histidine, arginine, lysine, proline), fragmentation of the peptide chain, aggregation of cross-linked reaction products, alteration of electrical charge (Davies, 2003). In some cases, oxidation of susceptible residues such as cysteine and histidine lead to the production of *oxo* groups that can be assayed to provide an index of

oxidative damage to proteins (Babior, 1997). Oxidation of specific amino acids "marks" the proteins for degradation by specific proteases and can lead to cross-linkings and to an increased susceptibility to proteolysis. Regarding the mode of action with biological ligands, lead is included in the class of metals preferentially binding with sulphur- and nitrogen-rich ligands (e.g. amino acids) (Patra *et al*., 2004). Pb^{2+} acetate and Pb^{2+} nitrate inhibited the protein synthesis in wheat seedlings at all tested concentrations (Table 3, Fig. 4).

Table 3. Effects of Pb^{2+} treatment on soluble protein content in 7 days old wheat seedlings.

Variant		Soluble protein level		
		mg g^{-1} fresh weight	%	decrease rate (–), in %
Lead acetate	control	11.42	100.00	0.00
	10µM	8.17	71.54	-28.46
	25 µM	8.06	70.57	-29.43
	50 µM	9.18	80.38	-19.62
	100µM	6.69	58.58	-41.42
Lead nitrate	control	11.42	100.00	0.00
	10µM	7.12	62.34	-37.66
	25 µM	9.54	83.53	-16.47
	50 µM	7.93	69.43	-30.57
	100µM	9.76	85.46	-14.54

The greatest declines were present in the variants treated with 100 µM lead acetate (approximately 40 % inhibition, comparative to control), 10 µM and 25 µM lead acetate (approximately 30% inhibition), and also in 10 µM and 50 µM lead nitrate treated variants - both with more than 30% diminution of soluble protein level.

Decreasing effect of lead on protein level was also evidenced in *Vicia faba* L. (Mansour and Kamel, 2005), *Phaseolus vulgaris* L. (Hamid *et al*., 2010) or in *Oryza sativa* L. (Maitra and Mukherji, 1977). One explanation for the decrease of soluble proteins might be their increased susceptibility to proteolysis by specific proteases as result of the changes provoked by Pb^{2+}-generated ROS in side chains of specific amino acids, situation proved in *Hydrilla verticillata* under lead acetate stress (Jana and Choudhary, 1982). Also, gene expression is susceptible to be altered by lead treatment; so, it is possible that the repression mechanism of genes coding for protein synthesis became functional, fact evidenced by low level of proteins, as was present in *Brassica juncea* L. (Singh *et al*., 2002). Excessive damage to proteins under Pb^{2+} treatment could result from the attack of lipid peroxidation intermediates (Pinto *et al*., 2003). Loss of nuclear genetic material by chromosome fragmentation, micronuclei or laggards (see section concerning chromosome aberrations) can also have repercussions on protein synthesis afferent to those genes lost in this way.

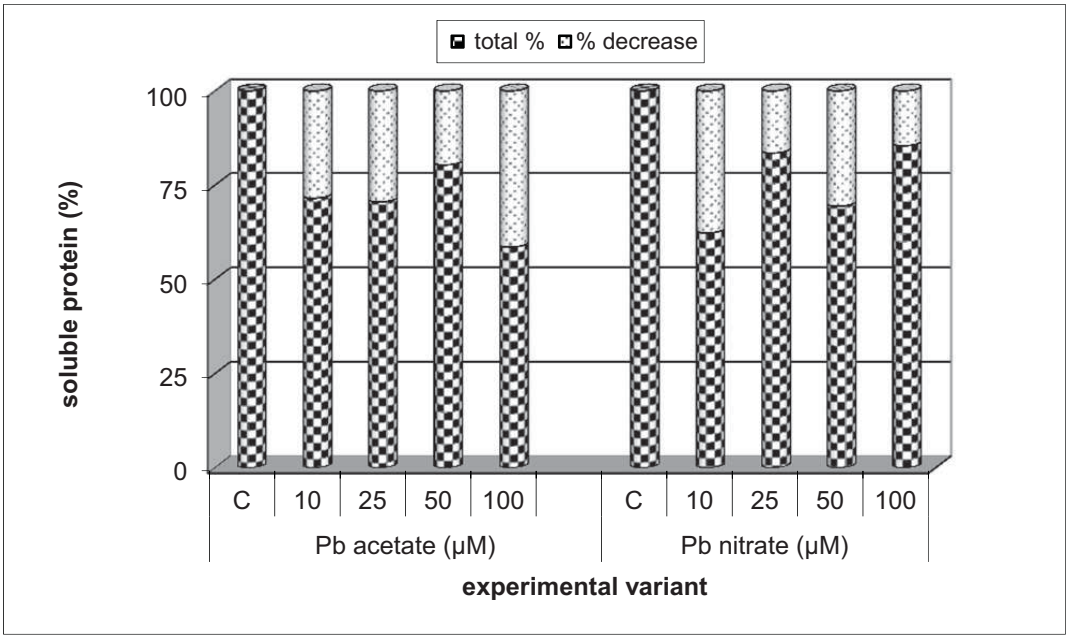

Fig. 4. Graphic representation of Pb^{2+} induced effects on soluble protein amounts in 7 days old wheat seedlings.

In literature, increases of some quantitative parameters including total protein amount were cited for common bean, alfalfa, oat and ryegrass, indifferently of lead concentration (Pinero *et al.*, 2002). In wheat and lens seedlings, the total protein content increased with the increase in lead concentration (Mesmar and Jaber, 1981). In other studies, different values of protein level have been noted in *Phaseolus mungo* and *Lens culinaris* seedlings, after lead treatment, depending on tested organs (Azmat and Haider, 2007). Increment of soluble protein amount can be a consequence of *de novo* synthesis of some stress proteins as result of exposure to exogenous factor (Gonçalves *et al*., 2007).

CONCLUSIONS

The results allow us to conclude that in *Triticum aestivum* cv. *Maruca* seedlings the protective mechanisms against Pb^{2+}-induced oxidative stress act by enhancing the antioxidant enzymes.
SOD and POD are mainly involved in the defence mechanism of wheat seedlings against Pb^{2+} toxicity.
For all tested concentrations of Pb^{2+} acetate and Pb^{2+} nitrate, a rise of SOD activity was registered.
Soluble protein level decreased in all variants, indifferently of compound type and Pb^{2+} concentration.
It was not established a direct relationship between Pb^{2+} concentration and enzyme activities.

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