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Comparative analysis of tissue compartmentalized heavy metal uptake by common forage crop: A field experiment

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ABSTRACT

Heavy metal contamination of agricultural soils is a severe cause of concern globally. The heavy metals can enter crops through roots and can result in biomagnification in the different plant tissues such as roots, stems and leaves. From plants these metals are transferred into animal and human systems resulting in serious health problems. Various physical and chemical methods are available for heavy metal removal from soil but phytoremediation is considered as one of the most sustainable and cost-friendly method. Although many studies have been carried throughout the world to assess phytoremediation potential of plants in controlled conditions, few studies are available on the metal uptake capabilities of plants growing in natural conditions. Therefore, the present study was conducted to assess the phytoremediation potential of *Trifolium alexandrinum* (Berseem), an important forage crop growing in intensively cultivated agricultural soils of Punjab, India with main focus on the accumulation and mobility of metals (Cr, Cd, Cu, Co, Fe, Mn, Pb and Zn) in various plant tissues like roots, stems and leaves. The maximum contents of Cd, Co, Fe and Pb were observed in roots whereas for Cr, Cu, Mn and Zn maximum content was observed in leaves of Berseem. Overall among the metals studied Fe content was highest in all tissues of Berseem, which could be due to the higher content of Fe in soil. Metal Bioaccumulation Factor (BAF) and Translocation Factor (TF) calculated for assessing metal uptake and transport by plant tissues were found to be above 1 for the studied metals (except Co and Fe), which indicated Berseem to be a suitable accumulator of these metals in natural conditions.

1. Introduction

Heavy metal contamination of soils is a very serious issue affecting plant, animal and human health throughout the globe. Indiscriminate anthropogenic activities such as industrialization, urbanization, excessive agrochemical application have contributed significantly to heavy metal contamination of soils. The soil physico-chemical characteristics such as pH, soil organic matter (SOM), texture, soil nutrients are very important in determining the retention and mobility of heavy metals in soils (Kavianpoor et al., 2012). Several conventional physical, chemical and biological approaches such as in situ vitrification, soil incineration, excavation and landfill have been employed for decontamination of soil. But these methods have limited applicability due to their high costs, intensive labor requirements, irreversible changes to soil structure and disturbance of soil microflora (Ali et al., 2013; Ma et al., 2016). Hence, there is a strong requirement for cost effective and environment friendly methods for cleanup of metal contaminated soils. Phytoremediation is one such approach, which employs plants for removal of metals from soil.

For phytoremediation, plants having high metal uptake capabilities are grown on metal contaminated soils. These plants either absorb and retain the metals in their roots (known as phytostabalization) or transport the metals to above ground tissues (known as phytoextraction). Usually plants having high metal extraction properties, high biomass, rapid growth and ability to accumulate > 1000 mg/kg of a metal in various tissues are considered ideal for phytoremediation (Baker and Brooks, 1989; Vamerali et al., 2010; Malik et al., 2010). *Trifolium alexandrinum* (Berseem) is one such plant. It is a leguminous crop belonging to family Fabaceae and having fast growth, high biomass and high metal extraction properties (Prasad, 2007; Ali et al., 2012). It flourishes best in neutral to alkaline soils and is usually grown in winter season where it has frost tolerance to temperatures as low as −6 °C (Muhammad et al., 2014). Although many studies have been conducted to assess phytoremediation potential of plants (including

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Berseem) in controlled conditions with metal spiked soils (Ali et al., 2012; Ma et al., 2016), these studies do not represent the actual metal accumulation capabilities of plants in open field natural conditions in the different plant tissues such as roots, stems, leaves and inflorescence. Thus, plants growing in natural habitats must be studied for assessing their phytoremediation potential (van der Ent et al., 2013).

Punjab is an important agrarian state situated in north-western part of India. Berseem is one of the main fodder crops grown in Punjab. The main sources of irrigation are river water and groundwater. However recent studies have indicated that the water of two main rivers of Punjab i.e. Beas and Sutlej are polluted due to industrial and urban activities (Singh et al., 2013; Kaur et al., 2014; Shrivastava, 2014; Kumar et al., 2016), which are further polluting the groundwater (Bhatti et al., 2015, 2016). Therefore, these polluted irrigation sources and intensive agricultural practices using agrochemicals are causing heavy metal contamination of soils of Punjab. In our earlier studies (Bhatti et al., 2015, 2016), physico-chemical characteristics and heavy metal contents in soils and crops (food and fodder) were analyzed in areas around Beas and Sutlej rivers. In these studies, main focus was overall contamination of the shoots of plants (including Berseem) without concentrating on the metal contents in different plant tissues. However, there is a strong need to assess the metal accumulation in various tissues of plants such as Berseem to understand their phytoremediation potential completely in order to provide a green solution for metals removal from soils. Hence, a detailed study was conducted in intensively cultivated areas around Harike wetland to assess the phytoremediation potential of Berseem grown in these areas by analyzing the metal uptake and accumulation in different tissues (roots, stem and leaves) of Berseem.

2. Material and methods

2.1. Study area

Punjab (Lat. 29°30 to 32°32′N and Long. 73°55 to 76°50′E) is a state located in the north-western part of India bordering Pakistan. The climate of Punjab is continental, semiarid to sub-humid with 435.6 mm annual rainfall. Punjab has two main crop seasons Kharif (fall) and Rabi (spring). Beas and Sutlej are the two main rivers of Punjab, and Harike wetland is the confluence point of these two rivers. The main sources of irrigation in areas around Harike wetland are the river water and groundwater. The sampling was done from the area surrounding Harike wetland, a Ramsar site where rivers Beas and Sutlej meet (shown in Fig. 1). The main occupation in the study area is agriculture, which involves extensive use of fertilizers, pesticides and weedicides, which are potent sources of heavy metals and contaminates the soil (Mortvedt, 1996; Milinović et al., 2008; Savci, 2012). There is also substantial industrial and urban activity (leather tanning, dyeing, electroplating, textile in cities such as Ludhiana, Jalandhar and Kapurthala. upstream of Harike wetland, which discharge sewage and industrial effluents in the rivers Beas and Sutlej, which contain several heavy metals such as Cr, Cd, Cu, Pb etc. (Kaur et al., 2017; Sharma and Walia, 2017). Thus, the irrigation sources i.e. river and ground water, are contaminated with heavy metals in this area, which further aggravates the problem of heavy metal contamination of soil (Singh et al., 2013; Kaur et al., 2014).

2.2. Sampling and preparation

Soil sampling was done during March–April 2015 (March 28, 2015, April 04–05, 2015). Six composite soil samples in triplicates were collected from fields under Berseem cultivation from the selected area which are shown in Fig. 1. At least five subsamples of soil were pooled to form a composite sample. Soil samples were taken from depths of 0–15 cm. Six composite samples of Berseem plants were collected in triplicates from corresponding soil sampling fields for heavy metal analysis. All soil and Berseem samples were stored in clean polythene bags and were brought to the laboratory. The soil samples were air-dried, ground and passed through 2 mm sieve for physicochemical and heavy metal analysis. The roots, stems and leaves of Berseem were separated in lab, washed with deionised water, oven dried at 70 °C and then ground to fine powder with pestle mortar (Bhatti et al., 2015).

2.3. Physico-chemical analysis of soil

Soil pH and conductivity were determined in 1:5 soil:water suspension. The mixture was shaken for 2 h and the supernatant was filtered and used for analysis of pH and conductivity using HM digital meter-COM-100 (New Delhi, India) and Equip-tronics EQ-614-A (Mumbai, India), respectively (Rodriguez Martin et al., 2013). Soil organic carbon content was determined by Walkley Black wet oxidation method (Nelson and Sommers, 1982). A factor of 1.72 was multiplied with organic carbon content to determine soil organic matter (SOM). Soil texture was determined by Hydrometer method (Jacob and Clarke, 2002), and an EDTA titration method was used for measuring Calcium (Ca) and Magnesium (Mg) (Lanyon and Heald, 1982). Total nitrogen (N) and available phosphorous (AP) were determined by the Kjeldahl method (Bremner and Mulvaney, 1982) and Olsen method (Olsen et al., 1954), respectively. The carbonates ($CaCO_3$) were analyzed using acid neutralization method (Hesse, 1971), and potassium (K) and sodium (Na) were measured by using a Systronics Flame Photometer-128, after digesting the samples in a diacid mixture ($HClO_4/HNO_3$ in a 4:1 ratio) (Bhat et al., 2014).

2.4. Heavy metal analysis

For heavy metal (Cd, Cr, Co, Cu, Fe, Pb, Mn and Zn) determination, one gram of soil was digested with 15 mL of aqua regia (HNO_3: HCl in 3:1 ratio) and 1 g each of ground root, stem and leaf samples of Berseem with 15 mL of triacid mixture (HNO_3:H_2SO_4:$HClO_4$ in 5:1:1 ratio) at 80 °C till a transparent solution was obtained (Allen et al., 1986). The digested samples were filtered and diluted with de-ionized water up to 50 mL and analyzed for different metals viz. Cadmium (Cd), Chromium (Cr), Cobalt (Co), Copper (Cu), Iron (Fe), Lead (Pb), Manganese (Mn) and Zinc (Zn) by flame Atomic Absorption Spectrophotometer (FAAS) (Agilent 240 FS AA model). The limits of detection of the instrument are given in Table 2. Properly washed glassware, double distilled water and analytical grade reagents were used throughout the study. The standard solutions of selected heavy metals were procured from Agilent (1000 mg/L) and were used to make solutions of varying concentrations by dilution of the standards. For quality assurance, the standards were run after every ten sample readings, to assure the working of machine with 95% accuracy as done by Arora et al., 2008. The samples were also run in triplicates per sample to maintain accuracy of the results.

2.5. Metal Bioaccumulation Factor (BAF)

Heavy metal accumulation in soil and Berseem were calculated on the basis of dry weight. The metal Bioaccumulation Factor is a ratio of heavy metal concentration in crop tissue to soil (Ali et al., 2013) and was calculated as follows:

$$BAF = C_{\text{plant tissue}}/C_{\text{soil}} \quad (1)$$

where $C_{\text{plant tissue}}$ and C_{soil} are the concentrations of heavy metal in Berseem tissues (roots, stems and leaves) and soil, respectively, on a dry weight basis.

2.6. Metal Translocation Factor (TF)

Translocation Factor (TF) is the ability of a plant to move the accumulated heavy metal from roots to above ground tissues (stems,

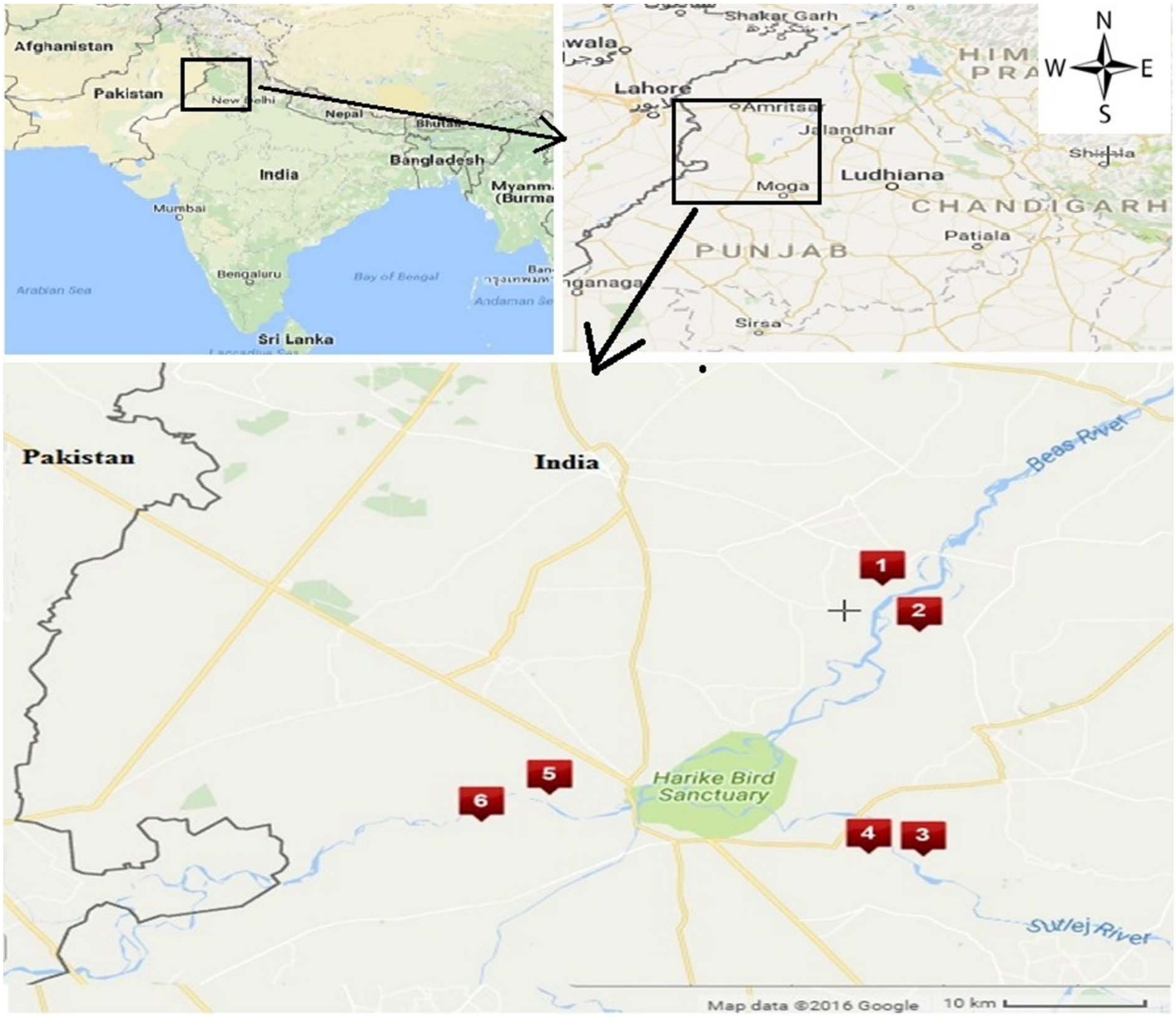

Fig. 1. Map of the sampling locations around Harike wetland.

leaves and inflorescences) (Ali et al., 2013), which is calculated as the ratio of heavy metal content in above ground tissues to roots or leaves to stem. In the present study, the Berseem root to aboveground tissues (stems and leaves) and stem to leaves TF was calculated as follows:

$$TF = C_{stem/leaves}/C_{roots} \text{ and } C_{leaves}/C_{stem} \quad (2)$$

where C_{leaves}, C_{stem} and C_{roots} are the concentrations of the heavy metal in Berseem leaves, stem and roots, respectively on a dry weight basis.

2.7. Statistical analysis

The physico-chemical analysis of soil and heavy metal contents of soil and Berseem samples were done in triplicates and the data is presented as mean ± standard error. One way ANOVA followed by Tukey's HSD test as post hoc was used to compare the means of heavy metal contents in soil and different tissues (roots, stems and leaves) of *T. alexandrinum*. Differences at $p < 0.05$ were considered statistically significant. Pearson correlation coefficients were calculated to analyze the correlation between physicochemical properties and heavy metals in soil and heavy metals in various Berseem tissues (roots, stems, and leaves) separately. The data was taken from natural conditions under random sampling, therefore considered normally distributed as done in similar works (Khan et al., 2013; Amin et al., 2013). Statistical analysis was done with the help of IBM SPSS version 16.0 (Chicago, USA), PAST and Microsoft excel computer software programs.

3. Result and discussion

3.1. Soil physico-chemical analysis

The physico-chemical properties of soil and heavy metal contents in soil and Berseem tissues (roots, stems and leaves) are represented in Tables 1 and 2, respectively. The soils analyzed in this study were found to be alkaline in nature, sandy in texture and having low soil organic matter (SOM). These characteristics were in line with the earlier works

Table 1
Average soil physico-chemical characteristics (Mean ± SD).

Parameters	Average
pH	8.18 ± 0.21
Conductivity (µS/cm)	229.88 ± 33.40
SOM (%)	4.23 ± 0.52
Sand (%)	86.89 ± 6.33
Silt (%)	8.27 ± 2.90
Clay (%)	4.84 ± 0.94
Ca (mg/Kg)	170.56 ± 34.40
Mg (mg/Kg)	446.10 ± 62.58
Na (mg/Kg)	342.22 ± 44.98
N (mg/Kg)	349.67 ± 42.60
AP (mg/Kg)	137.67 ± 64.91
K (mg/Kg)	1220.56 ± 131.12
$CaCO_3$ (%)	8.52 ± 3.45

Table 2
Average heavy metal contents in soil and *Trifolium alexandrinum* tissues (roots, stems and leaves).

	Cd	Cr	Co	Cu	Fe (× 1000)	Pb	Mn (× 100)	Zn (× 100)
Soil (mg/kg)	1.14 ± 0.27 a	22.31 ± 2.26 a	9.09 ± 0.80 a	18.73 ± 2.43 c	17.7 ± 1.92 a	6.58 ± 1.09 a	3.74 ± 0.45 a	0.59 ± 0.07 b
Root (mg/kg)	1.23 ± 0.21 a	14.10 ± 1.49 bc	3.16 ± 0.23 b	47.99 ± 3.32 ab	2.38 ± 0.45 b	4.91 ± 0.37 a	0.97 ± 0.12 bc	0.47 ± 0.04 b
Stem (mg/kg)	0.98 ± 0.19 a	11.78 ± 1.04 c	2.9 ± 0.28 b	33.71 ± 4.77 bc	0.52 ± 0.15 c	4.81 ± 0.64 a	0.37 ± 0.05 c	0.70 ± 0.14 b
Leaves (mg/kg)	0.97 ± 0.26 a	16.47 ± 1.43 b	2.78 ± 0.29 b	61.02 ± 11.40 a	1.45 ± 0.35 b	4.37 ± 0.93 a	1.08 ± 0.20 b	1.05 ± 0.21 a
Limits of detection of FAAS (μg/L)	1.5	5.0	5.0	1.2	7.3	14.0	1.0	1.6
Indian limits for soil[a]	3–6	–	–	135–270	–	250–500	–	300–600
Swedish limits for soil[b]	0.4	120	30	100	–	80	–	350
Chinese limits for fodder[c]	0.5	10	–	–	–	5	–	–
Background metal contents in sandy textured soils of Punjab[d,e]	0.35	11.7	–	10–21	7.1–16.8	4.8	1.50–4.14	0.13–0.47

Mean values of heavy metals followed by different letters in columns are significantly different (one-way ANOVA; Tukey's test, p ≤ 0.05) in soil and different tissues of *T. alexandrinum*.
[a] Awashthi (2000).
[b] SGV – Swedish guideline values for metal levels in polluted soils < https://www.naturvardsverket.se/Documents/publikationer/620-5053-2.pdf> (Bhagure and Mirgane, 2011).
[c] CERSPC (2009) - Chief Editor Room of Standard Press of China (CERSPC), 2009. Compilation of standards for feed industry. (Zhuang et al., 2013).
[d] Sharma et al. (1992).
[e] Saggoo and Gupta (2013).

done in these areas (Bhatti et al., 2015, 2016). The average contents of Ca, Mg and Na are 170.56 mg/Kg, 446.10 mg/Kg and 342.22 mg/Kg respectively and considered to be important nutrients for plants such as Berseem for vital physiological activities and nutritional requirements (Troeh and Thompson, 2005). The levels of N, P and K, which are the primary nutrients for plants were in ratio of 0.286:0.113:1. It was highly disproportionate to the ideal ratio of 4:2:1, due to excessive K contents in soils. The high carbonate content (8.52%) showed the calcareous nature of soil. Kaur et al. (2014) observed similar trend of alkaline and sandy soil in Amritsar district of Punjab, India, having higher Mg and lower Ca, Na and K contents than the present results. Galal (2016) analyzed agricultural soils under Berseem cultivation in Egypt and found them to be alkaline in nature but with much lower conductivity than in the current study. Statistically significant positive correlation (Table 3) was observed between soil pH and SOM, which was in line with earlier studies (Bhatti et al., 2016; Aciego Pietri and Brookes, 2008). The soil nutrients N, P, K and Mg were negatively correlated to sand and positively to clay showing high affinity and retention of these nutrients in clay (Boluda et al., 2011). Overall, the physico-chemical analysis revealed that the soils analyzed in the present study are alkaline with sandy texture and low SOM, resulting in a decrease in metal retaining capacity of the soils. So the metals can leach to lower layers of soil.

3.2. Soil heavy metal contents

The heavy metal contents observed in the soil of studied areas were low and below the national and international maximum permissible limits (Table 2). Such low levels are contradictory considering the intensive agricultural practices and industrial pressures on the soils of Punjab (Kaur et al., 2014; Kumar et al., 2016; Katnoria et al., 2011). However these lower levels of heavy metals may be due to the sandy texture of soil observed in these areas causing leaching of metals to lower layers and the very low SOM, which could not retain the heavy metals and facilitate the uptake of metals by plants. The metals such Fe and Mn are mainly of lithological origin, but anthropogenic activities are responsible for excessive contents of metals such as Cr, Cd, Cu, Pb, Zn (Rodriguez Martin et al., 2013). The geochemical baseline data on background metal values for Indian soils is yet unavailable (Mahanta and Bhattacharyya, 2011), therefore comparisons of the metal levels observed in this study were made with values reported in various studies from Punjab (Kaur et al., 2014), India (Tiwari et al., 2011; Mazumdar and Das, 2015) and other countries (Chang et al., 2014; Garcia-Salgado et al., 2012; Galal, 2016). Such comparisons revealed that the soil samples in the present work had higher contents of Mn than the contents of Mn observed by Kaur et al. (2014) in agricultural soils of Amritsar, Punjab. The Fe contents in soil samples in present study were higher than Fe contents observed by Mazumdar and Das (2015) in paper mill effluent contaminated areas around a wetland in Assam, India, and Cd contents in present study were higher than the Cd levels observed by Chang et al. (2014) in agricultural soils around Pearl river delta in China. The Cr contents in the present work were higher than the Cr levels reported by Garcia-Salgado et al. (2012) in mining affected soils from Spain and the Cr, Cu, Fe, Mn and Co levels in present work were higher than the levels observed by Galal (2016) in agricultural soils under Berseem cultivation from Egypt. Most of the soils from other areas were shown to be contaminated from various sources such as mine waste, industrial wastewater irrigation and polluted river water irrigation. The results of the present study therefore suggest that the soil showed higher contamination with these metals in comparison to soils from other areas. When compared to heavy metal contents in agricultural soils having similar sandy texture as observed in the present study, Punetha et al. (2015) reported higher levels of Cr, Mn and Zn and lower level of Fe industrially polluted agricultural soils of Moradabaad, Uttar Pradesh, India. Omoniyi et al. (2016) observed lower levels of Cu, Mn and Zn and higher levels of Cd and Pb in sandy textured agricultural soils around rivers Niger and Benue in Lokoja, Nigeria. The geochemical baseline data of metal contents in soils of the present study area is unavailable. However the metal contents recorded in earlier studies undertaken in similar sandy textured soils (Sharma et al., 1992; Saggoo and Gupta, 2013) in areas (South-western Punjab and Jalandhar) around the present study area can be taken as background data (Table 2). It was observed that the level of studied metals in the present study was higher than the levels reported in these previous studies. This increase in levels of the studied metals can be attributed to increased anthropogenic activities (industrialization, urbanization and intensive agriculture), which are potent sources of heavy metals in soils (Government of India, 2016; Sharma et al., 2017).

Further, heavy metal contents in soil are highly affected by soil pH, organic matter, cation exchange capacity and oxidation state of metals (Ghosh and Singh, 2005). A Positive correlation (Table 3) was also observed between pH and various heavy metals (except Cd, Mn and Pb), which was due to greater retention and decreased solubility of the heavy metals with higher pH because acids in soil decrease and various complexes are formed in soil with increasing pH (alkaline conditions), which reduces the availability of metals (Malik et al., 2010; Galal and Shehata, 2015). Statistically significant positive correlation was observed between Cr, Cu and Co, which could be due to their similar anthropogenic sources of contamination. The main anthropogenic source of heavy metal contamination of agricultural soils (such as the

Table 3
Pearson's correlation analysis of soil physico-chemical parameters and heavy metals.

	pH	Cond	SOM	Sand	Silt	Clay	Ca	Mg	Na	N	AP	K	$CaCO_3$	Cr	Cd	Cu	Co	Fe	Mn	Pb
Cond	0.27																			
SOM	0.92*	0.18																		
Sand	− 0.36	− 0.53	− 0.16																	
Silt	0.31	0.30	0.10	− 0.96**																
Clay	0.33	0.93**	0.25	− 0.57	0.33															
Ca	− 0.76	0.22	− 0.82*	0.29	− 0.35	0.06														
Mg	− 0.33	0.56	− 0.51	− 0.25	0.20	0.29	0.73													
Na	0.23	− 0.03	0.09	0.18	− 0.10	− 0.34	0.10	0.44												
N	0.02	0.06	− 0.16	− 0.37	0.18	0.01	0.37	0.49	0.44											
AP	− 0.35	0.50	0.09	− 0.47	− 0.17	0.20	0.82	0.93**	0.53	0.45										
K	− 0.23	− 0.04	0.10	− 0.31	0.02	0.40	0.38	0.68	0.85*	0.33	0.68									
$CaCO_3$	− 0.17	0.32	0.02	− 0.41	0.41	0.19	− 0.07	0.24	− 0.16	− 0.62	0.05	0.20								
Cr	0.15	− 0.59	− 0.12	− 0.45	− 0.64	0.68	0.27	− 0.42	− 0.51	− 0.37	− 0.26	− 0.33	− 0.14							
Cd	− 0.50	0.56	− 0.74	0.70	0.36	− 0.51	0.22	0.31	− 0.22	0.64	0.21	− 0.34	− 0.39	0.62						
Cu	0.27	− 0.98**	0.03	− 0.91*	− 0.43	0.63	− 0.27	− 0.64	0.04	− 0.19	− 0.52	− 0.01	− 0.30	0.84*	0.26					
Co	0.20	− 0.88*	− 0.11	− 0.97**	− 0.19	0.44	− 0.09	− 0.20	0.47	0.20	− 0.15	0.48	− 0.27	0.92**	0.48	0.89*				
Fe	0.37	− 0.89*	0.10	− 0.94**	− 0.21	0.45	− 0.14	− 0.27	0.45	0.28	− 0.20	0.38	− 0.43	0.84*	0.24	0.98**	0.93**			
Mn	− 0.20	− 0.78	− 0.35	− 0.76	0.24	0.01	− 0.08	− 0.16	0.01	0.16	− 0.31	0.24	− 0.01	0.66	0.32	0.79	0.80	0.75		
Pb	− 0.28	− 0.45	− 0.25	− 0.62	− 0.35	0.48	− 0.01	− 0.13	0.15	− 0.65	− 0.05	0.39	0.52	0.53	0.44	0.46	0.24	0.33	0.31	
Zn	0.21	− 0.42	− 0.10	− 0.59	− 0.72	0.79	0.58	0.25	0.56	0.39	0.51	0.54	− 0.51	0.42	0.35	0.59	0.44	0.59	0.17	0.26

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

soils in present study) is believed to be agrochemicals such as NPK fertilizers, pesticides, fungicides (Mortvedt, 1996; Milinović et al., 2008). Other sources of metal contamination include pollution of irrigation sources due to industrialization (electroplating, tanning, dying) and urbanization in the neighboring areas (Kaur et al., 2017).

3.3. Heavy metal contents in Berseem

3.3.1. Roots

Roots are the main tissues of plants which are directly exposed to heavy metals in soil. Plants absorb metals from soil by roots, which can either be stored in roots (Phytostabalization) or translocated to the shoots through xylem vessels, where they are stored in vacuoles (Phytoextraction) (Jabeen et al., 2009). A significant amount of metals can be immobilized underground in roots through sorption, precipitation, complexation or reduction in soils (Vamerali et al., 2010; Ali et al., 2013). Berseem has a shallow taproot system, which is helpful in metal accumulation due to high biomass. In the present study, the order of metal contents observed in roots was Fe > Mn > Cu > Zn > Cr > Pb > Co > Cd, which was in line with the elemental requirements of plants, as Fe, Mn, Cu and Zn are essential elements for plants for growth and physiological processes such as enzyme activation and therefore were in higher amounts than Cr, Pb and Cd, which are non-essential and toxic to plants (Nagajyoti et al., 2010). The heavy metal availability and retention in plants is highly dependent on soil properties, climatic conditions, plant genotype, active/passive transfer processes, metal species, metal binding components to roots, such as proteins and response of plants to elements (Tlustos et al., 2002; Zhuang et al., 2007; Garcia-Salgado et al., 2012; Caunii et al., 2015).

Higher contents of Cd, Cu, Pb and Zn in comparison to present work were observed in roots of Berseem in pot experiments done by Ali et al. (2012), which might be due to leaching of metals in sandy soils in the present study. Galal (2016) observed higher Cd, Fe, Pb and Zn contents and lower Cr, Cu and Mn contents than present study in roots of Berseem collected from farms in Egypt. Altaf et al. (2008) also recorded lower levels of Cr in roots of Berseem collected from tannery effluent irrigated soils from Kanpur, India. Mazumdar and Das (2015) observed lower contents of Fe, Pb and Zn in roots of wetland plant species *Alternanthera sessilis*, *Parthenium hysterophorus*, *Sonchus arvensis*, than Berseem roots in the present study. Cr content in roots of Berseem in the present study was higher than the contents observed by Garcia-Salgado et al. (2012) in roots of plants such as *Cynosorus echinatus*, *Jasione montana*, *Trisetum ovatum*. Lower contents of Mn in root than the present study were observed in Napier grass by Ma et al. (2016). Such studies revealed significant heavy metal accumulation potential of Berseem in comparison to other plant species growing in contaminated environments making it a potential capable for phytostabalization. Significantly positive correlation (Table 4a) was observed between Cr and Mn in roots, which might be due to similar absorption pathways in roots (van der Ent et al., 2013). Significantly negative correlation was observed between Cd and Cu, which showed a possible competition for absorption sites (Ghori et al., 2016). In addition to the heavy metal accumulation, Berseem is very useful in increasing fertility of soil because it is a leguminous crop and adds nitrogen to the level of 297–400 kg/ha in soil by nitrogen fixation (Muhammad et al., 2014). Thus in addition of phytostabalization of metals, Berseem is highly useful in nutrient addition to soil too. Phytostabalization also improves the characteristics of soil by increasing nutrient levels, organic matter content, cation exchange capacity and biological activity of soils (Vamerali et al., 2010).

3.3.2. Stem

Berseem has hollow, sparsely hairy, erect and 30–80 cm long stems. In the present study, the level of heavy metals (except Zn) in stems of Berseem was found to be lower than in the roots (Table 1). Lower contents of metals in the stems can be due to a low surface area of

Table 4
Pearson's correlation matrix of heavy metal contents of *Trifolium alexandrinum* (a) Roots (b) Stems (c) Leaves.

	Cr	Cd	Cu	Co	Fe	Mn	Pb
a) Roots							
Cd	0.46						
Cu	− 0.01	− 0.86*					
Co	0.41	0.67	− 0.48				
Fe	0.10	0.26	− 0.38	0.66			
Mn	0.87*	0.75	− 0.30	0.68	0.12		
Pb	− 0.57	− 0.45	0.10	− 0.86	− 0.49	− 0.73	
Zn	0.42	0.48	− 0.31	0.38	− 0.03	0.51	− 0.07
b) Stem							
Cd	0.04						
Cu	0.35	− 0.53					
Co	0.77	0.08	0.01				
Fe	0.54	0.66	0.10	0.16			
Mn	0.69	0.23	− 0.34	0.70	0.21		
Pb	− 0.15	0.21	0.26	− 0.49	0.57	− 0.62	
Zn	0.61	− 0.42	0.78	0.50	0.19	− 0.07	0.18
c) Leaves							
Cd	0.15						
Cu	0.86*	− 0.31					
Co	− 0.23	− 0.40	− 0.23				
Fe	0.86*	0.50	0.57	− 0.16			
Mn	0.92**	0.12	0.82*	− 0.05	0.92**		
Pb	0.18	− 0.19	0.35	0.11	0.34	0.46	
Zn	− 0.42	0.12	− 0.30	0.10	− 0.05	− 0.07	0.40

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

stems, less mobility of elements from roots or more elemental transport to the leaves due to transpiration pull (Tlustos et al., 2002). The heavy metals in stems are usually translocated to leaves where they are deposited in vacuoles. This transport of metals from roots to leaves via stems takes place through xylem vessels, mainly by transpiration pull and as such the stems act as only traffic way for this movement of metals. As a consequence the metal contents are lower in stems in comparison to roots and leaves (Ali et al., 2012). The overall trend of stem heavy metal contents was Fe > Zn > Mn > Cu > Cr > Pb > Co > Cd. In comparison to metal contents in stems of Berseem in the present study lower levels of Cu were observed by Ali et al. (2012) in Berseem. The Fe, Pb and Zn contents in stems in present study were also higher than their levels in stems of wetlands plants like *Diplazium esculentum*, *Sonchus arvensi*, *Ludwigia hyssopifolia*, *Solanum americanum*. Ma et al. (2016) observed higher contents of Zn, Cu, Cd, Pb and Cr and lower contents of Mn than the present study in stems of Napier grass. At most locations, Cd and Co have been found to be higher in roots because these are trace and unessential elements and thus retained in roots (Galal and Shehata, 2015). No significant correlation (Table 4b) was found between various metals in stems.

3.3.3. Leaves

Berseem has trifoliate, alternate and membranous leaves with visible green nerves. It has 1.5–3.5 × 0.6–1.5 cm oblong, elliptical to oblong lanceolate leaflets (Muhammad et al., 2014). In most of the samples analyzed, the contents of Cu, Cr and Zn in leaves were found to be more than in roots and stem, which can be due to efficient transfer of these metals from roots and their accumulation in leaves (Tlustos et al., 2002). The importance of Cu and Zn for plant physiological functions is well known, but high content of Cr in leaves, which is a non-essential element, shows the ability of Berseem to survive high concentrations of essential and toxic elements (Kabata-Pendias, 2004; Garcia-Salgado et al., 2012). The overall trend of heavy metal contents in leaves was Fe > Mn > Zn > Cu > Cr > Pb > Co > Cd. Higher levels of Cd, Zn and Pb were found in shoots and leaves of Berseem in works done by Ali et al. (2012) and Galal (2016) as compared to present study, whereas contents of Cu, Cr, Mn and Co were found to be less in leaves of Berseem in study of Galal (2016). The higher levels of metals observed in the study of Ali et al. (2012) can be explained by the pot studies carried out in their experiments where conditions are controlled. However, in comparison to the works of Ali et al. (2012) the present study had natural field conditions in which leaching of metals to lower soils layers occurs, which reduces the availability of metals. The Pb, Fe and Zn contents in leaves of Berseem in the present study were also higher than those observed in leaves of wetland plant species *Alternanthera sessilis*, *Sonchus arvensis*, *Eclipta prostrate*, *Ludwigia hyssopifolia*, *Nicotiana plumbaginifolia*. in works done by Mazumdar and Das (2015). The Cr and Cu contents in Berseem leaves in present study were also higher than those observed by Garcia-Salgado et al. (2012) in plant species such as *Cynosorus echinatus*, *Holcus mollis*, *Jasione Montana*. While working on phytoremediation potential of Napier grass Ma et al. (2016) observed lower contents of Zn, Mn and Cu and higher contents Pb, Cd and Cr in leaves of Napier grass as compared to the level of these metals in Berseem leaves in present study. Thus it is evident that Berseem has higher capability to accumulate the studied metals in its different tissues in natural conditions as compared to other plants (some having higher biomass) growing in experimental and natural conditions. The high deposition of metals in leaves and stems is due to well developed detoxification mechanism based on heavy metal sequestration into vacuoles by binding them on appropriate ligands (organic acids, proteins, peptides). This approach excludes metals from cellular sites where important processes such as cell division and respiration occur, thus proving to be an important protection mechanism (Singh et al., 2010; Caunii et al., 2015). Significant positive correlation (Table 4c) was observed among Cr, Cu, Fe and Mn in leaves, which could be due to similar routes of transfer and requirements in plant functions. Statistically significant variations were observed for Cr, Cu, Fe and Mn contents among soil and Berseem tissues (Table 2), which also showed different levels of uptake and accumulation of these metals in Berseem tissues.

Among the eight metals studied, maximum contents in different tissues were observed for Fe, which exceeded 1000 mg/kg in root and leaf samples (Table 2). Such high level of Fe might be due to high levels of Fe in the studied soil samples (Table 2). Heavy metal contents of Cu, Cd, Mn, Pb and Zn above 1000 mg/kg were reported in shoots of various plants in studies conducted in other parts of the world on metal contaminated soils (Yoon et al., 2006; Garcia-Salgado et al., 2012; Ma et al., 2016). However, the content of these metals was much lower than 1000 mg/kg in the present study, which might be due to comparatively lower level of these metals in soil of the study area. Among the Berseem tissues, the contents of Cd, Co, Fe and Pb were found to be maximum in roots whereas Cr, Cu, Mn and Zn were found to be maximum in leaves. The contents of Cd and Cr observed in Berseem stems and leaves (Table 2) were above the maximum permissible limits for fodder i.e. 0.5 mg/kg for Cd and 10.0 mg/kg for Cr (CERSPC, 2009). Since, Berseem is an important forage crop, the high levels of metals observed in the present study can pose severe risk to the livestock health (Rajaganpathy et al., 2011), thus ultimately affecting human beings. Therefore, such metal contaminated forage cannot be fed to animals, which would affect the dairy industry and rural economy. However, Berseem plants from metal contaminated soils can be used for other profitable purposes such as phytomining, a concept in which precious and semiprecious metals are recovered from plants or as a fuel for energy (Ali et al., 2013). The multiple harvests from Berseem in the same season further enhances its capability as an important tool for phytoremediation.

3.4. Metal Bioaccumulation Factor (BAF) and Translocation Factor (TF)

Metal Bioaccumulation Factor (BAF) is a ratio of metal concentration in various plant tissues to the metal content in soil. It is the most important feature in phytoremediation, which shows the metal uptake

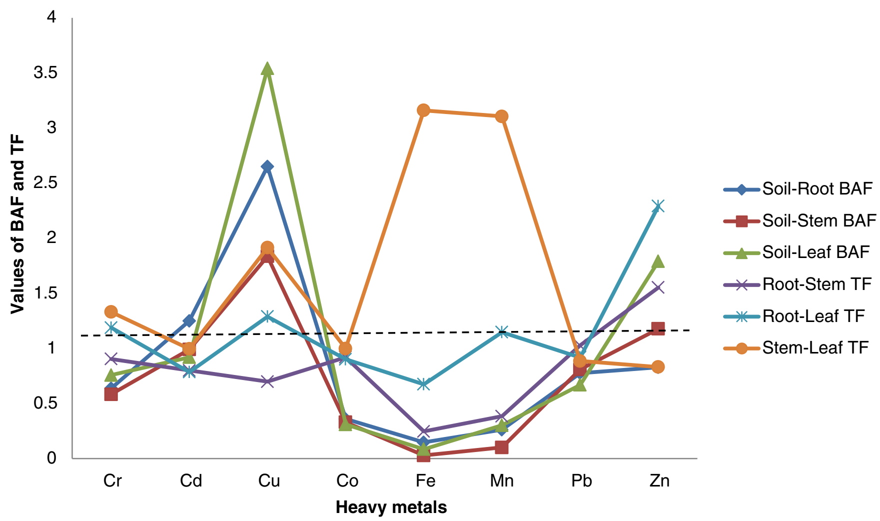

Fig. 2. Average Heavy metal Bioaccumulation Factor (BAF) and Translocation Factor (TF) in different tissues of Berseem (*Trifolium alexandrinum*).

by plants from soil, their mobilization in plant tissues and storage in aerial plant biomass. Translocation Factor (TF) shows the mobility and transport of heavy metals through different levels, which are mandatory to understand the mechanism of uptake of heavy metals in aerial tissues such as stems and leaves from roots. Both BAF and TF are required to assess a plant's potential for accumulation of metals and their values > 1 show that the plants have the ability to phytoremediate (Yoon et al., 2006; Zhuang et al., 2007; Caunii et al., 2015).

The average metal BAF and TF values are shown in Fig. 2. In the present study, the BAF values > 1 were observed for Cu, Cd and Zn, with maximum soil to leaf BAF (3.54) for Cu. BAF values > 1 in different tissues of Berseem for Cu and Zn is due to the their necessity for plants, but the BAF > 1 for Cd shows the high mobility of Cd in plants (Tlustos et al., 2002) and Cd accumulation potential of Berseem from studied areas.

The average Root-Stem, Root-Leaf and Stem-Leaf TF values > 1 (Fig. 2) were observed for Pb and Zn (Root - Stem), Cr, Cu, Mn and Zn (Root – Leaf) and Cr, Cu, Fe and Mn (Stem – Leaf). Pb is one of the most toxic and less mobile elements and its BAF and TF values > 1 in the present study revealed that Pb can show high accumulation and mobility under specific conditions such as those on this site (Tlustos et al., 2002; Badr et al., 2012). The soil-root, soil-stem and soil-leaf BAF values for Cd and Cu observed in the present study under open field natural conditions were higher than the values observed by Ali et al. (2012) in pot based experiments with metal spiked soils. The higher BAF values in present study signified the higher phytoremediation potential of Berseem in natural conditions. The root-stem and root-leaf TF values for Cd, Cu, Pb and Zn in the present study were also higher than the values observed by Ali et al. (2012). The higher TF values can be due to higher transpiration rates in natural field conditions as the plants are under open sunlight in the present study in comparison to the controlled field conditions in works of Ali et al. (2012). Similar to the present work, Galal (2016) also recorded BAF values > 1 for Cd and Zn in studies on Berseem growing in open field farms in Egypt. However, the TF values recorded by Galal (2016) were < 1 for most of the metals, which was contrary to our results. The TF values for Fe, Pb and Zn in the present work were higher than those observed by Mazumdar and Das (2015) in majority of wetland plant species such as *Alternanthera sessilis*, *Diplazium esculentum*, *Ipomoea aquatic*, *Eichhornia crassipes*, *Eragrostis atrovirens*, *Ricinus communis*. BAF and TF values in the present study for Cr, Cd, Cu, Pb and Zn were also higher than the values observed in plants *Arrhenatherum album*, *Cynosorus echinatus*, *Trisetum ovatum* by Garcia-Salgado et al. (2012). In phytoremediation studies on Napier grass Ma et al. (2016) recorded lower soil-root and soil-stem BAF values for Cu and lower soil-leaf BAF values for Cr and Zn than the present study. Also the TF values in the present study were higher for metals Mn and Zn than Ma et al. (2016). The comparative analysis of the present work with earlier studies mentioned above on Berseem and other plants growing in natural and controlled conditions revealed the significant potential of Berseem for Bioaccumulation and Translocation of the metals (especially Cr, Cd, Cu, Mn and Zn) to aboveground parts.

In this study, the levels of most metals were not found to be > 1000 mg/kg in shoots, which is a primary criterion for hyperaccumulators (Baker and Brooks, 1989), due to the low levels of heavy metals in the soil. But, despite the low levels of metals in soil, the BAF and TF values > 1 for most metals showed the high ability of Berseem to accumulate and tolerate these heavy metals. Therefore, the study proves Berseem to be a reliable plant for phytoextraction and phytostabalization of heavy metals in natural open field conditions. Since true metal accumulation potential of plants can be assessed in their natural habitats only, further research should focus on the heavy metal accumulation in different plants in open field conditions, instead of controlled lab conditions with metal spiked soils.

4. Conclusions

The observations of the present study revealed that among the different tissues of Berseem maximum contents of Cd, Co, Fe and Pb were found in roots and maximum contents of Cr, Cu, Mn and Zn were found in leaves, which showed the better phytostabalization proneness of Berseem for Cd, Co, Fe and Pb and phytoextraction proneness for Cr, Cu, Mn and Zn in natural open field conditions. Although heavy metal contents > 1000 mg/kg were not observed in Berseem tissues for most metals, but metal Bioaccumulation Factors (BAF) and Translocation Factors (TF) were found to be > 1 for all metals in the tissues of Berseem, which showed that it has high potential for phytoremediation. Since Cd and Cr contents in stems and leaves of Berseem were above maximum permissible limits for fodder, it was unsuitable as a feed for livestock. But it can be used for other economic purposes such as phytomining. Lastly, it is suggested that more studies should be carried out in open field natural conditions to assess the true metal accumulation potential of various plants.

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