Differential toxicity of Pb & Hg on the development of modular traits, photosynthetic and biochemical attributes in two varieties of a forage crop species *Trifolium alexandrinum* L.

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Abstract

Heavy metal stress as result of natural and anthropogenic activities is main environmental problem. Pb and Hg are among non-biodegradable metals thus remaining persistent in soil and water. The present study was carried out to assess growth and biochemical responses of two varieties (Desi and Misri) of *Trifolium alexandrinum* L. after application of varying levels (25mg/kg, 50mg/kg and 100mg/kg) of Pb and Hg in soil along with control. Seed germination, biomass of above and below ground tissues, number of flowers and leaves, leaf area and nodulation was observed. For biochemical attributes, green pigments, protein and amino acids, were determined. Both varieties (Desi and Misri) showed variable responses in relation to both Pb and Hg. Similarly, the pattern of character expression was independent for metal levels and types. Misri performed consistently better as it showed best threshold for most of the attributes studied. Hg was found to be more toxic as compared to the Pb as it induced more drastic decline in parameters studied. The study showed that biometric traits can be used as good predictors and the biochemical parameters cannot be used as useful biochemical markers as they showed no marked disparity.

Keywords: Biochemical Markers; Biometric Traits; Desi; Misri; *Trifolium Alexandrinum*.

1. Introduction

Heavy metal pollution is an international increasing environmental dilemma. The cultivated lands polluted with heavy metals due to anthropogenic activities are of serious concern [1, 2]. Supra optimal concentrations of both essential and non-essential metal ions are phytotoxic (Hamid et al., 2010). However, the responses of plants vary widely depending on their species and types of metals (Tomas et al., 2012).

Like many other developing countries, tremendous industrialization in Pakistan had resulted in disposal of industrial waste containing heavy metals into water bodies. The contamination of water bodies poses threats to both aquatic and terrestrial life forms (Marshall et al., 2007). In addition, the use of contaminated water for irrigation purposes can cause long-lasting metal contamination of agriculture soils (Singh et al., 2004; Sharma et al., 2007). Moreover, the use of municipal waste as fertilizer owing to high cost and shortage of fertilizers also resulted in metal pollution because the waste is heavily loaded with heavy metals such as Cu, Zn, Pb and Hg (Mushiaq and Khan, 2010). Pb and Hg are added to the environment as a result of both natural and anthropogenic activities. An incredibly use of automobiles is a major source of Pb pollution. Similarly, substantial amounts of mercury are introduced in the form of fertilizers and pesticides (Selin, 2009; Khan et al., 2011; Walter et al., 2011; Jalaluddin & Hamid, 2012). Pb and Hg pollution is of great concern because both of these metals are non-essential and non-biodegradable thus persists indefinitely in soil and water. Despite their non-essentiality, plants have a tendency to uptake and translocate them within their tissues when metal exists in higher concentrations (Elavrasi and Dhanam, 2012). Although metal tolerant species have shown a better threshold for Pb and mercury but exceeding amount of metal present in the plant tissues is phytotoxic. Metal toxicity is associated with visible damage to plants (Islam et al., 2008; Liu et al., 2010) as well as metabolic disorders which includes inactivation of enzymes, photosynthesis, respiration, chlorophyll biosynthesis, oxidative stress and inhibition of DNA synthesis thus disturbance of mitosis (Ros-sato et al., 2012).

Egyptian clover (*Trifolium alexandrinum* L.) is an important green fodder. Due to the highest fodder yield (100-125t ha⁻¹) it is also called as the ‘king of fodder’ and it is able to give 5-7 cuts of succulent forages (Khalil and Jan, 2000; Mahmood and Abbas, 2003). In addition, its high palatability, digestibility (about 65%) and protein enriched containing 20% crude protein (Malaviya et al., 2004). In Pakistan, forage crops are cultivated in over 10.3% of the total cultivated area of 22.6 million hectares. Thus, production of fodder in the country is low to support livestock including small ruminants (Agricultural Statistics of Pakistan 2009-10) and is the main hindrance for livestock and dairy development. Despite considerable significance of forage crops in the country, there has been given little attention to develop or improve forage crops which can be grown under a wide array of environmental situations and constraints. Therefore, it is imperative that intensive forage cultivation per unit area per season should be practiced (Dost et al., 1990, Bhatti et al., 1992) by exploiting high yielding varieties for metal polluted agricultural lands.

Keeping in view the above aspects, two local varieties of *Trifolium alexandrinum* L. were assessed for their performance under Pb...
and Hg pollution. The modular traits development, photosynthetic and biochemical attributes of the varieties were assessed to appraise their potential for metal contaminated soils of the country.

2. Materials and methods

The seeds of two varieties of *Trifolium alexandrinum* L., namely Desi and Misri were obtained from local market of Multan. The experiment was conducted under natural conditions during January-March, 2012 at Botanic Gardens, Bahauddin Zakariya University Multan, Pakistan. The sources of metals used were their respective salts i.e. Pb (NO3)2 and HgCl2. Germination experiment was performed in a laboratory under natural conditions and at room temperature (± 25 °C). Plastic germination trays were used for this purpose having 72 equal sized cells (each cell had diameter of 4cm). The hole below each cell was blocked using a water proof self-adhesive plastic tape. Sand was sieved through a 2mm sieve and washed with tap water to clear all the dirt and then air dried. Both salts (Pb(NO3)2 and (HgCl2) were completely mixed with sand in a ratio of 25, 50 and 100mg/kg. Total forty eight appropriately labeled cells were randomly filled with sand of different levels of both salts. There were made three replicates for each treatment. Thus each variety had a set of twenty four cells, twelve cells for each salt. Each cell was filled with 45g sand. Five seeds of each variety namely Desi and Misri were sown in each cell according to the following treatments for both Pb(NO3)2 and HgCl2 i.e., T0-Control (sand), T1, T2, T3=25mg/kg, T4=T5=50mg/kg and T6=T7=100mg/kg. Germination records were made through emergence data and data were presented as germination percentage using the following formula:

Germination Percentage = Number of Seeds Germinated \times 100

Total Number of Seeds Sown

After germination experiment, seeds were sown in earthen pots. For this compost soil was used. The soil was sieved through 2mm sieve. Forty earthen pots (length 15cm and width 16cm) were used and tagged for both salts and varieties. The hole at the base of each pot was blocked completely with muslin cloth to avoid leaching. Lead Nitrate (Pb(NO3)2) and Mercury Chloride (HgCl2) were thoroughly mixed in compost soil. Three replicates were made for each treatment. Each variety had a set of twenty four pots, twelve for each salt. The experiment was organized in a Complete Randomized Manner. Five seeds of both (Desi and Misri) varieties were sown in each pot according to the following levels of both salts (Pb(NO3)2 and HgCl2) i.e., T0-Control (sand), T1, T2-25mg/kg, T3-50mg/kg and T4-T7=100mg/kg. The plants were watered daily. After 16 weeks, the plants were harvested by taking out the whole plant from the pot. The soil was removed from the roots and washed with water to remove extra dirt. Then roots were separated from their respective shoots and were placed in labeled paper bags for further analysis. Different biometric and biochemical parameters were studied. The root and shoot length of each plant were determined by inch tape. The trifoliate leaf from each replicate of all the levels of both metals and varieties was taken and its area was determined by scanning and taking print on centimeter graded graph paper. Fresh and dry biomass of roots and shoots were determined by a digital balance. The number of leaves, flowers and root nodules were calculated of each plant. The contents of pigments were determined by taking out the whole plant from the pot. The soil was blocked using a water proof self-adhesive plastic tape. Sand was sieved through a 2mm sieve and washed with tap water to clear all the dirt and then air dried. Both salts (Pb(NO3)2 and (HgCl2) were completely mixed with sand in a ratio of 25, 50 and 100mg/kg. Total forty eight appropriately labeled cells were randomly filled with sand of different levels of both salts. There were made three replicates for each treatment. Thus each variety had a set of twenty four cells, twelve cells for each salt. Each cell was filled with 45g sand. Five seeds of each variety namely Desi and Misri were sown in each cell according to the following treatments for both Pb(NO3)2 and HgCl2 i.e., T0-Control (sand), T1, T2, T3=25mg/kg, T4=T5=50mg/kg and T6=T7=100mg/kg. Germination records were made through emergence data and data were presented as germination percentage using the following formula:

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Chlorophyll a (mg/g) = (12.7 (O.D 663) – 2.69 (O.D 645)) x V/1000 x W

Chlorophyll b (mg/g) = (22.9 (O.D 645) – 4.68 (O.D 663)) x V/1000 x W

Total Chlorophyll (mg/g) = 20.2 (O.D 645) x 8.02 (O.D 663) x V/1000 x W

Kirk and Allen (1965) formula was used to calculate carotenoids:

Carotenoids (mg/g) = O.D 480 + 0.114 (O.D 663) – 0.638 (O.D 645)

Total free amino acids were determined by Hamilton and Van Slyke (1943) method. 1ml of extract was mixed with 1ml of 10% pyridine and 1ml of 2% ninhydrin solution in 25ml test tubes. The tubes were heated in boiling water bath for 30 min and allowed to cool at room temperature. The volume of the mixture was made up to 50 ml with distilled water and the OD of the extract was read at 570 nm using a spectrophotometer (Biotecnology Medical Services, UV-1900, USA). A standard curve was developed with Leucine and free amino acids were calculated. Total protein content was determined by Bradford (1976) method. Optical densities were read at 595nm. A standard curve was made with the Bovine Serum Albumin and the protein contents were calculated. The data was then subjected to ANOVA General Linear Model by using Minitab 16 statistical software, to determine significant influences of varying levels of each metal on both varieties. The parameters having significant F ratios were subjected to Duncan Multiple Range Test (DMRT), (Duncan, 1955).

3. Results and discussion

The seed germination is the primary criteria to monitor effect of pollution (Shammugavel, 1993) because many environmental pollutants can affect seed germination which is the first developmental stage in the life cycle of a plant. Metal pollution appeared to cause a noticeable reduction in germination. Although, the varieties (Desi and Misri) depicted contrasting response in their emergence when exposed to varying levels of lead and mercury. The germination of Desi was less influenced by lead and mercury whereas the emergence of Misri was more in response to mercury only. Any alteration in the soil environment can influence optimal conditions which are crucially important for the successful establishment of plants and consequently the production of biomass or yield (Elavrasi and Dhanam, 2012). Increasing pollution dosages are well known to cause reduction in viability of seeds at different rates and the sensitivity also varied in different plants (Chaphekar, 1991). For root length, the two varieties; Desi and Misri had similar response to both metals as consistently longer roots were observed in response to Pb than Hg. Similar decline in root length was also reported in many other species. For example, in wheat (Datta et al., 2009; Mesmar and Jaber, 1991), Albizia lebbeck (Farooqi et al., 2009) and *Brassica juncea* (Donghua et al., 2000). The greater influence of Pb on roots can be attributed to its accumulation in the roots due to less mobility to aerial tissues. It has been well reported that Pb in higher concentrations can cause reduction in mitotic division in meristematic region of root of *Allium cepa* (Lerda, 1992) therefore less root elongation (Goldbold and Ketter, 1991; Sharifah and Hishashi, 1992). Same results have been reported by Peralta et al., (2001). Although, root growth inhibition by mercury has been reported but greater accumulation of mercury in the roots and it’s binding in cell wall may result in less toxicity (Wang and Greger, 2004). The results of this study are also parallel to these findings as root elongation was not much influenced by Hg.

There has been observed no contrasting responses of the varieties for shoot length as both Desi and Misri varieties showed no inter-specific disparity for both metals. Shoot length of both varieties was not influenced by Pb levels. The lesser toxicity of Pb might be due to its restricted penetration from roots to aerial parts of the
plants. According to Sharma and Dubey, (2005), the root endo-
dermis in maize and barley acts like a barrier for Pb uptake by the
shoots. However, by contrast Hg had caused decline in shoot
length though was less injurious to the roots. Thus it appears that
endodermis can become a week barrier against elevated level of
Hg. Similar Hg-induced stunted growth has been reported by
many workers in many plants (Cho and Park, 2000; Cargnelutti et
al., 2006; Ge et al., 2009; Costa et al., 2011). Mercury causes ul-
tra-structural damage that has inhibitory effect on plants growth
(Israr et al., 2006; Zhou et al., 2008; Shiyab et al., 2009a).

Plant growth and performance under heavy metal stress is fre-
quently predicted via fresh and dry biomass production which may
serve as an important indicator of plant success under stressful
environment. Since Trifolium is a multiply harvested crop there-
fore vegetative growth can be assessed through biomass of plants.
The below ground tissues growth can be adversely affected due to
heavy metal ions as roots are the first organs that come in contact
with contaminated soil. The above ground parts of plants are im-
portant as they support and accumulate photosynthetic products.
Both fresh and dry biomass of root in varieties did not vary greatly
as both varieties had more biomass in response to Pb than Hg. The
increasing trends for root and shoot biomass are in close agree-
ment to Wierzbicka, (1998), who reported an increase in the dry
weight of organs of corn plant with an increase in Pb content
which was due to an increase in the production of cell wall poly-
saccharides. However, no such trends were observed for Misri
thus showed a progressive decrease in biomass with increasing Pb
concentrations.

On the other hand, mercury (Hg) induced remarkable decline in
the fresh and dry biomass of root and shoot in both Desi and Misri
varieties. Thus, Hg appeared to be more injurious than Pb.
Our results showed that both Pb and Hg caused noteworthy de-
cline in number of leaves and flowers in both Desi and Misri vari-
eties. The decline in foliage and flower exhibited a similar trend
that was observed for biomass. The decline in leaf number is in in
accordance to Kabir et al., (2010) who reported same results for
Thespesia populnea L. in which increasing Pb dosages caused a
decline in foliage. Similarly, Pb at a lower dose is less toxic as
compared to the high dose for flowers induction. Khan and
Chaudhry, 2006; Tooke et al., 2005 reported that heavy metals
stress can delay or stop flowering.

Leaves as light capturing and photosynthetic organs are very vital
for the plants. Under Pb toxicity inhibition of leaf expansion has
been reported by Gabara and Gaoszewska, 1992; Pichalak et al.,
(2003). Lead induced decline in leaf area has also been stated by
Tanyolac et al., (2007). Leaf area was not influenced by Pb in both
varieties thus leaf expansion was not influenced remarkably by Pb
but Mercury had greatly influenced leaf area of the tested varie-
ties.

Nodulation is a characteristic of leguminous plants through which
atmospheric nitrogen is fixed and becomes available to plants. The
use of fungicides containing heavy metals have been reported to
reduce Rhizobium population and root nodules formation in Vigna
radiata, V. mungo, and Arachis hypogaea (Ghosh, 1995).
Fig. 1: Germination Percentage of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 2: Root length (cm) of Desi and Misri varieties of *Trifolium alexandrinum* after exposure to control and different levels of Pb and Hg.

Fig. 3: Fresh Weight of Root (g) Of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.
Fig. 4: Dry Weight of Root (g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 5: Shoot Length (cm) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 6: Fresh Weight of Shoot (g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.
Fig. 7: Dry Weight of Shoot (g) of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 8: Number of Leaves of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 9: Number of Flowers of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.
Fig. 10: Number of Root Nodules of Desi and Misri Varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 11: Leaf Area (Cm$^2$) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 12: Chlorophyll a (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.
Fig. 13: Chlorophyll b (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 14: Total Chlorophyll (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.

Fig. 15: Total Carotenoids (mg/g) of Desi and Misri varieties of *Trifolium alexandrinum* after Exposure to Control and Different Levels of Pb and Hg.
Fig. 16: Total Protein (mg/g) of Desi and Misri varieties of Trifolium alexandrinum after Exposure to Control and Different Levels of Pb and Hg.

Fig. 17: Amino Acids (mg/g) of Desi and Misri varieties of Trifolium alexandrinum after Exposure to Control and Different Levels of Pb and Hg.

4. Conclusion

While summing up the work reported here it can be concluded that both varieties (Desi and Misri) of *Trifolium alexandrinum* L. showed considerably variable responses to both Pb and Hg. The exposure of Pb and Hg at lower concentrations had no drastic effects on all parameters studied. However, the most elevated level of both metals caused more profound changes for growth and biometric parameters.

Misri performed better as compared to Desi as the former variety excelled for ten out of total seventeen parameters while the later showed tolerance for seven parameters in response to both Pb and Hg. Hg was found to be more toxic as compared to the Pb as it induced more drastic decline in almost all the biometric parameters studied.

Based on the results of the study it became evident that among biochemical attributes, pigments content did not depict any marked influence thus could not be used as biochemical marker for the appraisal of metal tolerance in these varieties. However biometric traits can serve as useful predictors.

The study clearly signified that the two varieties of *T. alexandrium* had shown their potential for Pb and Hg contamination. Thus, the varieties can be grown under contaminated soil conditions or in situations where sewerage water is being in use for irrigation. However, screening of crop germ-plasm should be carried out on a large scale. Moreover, mechanistic explanation of Pb and Hg tolerance needs to be addressed in future studies.

References


