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Antioxidant Enzyme Activities and Dry Matter of Rice Plant as Affected by Interactions of Lead, Phosphorus and Zinc

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Nutrient management can be an effective approach to enhance plant antioxidant defense system under heavy metal toxicity. A greenhouse experiment was conducted to examine the effects of the two- and three-way interactions of lead (Pb), zinc (Zn) and phosphorus (P) on the activity of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) enzymes and the total dry matter (TDM) of rice (*Oryza sativa* L. cv. Hashemi). This study was conducted as a factorial experiment in completely randomized design with three factors of Zn (0, 25 and 250 mg kg^{-1}), P (0, 50 and 500 mg kg^{-1}) and Pb (0, 200, 400 and 800 mg kg^{-1}) and three replications in calcareous soil. CAT, POD and SOD activity in the fresh leaves and TDM of the rice plant were determined. The effects of two- and three-way interactions of P, Pb and Zn on the antioxidant enzyme activities and TDM of rice plant were significant, and the type interaction was dependent on the levels of these factors. Soil Pb contamination significantly increased the CAT, POD and SOD enzyme activity but resulted in decreased TDM of the rice plant. Application of 250 mg Zn per kg of soil significantly increased activity of CAT and POD enzymes and TDM at the Pb level of 800 mg kg^{-1}. Phosphorus fertilization significantly increased CAT and POD activity and TDM under Zn- and Pb-contaminated conditions. To increase antioxidant enzyme activity and rice tolerance and growth in Pb-contaminated calcareous soils, combined application of P and Zn at 500 and 250 mg per kg of soil, respectively, can be recommended under similar conditions.

Key Words: catalase, enzymes, heavy metals, lead, peroxidase, phosphorus, rice, superoxide dismutase, total dry matter, zinc

INTRODUCTION

Food security depends on the increased production of cereals such as rice (*Oryza sativa* L.), an important source of calories, minerals and protein for humans. However, the accidental or anthropogenic release of lead (Pb) as a global contaminant and potent carcinogen into the environment may eventually result in soil, water, and air pollution, decrease in rice production and many health hazards (Fewtrell et al. 2003; Cannata et al. 2015; Caverzan et al. 2016). In various countries, the range of the maximum allowable concentrations of Pb in agricultural soils was 100–300 mg kg^{-1} (US-EPA 2005). Lead toxicity in plants generates reactive oxygen species (ROS), which can eventually cause cell damage in various tissues (Shanker et al. 2005; Liu et al. 2010). The main ROS molecules are singlet oxygen, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide (H_2O_2). Plants under oxidative stress display some defense mechanisms to protect themselves from the damaging effect of toxicity (Parida and Das 2005). Some of the ROS scavengers are antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Khan and Panda 2008). Levels of these antioxidant metalloenzymes can be used as indicators of metabolic stress induced by heavy metals in plants (Shanker et al. 2005).

Heavy metals like zinc (Zn) are important nutrients for plants, humans and animals, but depending on the mechanisms that plants use to protect themselves, heavy metals can be toxic at high concentrations (Verkleij et al. 2009). The maximum allowable Zn concentration for agricultural soils is 160 mg kg^{-1} (US-EPA 2005). Zinc accumulation in plant tissues at toxic concentrations (>300 mg kg^{-1}) causes physiological alterations and growth inhibition (Foy et al. 1978). Zinc status in soils and plants has a vital role in the accumulation of heavy metals in plants while Zn application in appropriate quantities can alleviate the physiological damage caused by oxidative stress (Wu and Zhang 2002).

Heavy metals may interfere with other nutrients and

thereby affect the mineral nutrition of plants. Nutrient management can be an effective approach to inhibit heavy metal toxicity in plants (Tu et al. 2000). Among the chemical fertilizers, nitrogen and phosphorus are the most highly used in the world. Phosphorus deficiency delays plant growth and maturity (Barker and Pilbeam 2007). Heavy metals in the soil hamper the metabolism of phosphorus in plants and inhibit the activity of phosphorolytic enzymes (Shah and Dubey 1998). P can protect plants from toxicity and oxidative stress caused by heavy metals, which suggest that P can produce an antagonistic response with heavy metals to mitigate oxidative stress in plants (Wang et al. 2009). Phosphorus increases SOD and CAT activity and reduces heavy metal toxicity in plants (Sarwar et al. 2010).

Rice ranks primarily as a cereal that is a good source of protein and energy. It is a staple food crop in Iran. According to the Rice Research Institute of Iran, per capita consumption of rice per year in Iran is 40.15 kg on the average (Jahed Khaniki and Zazoli 2005). Foods are the main source of heavy metal exposure in many developing countries (FAO/WHO 2011). Excessive levels of heavy metals such as Pb and Zn in agricultural soils and their uptake in rice and other food crops have become a long-term health risk (Williams 2006). Due to excessive concentrations of heavy metals, especially Pb in rice grains from different countries, rice can become enriched with Pb (Liu et al. 2013) in excess of the common safety threshold of 0.2 mg kg^{-1} for rice grain (FAO/WHO 2011).

This study was conducted to evaluate the main and interaction effects of P, Pb and Zn levels on total dry matter (TDM) and antioxidant enzyme activity of rice plants in calcareous soil. Rice antioxidant enzyme activity, e.g., SOD, CAT and POD, could be a key indicator to reflect environmental stress in studied conditions. Literature review showed that the effect of the three-way interaction of P × Zn × Pb on total dry matter and activity of SOD, CAT and POD enzymes in rice plants has not been studied previously.

MATERIALS AND METHODS

Soil Preparation and Analysis

Soil sample was taken at a depth of 0–25 cm for the pot experiment from an uncontaminated field near Espiran Village, northwest of Tabriz, Iran (38°5'57"N latitude, 46° 19'53"E longitude). The soil was classified as lithic Calcixerept according to the Soil Survey Staff (2014). After air drying, grinding, and passing through a 2-mm sieve, the soil was tested for its physical and chemical properties (Page et al. 1982; Dane and Topp 2002). Soil analysis results are presented in Tables 1 and 2. The soil texture was clay sandy loam and its available P and Zn were lower than the critical levels (Jones 2001; Alloway 2008). Three kilograms of the soil were placed in each pot. Zinc in the form of $ZnSO_4.7H_2O$ was added to obtain 0, 25 and 250 mg Zn per kg of soil. Pb in the form of $Pb(NO_3)_2$ was added to obtain levels of 0, 200, 400 and 800 mg Pb per kg of soil, and P in the form of $Ca(H_2PO_4)_2.H_2O$ was added to obtain 0, 50 and 500 mg P per kg of soil. The materials were purchased from Merck Co., Darmstadt, Germany, dissolved in deionized water, and poured into the soil slowly while mixing the soil at the same time. The thoroughly mixed soils were stored in pots and submerged in about 5 cm of water above the soil surface (in anaerobic conditions) for 2 wk before cultivation of the rice seedlings to achieve relative equilibrium. Reddy et al. (1984) reported that after soil waterlogging, the nitrate concentration of the soil solution is decreased by denitrification and reached zero after 2 wk. According to Havlin et al. (2007), the nitrate concentration of two different soils was decreased after anaerobic incubation and reached zero before 2 d. Results of our study showed that the nitrate concentration of soil solution in $Pb(NO_3)_2$ treatments will probably be negligible after 14 d incubation under flooded conditions and probably cannot significantly influence rice growth. Based on the soil test results, 152 mg of urea fertilizer per kg of soil was applied three times (before planting, 1 mo and 2 mo after planting, a total of 456 mg urea per kg of soil).

Table 1. Some chemical and physical characteristics of the soil used in the experiment.

Texture	Sand	Silt	Clay	CCE	OC	SP	pH (1:1)	EC (1:1) (dSm^{-1})
	(%)							
Clay sandy loam (USDA)	47	23	30	15.3	0.59	44.4	8.0	0.47

CCE – calcium carbonate equivalent, OC – organic carbon, SP – saturation percentage

Table 2. Concentration of total N and available elements in the soil used in the experiment.

Total N (%)	P	K	Na	Ca	Mg	Fe	Mn	Zn	Cu	Cd	Pb
	Available (mg kg^{-1})										
0.02	8.7	556	326	7235	798	3.9	7.0	0.50	2.2	0.04	Nil

Rice Plant Materials

The experiment was conducted in the research greenhouse of the University of Tabriz (38°01'55.0"N, 46° 23'36.2"E) in 2014 and 2015. Rice (*Oryza sativa* L. cv. Hashemi) seeds were obtained from the Rice Research Institute of Iran, Rasht, Gilan province, Iran. The seeds were germinated under moist conditions in an incubator at 26 ± 2 °C; 10 germinated seeds were planted in each pot. After seedling establishment, the plants were thinned to four per pot. The pot soil was maintained under waterlogged conditions with 5 ± 2 cm of water above the soil surface during the rice growth period. The pots were kept in a greenhouse with natural sunlight from early June to mid-October of 2014. The greenhouse temperature was between 20 ± 2 °C at night and 32 ± 2 °C during daytime. A big hydroelectric cooler was used to control the temperature. The relative humidity in the greenhouse was maintained at 70 ± 5% by watering the greenhouse floor.

Enzyme Activities

After 85 d of rice growth, the fresh leaves were sampled and washed with double-distilled water. Samples were homogenized in phosphate buffer (0.01 M, pH 7.0) containing polyvinylpyrrolidone (0.2% w/v) to stabilize the extract. The homogenate was centrifuged at 14,000 g at 4 °C for 20 min and the supernatant was used as the crude extract for the following assay. Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Winterbourn et al. (1976). One unit of SOD was defined as the amount of enzyme required to induce a 50% inhibition of NBT (*p*-nitro blue tetrazolium chloride) reduction as measured at 560 nm, compared with the control samples without enzyme aliquot. Catalase (CAT, EC 1.11.1.6) activity was assayed by monitoring the decrease in absorbance of H_2O_2 at 240 nm (Obinger et al. 1997). Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test (Ghamsari et al. 2007). Protein content was determined according to the method of Bradford with bovine serum albumin (BSA) as standard (Bradford 1976). At the end of the growth period, the rice shoots and roots were harvested, rinsed with distilled water, dried at 75 °C for at least 3 d and their dry weights determined. Then the total dry matter of the rice plant in each pot was calculated from the sum of the shoot and root dry matter.

Statistical Analysis

The study used a factorial experiment in completely randomized design with three replications and factors of Zn at three levels (0, 25 and 250 mg kg^{-1}), P at three levels (0, 50 and 500 mg kg^{-1}) and Pb at four levels (0, 200, 400, and 800 mg kg^{-1}). Data were statistically analyzed by using MSTATC software. Duncan's multiple range test at 0.05 probability was applied to compare the means of the measured attributes.

RESULTS AND DISCUSSION

Soil Properties

Selected physical and chemical properties of the soil are presented in Tables 1 and 2. The soil was calcareous, alkaline, non-contaminated and non-saline with clay sandy loam texture. The available P and Zn was low (Hazelton and Murphy 2007).

Results of ANOVA

The main effects of P, Pb and Zn, the two-way interactions of Pb × Zn, Pb × P and Zn × P, and the three-way interaction of Pb × Zn × P were significant on the total dry matter of the rice plant and activity of SOD, POD and CAT enzymes in rice leaves except for the main effect of Zn on POD enzyme activity (Table 3).

Total Dry Matter

In two-way interactions of Pb × Zn and Pb × P, comparison of means showed that Pb contamination of the soil decreased TDM in the rice plant. For example, application of 800 mg Pb per kg of soil decreased TDM by 26% compared with the control (Fig. 1a and b). Applications of 50 and 500 mg P per kg of soil increased plant TDM at different levels of Pb. At 800 mg Pb per kg

Table 3. Summary of ANOVA (mean squares) for total dry matter (TDM) and antioxidant enzyme activities of rice plant.

Source of variation	Degree of freedom (df)	SOD	CAT	POD	TDM
Pb	3	11.97**	2004.1**	0.98**	416.7**
Zn	2	27.14**	10.77**	0.005ns	438.8**
Zn x Pb	6	0.99*	19.67**	0.31**	6.16**
P	2	0.44**	31.55**	0.22**	528.6**
P x Pb	6	0.17**	37.39**	0.13**	5.55**
P x Zn	4	4.51**	53.93**	0.28**	9.79**
P X Zn x Pb	12	1.02**	37.29**	0.16**	6.16**
Error	72	0.43	1.45	0.003	1.04
CV(%)	—	14.43	5.06	5.37	3.40

ns: Non–significant; *: Significant at $p \leq 0.05$;
**: Significant at $p \leq 0.01$.

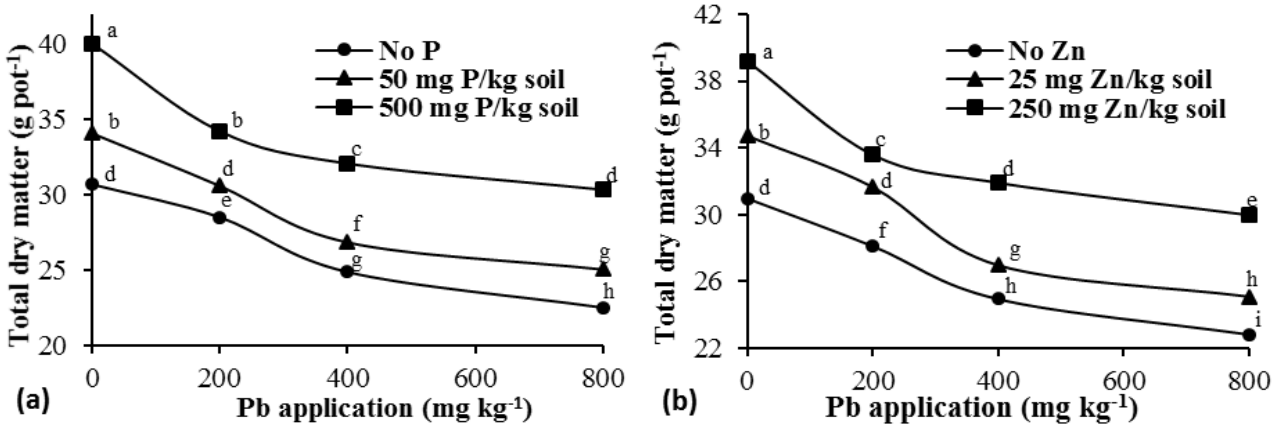

Fig. 1. Total dry matter (TDM) of rice in response to interactions of Pb × P (a) and Pb × Zn (b) (n=9).

of soil, application of 500 mg P per kg of soil increased rice TDM by 35% compared with the control (Fig. 1a). At all levels of Pb, application of P could alleviate the detrimental effect of Pb on rice biomass production. Nutrient supply including P was a dominant factor in rice growth in Pb-spiked soils and when P supply was limited, Pb toxicity could decrease rice growth. Heavy metal excess may lead to severe destruction and adversely affect the growth of cells and the entire plant. When P was available in appropriate amounts, the damage due to Pb and the toxicity of other heavy metals on plant growth could be reduced (Tu et al. 2000). The decrease in plant TDM as a result of proper P application under Pb contamination (Fig. 1a) concurred with reports by Su et al. (2006), Sarwar et al. (2010) and Yongqiang et al. (2010). There were significant linear positive relationships between P level (P) and rice TDM at all Pb levels. At 800 mg Pb kg^{-1} soil, the equation was TDM = 0.0141(P) + 23.38, r=0.98**. Maftoun and Moshiri (2008) and Amanullah and Stewart (2013) reported that by increasing P level, TDM in rice was significantly increased.

In Pb × Zn interaction, at both levels of Zn (25 and 250 mg kg^{-1}), TDM was significantly reduced by increasing Pb level. The decrease was 9% and 31% at 25 and 250 mg Zn and 800 mg Pb per kg of soil, respectively (Fig. 1b). Zn application could decrease Pb toxicity in the rice plant (Aravind et al. 2009). Based on a positive correlation between Zn level (Zn) and TDM in rice at all Pb levels in our study (TDM = 0.0239 (Zn) + 27.81, r=0.95**), Zn application could increase rice production under Pb-contaminated conditions (Fig. 1b). Khan and Qasim (2007) and Aravind et al. (2009) argued that by improving Zn nutrition, oxidative stress caused by Pb and toxicity due to other heavy metals can be improved to a great extent (Kloosterman et al. 2006). Also, Zn plays an important role in photosynthesis, protein and carbohydrate metabolism by activation of metallo-enzymes (Alloway 2008). The combined application of Zn and P increased TDM. Combined P and Zn application improved the growth of rice and could increase plant tolerance mechanisms against Pb toxicity by hormonal and non-hormonal mechanisms (Aravind et al. 2009). Balanced P and Zn nutrition by translocation of assimilates was reportedly essential for optimal yield (Shukla and Yadav 1982).

Our results showed that rice cv. Hashemi could grow in the Zn-spiked soil (250 mg Zn per kg of soil) without any Zn toxic effect on its TDM (Fig. 2a). The nontoxic effects of high level of Zn may be related to high pH of the soil solution (pH=8.0) that causes the precipitation of Zn as $Zn(OH)_2$. Another reason for this result may be the effect of waterlogging conditions on decreasing Zn bioavailability due to its precipitation as $ZnCO_3$, $Zn_3(PO_4)_2$ and ZnS (Havlin et al. 2007; Najafi et al. 2012). Comparison of the TDM means for the three-way interaction of Pb × Zn × P showed that the maximum TDM was in the $Pb_0P_{500}Zn_{250}$ treatment and the minimum was in the $Pb_{800}P_0Zn_0$ treatment (Table 4). In our study, the soil had alkaline pH; while Zn toxicity can occur in severe acid soils and at Zn concentrations > 300 mg kg^{-1} (Foy et al. 1978; Matsuo and Hoshikawa 1995), it seems that Zn was not toxic because toxicity symptoms of Zn in rice shoot were not observed. In a research similar to our study, no significant inhibitory effect on rice growth was observed below 600 mg Zn kg^{-1} (Matsuo and Hoshikawa 1995).

Superoxide Dismutase (SOD) Enzyme Activity

SOD activity was significantly increased by application of 800 mg Pb per kg of soil (Fig. 2b, 3a). The results are similar to those obtained by Dey et al. (2007). Mean comparison for the Pb × P interaction (Fig. 2b) showed that P fertilizer had no significant effect on SOD activity and application of 800 mg Pb per kg of soil significantly

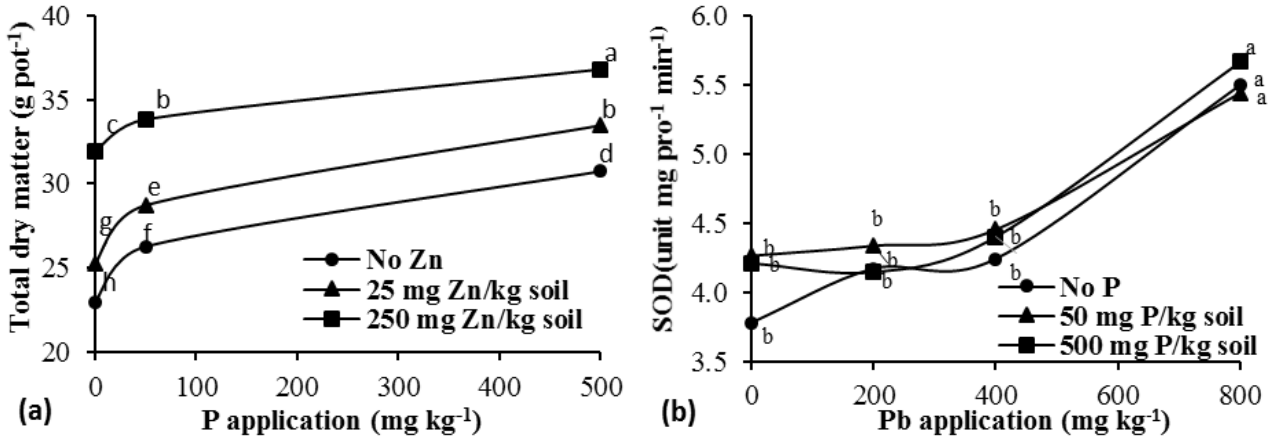

Fig. 2. Effects of P × Zn interaction on total dry matter (TDM (a) and P × Pb interaction on superoxide dismutase (SOD) activity (b) of rice (n = 9).

increased SOD activity at three levels of P fertilizer. High SOD activity was associated with the high potential to remove O_2–, as Sayantan and Shardendu (2013) indicated.

In Pb × Zn interaction, SOD activity increased with increasing Pb levels (Fig. 3a) as Verma and Dubey (2003) and Thakur et al. (2017) indicated in rice plant. SOD enzyme activity was increased by application of 25 mg Zn per kg of soil compared with the control (no Zn) (Fig. 3a). Mishra and Prakash (2009), Morina et al. (2010), and Bharti et al. (2014) reported similar results. The reason for this increase is that Zn as a micronutrient is needed as a co-factor in the functioning of SOD (Marschner 2011). However, when Zn was added at a high level (250 mg kg^{-1}), SOD enzyme activity was decreased in rice plant leaves compared with the control (no-Zn). At a high level, Zn acts as a heavy metal and its toxicity decreases SOD enzyme formation (Marschner 2011). Interaction between Zn and Pb depended on the levels of Zn and Pb. The effect of Pb × Zn interaction on SOD enzyme activity was synergistic at low Zn but antagonistic at high Zn (Fig. 3a).

In P × Zn interaction, at no-Zn fertilizer conditions, applications of 50 and 500 mg P per kg of soil significantly increased the SOD activity (Fig. 3b), as Sayantan and Shardendu (2013) indicated. The effect of P levels on the SOD activity at 250 mg kg^{-1} Zn was not significant but at 25 mg kg^{-1} Zn, application of 500 mg P per kg of soil decreased the SOD activity by almost 16%. SOD activity under 25 mg kg^{-1} Zn and no-Zn fertilizer conditions was greater than that under 250 mg kg^{-1} Zn conditions (Fig. 3b). Ozdener and Aydin (2010) also reported that SOD activity was decreased by Zn toxicity in leaves of *Eruca sativa* L. In general, the effect of P × Zn interaction on SOD activity in rice plant leaves was antagonistic at most levels of zinc application (Fig. 3b). Mean comparison for the three-way interaction of Pb × Zn × P showed that the maximum SOD activity was observed as a result of applying 800 mg Pb, 500 mg P and 25 mg Zn per kg of soil ($Pb_{800}Zn_{25}P_{500}$) whereas the minimum SOD activity was observed in the no-P, no-Zn and no-Pb conditions or the $Pb_0Zn_0P_0$ treatment (Table 4).

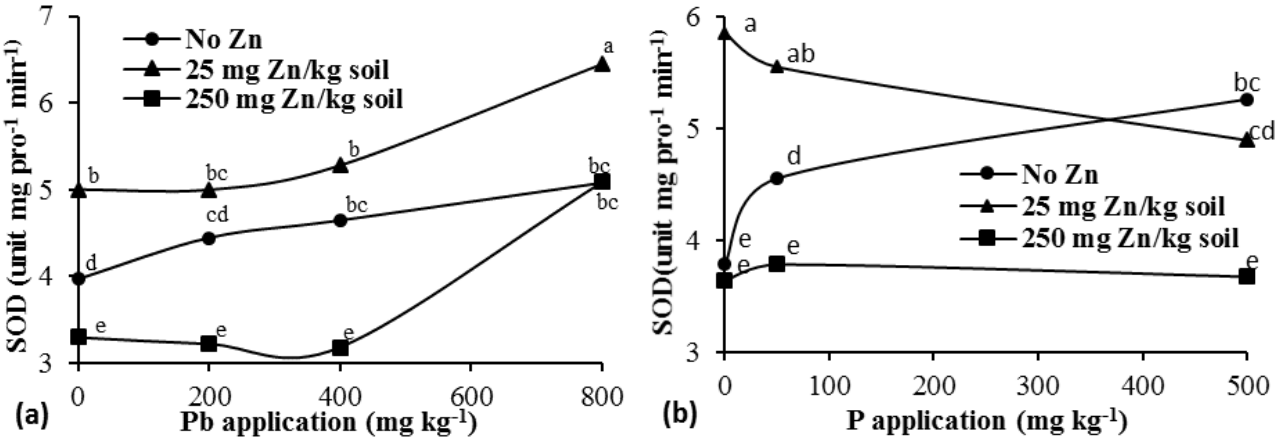

Fig. 3. Superoxide dismutase (SOD) enzyme activity of rice leaves in response to Pb × Zn (a) and P × Zn (b) interactions (n=9)

Table 4. The effects of Pb × P × Zn interaction on total dry matter (TDM) and antioxidant enzyme activities of rice plant.

Pb (mg kg^{-1})	Zn (mg kg^{-1})	P (mg kg^{-1})	SOD Activity (unit mg protein^{-1} min^{-1})	CAT Activity (μM H_2O_2 mg pro^{-1} min^{-1})	POD Activity (μM guaiacol mg pro^{-1} min^{-1})	TDM_1 (g pot)
		0	2.9^{ij}	12.5^{n}	0.8^{hij}	26.7^{kl}
	0	50	4.0$^{g–j}$	12.6^{n}	0.8^{hij}	29.4^{ijk}
		500	5.0^{c-h}	12.9^{n}	0.9^{h}	35.8^{c}
		0	5.3^{b-f}	13.5^{n}	0.8^{hij}	30.8^{ghi}
0	25	50	5.4^{b-f}	12.6^{n}	0.7^{l}	34.1^{d}
		500	3.4^{f-1}	12.8^{n}	0.8^{hij}	39.2^{b}
		0	3.2^{ij}	14.2^{n}	0.6^{nop}	33.6^{de}
	250	50	3.3^{ij}	12.8^{n}	0.6^{nop}	33.7^{b}
		500	3.3^{ij}	14.4^{n}	0.7^{lm}	45.2^{a}
		0	3.1^{ij}	20.1^{m}	0.9^{h}	24.9^{no}
	0	50	4.7^{d-h}	22.8^{jkl}	1.2^{e}	26.8^{lm}
		500	5.4^{b-f}	23.1^{ijk}	1.1^{fg}	32.7^{d-g}
		0	6.0^{a-d}	22.3^{jkl}	0.9^{h}	29.4^{ijk}
200	25	50	5.0^{c-h}	21.6^{klm}	0.8^{hij}	31.91^{e-h}
		500	4.0^{hij}	20.7^{lm}	0.7^{kl}	33.6^{de}
		0	3.4^{ij}	25.0^{ghi}	0.7^{lmn}	31.3^{f-l}
	250	50	3.2^{ij}	21.9^{hij}	0.6^{op}	33.1^{def}
		500	3.0^{ij}	22.3^{jkl}	0.6^{nop}	36.4^{c}
		0	3.9^{hij}	25.2^{ghi}	0.9^{hij}	21.1^{r}
	0	50	4.6^{e-h}	23.4^{ijk}	0.9^{hij}	23.3^{op}
		500	5.4^{b-f}	25.9^{gh}	0.9^{hij}	30.4^{hij}
		0	5.5^{a-f}	29.9^{de}	1.0^{g}	23.3^{opq}
400	25	50	5.7^{a-e}	26.6^{fg}	0.9^{hij}	25.8^{mn}
		500	4.6^{e-h}	25.1^{ghi}	0.8^{jk}	31.9^{e-h}
		0	3.3^{ij}	26.8^{fg}	0.6^{mno}	30.3^{hij}
	250	50	3.0^{ij}	24.2^{hij}	0.6^{op}	31.5^{fgh}
		500	3.1^{ij}	25.3^{ghi}	0.5^{p}	33.9^{d}
		0	5.37^{b-g}	33.1^{c}	0.8^{ijk}	18.3^{s}
	0	50	4.7^{d-h}	34.5^{c}	1.2^{g}	21.5^{qr}
		500	5.2^{c-h}	38.5^{b}	1.1^{f}	28.8^{jk}
		0	6.6^{ab}	34.6^{c}	1.0^{g}	21.6^{pqr}
800	25	50	6.1^{abc}	30.7^{d}	1.4^{c}	23.1^{opq}
		500	6.7^{a}	28.4^{ef}	1.7^{a}	30.6^{hij}
		0	4.6^{e-h}	26.7^{fg}	1.5^{b}	27.7^{kl}
	250	50	5.5^{a-f}	30.9^{d}	1.3^{de}	30.5^{hij}
		500	5.2^{c-h}	47.7^{a}	1.3^{cd}	31.7^{fgh}

Means in each column, followed by the same letters, are not significantly different by Duncan's multiple range test ($p \leq 0.05$).

By catalyzing dismutation reaction ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) and neutralizing the reactive $O2^-$ radicals, SOD is the first line of cell defense against reactive oxygen species (ROS) generated by heavy metal exposure. H_2O_2, which results from the action of SOD, is toxic to cells. Therefore, it is important that H_2O_2 be scavenged quickly by antioxidant systems like CAT and POD enzymes to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Guo et al. 2006). Enhanced activity of SOD, CAT and POD due to soil contamination with Pb was observed (Table 4). As a consequence, plants especially the sensitive ones with higher enzymatic defense system may have better protection mechanism against oxidative damage under heavy metal stress (Guo et al. 2006).

Catalase (CAT) Enzyme Activity

CAT enzyme activity was significantly increased by application of 200, 400, and 800 mg Pb per kg of soil compared with the control under no-P and no-Zn application conditions (Fig. 4a and b). These results are similar to those obtained by Chatterjee et al. (2004) and Liu et al. (2010) in rice plant where CAT activity was enhanced with increasing Pb concentration. The increase in catalase activity might be due to low protein formation and low availability of active iron because of direct competition between Pb and other nutrients for the same site (Chatterjee et al. 2004). However, Thakur et al. (2017) observed that CAT enzyme activity decreased in Pb-treated rice shoots.

Moreover, comparison of means showed that CAT activity in the P treatments and the no-P treatments began to significantly increase from 200 mg Pb per kg of soil. At 400 mg kg^{-1} Pb, CAT activity in the P-treated pots was lower than in the no-P treatments (Fig. 4a), similar to reports by Gunes et al. (2009) and Sayantan and Shardendu (2013). Our results showed that with an increase in P amendment, there was a reduction in the accumulation of Pb, in turn, reducing the stress of ROS formation. As the ROS level decreased, the activity of the enzyme responsible for its conversion also decreased. Because antioxidant enzyme processing is known to be substrate inducible, an increase in enzyme activities may be due to increased production of ROS as substrate resulting in the increased expression of genes encoding antioxidant enzymes (Tsang et al. 1991). At the highest level of Pb (800 mg kg^{-1}), application of 500 mg P per kg of soil significantly increased CAT activity which may result in higher protection against toxicity. This condition might be due to the greater toxic impact of the higher Pb levels, that the P fertilizer did not show any significant impact on Pb accumulation. Competition for carriers results in higher uptake and accumulation of Pb, in turn enhancing oxidative stress (Sinha et al. 2005). There was a significant linear positive relationship between P application level (P) and CAT activity at 800 mg Pb kg^{-1} soil (CAT activity = 0.0136(P) + 31.44, r = 0.999**).

Pb × Zn interaction effect on the CAT activity of rice leaves was mostly similar to the Pb × P interaction effect and was dependent on the levels of Pb, P and Zn. CAT enzyme activity was decreased by about 12% with application of 25 mg Zn per kg of soil at 800 mg kg^{-1} Pb condition compared with the control (Fig. 4b). It seems that applying 25 mg Zn per kg of soil decreased Pb concentration in rice leaves by competition for carriers, thus the plant needed lower CAT (Marschner 2011). Zinc had multiple roles in plant antioxidant features and in plant resistance against Pb toxicity (Kawano et al. 2001). With P application, the CAT activity increased under no-Zn and 250 mg kg^{-1} Zn conditions, while at 25 mg significantly kg^{-1} Zn, it was slightly decreased. It seems that this reduction was due to lower Pb concentration as a result of applying 25 mg Zn per kg of soil (Marschner 2011). The interaction effect of P × Zn on the CAT activity was dependent on P and Zn levels (Fig. 5a). Mean comparison for the three-way interaction of P × Pb × Zn showed that the highest CAT activity was in the $Pb_{800}Zn_{250}P_{500}$ treatment (500 mg P, 250 mg Zn and 800 mg Pb per kg of soil), and it was increased by increment of P and Zn levels and was the lowest in the control treatment ($Pb_0Zn_0P_0$). At high Pb levels, application of appropriate concentrations of P and Zn (500 and 250 mg per kg of soil, respectively) increased the CAT activity (Table 4). This result might be due to better plant nutrition and the role of these nutrients in enhancement of plant antioxidant enzymes (Marschner 2011). Catalase has one of the highest turnover rates for all enzymes with the potential to directly convert H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification in peroxisomes during stress conditions. SOD detoxifies superoxide anion free radicals (O_2^-) by forming H_2O_2, and then H_2O_2 can be eliminated by CAT and POD (Sairam and Srivastava 2001).

Peroxidase (POD) Enzyme Activity

Comparison of the POD enzyme activity means for P × Pb interaction showed that applications of 50 and 500 mg P per kg of soil increased the POD activity at 200 and 800 mg kg^{-1} Pb conditions. For example, at the Pb level of 800 mg kg^{-1}, applications of 50 and 500 mg P per kg of soil increased the POD activity by 83% and 18.8%, respectively, compared with the control (Fig. 5b). At the highest level of Pb, P application could alleviate Pb phytotoxicity due to increasing antioxidant enzyme

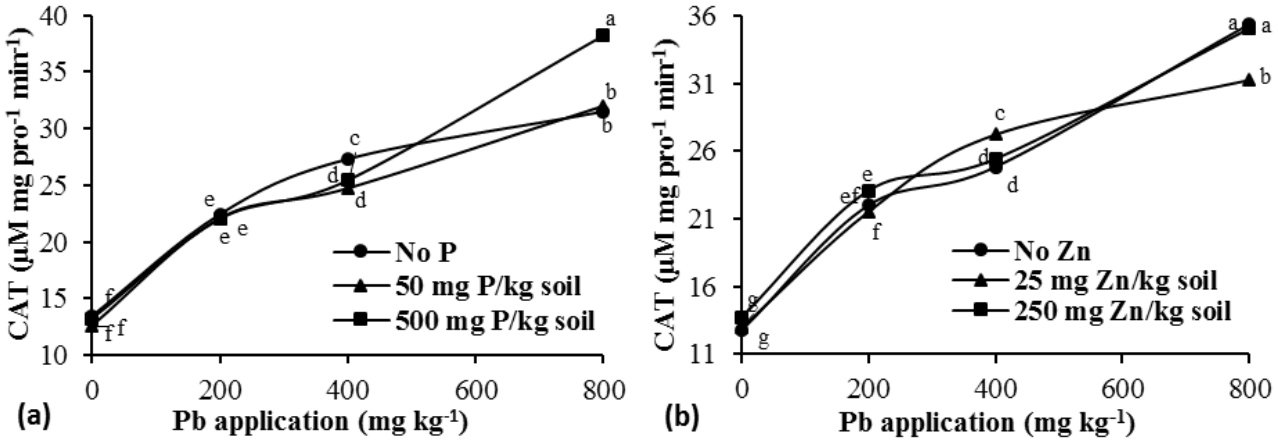

Fig. 4. Catalase (CAT) enzyme activity of rice leaves in response to Pb × P (a) and Pb × Zn (b) interactions (n=9).

activities. In rice production in Pb-spiked soils, nutrient supply was the dominant factor and when nutrient supply was limited, Pb stress could decrease rice growth and yield. When P was available in appropriate amounts, the damage of Pb and other heavy metals could be decreased (Tu et al. 2000). The increase in the POD activity by proper P application under Pb contamination (Fig. 5b) was in agreement with the report of Sayantan and Shardendu (2013). Comparison of the means showed that application of 500 mg P per kg of soil decreased POD activity at the Pb level of 400 mg kg^{-1} by nearly 29%. These results are similar to those of CAT activity.

At no-Zn conditions, POD enzyme activity was significantly increased by increasing the Pb level to 200 and 800 mg kg^{-1} but it was significantly decreased at 400 mg kg^{-1}. This result concurred with the report of Verma and Dubey (2003) that POD activity increased in rice seedlings grown at moderately toxic Pb level whereas a highly toxic Pb level led to a marked inhibition in enzyme activity. Stancheva et al. (2014) also reported that enhanced levels of Pb in soil increased levels of peroxidases in *Matricaria recutita* L. Thakur et al. (2017) observed that ascorbate peroxidase activity was increased in Pb-treated rice shoots. At both levels of Zn (25 and 250 mg kg^{-1}), POD activity did not change significantly with increase in Pb level to 200 mg kg–1 but it was decreased about 39% by application of 25 mg Zn per kg of soil at 400 mg kg^{-1} Pb condition compared with the control (Fig. 6a). These results showed that application of 250 mg Zn per kg of soil could decrease oxidative Pb toxicity in rice plant at Pb level of 400 mg kg^{-1} but at higher Pb concentration (800 mg kg^{-1}), application of 25 and 250 mg Zn per kg of soil significantly increased the POD activity. This might be due to the greater toxic impact of higher Pb level in soil (Verma and Dubey 2003). Kloosterman et al. (2006) and Aravind et al. (2009) argued that by improving Zn nutrition of the plant, oxidative stress caused by Pb and other heavy metals can be improved to a great extent that is aggravated by antioxidant enzymes. The combined application of Zn and P increased the POD activity; however, at low level of P (50 mg kg^{-1}) and high level of Zn (250 mg kg^{-1}), there was an antagonistic interaction between Zn and P (Fig. 6b). This might be due to precipitation of P as $Zn_3(PO_4)_2$ and decrease in P and Zn availability (Motalebifard et al. 2013), and the antagonistic interaction between P and Zn uptake by root and their translocation to shoot (Marschner 2011). Application of P and Zn could increase plant resistance mechanisms against Pb toxicity by hormonal and non-hormonal mechanisms (Aravind et al. 2009). Our results showed that rice plant (cv. Hashemi) could grow in Zn-spiked soil (250 mg kg^{-1}) without any symptoms of Zn toxicity. This result may be due to the enhancement in antioxidant defense system in rice and the decrease in the bioavailability of soil Zn under waterlogged conditions (Najafi et al. 2012). POD enzyme activity of rice leaves was highest at the highest level of Zn (250 mg kg^{-1}) in the treatments with 800 mg Pb and 500 mg P per kg of soil (Fig. 6a and b). Hu and Wenjiao (2015) observed a significant increase in POD enzyme activity in roots and leaves of *Kandelia obovata* L. plant under high-level Zn stress.

The three-way interaction of Pb × Zn × P showed that the maximum POD activity was observed in the $Pb_{400}Zn_{250}P_{500}$ treatment and the minimum in the $Pb_{800}Zn_{25}P_{500}$ treatment (Table 4). On the whole, the increase in POD enzyme activity, which has been described as an efficient enzyme for H_2O_2 scavenging in cells, may play a crucial role in ROS scavenging in rice plants treated with excess Zn (Table 4, Fig. 6a and b, Sgherri et al. 2003, Takahama et al. 2001). The POD enzyme is involved not only in scavenging H_2O_2 but also

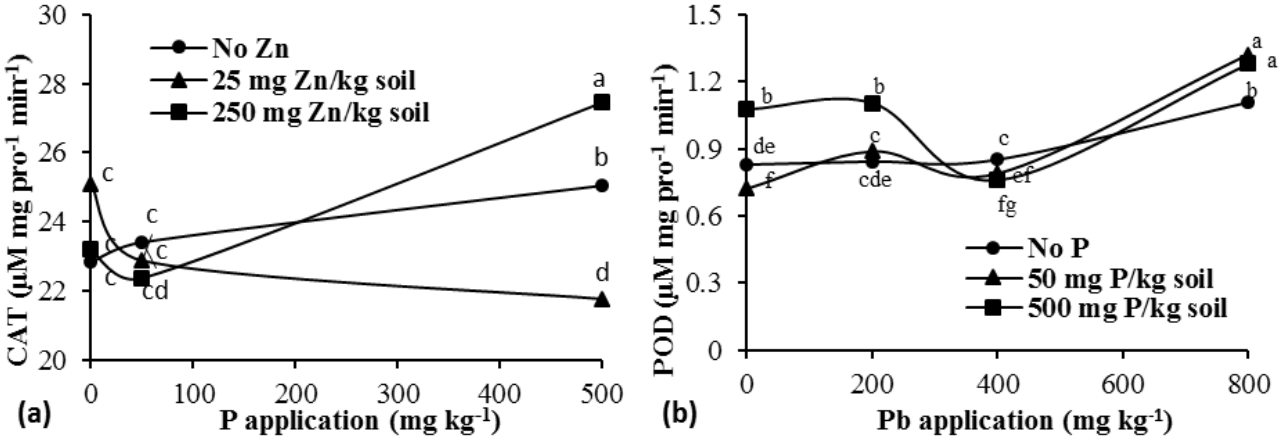

Fig. 5. Effects of P × Zn interaction on catalase (CAT) activity (a) and P × Pb interaction on peroxidase (POD) activity (b) of rice (n=9).

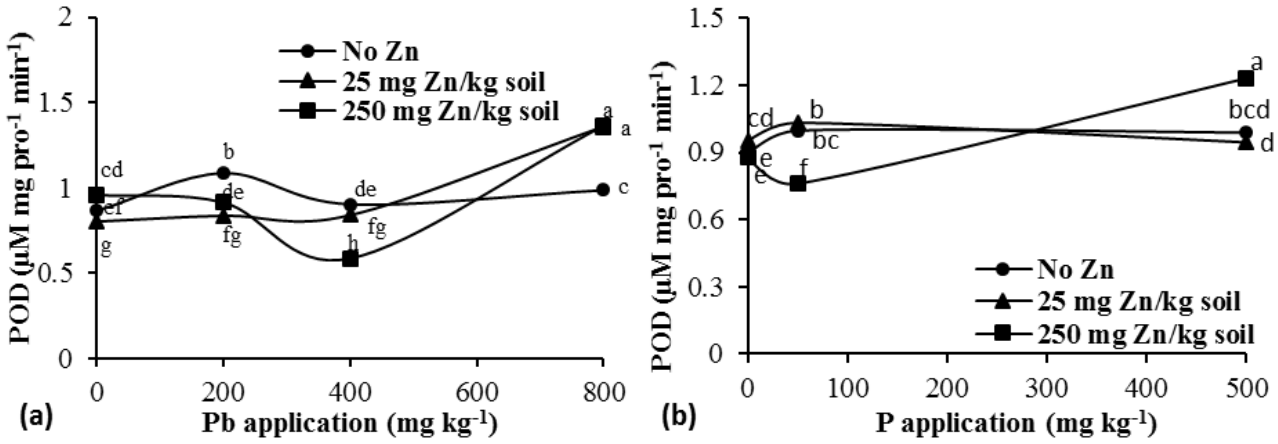

Fig. 6. Peroxidase (POD) enzyme activity of rice leaves in response to Pb × Zn (a) and P × Zn (b) interactions (n=12).

in various plant processes including plant growth, development, lignification (Hendriks et al. 1991), suberization, cross-linking of cell wall (Platisa et el. 2008), oxidation of phenolics (Largrimini 1991), and regulation of cell elongation (Mohammadkhani and Heidari 2008).

CONCLUSION

The main effects of P, Pb and Zn, and two-way interactions of Pb × Zn, Pb × P and Zn × P and three-way interaction of Pb × Zn × P were significant on the total dry matter (TDM) and the SOD, POD and CAT enzyme activities of rice plant except for the main effect of Zn on POD enzyme activity. Addition of P and Zn fertilizers to the soil increased plant TDM at all Pb levels and enhanced SOD, POD and CAT enzyme activities at Pb level of 800 mg kg^{-1}. The two-way interactions of P × Zn, Pb × Zn and P × Pb on SOD, CAT and POD activity were dependent on the levels of these elements. The effects of Pb stress on SOD, CAT and POD activity could be increased by P and Zn fertilization. The results showed that P and Zn requirements of rice plant were enhanced by increasing Pb toxicity. Fertilization of P and Zn significantly enhanced rice tolerance to Pb toxicity by mechanisms such as decreasing the concentrations of Pb in rice shoot, increasing rice plant TDM and the incidence of dilution effect, and increasing the SOD, CAT and POD activity. At 800 mg Pb kg^{-1}, increase in SOD and CAT enzyme activity by application of P amendment could be predicted by the regression equations SOD = 0.0001(P) + 1.37, r = 0.94** and CAT = 0.0034(P) + 7.56, r = 0.99**, respectively. Under no-Pb and no-Zn conditions, no-P fertilizer was required but in Zn-contaminated soil, application of 50 mg P per kg of soil could be suggested. Also, our results showed that rice plant (cv. Hashemi) could grow in Zn-spiked calcareous soils by controlling the level of ROS tightly even in soils with a total Zn concentration of about 250 mg kg^{-1}. In general, to achieve the maximum activity of SOD and POD enzymes in Pb-contaminated soils, the combined application of 25 mg Zn and 500 mg P may be recommended. Likewise, to achieve the maximum CAT activity and the maximum TDM, the combined application of 250 mg Zn and 500 mg P per kg of soil may be recommended under similar conditions.

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