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INFLUENCE OF RHIZOBACTERIA INOCULATION AND LEAD STRESS ON THE PHYSIOLOGICAL AND BIOCHEMICAL ATTRIBUTES OF WHEAT GENOTYPES

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ABSTRACT. Contamination of soils by lead (Pb) is of widespread occurrence as result of human, agricultural and industrial activities. A pot study was carried out to evaluate physio-biochemical responses (chlorophyll content, soluble protein, proline content and activities of enzymatic antioxidants) of 10 bread wheat genotypes to inoculation of plant growth promoting rhizobacteria (combination of *Azospirillum brasilense* and *Azotobacter chroococcum*) under Pb stress (0 and 65 mg kg^{-1}). Result revealed that lead stress averagely decreased grain yield of wheat cultivars by 41.4 %. Lead stress increased lipid peroxidation and induced a significant accumulation of proline in leaves. Protein content decreased from 8–25.4% in different cultivars in Pb-contaminated soils. Activities of antioxidant enzymes, such as, ascorbate peroxidase, superoxide dismutase and catalase were significantly increased in the presence of lead. An increase in total hydrogen peroxide (H_2O_2) content was noticed under lead stress in all cultivars, which was similar to production of malondialdehyde (MDA). However, promotion of growth was evident in most cultivars as a consequence of rhizobacterial inoculation, since plant growth promoting rhizobacteria could improve grain yield, proline content and membrane integrity, while significantly reduced the production of MDA and H_2O_2. Total chlorophyll content considerably declined with Pb stress. Between cultivars the best performance under lead stress was observed in Sardari, Shahriyar and Gaspard which had the highest yield and antioxidants activity. Obtained results showed that inoculation with *Azotobacter* and

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Azospirillium possibly through bioremediation strategy can stimulate plant growth under adverse environmental conditions, such as heavy metal contamination.

Key words: Antioxidant; Inoculation; Heavy metal; Proline; Reactive oxygen species.

INTRODUCTION

In recent years heavy metal accumulation in soil has been increased in proportion to the pace of worldwide industrialization. Increasing environmental pollution has serious consequences for plants, including crops. Contamination of soil and water by toxic heavy metals mostly is resulted from human activity and there are many records that agricultural land adjacent to industrial areas are polluted to varied extent by many toxic heavy metals (Rao, 1979). Metal smelting, gas exhaust, electroplating, mining operations, energy and fuel production power lines are some of the numerous human activities that contain quantities of toxic metals (Kumar *et al.,* 1995). On the other hand, agricultural intensification has greatly increased the productive capacity of agroecosystems, but has had unintended environmental consequences including degradation of soil and water resources, and alteration of biogeochemical cycles (Drinkwater and Snapp 2007).

Lead (Pb) is an environmental contaminant extremely toxic to plants and other living organisms including humans. Although lead is not included in essential elements for plants, it can be absorbed by plants when it is present in rhizosphere, especially in the around of the cities where the soil is polluted by automotive exhausts and in fields polluted with fertilizers containing heavy metal ingredients (Adriano, 2001). Increased Pb in soils may affect the soil productivity and even a very low concentration can inhibit some vital plant processes, such as photosynthesis, mitosis and water absorption with toxic symptoms of dark leaves, wilting of older leaves, stunted foliage and brown short roots (Patra *et al.,* 2004). When lead enters the plant cells, can induce the generation of reactive oxygen species (ROS) including hydrogen peroxide(H_2O_2), which unbalances cellular redox, inactivate enzymes, and cause a lipid peroxidation and totally result in a reduction of crop production (Moldovan and Moldovan, 2004). Malondialdehyde (MDA) is a product of lipid peroxidation by ROS and a most prominent indicator of oxidative stress in plants exposed to stress conditions (Yamamoto *et al.,* 2001). However, plants have enzymatic (catalase, ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase, etc) and non-enzymatic (ascorbate, glutathione,α-tocopherol) antioxidant systems to protect them against oxidative damage (Prochazkova *et al.,* 2001). Those detoxification processes are complex and highly compartmentalized in plant cells and it may show significant difference between the crop cultivars. Also, proline can accumulates in

many plant species in response to environmental stress. It protects folded protein structures against denaturation, stabilizes cell membranes by interacting with phospholipids, functions as a ROS scavenger, or serves as an energy and nitrogen source (Samaras *et al.,* 1995).

Due to the stability of heavy metals in the soil, it seems the only bioremediation methods can be effective in clean up the contaminated soils. Phytoremediation is a relatively new approach to removing contaminants from the environment. It may be defined as the use of plants to remove, destroy or sequester hazardous substances from the environment (Garbisu *et al.,* 2002). However, only a limited number of plant species can participate in phytoremediation and most of the commonly known heavy metal accumulators belong to the *Brassicaceae* family (Kumar *et al.,* 1995). Generally, plants with exceptionally high metal accumulating capacity often grow slowly and produce limited biomass, particularly when the metal concentration in the soil is high. Furthermore, excessive accumulation of heavy metals in plants can be toxic because these metals can modify essential protein structure or replace an essential element causing chlorosis, growth impairment, browning of roots, and inactivation of photosystems among others (Görhe and Paszkowski, 2006). However, there is an alternative way to maximize the chances of success of phytoremediation by utilizing plant growth-promoting rhizobacteria (PGPR), which are soil microbes that inhabit the rhizosphere (Glick, 2003). Some of the PGPR can fix atmospheric nitrogen and supply it to plants, or may synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells. Phytohormones produced by PGPR can enhance plant growth. Moreover, PGPR contain enzymes that modulate plant growth and development (Ma *et al.,* 2009). Results of previous research suggest that partnership between plants and PGPR can be utilized as a strategy to promote plant biomass production and heavy-metal removal from metal-polluted soils (Umrania, 2006; Tak *et al.,* 2013). The aim of present study is to assess the effect of PGPR application in lead contaminated soils on physiological and biochemical attributes of bread wheat genotypes.

MATERIALS AND METHODS

Plant material and growth conditions

In order to investigate the effects of seed inoculation with PGPR and lead stress a pot experiment was carried out at experimental field of the Department of Agronomy, Islamic Azad University, Karaj Branch, Iran, during October 2010-April 2011, with ten facultative bread wheat cultivars. Study was conducted in pots of 20 cm diameter and 30 cm in depth filled with ten kg of sandy loam gardening soil. The field was located at 50°49' East longitude and 35°46' North latitude, at an altitude of 1271 meter from

sea level, where the climate is semi arid and cold temperate. The soil was non-saline, with pH 6.8 and 0.95% organic matters. The total nitrogen and phosphorus content were about 0.67 mg/kg and 0.085 mg/kg, respectively. It contains (μg/g air dry soil) 6.7 Mn, 254 K, 72 Mg, 13.6 Zn, 0.27 Pb. The experiment comprised three replicates and was laid out as factorial (10×2×2) based on randomized block design. Seeds of ten bread wheat genotypes (*Triticum aestivum* L.) including Azar2 (V1), Gaskozhen (V2), Backcross Roshan (V3), Zarin (V4), MV17 (V5), Pishgam (V6), Alamout (V7), Shahriar (V8), Gaspard (V9) and Sardari (V10) used in this study were obtained from Seed and Plant Improvement Institute (SPII), Karaj, Iran. Second factor was PGPR inoculation, since seeds were divided into two groups and first group as control (without PGPR inoculation) and the rest were inoculated in combination with *Azospirillum brasilense* and *Azotobacter chroococcum* before sowing. Fifteen seed were planted in each pot at a depth of 4 cm. By thinning we maintained only eight seedlings in each pot in order to avoid imbalanced uptake of nutrients by plants. The third factor was lead stress with two levels (0 and 65 mg kg-1). Forty days after sowing the heavy metals stress was imposed by application of lead (II) acetate [$Pb(C_2H_3O_2)_2$] in liquid form mixed with distilled water following the method used by (Stoeva and Bineva, 2003). Both control and Pb treated pots were irrigated daily with tap water carefully to avoid leach out of solution from treated pots. Growth was expressed as the relative growth rate (RGR) from the mean natural logarithm-transformed plant weights, as described by Hoffmann and Poorter (2002). Five plants from each pot were harvested during the booting stage and upper fully expanded leaves were used for physiological and biochemical analysis. At maturity stage, spike length, 100-seed weight and grain yield plant-1 were evaluated. Tolerance index (Tol) was calculated according to modulated Wilkins's equation (Wilkins, 1957) where GYPb and GYC represented the mean grain yield of plants in Pb treatments and controls, respectively: Tol = GYPb / GYC ×100.

Total chlorophyll determination

Chlorophyll was extracted and determined from expanded young leaves according to Inskeep and Bloom (1985). Known fresh weight (about 0.1 g) of leaves were immersed in 10 ml N, N-dimethylformamide (DMF) and kept overnight at 4°C. After incubation, chlorophylls contents were determined in the extract by UV-spectrophotometer.

Leaf membrane stability

Leaf membrane stability index (MSI) was measured as ion leakage. For this purpose the washed leaves were cut into 1 cm pieces and placed in a glass beaker containing 10 mL deionised water. The beakers were kept at 30°C for 3 h and the conductivity of solution was measured by an electrical conductivity meter. The same samples were boiled for 2 min and then their conductivity was measured again, when the solution was cooled to room temperature. The percentage of membrane stability was calculated as follows, MSI (%) = {1- (C1/C2)} x 100. Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively (Sairam *et al.,* 1997). Lipid peroxidation in leaves was measured in terms of malanodialdehyde (MDA), a product of lipid peroxidation content determined by the thiobarbituric acid (TBA), according to the method of Heath and Packer (1968).

Determination of soluble protein and proline concentration

Proline concentration was determined using the method of Bates *et al.* (1973). Fully expanded young leaves (0.5 g) were ground in 3% (w/v) aqueous sulphosalicylic acid and proline was estimated by ninhydrin reagent. The absorbance of the fraction with toluene aspired from the liquid phase was read at 520 nm. The proline concentration was determined after the realization of a standard curve; it was expressed in µmol/g fresh weight. Proteins were estimated by the method of Bradford (1976)**.** In the Bradford assay, protein concentration is determined by quantifying the binding of the dye, Coomassie Brilliant Blue G-250, to the unknown protein solution, as compared to known standards. Tubes containing 100 µl aliquots of known concentrations of bovine serum albumin (BSA: 0.156 mg l^{-1} to 10 mg l^{-1} in 0.15 M NaCl), were prepared. Blank tubes containing 100 µl of 0.15 M NaCl were also prepared. One ml Coomassie Brilliant Blue solution was added to each tube and the mixtures vortexed. The reactions were left at room temperature for 2 min. The absorbance at wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted (Copeland, 1994). Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up as above and the absorbance at 595 nm determined. The protein concentration of the extracts was determined from the standard curve, using spectrophotometer.

Determination of antioxidative enzyme activity

About 0.2 g leaf tissues were homogenized in an ice cooled mortar with 5 mL of 50 mmol l^{-1} Na-phosphate buffer (pH 7.8) containing 0.1 mmol l^{-1} Na_2-EDTA and 1% (W/V) polyvinyl-polypirrolidone (PVPP). The extract was centrifuged at 10 000 r min^{-1} for 15 min at 4°C, and the supernatant was prepared for the determination of soluble protein content and enzyme activity. Catalase activity was assayed according to Aebi (1984) where decomposition of H_2O_2 is followed spectrophotometrically at 240 nm. One unit of enzyme activity is equal to 1 µmol of H_2O_2 decomposed per min. APX activity was determined according to the method Chen and Asada (1989). SOD activity was estimated by recording the decrease in absorbance of superoxidenitro blue tetrazolium complex by the enzyme (Sen-Gupta *et al.,* 1993). Total hydrogen peroxide (H_2O_2) content was measured according to Ohwada and Sagisaka (1987).

A similar laboratory experiment was conducted in a germinator to evaluate the effect of mentioned treatments on the germination characteristics. For this purpose seeds were germinated in 12 cm diameter glass Petri dishes at 25±1 °C in a dark growth chamber with 45% relativity humidity. Germinating seed were counted daily, and terminated when no further germination occurred. Mean germination time (MGT) was computed according to Ellis and Roberts (1981) as $MGT=\sum Ti\ Ni/\sum Ni$, where N*i* is the number of newly germinated seeds at time T*i*. Data was subjected to analysis of variance (ANOVA) by SPSS version 17 computer package (SPSS Inc., Wacker Drive) and Microsoft Excel was used for Standard Error. The significance of difference between means was computed following the LSD. Cluster analysis was performed by the un-weighted pair group method with arithmetic average (Ward) using Pearson correlation. Principal component analysis (PCA), based on the rank correlation matrix and biplot analysis,

were performed by STATISTICA ver. 8 and Minitab ver.16.

RESULTS AND DISCUSSION

The results pertaining to effect of Pb and PGPR treatments on germination characteristics of wheat cultivars are depicted in *Table 1*. The results showed that Pb stress significantly reduced germination percentage and seedling dry weight while increased mean germination time (MGT) in all investigated cultivars compared with control. A comparison among the wheat cultivars indicated that the lowest germination percentage under lead stress was observed in Sardari, Alamout, MV17 and Gaskozhen cultivars without rhizobacterial inoculation, since PGPR inoculation could significantly improve the germination. Seedling dry weight reduced up to 60% by lead stress and the largest decrease was observed in cv. Alamout. Rhizobacterial inoculation under Pb stress could increase seedling dry weight by more than one fold, while incremental effect of rhizobacterial inoculation under heavy metal-free conditions was only 13%. PGPR inoculation could significantly reduce MGT by 19% under Pb stress. However under heavy metal-free conditions decreasing effect of PGPR inoculation on MGT was only near to 10% (*Table 1*).

Result of variance analysis for total chlorophyll content revealed that the interaction between lead treatments and cultivars was statistically significant at the 5% level. It was observed that Pb stress significantly reduced chlorophyll content by 68% in comparison with control. Although the largest decrease was recorded in Sardari and Pishagam cultivars, the highest chlorophyll content under Pb stress was observed in the same cultivars (*Table 2*). Similar results as in the chlorophyll content were found for total soluble protein content as protein content significantly reduced (16%) in leaves under heavy metal stress. However, the greatest decrease was recorded in Shahriar, Alamout and Pishgam. Conversely, proline content of the leaves was greatly enhanced with lead stress with various intensities in different cultivars (*Fig. 1*). In addition seed inoculation with PGPR in cv. Azar 2 and cv. Shahriar could significantly improve proline content in leaves of plant under Pb stress. Nevertheless, the rhizobacterial inoculation in Sardari, MV17 and Zarin cultivars reduced the proline content when compared with control plants. Exposure of plants to lead resulted in an increase of hydrogen peroxide concentration. Accumulation of H_2O_2 in different cultivars showed the exclusive patterns. The largest increase was recorded in Zarin, MV17 and Pishgam cultivars, while Gaspard and Sardari cultivars showed the lowest increase (*Table 2*).

Table 1 - Effect of Pb and PGPR on germination traits of wheat varieties

Cultivars	Germination percenrage				MGT (day)				Seedling Dry Weight (mg)			
	with PGPR		without PGPR		with PGPR		without PGPR		with PGPR		without PGPR	
	Pb	control	Pb	control	Pb	control	Pb	control	Pb	control	Pb	control
Azar2	75.27	99.33	75.44	95.66	4.62	4.26	5.46	3.67	3.15	5.92	1.75	5.61
Gaskozhen	66.33	96.52	66.82	100	4.02	5.11	5.40	3.47	3.84	7.50	1.51	7.33
Roshan	75.83	98.42	75.34	90.27	3.91	3.98	5.21	4.12	4.23	6.41	1.42	8.21
Zarin	83.75	90.19	83.58	98.13	3.59	4.25	5.02	4.25	3.28	6.38	1.77	6.57
MV17	65.26	94.66	65.87	100	4.68	5.41	5.54	3.96	2.85	6.57	1.29	7.27
Pishgam	71.83	93.0	71.66	96.75	4.21	4.62	5.34	4.22	4.21	6.82	1.83	8.72
Alamout	65.72	96.58	65.27	90.0	4	4.33	5.13	4.52	3.52	7.42	1.37	10.14
Shahriar	70.0	87.42	70.33	95.23	4.76	5.29	5.17	4.72	3.49	5.07	1.23	6.59
Gaspard	74.66	87.86	74.28	87.0	4.47	5.33	4.82	4.97	2.83	4.97	1.28	6.22
Sardari	65.80	98.12	65.49	95.33	4.90	4.57	5.50	4.15	2.67	5.34	1.15	5.63
LSD P < 0.05	4.53				0.24				0.62			

Table 2 - Effect of Pb stress on biochemical traits of wheat varieties

Cultivars	Protein (µg g^{-1} fw)		SOD (U mg^{-1} Protein)		CAT (U mg^{-1} Protein)		H_2O_2		MSI (%)		CHL (mg g^{-1} fw)	
	Pb	control	Pb	control	Pb	control	Pb	control	Pb	control	Pb	control
Azar2	29.66	34.88	17.19	9.073	60.92	20.47	4.82	2.09	25.92	62.98	0.377	0.5500
Gaskozhen	33.20	36.90	16.10	10.820	50.25	21.52	4.83	2.21	44.46	64.53	0.416	0.6020
Roshan	31.47	36.50	14.15	9.470	66.58	20.04	4.83	2.21	32.97	70.36	0.406	0.6560
Zarin	41.50	45.19	18.50	14.070	59.75	17.07	4.92	1.31	53.80	73.03	0.411	0.5810
MV17	41.89	46.69	18.60	15.590	85.18	21.35	4.89	1.57	56.97	74.96	0.415	0.6065
Pishgam	39.90	48.43	20.89	17.520	97.21	27.99	4.15	1.04	65.14	80.77	0.436	0.6670
Alamout	34.67	44.04	17.51	14.100	72.65	24.92	4.26	1.50	51.95	75.05	0.368	0.5350
Shahriar	45.27	60.57	22.96	17.880	104.50	25.30	3.03	1.63	62.75	83.55	0.345	0.5400
Gaspard	41.11	48.46	21.07	16.950	83.67	35.32	3.13	1.12	60.72	82.74	0.435	0.5205
Sardari	45.69	52.63	24.15	20.430	112.12	22.60	2.34	1.27	75.09	89.06	0.559	0.8594
LSD P < 0.05	3.69		2.74		10.53		0.56		9.35		0.086	

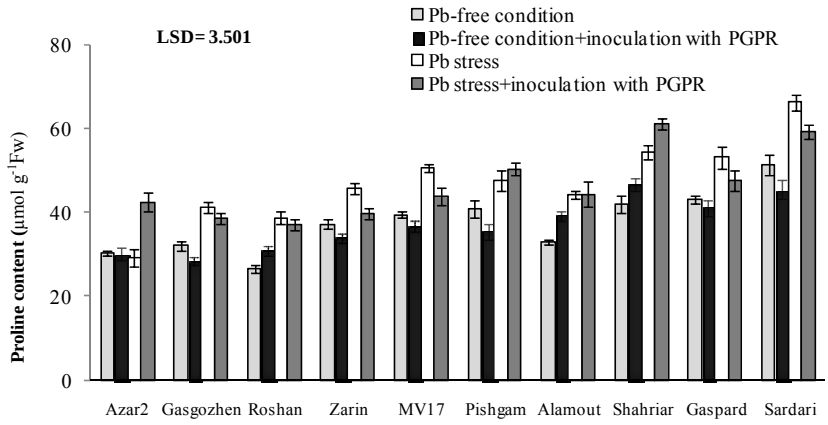

Figure 1 - Effect of PGPR inoculation and Pb stress on proline content in leaves of different wheat cultivars. The values and standards errors (vertical bars) of three replications are shown.

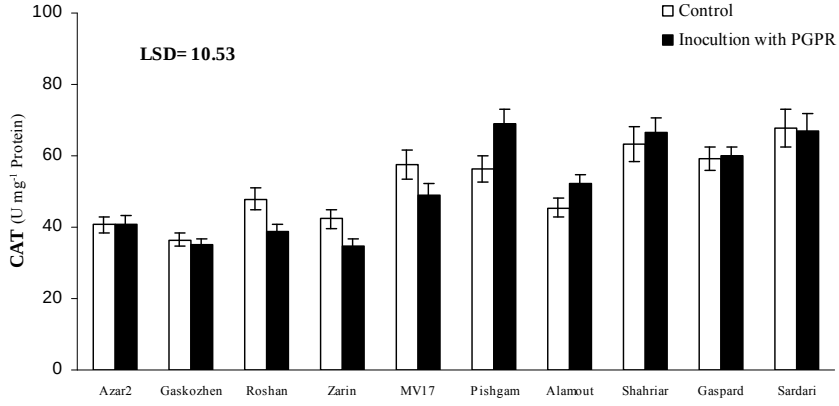

Figure 2 - Effect of PGPR inoculation on catalse activity in leaves of different wheat cultivars. The values and standards errors (vertical bars) of three replications are shown.

Analysis of enzymatic antioxidants showed that the activity of SOD and CAT significantly increased by heavy metal stress. SOD activity under Pb stress conditions averagely increase by 31% and the greatest increase was recorded in cv. Azar 2 (87%). A similar trend was observed for CAT activity, since the activity of this enzyme increased more than twice under lead stress when compared with pb-free condition. The largest increase was observed in Sardari, Shahriar, Pishgam and MV17 cultivars. The result of interaction between

rhizobacterial inoculation and cultivar is shown in *Fig. 2*. In the present study seed inoculation with rhizobacteria decreased CAT activity in Roshan and MV17 cultivars. However, Pishgam cultivars showed a significant increase by PGPR.

Investigation the effects of Pb stress and PGPR on APX activity in different cultivars is shown in *Fig. 3*. Although Pb stress induced the activity of APX, the bacterial inoculation effect was dissimilar in different cultivars. Rhizobacterial inoculation could significantly increase APX activity in cvs. Sardari and Zarin under Pb stress. The highest APX activity was recorded in cv. Pishgam under Pb stress without PGPR which may refer to high scavenging capacity of this genotype.

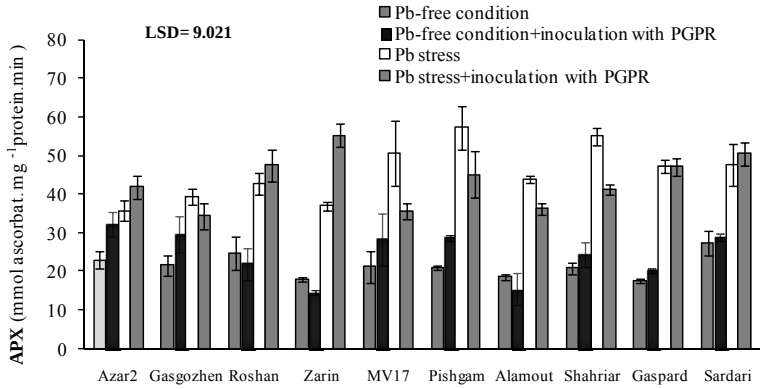

Figure 3 - Effect of PGPR inoculation and Pb stress on ascorbate peroxidase in leaves of different wheat cultivars. The values and standards errors (vertical bars) of three replications are shown.

Changes in lipid per oxidation and malanodialdehyde production in leaves of wheat cultivars under lead stress and PGPR inoculation is shown in *Fig. 4*. Metal stress significantly increased lipid peroxidation and rhizobacterial inoculation reduced malanodialdehyde production in some cultivars like as Azar 2, Gasgozhen, Zarin, Gaspard and Sardari (*Fig. 4*). The results in relation to the effect of Pb on membrane stability measured in terms of solutes leakage showed that the plasma membrane in Sardari, Pishgam and MV17 cultivars appropriately was protected from the destructive effects of lead stress. This was consistent with the results obtained from the malondialdehyde assessments. However, membrane stability in cv. Azar 2 and cv. Roshan stress drastically affected by Pb stress (37%).

Results indicated that grain yield under Pb stress averagely decrease about 4.41% in comparison with control. Furthermore rhizobacterial inoculation could increase grain yield up to 6 percent. Although, the main effects were significant, their

interactions were not statistically significant. Changes pattern of spike length was the similar to grain yield. Variance analysis for 100-grain weight revealed that the interaction of rhizobacterial inoculation × lead was significant, so that PGPR inoculation could significantly improve grain weight under both Pb stress and stress free condition. Means comparison showed that the highest 100-grain weight was obtained from PGPR inoculated plant under Pb-stress free condition (*Fig. 5*). The lowest grain weight was recorded for intact plants of cv. Shahriar under Pb stress (*Fig. 6*). Treatments could affect rate of stem elongation (RGR) and tolerance index. The rhizobacterial inoculation under Pb-stress free condition could improve relative growth rate in all cultivars, except cv. Pishgam and cv. Azar 2. The best performance in terms of tolerance index was related to cv. Sardari and Shahriar.

Based on biochemical and physiological traits, 10 wheat cultivars were clustered into two groups including resistant and susceptible groups. Group A included Sardari, Shahriar, Gaspard and Pishgam which were resistant to Pb stress. Group B included Zarin, Alamout, MV17, Gaskozhen, Roshan and Azar 2 as susceptible to lead stress (*Fig. 7*). To better understand the relationships, similarities and dissimilarities among the physiological and biochemical traits, principal component analysis (PCA), based on the rank correlation matrix was used. Based on PCA results CAT, APX, TOL, H_2O_2 and MDA could be introduced as group 1= G1 indices and positively correlated with each other. The PCs axes separated chlorophyll content, relative growth rate (RGR), mean germination time, germination percent and seedling dry weight in the second group (G2) and spike length, grain yield and MSI in a third group (G3) (*Fig. 8*). As the cosine of the angle between investigated traits indicated H_2O_2 and MDA negatively correlated with grain yield and MSI. However, antioxidant enzymes activity and proline content showed a positive correlation increased with ROS production.

In the present study the most prominent of Pb toxicity was found to be the inhibition of germination. Seed germination is the basic phase in the growth of any plant and is energy consumable process. During the seed germination some of the hydrolyzing enzymes become active. These include amylases, invertases, proteases and lipases that hydrolyse polysaccharides, proteins and lipids respectively into their consumable monomers (Bewely and Black, 1982). The stored materials in the cotyledons and the endosperm are hydrolysed and transferred to the growing embryo. This involves the activation and synthesis of several hydrolyzing enzymes (Surekha and Duhan, 2012). Inhibition of seed germination, hydrolyzing enzymes and seedling growth treatment of some heavy metals has been reported in many plants (Sharma *et al.,* 1995; Jain *et al.,* 1998).

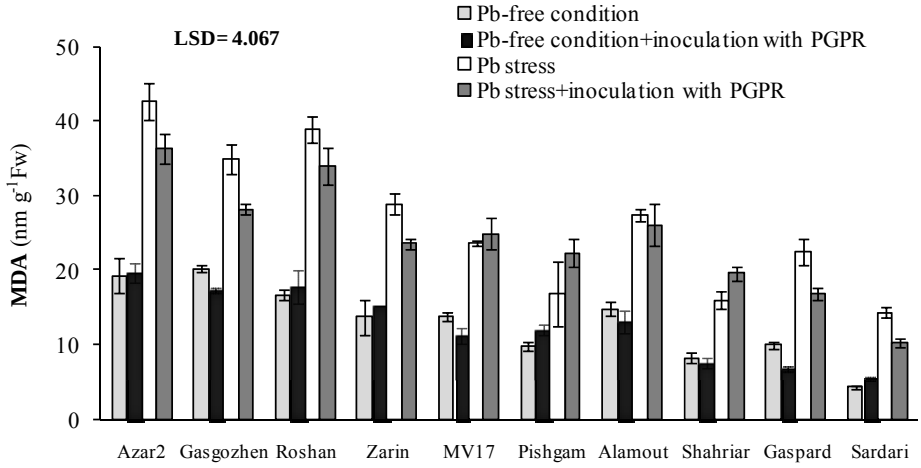

Figure 4 - Effect of PGPR inoculation and Pb stress on malanodialdehyde in leaves of different wheat cultivars. The values and standards errors (vertical bars) of three replications are shown.

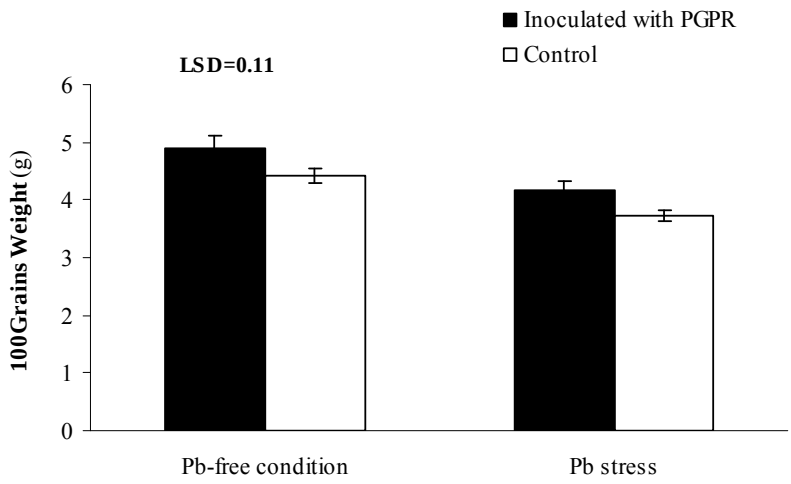

Figure 5 - Effect of PGPR inoculation and Pb stress on 100-grain weight. The values and standards errors (vertical bars) of three replications are shown.

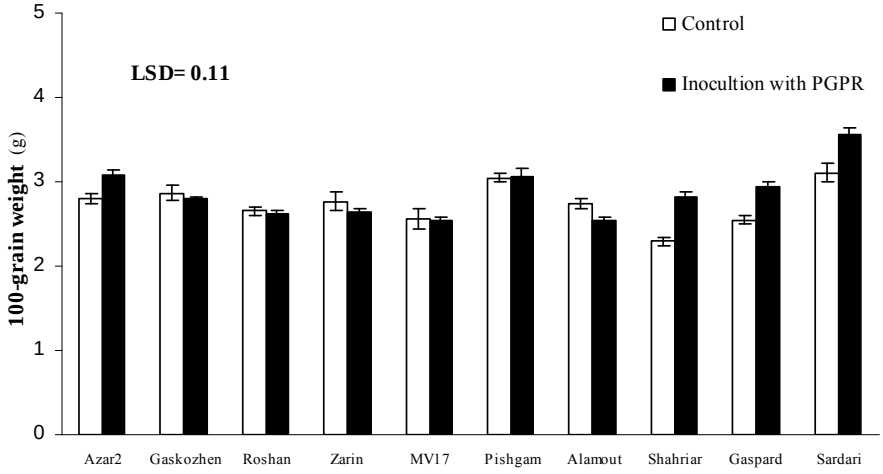

Figure 6 - Effect of PGPR inoculation on 100-grain weight of different wheat cultivars. The values and standards errors (vertical bars) of three replications are shown.

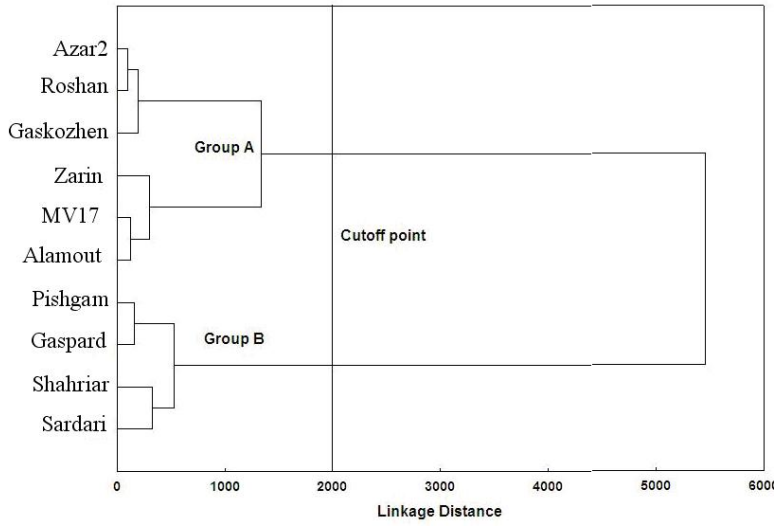

Figure 7 - Dendrograms established from Pearson correlation between cultivars using Ward method based on biochemical and physiological traits.

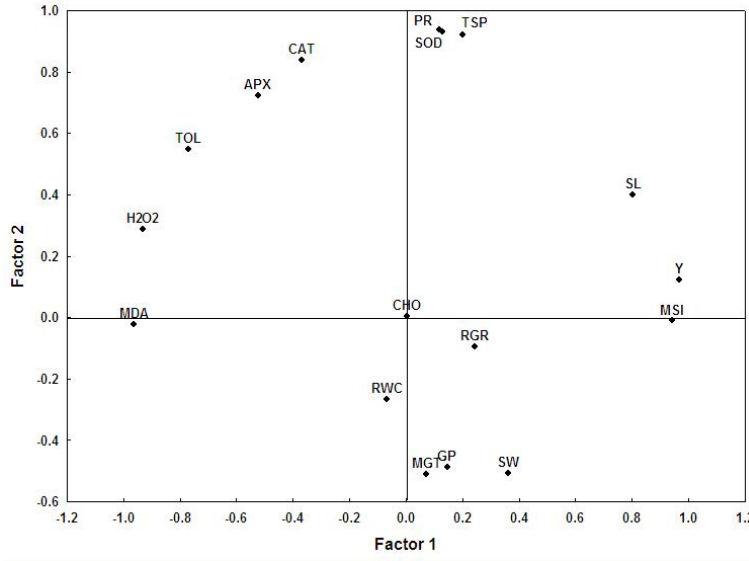

Figure 8 - Principal component analysis of biochemical and physiological traits of wheat cultivars under pb stress and rhziobacterial inoculation.
GP: germination percentage,;MGT: mean germination time; SW: seedling dry weight; RGR: relative growth rate; MSI: membrane stability index; Y: grain yield; SL: spike length; CHO: chlorophyll content; TSP: total soluble protein; SOD: super oxide dismutase activity; PR: praline content; CAT: catalase activity; APX: ascorbate peroxidise; TOL: tolerance index; H_2O_2: hydrogen peroxide concentration; MDA: malanodialdehyde concentration; RWC: relative water content.

The heavy metal stress can induce generation of reactive oxygen species and my cause oxidative stress. Much of the reactive oxygen species produced through contacts between heavy metal and biological membranes (Dietz *et al.,* 1999). In order to cope with highly toxic metals, or to maintain the level of essential metals within physiological ranges, plants have evolved complex mechanisms that serve to control the uptake, accumulation and detoxification of metals. To mitigate and repair the damage initiated by ROS the induction of the activities of a particular group of enzymes i.e., antioxidant enzymes play an important role in the cellular defense strategy against oxidative stress caused by toxic heavy metal concentrations. In the current study activity of CAT and SOD and APX enzyme increased in leaves when subjected to lead stress. The degree of resistance of living systems, the level of their reliability, and the process of aging significantly depend on the activity of the antioxidant enzymes (Surekha and Duhan, 2012). However, H_2O_2 production rate increased by lead stress and an increase observed in MDA content in Pb-contaminated soils. The

enhancement of MDA production in plant without PGPR inoculation and lowing content in inoculated plant may refer to stimulatory effect of rhizobacteria on protective mechanism of plants.

Chlorophyll content is often measured in plants in order to assess the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic productivity (Zengin and Munzuroglu, 2005). Heavy metals inhibit metabolic processes by inhibiting the action of enzymes, and this may be the most important cause of this inhibition. Pb inhibits chlorophyll synthesis by causing impaired uptake of essential elements such as Mg and Fe by plants (Sharma and Dubey, 2005). An enhancement of chlorophyll degradation occurs in Pb-treated plants due to increased chlorophyllase activity (Drazkiewicz, 1994). On the other hand, decreased chlorophyll content associated with heavy metal stress may be the result of inhibition of the enzymes responsible for chlorophyll biosynthesis. Heavy metal stress may restrain chlorophyll biosynthesis by inhibition of protochlorophyll reductase and aminolevulinic acid (ALA) synthesis (Sharma and Dubey 2005). At 50 mM Pb treatment level the concentration of Pb inside the leaf might have been high enough to directly inhibit chlorophyll synthesis (Sengar and Pandey, 1996).

Lead stress caused a significant reduction in total soluble protein. Abiotic stress may inhibit a synthesis of some proteins and promote others (Ericson and Alfinito, 1984) with a general trend of decline in the overall content. Our studies coincide with Bhardwaj *et al.* (2009) and John *et al.* (2008) who also reported a decrease in *Phaseolus vulgaris* and *Lemna polyrrhiza* leaves with Pb stress. Protein content under heavy metal influence may be affected due to: (i) Enhanced protein hydrolysis resulting in decreased concentration of soluble proteins, (ii) Catalytic activity of lead; (iii) Reduction in protein synthesis under all stress condition (Bhattacharyya and Choudhuri, 1997). When a plant is subjected to abiotic stress, a number of non-specific defense systems are also activated. One of the most important systems is synthesis of osmolytes like as proline. It has been shown that free proline acts as an osmoprotectant, protein stabilizer, metal chelator, inhibitor of lipid peroxidation and free radical scavenger (Alia and Matysik, 2001).

The rhizosphere is defined as the zone of soil in which microbes may influence root system as root growth-stimulators or growth inhibitors. The results of the physiological and biochemical characteristics evaluated that rhizobacterial inoculation positively affect Pb tolerance in some wheat cultivars. Under environmental stress plants produce high levels of ethylene which can make internal stress. Moreover, much of the growth inhibition that occurs as a consequence of an environmental

stress is the result of the response of the plant to the increased levels of "stress ethylene" which exacerbates the response to the stress. However, some of the PGPR have high amount and activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can decrease ethylene levels in plants and thereby provide some protection against the inhibitory effects of various stresses. ACC deaminase-containing plant growth-promoting bacteria have been used to protect plants against growth inhibition caused by the presence of a variety of different metals and under other stress condition (Glick, 2010). Moreover, PGPR have high capacity for indole-3-acetic acid (IAA) synthesis. One of the main effects of bacterial IAA is the enhancement of lateral and adventitious rooting leading to improved mineral and nutrient uptake and root exudation that in turn stimulates bacterial proliferation on the roots (Glick, 2003). Utilization of IAA-overproducing rhizobacteria could increase tolerance of *Medicago truncatula* against salt stress. Plants inoculated with this mutant accumulated a high amount of proline, and showed enhanced levels of the antioxidant enzymes superoxide dismutase, peroxidase, glutathione reductase, and ascorbate peroxidase compared with plants inoculated with the parental strain (Bianco and Defez, 2009).

CONCLUSIONS

Result revealed that imposition of heavy metal toxicity induces an oxidative stress as evidenced by oxidative damage and antioxidant activity. The results of the present study clearly showed the beneficial role of rhizobacteria under lead stress condition. The higher grain yield and antioxidative responses was observed by PGPR inoculation which indicate the possibility of improved associations using Pb resistant wheat cv. Sardari and Shahriar along with rhizobacteria. Yield improvement by inoculation with associative bacteria requires the most successful Pb-resistant plant genotype selection. Although the activity of antioxidant enzymes significantly increased by lead stress, it was not sufficient for complete scavenging of ROS. In conclusion, inoculated plants of cv. Sardari and Shahriar that showed the best performance under pb-stress can be suggestible for lead contaminated soils. An important field for further research would be the identification of tolerance mechanism in rhizobacteria and selection the pb-resistant bacteria. The knowledge gained in such studies could facilitate both selection and the breeding of heavy metal–tolerant cultivars.

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