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FAILURE OF SURVIVAL STRATEGIES IN ADAPTION OF HEAVY METAL ENVIRONMENT IN *LENS CULINARIS* AND *PHASEOLUS MUNGO*

SABA HAIDER[1] AND RAFIA AZMAT[2*]

[1]*Department of Botany, Jinnah University for Women 5C Nazimabad Karachi, 74600, Pakistan*
[2]*Department of Chemistry, University of Karachi, 75270, Pakistan.*
[*]*Corresponding author e-mail: rafiasaeed200@yahoo.com*

Abstract

Lead (Pb)-treated *Lens culinaris* and *Phaseolus mungo* seedlings leaves showed considerable reduction in the size with enhance proline and phenol contents while peroxidase and lignin activity was Pb^{2+} dose dependent. The reduced leaves sizes of both seedlings were correlated with an increase in Pb^{2+} levels, and activities of peroxidase and lignin deposition in it. The intensification of activities of peroxidase and phenol in the Pb^{2+} treated plants were accompanied by an increase in the biosynthesis of the lignin contents as their function is of scavenging ROS radical. A strong correlation (r^2=0.8570) was observed between Pb^{2+} and lignin deposition in the *Lens culinaris* whereas it was non-significant in *Phaseolus mungo* (r^2=0.466). Increased in the lignin contents in the *Lens culinaris* as a chemical adaptation of the cell walls of various leaves tissues for endurance while decrease in the lignin contents in *Phaseolus mungo* at high dose of Pb^{2+}may be attributed with the decline in the peroxidase activity. Investigations revealed that although plants adopt several biochemical strategies for their survival but toxicity of $Pb^{2+}$was significant due to which plant fails to continue in stay alive.

Introduction

In response to heavy metal toxicity plants itself develop own immune system by acquiring different mechanism. Plants growing in metal contaminated sites show altered metabolism, growth reduction, lower biomass production, and metal accumulation. They develop certain physiological and morphological alteration for their survival in stress environmental situation like proline content has been reported to accumulate in such stress condition (el-Enany & Issa, 2001). Koeppe & Miller (1970) investigated that the lead toxicity affected the mitochondria and inhibited the electron transport in corn. Lead also promotes the production of protein (Haider *et al.,* 2007). Siripornadulsil *et al*., (2002) reported that the plant tolerance capability increased due to adaptation. Recently proline get esteem value in response to aboitic stress. That performs valuable function in higher plant conditions such as osmolyte, induction of tolerance and chelation. Kastori *et al*., (1992) demonstrated that proline accumulation in metal induced sunflower was a direct consequence of metal uptake. They also indicate that proline act as an osmoregulator under the stress condition (Bassi& Sharma 1993).

Horvath, (2009) reported that the lignin content and its structure presented potential advantages including improved pulping efficiency, lower chemical and energy consumption, and reduced environmental impacts. However, decrease in the lignin content and changes in the lignin structure could lead to the modifications in the plants structure. Lignin is a complex aromatic hetropolymer and a second most important polymer which is excessively found in the xylem cell wall after the cellulose. Lignin perform various function in the plant cell i) Provide the mechanical support, ii) improve the cell sap conduction, iii) inhibit water loss, iv) act as a filler to fill the space in the cell wall, which exist in between the cellulose hemicellulose and pectin substances in water conducting tissues. Mostly the water conducting tissues are lignified. In the lignin synthesis various components are engaged like hydroxycinnamyl alcohol, P–coumaryl alcohol, conferyl alcohol and sinaply alcohol (Gavnholt & Larsen 2002), as well as some enzyme are involved in the lignifications. Mostly these enzymes become more activated in stress condition. Peroxidase, laccase, coniferyl alcohol oxidase, phenol oxidase and sometime cytochrom oxides are utilized in the biosynthesis of the lignin content (Bao *et al*., 1993). Lavid *et al*., (2001) suggested that in aquatic plant**s** *Nymphoides peltata* (Menyanthaceae) and *Nymphaea* (Nymphaeaceae*)* the central tool for cadmium gathering was built on the tricking of cadmium crystals by polymerized phenols in specialized epidermal structures which were related to the peroxidase and polyphenol oxidase activities. *Nymphaeae*, with greater peroxidase activity and more polyphenols, was more resistant to this heavy metal than *N. peltata*. Peroxidase was involved at the last stage of catalytic reaction; early investigation showed that the peroxidase activity increased under abiotic tension which ultimately increased the lignin concentration. Peroxidase classified into two main categories acidic and alkaline, mutually involved and stimulated their activity under the abiotic exposure. Barceló *et al*., (2003) investigated although peroxidase is a constitutive enzyme in grapevines; its levels were powerfully moderated during plant cell development and in response to both the biotic and abiotic environmental factors beside this basic, peroxidase also take part in the lignifications (Liu & Ger, 1997). Many scientific researchers also proved that peroxidase is an important enzyme for manufacturing of lignin content (Paleg *et al*., 1984; Christensen *et al*., 1998).

It was imperative to know how plants live and add metals from the contaminated environment, the bio-molecular chemical analysis of plants under metal anxiety can give better results to describe the underlying processes of acclimation and variation in a several ways. Therefore this study was undertaken to understand the chemical adaptation for survival of two economically important leguminous plants viz., *Lens culinaris* and *Phaseolus mungo* under Pb^{2+} stress.

Materials and Method

Phaseolus mungo and *Lens culinaris* were grown in hydroponic to which $PbCl_2$ was added in the range of 50ppm, 100ppm, 150ppm, 200pp, and 250ppm and one set was taken as control. Observations were recorded after two weeks. Plants weight was measured separately then further preceded to the estimation process of lignin and proline contents.

Estimation of proline: Amino acid (proline) was analyzed by ninhydrine method as described by Bates *et al.,* (1973) on Schimadzo UV/Visible Spectrophotometer 180 A using pure proline as a standard for which 0.3gm fresh leaves of treated and untreated plant were homogenized in 10ml of 30% sulphosalicylic acid, then homogenate was centrifuged for 30 min. at 4000rpm. Absorbance was measured at 570nm.

Estimation of the lignin: lignin contents were estimated in the leaves of *Phaseolus mungo* and *Lens culinaris* in both sets of treated and control plants and successively extracted with 8ml of ethanol benzene mixture (1:2) on water bath at 95°C for two hours and finally washed with 96% of 0.3 ml ethanol and ether. The residue was hydrolyzed with 10ml 0.5 N NaOH for 30 hours at 80°C. Cooled hydrolyzed was centrifuged at 4000rpm for min. 2, 6 dichloroquinone added in the supernatant and an absorbance were noted at 610nm in spectrophotometer (Chen *et al.,* 2002).

Peroxidase: Enzyme peroxidase was analyzed by the process as described by Guthrie (1931) in which indophenol development from the oxidation of alpha-naphthol and paraphenylenediamine hydrochloride is active. At neutral condition autoxidation of this mixture was very prompt while at pH 4.5 it was effectively slow. The citrate buffer at pH 4.5 was used to eliminate interference of catalase and oxidase. The substrate practiced in this study consisted of 200 ml of buffer, 200 ml of water, 1 grain of para-phenylenediamine hydrochloride, and 20 ml of 4% Alpha-naphthol in 50% alcohol then filter, then 15 ml of this substrate was directly placed in a 50-ml centrifuge tube containing one ml of plant extract. Three ml of N/20 H_2O_2 were then added. The reaction was stopped after 15 minutes with three ml of 0.1% KCN. The indophenol formed was extracted with 15 ml of toluene with shaking, and the two liquefied layers were separated by centrifuging. The toluene layer was then poured off and determined in a photoelectric colorimeter.

Phenols: Total Phenols were determined through spectrophotometry in the visible spectral range based on the oxidation of phenolic compounds by the Folin-Denis reagent as described by Mechikova *et al*., (2007)

Statistics: Data obtained were subjected to statistical analysis.

Results and Discussion

This investigation highlights the effect of Pb^{2+} on some biochemical adaptations in physic-morphological parameters on *Lens culinaris* and *Phaseolus mungo* for survival in stress. Results showed an increase in the proline (Fig. 1) and phenol contents at all applied doses of the Pb^{2+} in both species whereas lignin and peroxidase showed a direct relation in *Phaseolus mungo*. The reduction in the shoot length as reported earlier by (Azmat *et al.,* 2006) may be the consequence of the lignin and the peroxidase activity in the leaves of the both seedlings (Tables 1 and 2). A strong correlation (r^2=0.8570) was observed between Pb^{2+} and lignin deposition in the *Lens culinaris* while it was non-significant in *Phaseolus mungo* (r^2=0.466) (Fig. 2). The reduced leaves area and roots were the general symptom of heavy metal toxicity which always developed due inhibition in the cell division (Azmat *et al.,* 2006). Initially lignin content increases with an increase in Pb^{2+} concentration followed by the drastic drop in the lignin activity at a concentration range of 150 to 250 ppm in *Phaseolus mungo* that may be related with ROS activity. This may put down the lignin contents attributed with dive in peroxidase activity from 100 to 250ppm or the decline in the lignin contents at high dose of Pb^{2+} may be due to the cell injury owing to which cell fail to deposit or synthesized the lignin in the *Phaseolus mungo* (Table 1).

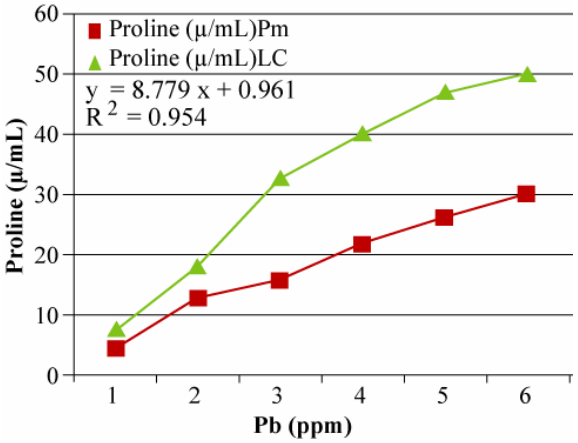

Fig. 1. Effect of Pb on proline contents of *Lens culinaris* and *Phaseolus mungo.*

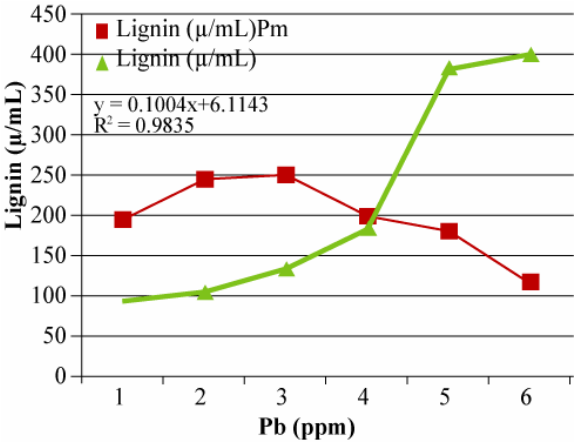

Fig. 2. Effect of Pb on lignin contents of *Lens culinaris* and *Phaseolus mungo*.

Table 1. Secondary metabolites of *Phaseolus mungo* (Shoot) under Pb stress for survival of plant.

Pb (ppm)	Proline (µ/ml)	Lignin (µ/ml)	Peroxidase mg/ml	Phenol (µ/ml) (µ/ml)
0	4.51 ± 0.036	192.5 ± 9.501	1356.68 ± 13.31	111.7 ± 0.730
50	12.96 ± 0.0447	244.7 ± 1.089	1471.88 ± 15.11	186.3 ± 0.681
100	15.81 ± 0.0244	250.3 ± 1.598	1003.88 ± 14.11	243.7 ± 1.368
150	21.96 ± 0.0707	200.0 ± 0.951	922.62 ± 11.45	478.8 ± 2.966
200	26.38 ± 0.115	180.6 ± 1.979	1651.88 ± 17.23	620.9 ± 3.136
250	30.37 ± 0.074	116.1 ± 1.966	1275.42 ± 13.32	709.8 ± 2.329

Table 2. Secondary metabolites of *Lens culinaris* (shoot) under Pb stresss for survival of plant.

Pb (ppm)	Proline (µ/ml)	Lignin (µ/ml)	Peroxidase mg/ml	Phenol (µ/ml) (µ/ml)
0	07.56 ± 0.050	95 ± 2.677	1105.21 ± 19.23	80.8 ± 1.225
50	18.184 ± 0.050	105 ± 1.536	1251.02 ± 21.43	160.7 ± 0.7598
100	32.91 ± 0.122	134 ± 3.452	1020.50 ± 15.34	258.5± 1.386
150	40.192 ± 0.208	183 ± 1.277	1020.60 ± 12.23	484.6 ± 1.277
200	47.071 ± 0.156	384 ± 2.225	1425.10 ± 14.32	698.1 ± 0.968
250	50.230 ± 0.120	400.15±5.340	1516-90 ± 18.56	651.4 ± 2.110

Increase in the lignin contents within the leaves of plant probably responsible for reduced photosynthesis followed the reduced leaf area and chloroplast pigments as reported earlier by (Haider *et al.,* 2007), also directly related with the peroxidase activity and phenol contents. A significant correlation (r^2=0.476) in between the lignin and the leaves area showed that the lignin deposition play an adverse rule because it also block the process of osmosis due to which stomatal movements effected. This result in an increase in number of stomata (Azmat *et al.,* 2009a & b) to regulate the exchange of CO_2 and O_2 gases toward an increase in the rate of photosynthesis.

Results reported in the Tables 1 and 2 showed higher contents of phenols in both species as a result of Pb^{2+} stress. This higher phenol concentration may be for scavenging the oxidative stress to overcome the direct effect of metal on the plant growth and are responsible for lignin synthesis as secondary metabolites. The biosynthesis of the lignin was a direct consequence of an increase in peroxidase activity and phenol contents in the two species under investigation. Lignifications in the cell wall due to enzyme activity, may involve in the destruction of photosynthesis apparatus due to aging and senescence as reported earlier by Moerschbacher *et al.,* (1988) and Haider *et al.,* (2007). The increase in biosynthesis of the lignin may also be related with transpiration of water molecule during stress as the lignin is a cementing material, very unaffected to degradation, being held organized with strong chemical bonds; consist of share of internal H bonds essentially not one compound but numerous. All are complex, amorphous, three-dimensional polymers that have in common a phenyl propane structure, that is, a benzene ring with a tail of three carbons (Lavid *et al*., 2001; Nelsen *et al*., 1983). The deposition of the lignin in the leaves tissues may provide the strength to the photosynthetic apparatus in stress to increase the working capability for survival in stress condition but side effect of lignin was dominant attributed with the reduced leaf area as observed in the current investigation due to which photosynthetic activity was affected (Haider *et al.,* 2007). It is bonded in complex and several ways to carbohydrates, mostly between the cells, but also within the cells, and in the cell walls. Its functions to regulate the transport of liquid in the living plant (partly by reinforcing cell walls and keeping them from collapsing, partly by regulating the flow of liquid). It was investigated that the stress-induced responses mediate a coordinated increase in the activities of lignifying enzymes including phenylalanine ammonia lyase and peroxidase activity leading to an enhanced deposition of lignin-like polymers (stress lignin) (Moerschbacher *et al*., 1988; Bruce & West, 1989) and a hydroxyl proline-rich protein component is present in precursor genes (Bassi & Sharma, 1993). Regression analysis revealed a linear dependence of the increase in the lignin in the species

related to the reduction in leaves growth with an increase in the concentration of lignin was establishes under Pb^{2+} toxicity. The increasing phenol concentration and decline lignin contents in *Phaseolus mungo* might affects the peroxidase activity negatively (Table 2) while in *Lens culinaris* leaves; an increase in activity was associated with reduced chlorophyll contents and leaf area (Haider *et al.,* 2007). Lignin deposition blocks the diffusion of heavy metal to provide the protection from heavy metal injury but it also blocks the diffusion of essential mineral ions as reported earlier in same species (Azmat & Haider, 2007).

Enhance ratio of proline accumulative in both plants, due to Pb^{2+} toxicity was correlated with the increasing tolerance capability of plants. Proline is enormously studied molecule in the context of the plant responses to aboitic stresses, such as salinity, water deficiency, low and high temperature or heavy metal stresses (El-Enany et al., 2001). In metal stress, it is tempting to assume a function for proline in an increased metal tolerance (Bassi & Sharma. 1993). Proline may bind with metal due to its chelating ability, provide the strength against free radicals and involved in various defensive mechanism to overcome the toxic effect of metal because proline is good osmo or redox regulator, ROS scavenger, activator of antioxidant enzyme and chelate formatter (Klein & Itai, 1989; Aziz & Khan, 2003; Azmat & Khan, 2011; Azmat & Akhter, 2010; Alia & Pardha, 1991). Earlier investigation (El-Enany et al., 2001) showed that the plants have the ability to with it has been standing the water deficit stress by osmoregulation which was conduct by proline activity (Azmat & Khan, 2011). It was established that accumulation of proline was marked as an increase in the number of stomata with reduced size which may be for the osmoregualtion of exchange of gasses (Azmat *et al*., 2009a & b). In past era many researchers also reported analogous outcome in heavy metal stress in Cd and Ni induced Plants (Alia & Pardha, 1991). In Pb stress condition proline accumulation mechanism still controversial. It may be attributed with the interruption in the activity of electron transport system by which plant suffered from oxidation damage because in stress condition electron transport system has been disrupted (Zagoskina *et al*., 2007). In addition, proline can now be added to an exclusive list of non-enzymatic antioxidants that microbes, animals, and plants need to stand action, the inhibitory effects of reactive oxygen species (ROS) (Chen & Dickman, 2005). Increase in the concentration of proline in stress condition results in an increase in the plant tolerance, may therefore be developed by the boost up in the AOX, ROS, Oxidative stress (excess ROS). At this stage plant developed this own immune system (Maggio *et al*., 2002; Sawhney *et al.,* 1990; Ali *et al.,* 2007). Increase in amino acids concentration as reported earlier in these species by (Azmat *et al.,* 2007), indicated that it may have elevated proportion of proline (Choudhary *et al.,* 2006, Jetley *et al*., 2004). This may also be related with Pb toxicity or amino acid modification and fragmentation of the peptide chain could be recognized to accumulation of proline. Although under the abiotic stress or lead stress plants developed their own defensive strategy for survival in which proline accumulation is one of that but results also revealed that proline showed inverse relationship with the growth rate of plants in both species including biomass reduction.

Mechanism of survival: It is well known fact that abiotic anxieties intensely disturb the plant cell bioenergetics. Therefore, the mitochondria may play a principal role in the cell variation to abiotic stresses, which are known to induce oxidative stress at the cellular level. These mitochondria may regulate the ROS generation through energy-dissipating systems. ROS are also produced continuously as by products of various metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes (Pastore *et al.,* 2007*)*. Approximately in all higher plants and algae, photosynthesis takes place in chloroplasts, which cover a highly organized thylakoid membrane structure that harbors all the components of the light capturing photosynthetic apparatus and provides all structural properties for prime light harvesting (Gill & Tuteja, 2010). It has been observed under the heavy metal exposure plant adapted the protective mechanism for survival which may involves increased in the secondary metabolites like lignin, which stimulated in Pb^{2+} stress condition (Moerschbacher *et al*., 1988). Lignin deposition point is cell wall specially tracheid and vessel of xylem in cell wall because cell wall is main platform for lignifications and antioxidant enzyme also located in the cell which appeared as thick margined leaved in *Lens calinaris* due to the accumulation of lignin content (Azmat *et al*., 2009b). It was concluded that lignin biosynthesis was the direct consequence of phenol and peroxidase activity for protection of plant and the ROS molecules are scavenged by various antioxidative defense mechanisms like proline which also act as a scavenger. Phenols are act as effective antioxidants. During heavy metal stress phenolic compounds can act as metal chelators and on the other hand phenolics compounds can directly scavenge molecular species of active oxygen. These characteristic features became severe for plant growth in response to disrupt the entrance of CO_2 and other important gases which ultimately affect the diverse physiological function and finally reduced the growth rate index. These secondary metabolites in the plants acquire homeostatic mechanisms that tolerate them to keep acceptable concentrations of essential nutrients and metal ions in the cellular compartments and to reduce the damaging effects of an excess of nonessential ones(Bahl, *et al*., 1995)

Conclusion

It was well established fact that ROS is efficiently quenched by betacarotene, phenol or plastoquinone or proline or peroxidase. Current investigation conclude that although in abiotic stress condition, increased the lignin content in both seedlings may be for the protection of plants but at high concentration of Pb^{2+} its became destructive because it may prevent the absorption of CO_2

in plant cell and release of oxygen due to which reactive oxygen species (ROS) generated and caused the oxidative stress. These results also demonstrate that at low concentration of Pb^{2+} proline action might be fulfilled and plant grow normally but at high concentration of Pb^{2+}, proline, phenol, peroxidase with lignin deposition could not neutralize or reduced harmful effect of Pb^{2+} and proline action was not enough for plant survival. It was suggested that Pb^{2+} is lethal to the *Lens culinaris* and *Phaseolus mungo* and it was established fact that all chemical and morphological adaption by the both species were failed for the survival of plants in heavy metal environment and antioxidant defense machinery fails to protect plants against oxidative stress damages.

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