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Effect of temperature on oxidative stress induced by lead in the leaves of *Plantago major* L.

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A b s t r a c t. Fluctuation of the summer day-time temperatures in the mid-latitudes in a range from 16 to 30°C should not have irreversible negative effects on plants, but may influence metabolic processes including the oxidative stress. To test the effect of moderately high temperature on oxidative stress induced by lead in the leaves of *Plantago major* L.; the plants were incubated in a water solution of 0, 150, 450, and 900 μM $Pb(NO_3)_2$ at 20 and 28°C. Plant reactions were evaluated by the content of thiobarbituric acid reactive substances and ascorbate peroxidase and glutathione reductase activities in leaves after 2, 24, 48, and 72 h. The Pb concentration in the leaves rose with the increase in the Pb content and was higher at 20°C. The increase in stomatal resistance caused by Pb was higher at 28°C. The contents of TBARS increased after 2 h of plant exposure to Pb and the increase was the highest at 900 μM Pb, 28°C. The AsP activity increased up to 50% after 24 h of Pb-treatment at 28°C; the highest increase in glutathione reductase activity was observed after 72 h at 20°C. Thus, the moderately high temperature 28°C compared with optimal 20°C caused a decrease in Pb accumulation in *Plantago* leaves but amplified the negative effects of lead, especially in the beginning of stress development.

K e y w o r d s: ascorbate peroxidase, glutathione reductase, lead, heat stress, oxidative destruction, *Plantago major* L.

INTRODUCTION

Lead (Pb) is a heavy metal toxic to most living organisms exerting adverse effects on the morphology, growth, and photosynthetic processes of plants. It also causes inhibition of enzyme activities, water imbalance, and alterations in membrane permeability and disturbs mineral nutrition (Kosobryukhov *et al*., 2004; Sharma and Dubey, 2005).

Pb concentration in tissues greater than 30 ppm is toxic to most plant species (Xiong, 1997). Usually Pb is accumulated in plant roots in much higher concentrations than in stems and, especially in leaves (Islam *et al*., 2008; Sharma and Dubey, 2005; Xiong, 1997). However, in the red beet, field pumpkin, chicory, common bean, white cabbage, and parsnip, the maximum Pb content was found in leaves (Sêkara *et al*., 2005). Pb accumulation in *Plantago major* (Voskresenskaya *et al*., 2013) was shown to be similar in the roots and leaves of plants grown on heavily contaminated soils.

Pb causes multiple direct and indirect negative effects on plant growth and metabolism. In particular, treatment of moss with 100 and 1000 μM Pb resulted in a decrease in the dry mass and total chlorophyll content, a significant increase in the reactive oxygen species (ROS) production and lipid peroxidation, and accumulation of ascorbate and glutathione (Choudhury and Panda, 2005). At high concentrations of heavy metals in plant tissues, the oxidative stress is characterized by inhibition of the major antioxidant metabolism (Choudhury and Panda, 2005; Sharma and Dubey, 2005).

Photosynthesis is one of the most Pb-sensitive processes in plants. Photosynthesis is adversely affected by Pb, mostly due to the metal-induced reductions of the concentration of photosynthetic pigments (Mishra *et al*., 2006), inhibition of the electron transport chain (Gajic *et al*., 2009), and changes in the chloroplast structure and stomatal status

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(Islam *et al*., 2008). Pb-induced inhibition of photosynthesis of plant seedlings was shown to be accompanied with stomatal closure and a lowered transpiration rate, especially at higher levels of Pb (Islam *et al*., 2008). According to Ahmad *et al*. (2008) and Islam *et al*. (2008), there is a strong relationship between Pb application and a decrease in plant photosynthesis, which is believed to be a result of stomatal closure. The net photosynthetic rate in the leaves of rice *Oryza sativa* L. was significantly inhibited by Pb treatments with a simultaneous decrease in stomatal conductance and transpiration rate (Li *et al*., 2012).

One of the plant responses to environmental stress is generation of the excessive amount of ROS, such as the superoxide anion-radical, singlet oxygen, hydrogen pero xide, and hydroxyl radical. Reactive oxygen species (ROS) induce oxidative damage to organic compounds including membrane lipids in cells (Halliwell and Gutteridge, 1999). Malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS) are one of the major products of the decomposition of polyunsaturated fatty acids in biomembranes and has been widely used as an indicator of oxidative processes (Balakhnina *et al*., 2005; 2013; Reddy *et al*., 2005; Ruley *et al*., 2004; Verma and Dubey, 2003). A number of heavy metals, including Pb, are known to induce the overproduction of ROS and consequently an increase in peroxidative processes, a decrease in saturated fatty acids, and an increase in the unsaturated fatty acid contents in membranes of several plant species (Erdei *et al*., 2002; Geebelen *et al*., 2002; Girard *et al*., 2002; Malecka *et al*., 2001).

There is good evidence that the alleviation of oxidative damage and increased resistance to environmental stresses often correlate with an efficient antioxidant system (Suzuki *et al*., 2012). The main antioxidant enzymes superoxide dismutase (SOD), peroxidase, and catalase are known to be stimulated to counteract the oxidative processes, and their activities could serve as an important indicator of an antioxidant defense mechanism (Balakhnina *et al*., 2005; 2013; Liu *et al*., 2009; Ruley *et al*., 2004; Wang *et al*., 2007).

Field grown plants are often subjected to fluctuating air and soil temperature that has profound effects on the plant metabolism (Chaitanya *et al*., 2001; Nosalewicz *et al*., 2013). There are a number of publications demonstrating irreversible damage to the plants caused by dangerously low or high temperatures (Chaitanya *et al.*, 2001; Gulen and Eris, 2004; Wahid *et al*., 2007). For most plants, the threshold temperatures range from 30 to 40°C. A decrease in the protein content was shown for seedlings of strawberry exposed to gradual heat stress (from 25 to 45°C) and shock heat stress (45°C without adaptation) accompanied by an increase in total peroxidase activity at high temperature treatment (Gulen and Eris, 2004). Heat stress was also reported to induce or enhance active oxygen species-scavenging enzymes like SOD, catalase, peroxidase, and several low-molecular antioxidant compounds (Chaitanya *et al*., 2002).

The summer day-time temperatures in the mid-latitudes usually range from 16 to 30°C. Fluctuation of the temperature in this range is not dangerous for most plants. In particular, the zone of temperature optimum for *Plantago major* plants shifts from 19.0 to 21.2 ± 3°C during plant ontogenesis (Atkin *et al*., 2007; Mudrik *et al*., 2003). Fluctuations of the temperatures in a range of 16-30°C should not have irreversible negative effects on plants, but they may influence the intensity of transpiration, nutrient absorption, photosynthesis, and a number of other metabolic processes including the oxidative stress. However, studies analyzing the effect of moderately elevated temperatures to oxidative stress induced in plants by heavy metals are absent in the literature.

The present study aimed to test the effect of temperature on lead accumulation in *Plantago major* L. leaves and changes in the stomatal status and oxidative stress development. Oxidative stress was assayed by thiobarbituric acid reactive substances (TBARS) content and ascorbate peroxidise (AsP) and glutathione reductase (GR) activities in leaves.

MATERIALS AND METHODS

Plantago major L. plants grown in natural conditions (Felin region, Lublin, Poland) were used as a plant material. For this experiment, we selected plants having five fully developed leaves and carefully dug them out from the soil. After thorough washing of roots, they were planted in 1.5 L plastic pots (six plants per pot; 108 pots in total) with 50% Hoagland solution (Hoagland and Arnon, 1938). The pots with plants were placed in two growth chambers where the light period was 12 h; the light intensity was 260 μmol photons m^{-2} s^{-1}. Relative air humidity was 45 ± 5% during the day and 70 ± 5% during the night, and air temperature was kept at 20/18 ± 2°C during the day/night time.

After a 3-day long period of plant adaptation, the conditions of plant growth were changed. The nutrient solution was replaced by distilled water (control) or water solution of 150 μM $Pb(NO_3)_2$ (50 ppm Pb^{+2}), 450 μM $Pb(NO_3)_2$ (149 ppm Pb^{+2}), and 900 μM $Pb(NO_3)_2$ (298 ppm Pb^{+2}). The air temperatures in the chambers with plants were differentiated and maintained at the level of 20/20 ± 2°C and 28/28 ± 2°C during the day/night time. As a result, the experimental treatments were as follows:

20°C+: 0, 150, 450, and 900 μM Pb, respectively;

28°C+: 0, 150, 450, and 900 μM Pb, respectively.

There were 72 pots in total: 9 pots for each of the experimental treatments. Plant stress reactions were evaluated after 2, 24, 48, and 72 h starting from replacement of the nutrient solution.

Leaf fragments were dried to constant weight at 105°C for 24 h, and then ashed at 560°C for 16 h. The ash was dissolved in a mixture of HNO_3-$HClO_4$ (4:1 v/v) and heated at 150°C for 6 h. After cooling, the extract was diluted

with 1 N HCl and used for lead content determination with a flame atomic absorption spectrometer (AAS) (Perkin Elmer SIMMA 6000, Perkin-Elmer Corp, Norwalk, CT).

The rates of net CO_2 assimilation (P_N) and stomatal conductance were measured on intact, fully mature top leaves with an LCPro+ Portable Photosynthetic System (ADC BioScientific, UK) in the growth chamber *ie* at the light intensity of 260 μmol photons m^{-2} s^{-1}. The stomatal resistance (r_s) was measured with an automated AP-4 porometer (Delta-T). Chlorophylls *a* and *b* were measured in 100% acetone extracts of the leaves according to (Lichtenthaller and Wellburn, 1983).

Ascorbate peroxidise (AsP) and glutathione reductase (GR) activities and thiobarbituric acid reactive substances (TBARS) content were determined as described earlier (Balakhnina *et al*., 2009, 2012a, 2012b) in enzymatic extracts and in crude homogenates of the top leaves. Middle fragments (0.5 g) of the fully developed leaves without the midrib were homogenized manually in a mortar with 4.5 ml of cooled 30 mM K/Na phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 2% PVP (M.м. = 25 000). The homogenates were filtered through a nylon cloth. A part of the filtrate, defined by us as a crude homogenate, was used for assessment of the intensity of oxidative process on the basis of the TBARS content. The second part of the filtrate was centrifuged at 4 000 g for 20 min. In the supernatant, described as an enzymatic extract, the AsP and GR activities were determined.

The thiobarbituric acid reactive substances (TBARS) content was assessed by the method of Uchiyama and Mihara (1978). 3 ml of 1% phosphoric acid, 1 ml of 0.6% aqueous solution of thiobarbituric acid, and 0.1 ml of aqueous $FeSO_4 \times 7\ H_2O$ (2.8 mg ml^{-1}) were added to 0.3 ml of the crude homogenate. The reaction mixture was heated to 100°C in a water bath during an hour. After cooling and addition of 4 ml butanol-1, the reaction mixture was mixed and centrifuged at 3 000 g for 10 min. The absorbance of TBARS was measured at 532 nm and 600 nm using a Shimadzu UV-VIS 160A (Kyoto, Japan) spectrophotometer. The concentration of TBARS was calculated using a coefficient of extinction equal to 1.56×10^{-5} M cm^{-1}.

The activity of AsP was estimated by measuring the decreased absorption at 310 nm during ascorbate oxidation (extinction coefficient of 2.8 mM cm^{-1}) (Nakano and Asada, 1981). The reaction mixture (1 ml) contained 25 mM K/Na phosphate buffer (pH 7.4), 0.5 mM ascorbate, 0.1 mM EDTA, and the enzymatic extract. The reaction was started by the addition of 0.1 ml of 1 mM hydrogen peroxide.

The activity of GR was determined by glutathione-dependent oxidation of NADPH (Foyer and Halliwell, 1976). The reaction mixture contained 30 mM K/Na phosphate buffer, pH 7.4, 0.15 mM NADPH, 3 mM $MgCl_2$, and 0.5 mM oxidized glutathione (GSSG). Corrections were made for NADPH oxidation in the absence of GSSG. The reaction was initiated by addition of the enzyme extract. The measurements were performed at 340 nm; a 6.22 mM cm^{-1} coefficient of extinction was used for calculations.

All the results were calculated per one gram of fresh mass. Several (4÷6) independent biological replications were made for the measurements. Each value represents the mean ± standard error. Statistical analysis of the results was done using confidence tests with a one-way analysis of variance ANOVA (STATISTICA 10). Means were compared by the ANOVA LSD (least significant difference) test.

RESULTS

The concentration of Pb in the leaves of *Plantago major* L. plants that were not treated by Pb was about 5.0-5.6 mg kg^{-1} dry mass. The Pb concentration in the leaves of Pb-stressed plants increased with the increase in the Pb content in the growth medium and time of exposure (Table 1). At the temperature that is optimal for photosynthesis (20°C), the accumulation of Pb in the leaves of treated plants was higher than that at 28°C.

To confirm that the Pb-induced inhibition of photosynthesis of plants in our experiments is accompanied with stomata closure, we measured the net CO_2 assimilation rate (P_N) and stomatal conductance in the leaves of plants exposed to the optimal 20°C temperature without (0 μM Pb) and with (450 μM Pb) lead treatment for 72 h. Data presented in Table 2 show that the Pb treatment caused

Table 1. Pb content in the top leaves of *Plantago major* L. plants treated with 150, 450, and 900 μM Pb for 72 h at temperatures of 20 and 28°C

Treatment (μM Pb)	Pb (mg kg^{-1} dry mass (%))			
	24 h		72 h	
	20°C	28°C	20°C	28°C
0	5.6 ± 0.33a (100)	5.4 ± 0.37a (100)	5.2 ± 0.31a (100)	5.0 ± 0.35a (100)
150	17 ± 1.02a (304)	10 ± 0.60b (185)	28 ± 1.96c (538)	15 ± 0.99a (300)
450	26 ± 1.82a (464)	13 ± 0.78b (241)	47 ± 3.10c (904)	22 ± 1.54a (440)
900	39 ± 2.67a (696)	26 ± 1.82b (481)	61 ± 3.66c (1173)	34 ± 2.24a (608)

Different letters indicate significant differences between columns at $p<0.05$.

T a b l e 2. Net CO_2 assimilation rate (P_N), chlorophyll content and stomatal conductance in the top leaves of *Plantago major* L. plants treated with 450 μM Pb for 72 h at temperature of 20°C

Treatment (μM Pb)	P_N (μmol CO_2 kg^{-1} dry mass s^{-1})	Chlorophyll (*a* + *b*), (mg g^{-1} dry mass)	Stomatal conductance (mmol m^{-2} leaf area s^{-1})
0	131 ± 7a	2.60 ± 0.14a	142 ± 6a
450	93 ± 5b	2.31 ± 0.09a	104 ± 5b

Different letters indicate significant differences between treatments at $p<0.05$.

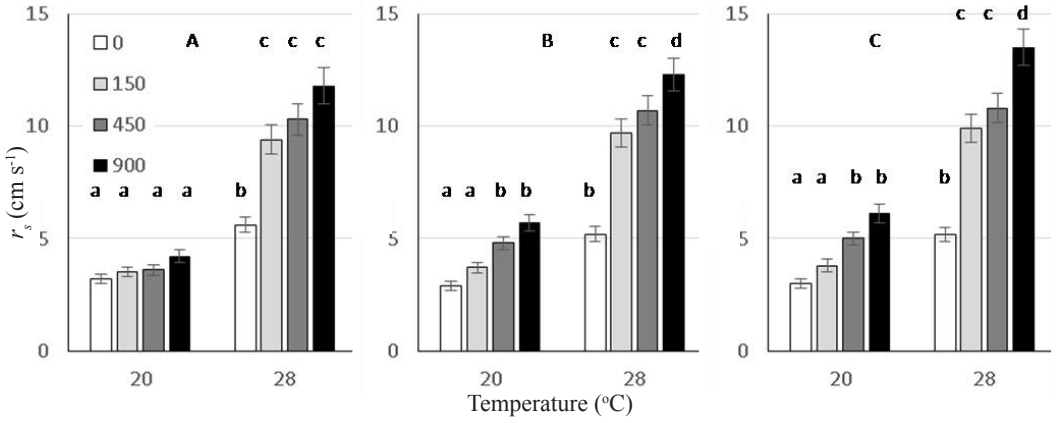

Fig. 1. Stomatal resistance, r_s, in the top leaves of *Plantago major* L. plants treated with 150, 450, and 900 μM Pb for 24 (A), 48 (B) and 72 h (C) at temperatures of 20 and 28°C. Different letters indicate significant differences at $p<0.05$.

a simultaneous decrease in P_N (about 42%) and stomatal conductance (about 37%), while the chlorophyll content did not change significantly.

The stomatal resistance (r_s) values in the treatments without lead in the growth medium were higher in plants incubated at 28°C by about 73-79% compared with the r_s values recorded under exposure to the optimal temperature of 20°C (Fig. 1).

Addition of lead did not cause a significant increase in r_s at temperature 20°C after 24 h incubation. Prolongation of incubation for 48 and 72 h with Pb concentrations of 450 and 900 μM led to an increase in r_s, which was about twofold with 900 μM Pb. Stomatal response to Pb stress at 28°C was observed after 24 h at Pb concentrations of 150, 450, and 900 μM. Prolongation of incubation, in particular with 900 μM Pb for 72 h, led to a 2.6 fold increase in r_s compared with the non-treated variant. The values of r_s in plants exposed to 28°C in all treatments were higher than the values of r_s in plants exposed to 20°C.

Plants exposed to 28°C without lead in the growth medium showed a 26% increase in the TBARS contents compared with plants at 20°C, after 2 h exposure (Table 3). Then, after 24- and 72-h exposure, the difference in the contents of TBARS between the plants at both temperatures decreased. Pb treatment stimulated the intensity of oxidative processes in all variants. The increase in the TBARS contents in Pb treated plants was observed just after 2 h of treatment. The highest value of TBARS contents was found in plants treated with 900 μM Pb at 28°C.

The activity of an antioxidant enzyme (AsP) in the leaves of plants, which were not treated with Pb, did not vary significantly depending on the temperature of incubation. Short-term 2 h Pb treatment did not increase AsP activity at both temperatures (20 and 28°C). After 24 h of Pb treatment, the AsP activity increased at both temperatures at Pb concentrations of 450 and 900 μM, and the increase was more pronounced at 28°C. Moreover, with the 150 μM Pb concentration, the AsP activity increased significantly ($p<0.05$) only in plants exposed to 28°C. After 72 h of Pb treatment, the increase in AsP activity became smaller than after 24-h treatment.

The glutathione reductase (GR) activity in the leaves of Pb non-treated plants exposed to 28°C was higher by 23-40% than in the leaves of plants exposed to 28°C. After 24-h treatment, an increase in GR activity was observed in variants with 450 and 900 μM Pb at 28°C. All Pb doses (150, 450 and 900 μM) led to a significant increase in GR activity after 72 h of exposure to Pb at 20°C. Incubation at 28°C for 72 h caused a significant increase in GR activity with 150 and 450 μM Pb, but not with 900 μM Pb.

DISCUSSION

The Pb concentration (about 5 mg kg^{-1} dry mass Table 1) in the leaves of *Plantago* plants incubated in medium without Pb was in agreement with normal concentrations of Pb in plants (0.1-10 mg kg^{-1} dry mass) according to Kabata-Pendias and Pendias (1992), *ie* with metal concentrations in plants growing in uncontaminated soils (Shen and Liu,

T a b l e 3. Content of thiobarbituric acid reactive substances (TBARS) and ascorbate peroxidase (AsP) and glutathione reductase (GR) activities in the top leaves of *Plantago major* L. plants treated with 150, 450, and 900 μM Pb for 2 h, 24 h, and 72 h at temperatures of 20 and 28°C

Treatment (μM Pb)	2 h		24 h		72 h	
	20°C	28°C	20°C	28°C	20°C	28°C
	TBARS, nmol g^{-1}fm (%)					
0	31±1.9 a (100)	39±2.4 b (100)	32±2.2a (100)	35±2.4a 100)	32±2.1 a (100)	33±2.0 a (100)
150	41±2.8 a (132)	48±2.9 b (123)	39±2.5 a (122)	45±2.8 ab (129)	38±2.4 a (119)	45±2.7 ab (136)
450	45±2.8 ab (145)	69±4.5 c (177)	43±2.3 a (134)	49±3.2 ab (140)	42±2.3 a (131)	50±3.3 b (152)
900	51±3.4 a (165)	72±4.9 c (185)	48±3.1 a (150)	58±3.8 b (161)	49±3.1 a (153)	61±4.0 b (185)
	AsP activity, μmol g^{-1} fm min^{-1}					
0	17.0±1.1a (100)	20.1±1.3ab (100)	19.0±1.2a (100)	22.0±1.3ab (100)	21.0±1.3ab (100)	23.0±1.5b (100)
150	17.7±1.2a (104)	20.3±1.3ab (101)	22.5±1.5ab (118)	34.3±2.4c (156)	24.0±1.6b (114)	29.0±1.7c (126)
450	18.7±1.2a (110)	20.6±1.4a (102)	25.8±1.5b (136)	34.1±2.0d (155)	27.0±1.7b (129)	30.0±1.9c (130)
900	17.3±1.1a (102)	22.0±1.4a (109)	28.0±1.8b (147)	34.7±2.1c (158)	28.0±1.9b (133)	31.0±1.9b (135)
	GR activity, μmol g^{-1} fm min^{-1}					
0	0.40±0.02a (100)	0.56±0.03b (100)	0.46±0.02ab (100)	0.58±0.03b (100)	0.48±0.02ab (100)	0.59±0.03b (100)
150	0.43±0.03a (108)	0.57±0.03b (102)	0.50±0.03ab (109)	0.62±0.04b (107)	0.74±0.05c (154)	0.87±0.06c (147)
450	0.44±0.03a (110)	0.65±0.04b (116)	0.55±0.04ab (120)	0.71±0.04c (122)	0.83±0.05d (173)	0.73±0.04d (124)
900	0.51±0.03a (128)	0.67±0.04b (120)	0.56±0.04a (120)	0.78±0.05c (134)	0.84±0.05c (175)	0.68±0.04b (115)

Different letters indicate significant differences between columns at $p<0.05$.

1998; Voskresenskaya *et al*., 2013; Yoon *et al.,* 2006). The strong increase in the Pb content in the leaves of the Pb-treated plants and the relation between Pb accumulation and Pb concentration in the medium and exposure time were in agreement with other investigations (Filipović-Trajković *et al*., 2012).

Our results showed that, along with the increase in the Pb content in the leaves of the Pb-treated plants, accumulation of Pb by plants incubated at 20°C was higher than in those incubated at 28°C. It was also shown that Pb uptake by plants incubated in the water solution containing Pb(II) ions increased with the increase in temperature but decreased again if the temperature was rising above 25°C for *Cucumis sativus* (Takeda *et al*., 2006) or 30°C for *Lemna minor* L. (Uysal and Taner, 2011). We suppose that the lower lead accumulation in the leaves at moderately high temperatures compared to that at 20°C was caused by an increase in stomatal resistance (Fig. 1), a decrease in root pressure, and a corresponding decrease in transpiration and transport of minerals.

In our study, the response of plants to 450 μM (150 ppm) Pb in the solution for 72 h is associated with the inhibition of photosynthesis and a simultaneous decrease in stomatal conductance. This result is in agreement with a number of papers showing Pb-induced inhibition of photosynthesis accompanied by stomatal closure (Ahmad *et al.,* 2008; Islam *et al.,* 2008; Kosobryukhov *et al*., 2004; Li *et al.,* 2012). Unlike photosynthesis, the decrease in the chlorophyll content in *Plantago* leaves in response to Pb treatment was not observed in the present work. Probably, the doses of Pb (the content in the medium and time of treatment) were not high enough to cause pigment destruction. Bafeel (2010) and Malar *et al.* (2014) have demonstrated on the leaves of *Lepidium sativum* (cress) and water hyacinths *Eichhornia crassipes*, respectively, that a significant (above 50%) decrease in the chlorophyll content occurred only under a strong Pb stress (above 400 ppm Pb lasting for several days).

The moderately high temperature of incubation (28°C) increased stomatal resistance compared to that at 20°C, which was shown to be optimal for *Plantago* photosynthesis (Kosobryukhov *et al*., 2004). The inhibitory effects of temperatures increasing from 22 to 32°C on the photosynthesis rate and transpiration were described by Lipiec *et al.* (2013). In our experiment, Pb treatment led to an increase in stomatal resistance and this increase was significantly more pronounced at 28 than at 20°C. The effect of Pb on stomatal status is supposed to be due to Pb action on the content of ABA (Sharma and Dubey, 2005). A unified hypothesis regarding Pb-induced stomatal closure originates from the inhibition of an energetic system or alterations of K^+ fluxes through membranes (Bazzaz *et al.,* 1974). As a result, the transpiration rate is decreased. We suppose that the transpiration rate and heavy metal accumulation in the leaves of *Plantago* plants are affected by the 8°C increase in air temperature *ie* regulation of stomatal closure can be considered as one of the protective responses of plants to prevent accumulation of lead in the leaves.

Pb induced stimulation of destructive processes (tested by TBARS content) strongly depending on the Pb concentration in the medium. Similar results were shown by Gupta *et al*. (2009), who observed a linear increase in the MDA concentration with increasing Pb levels independently of duration of exposure for maize shoots and two cultivars of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.) (Reddy *et al*., 2005). The increase in the MDA concentration indicates prevalence of oxidative stress and perhaps this may be one of the possible mechanisms by which Pb toxicity could be manifested in the plant tissues.

As for the co-action of air temperature changes and Pb in inducing destructive processes in Pb-stressed plants, the data in literature are practically absent.

In the present work, increased air temperature (28 °C) caused the destructive processes in *Plantago* leaves of Pb non-stressed plants already after the first 2 h of the experiment. These processes were eliminated by the increase in the incubation time, allowing plant adaptation to the growth conditions. The highest value of the TBARS content in Pb-stressed plants was found in the leaves of plants treated by 900 μM Pb at the temperature of 28°C for 2 h, *ie* at the time when temperature itself caused the highest increase in the TBARS content. Such synergism in the negative action of Pb and temperature was less pronounced but was not eliminated with time of incubation longer than 2 h. In all the tested time points, the intensity of oxidative processes in Pb-treated plants was higher at 28 than at 20°C.

Unlike the TBARS content, the activity of AsP in the *Plantago* leaves of the Pb non-treated plants was not altered significantly by the temperatures applied in the experiment. Moreover, short-term (2 h) Pb treatment, even with a high Pb concentration of 900 μM did not cause significant changes in AsP activity. Ruley *et al*. (2004) showed that the activity of AsP in *Sesbania drummondii* seedlings incubated in the presence of 500 mg l^{-1} $Pb(NO_3)_2$ in Hoagland medium remained unchanged for the first 2 weeks and only then increased significantly. In our experiment, stimulation of the activity of this antioxidant enzyme was observed after 24 h of Pb-treatment. At this time, the Pb-induced increase in AsP activity was strong (above 50%) at all Pb concentrations at 28°C, but was less pronounced at 20°C, especially at Pb concentration of 150 μM. We assumed that higher stimulation of Asp activity in the Pb-stressed plants at 28°C compared with 20°C was associated with more intensive destructive processes that were induced by Pb in *Plantago* leaves at 28°C. The fact that after 72 h of Pb-treatment the increase in the AsP activity became lesser than after 24 h treatment allows an assumption that induction of other protective mechanisms started *eg* activation of the second antioxidant enzyme of the ascorbate-glutathione cycle such as GR.

Indeed, the highest increase in GR activity in the leaves of Pb-stressed plants was observed after 72 h of Pb treatment at 20°C. In the leaves of *Sesbania drummondii,* the activities of antioxidant enzymes such as SOD, AsP, quaiacol peroxidase and catalase were shown to be significantly elevated upon exposure to 500 mg l^{-1} Pb (Ruley *et al*., 2004). The authors concluded that *Sesbania* plants were able to tolerate Pb-induced stress using an effective antioxidant defense mechanism. Garlic *Allium sativum* L. leaves showed an increase in SOD and peroxidase activities at a Pb concentration of 10^{-3} M (Liu *et al*., 2009). In *Zea mays* seedlings exposed to Pb (0-200 μM), activities of SOD and catalase and the content of ascorbic acid increased linearly with increasing Pb levels (Gupta *et al*., 2009).

The antioxidant enzymes are the main component of the plant defense system against heavy metal toxicity. The sequence of activation of the antioxidant enzymes in plant

roots and leaves during stress development was described in our earlier studies (Balakhnina *et al.,* 2012a). Here it should be noted that the negative effects of lead were amplified at moderately high temperature.

CONCLUSIONS

1. The moderately high temperature reduces the accumulation of lead in *Plantago major* L. leaves due to increased stomatal resistance.

2. The negative effects of Pb, especially in the beginning of stress development were increased by modearately high temperature (28°C) leading to earlier suppression of plant adaptive potential.

3. Adaptive response to stress development of *Plantago* plants at Pb pollution has the following sequences: stimulation of Asp activity at the beginning of stress development is shifted by activation of the GR as the second antioxidant enzyme of the ascorbate-glutathione cycle.

Conflict of interest: The Authors do not declare conflict of interest.

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