PONGAMIA PINNATA (L.) PANIGRAHI AQUEOUS EXTRACT ALLEVIATES MERCURIC CHLORIDE INDUCED STRESS ON SEEDLING GROWTH OF MAIZE

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Abstract. Aqueous extract of *Pongamia pinnata* (PALE) was accessed to alleviate mercuric chloride ($HgCl_2$) induced stress on seed germination and early seedling growth of *Zea mays* L. Maize seeds were soaked in 5 and 2.5% PALE prior to sowing in the pots and $HgCl_2$ was applied at rate of 1 and 0.5 mg kg^{-1} to the soil to induce an artificial stress for the germination and seedling growth. The results showed that seed germination (%), root dry weight and photosynthetic pigments were significantly reduced by $HgCl_2$ at 1 mg kg^{-1}, which resulted in accumulation of phenolic compounds in maize leaves. It was found that the total soluble phenolic content was negatively correlated with shoot dry weight ($r = -0.656$, $p = 0.001$). On the other hand, seed soaked with PALE significantly alleviated adverse effects of $HgCl_2$ stress on seed germination and growth attributes of maize, thus it was found to be effective to protect maize plants from adverse effects of mercury and can be recommended for application in mercury contaminated areas of farmer's field.
Keywords: *mercury stress, phenolic, Pongamia pinnata, phytoremidiation, PALE, Zea mays, $HgCl_2$*

Introduction

The term 'heavy metal' is often used to cover a diverse range of elements, which are considered to be toxic to human health as being an important class of pollutants. Although heavy metals are naturally present in the soil (Zakir et al., 2014), but the geologic and anthropogenic activities have caused them to increase to the toxic level that are harmful to both plants and animals (Alyemeni and Almohisen, 2014). Heavy metals adversely affect the development of both vegetative and generative parts of the plants (Jeliazkova and Craker, 2003) leading to losses in yield (Chatterjee and Chatterjee, 2000). Therefore, the remediation of heavy metal polluted soils cannot be over emphasized (Chibuike and Obiora, 2014).

Heavy metals block functional groups of metabolically important molecules, alter hormonal balance, reduce nutrient assimilation, protein synthesis, and DNA replication (Nagajyoti et al., 2010). They inhibit chlorophyll synthesis by substitution of magnesium in chlorophyll and also water uptake (Boening, 2000). Among the various types of heavy metals, mercury accumulates in all parts of the plant but mostly in roots indicating that roots serve as a barrier to mercury uptake (Bibi et al., 2016).

To reclaim the soils contaminated with heavy metals, various physical and chemical approaches have been adopted but these are expensive and make the soil unsuitable for plant growth (Marques et al., 2009). Biological approach (bioremediation) on other hand is the best for establishment / reestablishment of plants on polluted soils. Being a natural process, it is an environment friendly method. Phytoremediation is a part of bioremediation in which plants are used for the treatment of polluted soils (Cuypers et

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al., 2010). The different mechanisms used for phytoremediation are phytostabilization, phytovolatilization and phytoextraction (Chaney et al., 1997). The amount of phytochemicals such as plant phenolics are reported to increase under the stress of heavy metals. This increase of phenolic content is correlated with increased activity of enzymes involved in biosynthesis of phenols under heavy metal stress (Winkel-Shirley, 2002; Rizvi, 2006; Bibi et al., 2016).

Pongamia (*Millettia pinnata*), formerly known as *Pongamia pinnata*, is a tree/shrub widely cultivated in Pakistan. It can tolerate a wide range of soil types including saline, alkaline, sandy, heavy clay and rocky soils and waterlogged soils. Pongamia is used in land reclamation and as a soil stabilizer (Shirbhate and Malode, 2012). Keeping in view the harmful effects of heavy metals on plants and the role of phytochemicals in heavy metal stress tolerance of plants, the present investigation was carried out to alleviate mercury induced stress on maize plants by *Pongamia pinnata* leaf extract.

Material and methods

The healthy and fully expended leaves of *Pongamia pinnata* (200 g) were collected (20 gm per tree) from the campus of the University of Science and Technology, Bannu, mixed together to form a composite sample, dried under shade for 2 to 3 weeks and finely ground in an electric grinder. The elemental composition of *Pongamia Pinnata* leaf was analysed following the method described by Rashid (1986). The powdered leaf material was digested in a solution of nitric acid and perchloric acid (2:1) and heated at 150 °C. After cooling it to room temperature, the volume was raised to 50 ml using deionized water and filtered. Elemental analyses were carried out using an Atomic Absorption Spectrophotometer (Model: Perkin Elmer Analyst-200, USA).

Preparation of Pongamia pinnata leaf extract

Formal maceration techniques were used in the preparation of extracts. The *Pongamia pinnata* leaf powder (20 g) was soaked in 200 mL of distilled water and was kept there for 48 h. It was filtered first with fine porous cloth and then with a Whatman No. 1 filter paper. The aqueous extract obtained was designated as a 10% (w/v) stock solution. This stock solution was further diluted to 5 and 2.5% (w/v) in distilled water and were denoted as PALE.

Bioassay

The seeds of maize (*Zea mays* L., variety Azam) were obtained from the Agriculture Research Centre Sara-e- Nourang, Lakki Marwat, KP, Pakistan. Finely ground uniformed soil (sand and clay 1:1) was filled in plastic pots (8×12 cm^2) for sowing of maize seeds in order to facilitate easy penetration of roots and uprooting of plants at the time of harvest. The soil used as culture medium was analyzed for pH, content of available nitrogen and phosphorous using standard protocols. Determination of soil pH was carried out by the method of McLean (1982). For electrical conductivity (EC) determination, the saturated paste of soil was prepared by suspending soil into water and thoroughly mixed by using magnetic stirrer for 30 min. After settling, the EC was noted by using conductivity meter (Page et al., 1982). Phosphorous content of the soil was determined according to the method of Olsen and Sommers (1982). For determination of total nitrogen content 10 g of soil was digested in micro-Kjeldahl flask

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with 20 mL of concentrated H_2SO_4 and 10 g digestion mixture. After digestion, the flasks were allowed to cool. The contents were transferred to a 50 mL flask and distilled. The distillation was carried out in the presence of 20 mL of 40% NaOH and collected in 5 mL boric acid. The distillate was titrated against 0.1 N H_2SO_4 using $KMNO_4$ as indicator. The particle size of the soil was determined at Agriculture Research Station Sari Naurang Lakki Marwat, Pakistan. The physico-chemical properties of the soil are shown in *Table 1*.

Table 1. *Physico-chemical properties of the soil used in the experiment*

Parameters	Values
pH	7.5
E.C.	3.10 dS m^{-1}
Total nitrogen content	0.41% dry weight
Total phosphorous content	29 mg kg^{-1}
Clay (<0.002 mm)	34%
Fine silt (0.002–0.02 mm)	13%
Fine sand (0.05–0.2 mm)	23%
Coarse sand (0.2–2 mm)	30%

To impose artificial stress of mercury, $HgCl_2$ was added at 1 and 0.5 mg kg^{-1} soil. The experiment was carried out statistically in completely randomized design (CRD) with three replications for each treatment (various levels of mercury and PALE) in a glass house in the Department of Botany, University of Science and Technology, Bannu, KP Province, Pakistan. Maize seeds were soaked in various treatment levels of PALE for three hours before sowing them in pots. The following samples were examined: Control pots where seeds soaked in distilled water were sown, Pots with soil contaminated with $HgCl_2$ at 1 mg kg^{-1} soil, Pots with soil contaminated with $HgCl_2$ at 0.5 mg kg^{-1} soil, Pots with soil contaminated with $HgCl_2$ at 1 mg kg^{-1} soil where seeds soaked in 5% PALE were sown, Pots with soil contaminated with $HgCl_2$ at 1 mg kg^{-1} soil where seeds soaked in 2.5% PALE were sown, Pots with soil contaminated with $HgCl_2$ at 0.5 mg kg^{-1} soil, where seeds soaked in 5% PALE were sown, Pots with soil contaminated with $HgCl_2$ at 0.5 mg kg^{-1} soil, where seeds soaked in 2.5% PALE were sown.

When 90% of the seeds were germinated in control pots, the seeds germination (%) was calculated according to the following formula:

$$\text{Seed germination } (\%) = (\text{Seeds germination / Total number of seeds grown}) \times 100$$

The plants were harvested 28 days after sowing and were analyzed for the following morphological, physiological and biochemical growth attributes. The shoot and root weight were determined using electronic balance. Root length was determined using a measuring tape. The leaf photosynthetic pigments were analysed according to the method described by Arnon (1949). The total soluble phenolic content of the maize leaves were determined by the Folin-Ciocalteau method (Wolfe et al., 2003). The extract (200 µL) was mixed with the Folin-Ciocalteau reagent (Sigma-Aldritch, Cat # F9252-100ML) and incubated for five minutes at room temperature in dark. The

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mixture was added to a 1.5 mL sodium carbonate (7%, w/v) solution and incubated in dark for 90 min. The optical density of the mixture was determined at 760 nm using a spectrophotometer (Hitachi's U-5100 Tokyo, Japan). Standard curve made of various concentrations of gallic acid solutions was matched with the sample measurements. The concentration of total phenolic content was expressed as µg gallic acid equivalents g^{-1} sample.

Statistical analyses

Analysis of variance technique (ANOVA) was used for the analysis of data and the Least Significant Difference (LSD) test (Steel and Torrie, 1980) was used for the comparison of treatment means. Student Statistix (version 8.1 USA) software package was used for the determination of coefficient of correlation.

Results and discussion

The Pongamia leaf was analysed for elemental composition and total soluble phenolic content (*Table 2*). Among macronutrients, Na (204 µg $g-^{-1}$), K (197 µg g^{-1}), Ca (67 µg g^{-1}) and Mg (89 µg g^{-1}) were determined. Among micronutrients Fe (42 µg/g), Mn (22 µg/g) and Zn (35.22 µg/g) were analysed. The total soluble phenolic content of Pongamia leaf was 139 mg gallic acid g^{-1} d.w. Plant extracts were characterized by the presence of natural phenolics and various types of nutrients having beneficial effect on plant growth (Ullah et al., 2014; Khattak et al., 2015).

Table 2. *Nutrient composition of Pongamia pinnata leaf*

Parameter studied	**Value (µg g^{-1} d.w)**
Na^{+}	204
K^{+}	197
Mg^{2+}	67
Ca^{2+}	80
Fe^{2+}	42
Mn^{2+}	22
Zn^{2+}	35
Total soluble phenolic content (µg gallic acid g^{-1} d.w)	139000

The data depicted that soil contamination with higher concentration of $HgCl_2$ (1 mg kg^{-1} soil) significantly reduced the seed germination compared to control (*Table 3*). On the other hand, seeds soaked with PALE significantly ameliorated adverse effects of $HgCl_2$ on seed germination (%). A negative impact of $HgCl_2$ stress on shoot fresh and dry weights was observed (*Table 3*). However, the exogenous application of PALE minimized adverse effects of $HgCl_2$ on shoot fresh and dry weights. Root length was also significantly reduced by $HgCl_2$ at 1 mg kg^{-1} soil as compared to untreated control (*Table 3*). The application of PALE (5%) exhibited stimulatory effects and minimized adverse effects of $HgCl_2$ on root length. The soil contamination with $HgCl_2$ significantly decreased root fresh weight as compared to control ($p < 0.05$). The PALE effectively protected fresh weight of roots from adverse effects of $HgCl_2$ induced stress. At higher concentration of $HgCl_2$ (1 mg kg^{-1} soil), the beneficial effect of PALE on root fresh

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weight was also found to be stronger. The $HgCl_2$ has no significant effect on root dry weight (*Table 3*).

Table 3. *Effect of PALE on morphological growth attributes of maize under mercuric chloride stress*

	Treatment	Germination (%)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Root length (cm)
T0	**Control (**pots where seeds soaked in distilled water were sown)	100.0 a	1.286 a	0.214 a	0.780 a	0.077 ab	68.22 ab
T1	Pots with soil contaminated with **$HgCl_2$ at 1 mg kg^{-1} soil**	83.2 b	0.440 c	0.013 c	0.420 d	0.057 ab	53.42 d
T2	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil**	99.4 a	0.905 ab	0.096 bc	0.440 cd	0.112 a	63.05 bc
T3	Pots with soil contaminated with **$HgCl_2$ 1 mg kg^{-1} soil and** seeds soaked in **5% PALE** were sown	100.0 a	0.873 b	0.137 ab	0.603 bc	0.064 ab	72.22 a
T4	Pots with soil contaminated with **$HgCl_2$ 1 mg kg^{-1} soil and** seeds soaked in **2.5% PALE** were sown	94.4 a	0.817 bc	0.141 ab	0.534 cd	0.062 ab	57.75 cd
T5	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil and** seeds soaked in **5% PALE** were sown	94.4 a	0.910 ab	0.095 bc	0.743 ab	0.048 ab	65.49 abc
T6	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil and** seeds soaked in **2.5% PALE** were sown	100.0 a	0.941 ab	0.140 b	0.730 ab	0.042 b	71.70 ab
LSD		**9.03**	**0.400**	**0.0875**	**0.1659**	**0.0697**	**8.86**

Means sharing common English letters are statistically similar (p = 0.05, n = 3)

Mercury stress results in abnormal seed germination with a significant reduction in germination indices (Patra and Sharma, 2000). Mercury reduces mitotic activity in plant organs, therefore results in lower biomass production (Patra et al., 2004). The beneficial effect of PALE on seed germination and morphological growth characters of maize under $HgCl_2$ could be attributed to its phytochemical composition. Previous studies have shown that the aqueous leaf extract of *Moringaoleifera* was highly effective and protected maize plants from adverse effect of $HgCl_2$ (Bibi et al., 2016).

Results showed that $HgCl_2$ at 1 mg kg^{-1} soil significantly reduced chlorophyll *a* content as compared to control (*Table 4*). The application of PALE at 5% concentration significantly ameliorated adverse effects of $HgCl_2$ stress on chlorophyll *a* content. All the treatments showed non-significant effect on chlorophyll *b* content (*Table 4*). The reduction of chlorophyll content under mercury stress has been reported in *Marrabiumvulgare* (Moreno-Jiménez et al., 2007). Mercury disrupts the photosynthetic machinery by inactivating the magnesium ion of the chlorophyll molecule (Patra et al., 2004).

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Table 4. *The effect of PALE on physiological attributes of maize under mercuric chloride stress*

Treatment		Chl *a* (mg/g f.w)	Chl *b* (mg/g f.w)	Total soluble phenolic content (mg gallic acid eq./g f.w)
T0	**Control (**pots where seeds soaked in distilled water were sown)	12.88 a	6.224 a	34.94 c
T1	Pots with soil contaminated with **$HgCl_2$ at 1 mg kg^{-1} soil**	4.98 c	3.665 b	64.52 b
T2	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil**	11.66 ab	4.667 ab	45.46 c
T3	Pots with soil contaminated with **$HgCl_2$ 1 mg kg^{-1} soil and** seeds soaked in **5% PALE** were sown	12.54 a	7.120 a	106.88 a
T4	Pots with soil contaminated with **$HgCl_2$ 1 mg kg^{-1} soil and** seeds soaked in **2.5% PALE** were sown	10.37 b	6.443 a	105.15 a
T5	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil and** seeds soaked in **5% PALE** were sown	13.19 a	6.637 a	67.10 b
T6	Pots with soil contaminated with **$HgCl_2$ 0.5 mg kg^{-1} soil and** seeds soaked in **2.5% PALE** were sown	10.24 b	5.063 ab	49.41 bc
LSD		**2.025**	**2.502**	**17.803**

Means sharing common English letters are statistically similar (p = 0.05, n = 3)

In the present investigation, it was found that $HgCl_2$ induced stress caused accumulation of phenolic compounds in the leaves of maize (*Table 4*). Total soluble phenolic content was found to be higher when $HgCl_2$ was applied at 1 mg kg^{-1} soil. However, soaking seeds in PALE resulted in further augmented accumulation of phenolics in maize leaves. The most effective concentration of PALE was 5%. Plants accumulate phenolic compounds when exposed to heavy metal stress (Klepacka et al., 2011). Accumulation of phenolic under heavy metal stress has also been reported in *Phaseolus vulgaris* (Hamid et al., 2010), *Boerhavia diffusa* (Abdussalam et al., 2015) and *Trigonellafoenum-graecum* (Askari and Azmat, 2013). Phenolic compounds protect cell biomolecules from oxidative damage by scavenging reactive oxygen species and prevent lipid peroxidation. This is achieved by the reduced forms of phenolic compounds whereas in oxidized state, these may function as prooxidants (Sakihama et al., 2000). Antioxidant activity of Pongamia extract was correlated with its flavonoid and phenol content (Behera et al., 2012). The higher increase in phenolic compounds by seed soaked with PALE under $HgCl_2$ stress indicates the possible role of bioactive compounds present in the extract which contributed to the mercury stress tolerance of maize.

It was observed that Hg stress resulted in the accumulation of phenolic compounds with a reduction in the content of photosynthetic pigments. On the other hand PALE not only caused accumulation of phenolic compounds but also improved the content of photosynthetic pigments. This suggested that exogenous application of PALE reduced chlorophyllase activity responsible for chlorophyll degradation. Phenolic compounds

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either stimulate or inhibit the content of photosynthetic pigments that is why phenolics are considered as compounds with bifunctional role, one of stimulation and another of inhibition (Yang et al., 2004). This may be attributed to the fact that phytochemicals present in PALE have stimulatory effect on photosynthetic pigments in maize.

The correlation of chlorophyll *a* was found to be positive with chlorophyll *b* (r = 0.691, p = 0.000), germination (%) (r = 0.581, p = 0.005), root fresh weight (r = 0486, p = 0.025) and shoot fresh weight (r = 0.571, p = 0.006). The total soluble phenolic content was negatively correlated with shoot dry weight (r = -0.656, p = 0.001). The correlation of root fresh weight was also positive with shoot fresh weight (r = 0.491, p = 0.023).

Conclusion

The growth of maize was inhibited when grown on $HgCl_2$ contaminated soil due to the toxicity of mercury. This mercury stress was associated with the accumulation of phenolic compounds. It was found in the present studies that exogenous application of PALE was effective in alleviating the adverse effects of $HgCl_2$ on maize plants grown under $HgCl_2$ contaminated areas, thus, PALE can be used in bioremediation of contaminated soils for better plant growth. *Pongamia pinnata* is a drought tolerant and nitrogen fixing plant species. Its oil has potential for use as a feedstock for biodiesel production and can survive under harsh environmental conditions. Therefore, it is a more suitable tree species for exploitation as a source of future bioregulators for improving growth and productivity of crop plants.

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