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BRIEF COMMUNICATION

Lead induced oxidative stress and alteration in the activities of antioxidative enzymes in rice shoots

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Abstract

Physiological responses of *Oryza sativa* L. to lead excess (10 and 50 μM) were studied in a hydroponic system after 48- and 96-h exposure. Accumulation of Pb in stressed rice shoots was concomitant with an increased metal concentration in the growth media and duration of exposure. The Pb stress resulted in an enhanced lipid peroxidation accompanied by altered activities of antioxidants. A substantial increase in α-tocopherol content of the Pb stressed rice shoots was observed suggesting its important role as an antioxidant. Among the antioxidant enzymes studied, activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) increased in the Pb-treated rice shoots, whereas that of catalase (CAT) declined. Activity of an important ascorbate-glutathione cycle enzyme, glutathione reductase (GR), also increased significantly in the Pb-treated shoots. The results suggest that Pb toxicity resulted in induction of oxidative stress in rice shoots, and α-tocopherol accumulation and upregulation of SOD, APX, and GR activities play an effective role in acclimatization to Pb stress.

Additional key words: α-tocopherol, ascorbate peroxidase, catalase, glutathione reductase, lipid peroxidation, *Oryza sativa*, superoxide dismutase.

Lead is the second most hazardous heavy metal (HM) pollutant after arsenic (Shahid *et al.* 2014). An elevated Pb concentration in soil has been reported to inhibit seed germination and retard plant growth (Kumar *et al.* 2013). Deleterious effects of Pb on plants include a reduced stomatal conductance and chlorophyll biosynthesis causing disturbed photosynthesis and transpiration (Xiong 1997). Besides, Pb induced toxic effects could be attributed to a lowered mineral nutrition (Kannan and Keppel 1976) and inhibition of activities of many enzymes (Kumar *et al.* 2013). The phytotoxic effects of Pb may be also ascribed to its ability to induce oxidative stress in plants (Reddy *et al.* 2005, Qureshi *et al.* 2007). Heavy metals, including Pb, increase production and accumulation of reactive oxygen species (ROS) such as a superoxide radical, a hydroxyl radical, singlet oxygen, and hydrogen peroxide. The ROS react rapidly with many biomolecules including lipids resulting in their peroxidation, *e.g.*, in membranes (Israr *et al.* 2011). Lipid peroxidation is considered as the most reliable indicator of oxidative stress; it results in altered membrane integrity, enhanced permeability, and inactivation of membrane associated enzymes (Schutzendubel and Polle 2002). Plants have developed an integrative defense system against oxidative stress consisting of both enzymatic and non-enzymatic antioxidants. The main components of this defense system are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). (Thounaojam *et al.* 2012). Under stress conditions including HM stress, activities of antioxidant enzymes have been reported to alter greatly (Thakur *et al.* 2016). Besides antioxidative enzyme activities, we also investigated a potential role of α-tocopherol against Pb-induced oxidative stress. Previous reports suggest the role of α-tocopherol as a membrane lipid protectant against photooxidation and as a scavenger of lipid peroxides and reactive oxygen radicals (Gajewska and Skłodowska 2007). However,

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase, GR - glutathione reductase; HM - heavy metal; MDA - malondialdehyde; ROS - reactive oxygen species; SOD - superoxide dismutase.
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there is the scarcity of information about the participation of tocopherols against HM-elicited oxidative stress.

A substantial increase in concentration of Pb in environment during recent years categorizes it as one of the most abundant and toxic pollutants. Rice is one of the most important food crops worldwide. The present work was carried out to investigate the sequence of metabolic responses in rice shoots to Pb-induced oxidative stress and, in turn, the alterations in behavior of the antioxidant enzymes and α-tocopherol after metal exposure. In parallel, shoot growth parameters and Pb uptake were also measured.

Viable rice (*Oryza sativa* L. cv. MR 219) seeds were sown in plastic pots containing Hoagland nutrient solution (1/4 strength, pH 5.5) for germination following surface sterilization with 0.1 % (m/v) solution of mercuric chloride. The pots were shifted to a growth chamber set at a temperature of 26 ± 2 °C, an 80 % relative humidity, a 14-h photoperiod, and an irradiance of 60 - 80 μmol m^{-2} s^{-1}. Lead treatment in the form of $Pb(NO_3)_2$ of desired concentrations (10 and 50 μM) was given to the five-d-old seedlings. The plants were harvested after 48 and 96 h of Pb exposure for various analyses.

Shoot length and fresh mass were measured to characterize the plant growth. Lead accumulation in rice shoots was determined according to the method of Humphries (1956) using an atomic absorption spectrometer (*Polarized Zeeman Z-5000*, *Hitachi*, Tokyo, Japan). Tocopherol content was measured following the fluorometric method of Taylor *et al.* (1976). Saponification of a tissue homogenate was carried out with KOH in the presence of ascorbic acid at 70 ºC. Hexane was added to extract non-saponifiable lipids. The organic layer was measured at 290 and 330 nm using α-tocopherol as a standard. Malondialdehyde (MDA) content was measured according to the method of Dhindsa *et al.* (1981).

Rice plant shoots were homogenized in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM ascorbate, 0.1 mM ethylene diamine-tetraacetic acid (EDTA), 1 % (m/v) polyvinylpyrrolidone (PVP) and 0.5 % (v/v) *Triton X-100*. After centrifugation at 10 000 *g* and 4 °C for 20 min, the supernatant was used to measure the activities of SOD, CAT, APX, and GR. Determination of SOD activity was performed following the method of Beyer and Fridovich (1987). One unit of SOD activity is the amount of the enzyme required to inhibit photoreduction of nitro blue tetrazolium (measured at 560 nm) by 50 %. Catalase activity was determined following the method of Aebi (1974). Decomposition of H_2O_2 was monitored at 240 nm using the coefficient of absorbance of 39.4 mM^{-1} cm^{-1}. The amount of CAT required to liberate a half of the peroxide oxygen from 10 mM H_2O_2 solution in 100 s at 25 °C is defined as one unit. Ascorbate peroxidase activity was determined following the method of Nakano and Asada (1981). A change in absorbance at 290 nm was monitored for 3 min to measure the oxidation of ascorbic acid. The activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}. The amount of the enzyme necessary to decompose 1 μmol of the substrate per min at 25 °C is defined as one unit. Activity of GR was measured following the method of Rao *et al.* (1996). The enzyme activity was determined using the coefficient of absorgance of 6.2 mM^{-1} cm^{-1}. The amount of the enzyme necessary to decompose 1 μmol of NADPH per min at 25 °C is defined as one unit. Protein content was estimated according to Bradford (1976).

The results presented are means of three independent experiments. All the experiments were performed in triplicate. Data were statistically analyzed by two-way analysis of variance (*ANOVA*) using *SPSS v. 20.0* (*SPSS Inc*., Chicago, IL, USA). To detect the significance of differences between individual means, Tukey's post hoc test was applied.

The lead treatment resulted in a substantial reduction in shoot growth and fresh mass. Shoot length and shoot fresh mass suppressions were concentration and exposure duration dependent. Exposure of the rice seedlings to 10 μM Pb for 48 and 96 h led to 16 and 25 % inhibition of shoot length, respectively (Table 1). Lead at a concentration of 50 μM inhibited shoot length by 26 and 44 %, respectively (Table 1). Similarly, up to 38 and 63 % decline in rice shoot fresh mass was noticed after 96-h treatment at 10 and 50 μM Pb, respectively (Table 1).

The amount of Pb accumulated in rice shoots increased linearly with both the increase in Pb concentration in the growth medium and the period of exposure (Table 1). It increased dramatically after 96-h treatment as compared to 48-h. The reason behind the low amount of Pb accumulation after the short exposure might be the binding of metal ions in root cells to avoid them to be transported into shoots. This might be one of the first lines of defense strategy used by plants under HM stress. This duration dependent increase in accumulated metal content has also been reported in previous studies (Singh *et al.* 2006).

The α-tocopherol content in rice shoots was enhanced following the Pb exposure. After 48- and 96-h treatment with 10 μM Pb, it was 8 and 33 % higher, respectively, than in the control (Table 1). At 50 μM Pb, α-tocopherol content increased significantly and the values were 31 and 69 % higher than in the control after 48 and 96 h, respectively (Table 1). The increased α-tocopherol content in rice shoots can be attributed to an elevated lipid peroxidation suggesting its possible involvement in protecting the plant against Pb induced oxidative stress. In context to increase in α-tocopherol content under HM stress and its possible role as lipid peroxides and a singlet oxygen scavenger, our results are in agreement with previous available reports (Krieger-Liszkay and Trebst 2006, Gajewska and Skłodowska 2007). Lead induced enhanced ROS generation is well established in literature (Lamhamdi *et al.* 2011). Malondialdehyde content in stressed rice shoots increased with the increase in Pb concentrations in the growth medium. The increase in MDA content was significant

after the 96-h exposure period at both Pb concentrations indicating that the oxidative stress increased with duration of treatment. Content of MDA increased by 47 and 96 % after 96-h treatment at 10 and 50 μM Pb, respectively (Table 1). The increased oxidative damage might be related to the increased lead accumulation. Our observation in relation to the Pb toxicity induced oxidative stress is in conformity with previous available reports (Reddy *et al.* 2005).

Table 1. Effects of Pb treatment (10 and 50 μM) on shoot length, shoot fresh mass, Pb content, α-tocopherol content, malondialdehyde (MDA) content, and activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in rice shoots after 48- and 96-h exposure. Means ± SEs, n = 6. Upper case letters represent significant differences among different treatments at the same exposure period, and lower case letters represent significant differences among thre same treatment at different exposure periods (Tukey's test; $P < 0.05$).

Parameter	Pb [μM]	48 h	96 h
Shoot length	0	5.8 ± 0.4Ab	7.9 ± 0.3Aa
[cm]	10	4.9 ± 0.1Bb	5.9 ± 0.1Ba
	50	4.3 ± 0.02Ca	4.5 ± 0.3Ca
Shoot fresh mass	0	21.3 ± 1.8Ab	33.6 ± 1.6Aa
[mg]	10	16.6 ± 0.4Bb	21.3 ± 0.7Ba
	50	12.1 ± 0.3Ca	12.9 ± 0.7Ca
Lead content	0	-	-
[μg g^{-1}(d.m.)]	10	58.4 ± 2.1Bb	97.3 ± 5.2Aa
	50	99.3 ± 4.1Ab	188.6 ± 8.3Ba
α-tocopherol	0	142.6 ± 6.3Bb	176.3 ± 12.4Ca
content	10	154.3 ± 5.4Bb	234.1 ± 7.3Ba
[μg g^{-1}(d.m.)]	50	186.1 ± 5.9Ab	298.1 ± 7.8Aa
MDA content	0	23.9 ± 1.3BCb	32.3 ± 1.6Ca
[nmol g^{-1}(f.m.)]	10	31.8 ± 2.1Bb	47.6 ± 1.9Ba
	50	42.1 ± 1.1Ab	63.2 ± 2.4Aa
SOD activity	0	7.9 ± 1.3BCb	10.5 ± 0.2Ca
[U mg^{-1}(protein)]	10	9.4 ± 0.4Bb	15.4 ± 0.5Ba
	50	12.3 ± 0.6Ab	18.1 ± 0.6Aa
CAT activity	0	6.4 ± 0.2Ab	7.6 ± 0.3Aa
[U mg^{-1}(protein)]	10	5.9 ± 0.1ABa	5.2 ± 0.2Bb
	50	6.8 ± 0.3Aa	4.9 ± 0.4BCb
APX activity	0	3.3 ± 0.2BCb	4.8 ± 0.6Ca
[U mg^{-1}(protein)]	10	3.8 ± 0.1Bb	8.0 ± 0.3Ba
	50	4.6 ± 0.2Ab	9.5 ± 0.3Aa
GR activity	0	1.3 ± 0.08Ca	1.4 ± 0.09Ca
([mg^{-1}(protein)]	10	1.7 ± 0.03Ba	2.2 ± 0.04Ba
	50	1.9 ± 0.03ABb	2.7 ± 0.02Aa

Plants employ both enzymatic and non-enzymatic mechanisms to scavenge enhanced ROS content to overcome oxidative stress (Gill and Tuteja 2010). Superoxide dismutase is one of the most important first lines of defense enzymes against ROS; it dismutates superoxide radical to H_2O_2 and oxygen. In the present study, a significant increase in SOD activity was noticed especially after the longer (96 h) Pb exposure. Shoot SOD activity increased by 19 and 46 % at 10 μM Pb after 48 and 96 h of exposure, respectively, as compared to the control (Table 1). At 50 μM Pb, SOD activity further increased up to 56 and 72 % after 48 and 96 h, respectively, as compared to the control (Table 1). This increased SOD activity in the Pb treated rice shoots might be considered as an alteration in the plant antioxidative defense system against the increased superoxide content. Besides, increased SOD activity is also of relevance in maintaining the overall defense system of rice plants under Pb induced oxidative damage. Activity of SOD has been reported to increase under various types of abiotic stresses including HM stress. For example, a similar induction was reported under HM stress (Thakur and Sharma 2015), salinity (Meloni 2003), and drought (Zhang and Kirkham 1994).

Catalase is an antioxidative enzyme which is involved in scavenging H_2O_2 by degrading it into water and O_2 in stressed plants. Activity of CAT in shoots of the Pb treated plants was not altered much at both used Pb concentrations after 48-h exposure (Table 1), whereas after 96 h, CAT activity decreased by 32 and 35 % at 10 and 50 μM Pb, respectively (Table 1). The interference of Pb ions in synthesis or assembly of enzyme subunits and inactivation of the enzyme protein due to ROS might be possible reasons for the decline in CAT activity (Rascio 2011). Thus in the present study, the decline in CAT acvity might be responsible for the increase in the H_2O_2 content and in turn for the H_2O_2 induced lipid peroxidation. The response of CAT activity has been reported to differ in a wide range depending on the plant organ/species and the HM involved. For example, Rucinska *et al.* (1999) reported an increased CAT activity in *Lupinus luteus* roots under low Pb concentrations, whereas the activity declined at higher concentrations.

Like SOD, APX activity increased under both the Pb concentrations and both the exposures. After 48 h, the increase in APX activity was 15 and 39 % as compared to the control at 10 and 50 μM Pb, respectively, whereas at 50 μM Pb, the increase was 66 and 98 %, respectively (Table 1). The increased APX activity reported in the Pb stressed rice shoot in the present investigation indicates its important role in the plant defense mechanism against oxidative stress. Ascorbate peroxidase detoxifies H_2O_2 using ascorbate as a substrate. A similar increase in APX activity under Pb stress has also been reported in previous reports (Lamhamdi *et al.* 2011). Also, APX is a major component of the ascorbate-glutathione cycle.

Activity of GR also increased significantly after 96-h treatment. At 50 μM Pb, GR activity increased by 93 %, whereas this increase was only 57 % in the case of 10 μM Pb (Table 1). The ascorbate-glutathione cycle is an integral part of the overall antioxidant defense system in plants. Thus, the increased APX and GR activities in the Pb treated rice shoots could be an important step to cope with the Pb induced ROS burst. The role of GR is also indispensible in maintaining a high reduced glutathione to oxidized glutathione ratio (Noctor and Foyer 1998). The increased GR activity in the present study corroborates to some previous reports of GR activity upregulation under HM imposed oxidative stress (Reddy

et al. 2005).

On the basis of the findings of the present study, it is concluded that both the Pb concentrations (10 and 50 μM) used were toxic for the rice plants. A direct relation was established between Pb accumulation and its inhibitory effects on growth of the rice shoots. The increased Pb concentration in the rice shoots resulted in the oxidative stress, and the severity of the stress was enhanced with both the increase in metal concentration and the duration of exposure. To mitigate the ROS caused damage, the activities of SOD, APX, and GR increased. In addition, the substantial increase in rice shoot α-tocopherol content under the Pb stress suggests its role in defence of rice plants.

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