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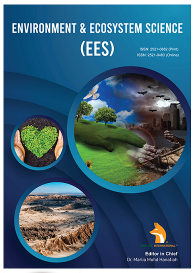

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RESEARCH ARTICLE

EFFECTS OF COPPER TOXICITY ON DIFFERENT GROWTH ATTRIBUTES OF *PHLOX DRUMMONDII*

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ABSTRACT

Heavy metal contamination is one of the major problems prevailing in environment. Copper in high concentration is considered to have serious effects on plant growth parameters which results in chlorosis, disturbed mineral uptake and stunted growth. A pot experiment was conducted to evaluate the ability of *Phlox drummondii* to tolerate and accumulate high copper doses. Plants were exposed to copper toxicity at three different concentrations (10 ppm, 20 ppm and 30 ppm) by using copper sulfate ($CuSO_4.5H_2O$). Results showed that chlorophyll-a, chlorophyll-b, total chlorophyll and carotenoids of treated plants decreased significantly ($p<0.05$) with the increase of copper concentration. Selected copper levels showed no effect on plant height, number of leaves, leaf area and ascorbic acid ($p>0.05$). Relative water content increased significantly ($p<0.05$) as compared to control plants. Copper uptake by roots of treated plants was greater as compared to control plants indicating *Phlox drummondii* ability to grow well in the copper contaminated soils and could be classified as copper tolerant plant. Copper tolerance by phlox was associated with its capacity to absorb and accumulate in roots preventing translocation of metal to other photosynthetic tissues. Therefore, *Phlox drummondii* has the characteristic to be used as hyperaccumulator by vegetating in copper contaminated soils. Further studies at genetic level would play a key role in understanding the tolerance mechanism of *Phlox drummondii* towards copper contamination.

KEYWORDS

Phlox drummondii, Copper, Ascorbic acid, Toxicity, Bioremediation.

1. INTRODUCTION

Contamination of soil due to heavy metals and other pollutants is a major threat for agricultural and environmental quality of developed and developing countries. According to a European study high levels of copper, lead and mercury have been found in 15-21cm of farming soils (Toth et al., 2016). Increasing metal accumulation in soils for growing crops not only affect crop yield but also cause detrimental health hazards. Soil and plants are contaminated with copper and other metals as a result of pesticides, fungicides, industrial effluents, mining, burning, transport, power generation and improper disposal of organic wastes in water or on land (Grytsyuk et al., 2006; Miotto et al., 2014; Pietrini et al., 2019). Metals are considered problematic due to their non-degrading nature as they require physical or chemical removal from polluted water and soil (Chaignon and Hinsinger, 2003).

Copper is an essential micronutrient of plants as it is involved in plant metabolism (Li et al, 2004). But higher plants largely take up copper in Cu^{+2} and Cu^{+} forms via roots whereas some plant absorbs copper by leaves and shoots thus becoming a major threat for human health and ecosystem (Mattioni et al., 1997; Oteef et al., 2015). Copper along with other heavy metals such as cadmium, zinc and lead are the main elements present in field soils of major foods, fruits, vegetables and ornamental plants of industrial-polluted developed and developing countries (Oteef et al., 2015). In recent researches exposure of metal toxicity is studied for various heavy metals in soil and water but very few have mentioned the adverse effects of copper regarding growth and production in overall plants (crops and ornamental plants) (Qureshi et al., 2016).

Ornamental plants are mainly known for their aesthetic qualities and are becoming rapidly growing trade with economic potential (Anderson et al., 2010; De, 2017). They provide shadow and purify wind. Ornamental plants have phytoremediation potential along with beautifying the environment and this property makes them quite unique from other hyper-accumulators (Shibata, 2008). As ornamental plants are grown on numerous sites, they share varying degrees of man-made stresses such as pollution, compaction, water loss and others (Davidson and Miller, 1990). In urban areas ornamental plants are continuously exposed to heavy metals' pollution. They are more exposed to copper pollution particularly along road side due to significant increase in vehicular traffic, pesticides, soil and water pollution. The objective of the present study was to evaluate the effects of different doses of copper on *Phlox drummondii* plant growth and its ability to absorb and tolerate metal contamination.

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2. MATERIALS AND METHODS

2.1 Plant material

A pot experiment was conducted to analyze the effects of copper stress on *Phlox drummondii* (Polemoniaceae) growth attributes. Prior to start the experiment, soil physicochemical properties of soil like pH, moisture content and electrical conductivity were determined. Cleaned soil was filled in twenty-four earthen pots with the diameter of 25cm whereas height of pots was 10cm. Seedlings were grown in containers. Selected matured seedlings (sufficient root and shoot material, equal sized) were shifted to pots. Six pots were used as control with no copper treatment in the same way six pots were used for each copper level. Copper treatments were given in three different concentrations i.e. 10 ppm, 20 ppm and 30 ppm by using copper sulpahte pentahydrate ($CuSO_4.5H_2O$). When the plants attained sufficient height, they were given first treatment whereas other two treatments were given after every two-week interval in order to increase the probability of copper uptake by plants. Different growth attributes such as no. of leaves, leaf area, chlorophyll content, ascorbic acid, carotenoids and plant height were analyzed and the measurements were taken after every two-week interval. At the harvest period above and below ground biomass, relative water content and metal concentrations were also determined.

2.2 Estimation of chlorophyll and carotenoid content

Chlorophyll content in the leaf tissues was determined according to the method described by (Arnon, 1949). Briefly 0.3 g of fresh leaf samples were extracted with 10 ml of ethanol and then absorbance of the extract was measured by UV/VIS Spectrophotometer at 663, 645 nm and 480 nm. Chlorophyll was estimated using the following formula:

Chlorophyll [a] (mg/ml) = (0.0127) × (A.663) – (0.00269) × (A.645)

Chlorophyll [b] (mg/ml) = (0.0229) × (A.645) – (0.00468) × (A.663)

Total chlorophyll (mg/ml) = (0.0202) × (A.645) +(0.00802) × (A.663)

Carotenoid content was determined using the formula of Kirk & Allen (1965):

Carotenoid (mg/g) = [A.480 + (0.114 × A.663 – 0.638 ×A.645)].

2.3 Measuring number of leaves and plant height

Number of leaves was measured by simply counting the leaves of each plant. Plant height of each plant was measured by using measuring tape from the base of the plant to the tip of the last leaf (Ayolagha and Peter, 2011).

2.4 Measurement of leaf area

Graphical method was followed to measure leaf area. For this purpose, leaf was carefully removed from the plant and placed on a graph paper. Then outline of leaf was carefully drawn on the graph paper and area of leaf was measured by counting the number of grids covered by leaf (Patil, 2011).

2.5 Estimation of ascorbic acid

Titration method was used for estimation of ascorbic acid in leaves of *Phlox drummondii* according to the method (Reiss, 1993).

2.6 Estimation of biomass

Fresh biomass of each plant was determined immediately on weighing balance after harvest. For dry biomass, plants were dried in hot air oven at 65°C for 48 hours and after that their weight was determined on weighing balance (Malar et al., 2014).

2.7 Estimation of relative water content

The relative water content (RWC) was determined using the formula (Chen et al., 2009).

$$\text{Relative water content (\%)} = \text{Fresh weight} - \frac{\text{Dry weight}}{\text{Fresh Weight}} \times 100$$

2.8 Estimation of copper content in above and below ground biomass

Method of was used to analyze copper content in plants at the time of harvest using atomic absorption spectrophotometer (Ansari et al., 2004).

2.9 Statistical analysis

Significant differences of treated plants against the untreated plants were analyzed by one-way ANOVA, using IBM SPSS Version 20 software. The data for all the parameters was presented as mean, standard deviation and significance was tested at $p<0.05$. All figures were made using Microsoft Excel 2007.

3. RESULTS

3.1 Selected soil analysis

The electrical conductivity (EC), moisture content and pH of the selected soil were 139 μS/cm, 9.53% and 6.8 respectively.

3.2 Effect of copper on biochemical parameters of *Phlox drummondii*

3.2.1 Effect of copper on chlorophyll and carotenoid content

Figure 1 showed that increasing the copper levels in the soil decreased chlorophyll-a, chlorophyll-b and total chlorophyll plant leaves of *Phlox drummondii*. Lowest amount of chlorophyll content was found for plants grown in the soil containing 30 ppm copper treatment. Chlorophyll-a, chlorophyll-b and total chlorophyll decreased significantly as compared to the control plants (Table 1). Table 1 showed that level of carotenoid content was significantly reduced. The lowest declined value for carotenoid content was found at 11th week for the 30ppm copper concentration (Figure 1).

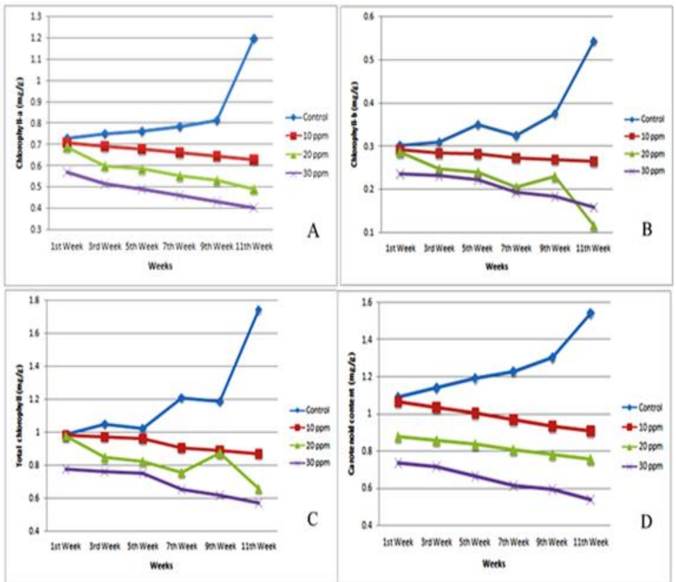

Figure 1: Weekly comparison of copper treatments on chlorophyll-a (A), Chlorophyll-b (B), Total chlorophyll (C) and carotenoids content (D) against control plants

3.2.2 Effect of copper on ascorbic acid

The level of ascorbic acid content in *Phlox drummondii* increased as well as decreased. In control plants ascorbic acid content increased constantly throughout the growth cycle of *Phlox drummondii* same trend was observed in case of all treated *Phlox drummondii* but by weekly comparison it was observed that the amount of ascorbic acid content is less among treated plants as the concentration of copper increased. This increased level of ascorbic acid content was statistically insignificant ($p>0.05$) (Table 1).

Table 1: Results of ANOVA test for biochemical parameters of Phlox drummondii						
Parameters	**Copper concentration (ppm)**	**Mean (mg/g) `**	**S.D.**	***df***	**F**	***p***
Chlorophyll (a)	Control	.857	.216	23	11.035	0.001
	10 ppm	.670	.029			
	20 ppm	.576	.067			
	30 ppm	.480	.059			
Chlorophyll (b)	Control	.368	.090	23	11.874	0.002`
	10 ppm	.278	.010			
	20 ppm	.237	.028			
	30 ppm	.205	.030			
Total chlorophyll	Control	1.27	.321	23	11.983	.001
	10 ppm	.931	.048			
	20 ppm	.823	.107			
	30 ppm	.689	.086			
Carotenoid content	Control	1.25	.160	23	42.431	.000
	10 ppm	.985	.061			
	20 ppm	.820	.046			
	30 ppm	.644	.076			
Ascorbic acid content	Control	.707	.090	23	2.798	.066
	10 ppm	.770	.075			
	20 ppm	.654	.083			
	30 ppm	.618	.130			

S.D= standard deviation; df= degree of freedom; p= significance

3.3 Effect of copper on growth parameters of *Phlox drummondii*

3.3.1 Plant height, number of leaves and leaf area

Plant height, number of leaves and leaf area in control plants were somewhat higher than treated plants. However, this difference between treated and control plants was not statistically significant ($p>0.05$) (Table 2). Figures 2 show weekly effects of copper on growth parameters of *Phlox drummondii.*

Table 2: Results of ANOVA test on growth parameters of Phlox drummondii						
Plant parameters (units)	**Copper concentration (ppm)**	**Mean**	**S.D**	***df***	**F**	***p***
Plant height (cm)	Control	16.74	9.13	23	.085	.967
	10 ppm	15.43	8.29			
	20 ppm	15.02	7.93			
	30 ppm	14.43	7.46			
Number of leaves`	Control	51.10	23.48	23	.068	.976
	10 ppm	47.80	20.14			
	20 ppm	46.56	19.38			
	30 ppm	46.36	19.31			
Leaf area $(cm)^2$	Control	3.963	1.17	23	.203	.893
	10 ppm	3.671	1.03			
	20 ppm	3.624	1.03			
	30 ppm	3.495	1.04			

S.D= standard deviation; df= degree of freedom; p= significance

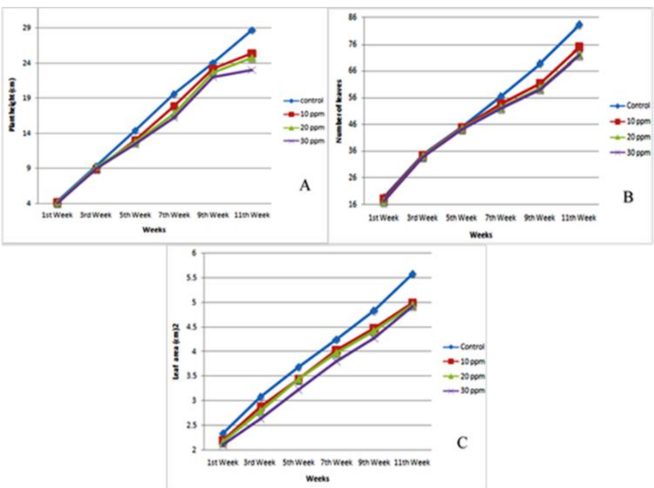

Figure 2: Weekly comparison of copper effect on number of leaves (A), plant height (B) and leaf area (C) against control plants

3.3.2 Biomass of treated plants

Table 3 show that above ground biomass decreased significantly ($p<0.05$) with the increase of copper concentration as compared to control plants. Figure 3 & 4 shows percentage reduction of fresh and dry weight of above and below ground biomass respectively. Treated plant also showed significant ($p<0.05$) decrease in fresh and dry weight of below ground biomass as compared to control plants (Table 3). Over all, maximum percentage reduction was shown by 30 ppm copper concentration.

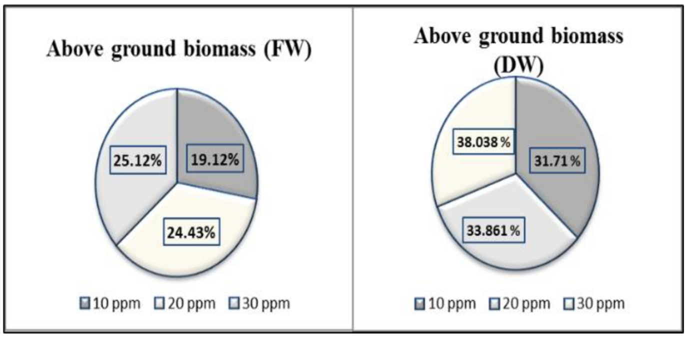

Figure 3: Percentage reduction in fresh/dry weight of above ground biomass

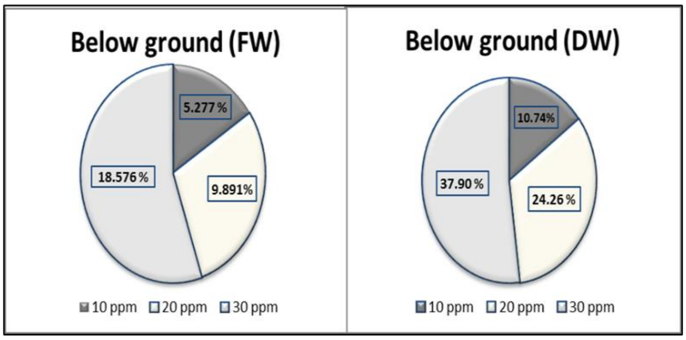

Figure 4: Percentage reduction in fresh/dry weight of below ground biomass

Table 3: Result of ANOVA test for copper effect on biomass of Phlox drummondii						
Parameters	**Copper concentration (ppm)**	**Mean (g)**	**S.D**	***df***	**F**	***p***
Above ground biomass (FW)	Control	31.66	3.08	23	5.643	.006
	10 ppm	25.60	3.14			
	20 ppm	23.92	3.89			
	30 ppm	23.70	4.93			
Above ground biomass (DW)	Control	4.694	.799	23	7.912	.001
	10 ppm	3.596	.32			
	20 ppm	3.506	.61			
	30 ppm	3.205	.39			
Below ground biomass (FW)	Control	.3316	.01	23	3.284	.042
	10 ppm	3.671	1.03			
	20 ppm	3.624	1.03			
	30 ppm	3.495	1.04			

S.D= standard deviation; df= degree of freedom; p= significance; FW= fresh weight; DW= dry weight.

3.4 Relative water content (RWC)

Plants with 30 ppm copper treatment showed highest values of relative water content for above and below ground biomass i.e. 86.48% and 56.68% respectively (Table 4). Results for RWC were statistically significant ($P<0.05$).

Table 4: Results of ANOVA test for copper effect on relative water content of Phlox drummondii

Parameters	Copper concentration (ppm)	Mean (%)	S.D	*df*	F	*p*
relative water content of below ground biomass	Control	40.62	3.73	23	8.826	.001
	10 ppm	43.18	6.08			
	20 ppm	43.59	6.34			
	30 ppm	56.68	7.13			
relative water content of above ground biomass	Control	83.94	1.91	23	4.038	.021
	10 ppm	84.04	1.80			
	20 ppm	86.43	1.22			
	30 ppm	86.48	1.89			

df= degree of freedom; *p*= significance

3.5 Copper accumulation by *Phlox drummondii*

Table 5 show that amount of copper in above ground parts (shoot) was less as compared to below ground parts (roots). Amount of copper accumulation by treated plants was greater than copper accumulated by control plants.

Table 5: Copper accumulation by shoots and roots of Phlox drummondii

Treatments	Cu accumulation by plant shoots (mg/kg)	Cu accumulation by plant roots (mg/kg)
Control	2.352	1.346
10 ppm	8.319	12.87
20 ppm	12.06	18.86
30 ppm	14.78	25.78

4. Discussion

Copper is among the toxic heavy metals. In plants, when copper is given in high concentration, they show toxic effects like stunted growth, chlorosis, disturbs mineral uptake, cell oxidative damage, formation of reactive oxygen species, and increasing membrane permeability (Madejonb et al., 2009). This cause change in plant antioxidative enzyme system. On the other hand lack of copper can affect growth, metabolism, and developmental processes of plants (Scandalios, 1993). Heavy metals particularly copper cause a decline in concentration of chlorophyll (a, b & total) which are part of photosynthetic pigments thus it interferes with functionality of chlorophyll (Adrees et al., 2015). In present study copper reduced the chlorophyll contents in Phlox drummondii similarly it was also reported for cauliflower (Chatterjee et al., 2000). Chlorophyll content may be reduced as the result of an inhibited photosynthetic electron transport or by decomposition of the chloroplast membrane with higher copper concentration (Bohner et al., 1980; Sandmann and Boger, 1980).

Carotenoids are the pigments which are mostly produced by all photosynthetic and non-photosynthetic organisms (Demmig-Adams et al., 1996). In present study copper treatment may have affected these photosynthetic pigments (carotenoids) and resulted in reduced activity of photosynthesis and the dark respiration rate (Shahbaz et al., 2010). Similarly, copper toxicity resulted in reduced carotenoid content in Ludwigia Perennis L. The highest decline (44.64%) was recorded at higher concentration (10ppm) of copper (Jangid and Shringi, 2013). In present experiment, ascorbic acid content (AsA) increased with the growth of plant which may be one important reason for the tolerance of Phlox drummondii against copper accumulation. Similarly studied effect of excess copper on maize leaves and in their study and concentration of copper were 0, 2, 4 and 8 mM (Wang et al., 2011). Ascorbic acid content was increased up to 4mM and then decreased in higher concentration plants. Ascorbic acid has an important role in protecting organisms and also plays a role in the regular metabolism of plants from the destructive effects of oxidative stress. It acts as both primary as well as secondary antioxidant, as primary antioxidant it reacts directly with reactive oxygen species, and as secondary antioxidant it reduces the oxidized form of tocopherol thus preventing membrane damage (Padh, 1990; Asada, 1992).

The number of leaves were slightly affected by copper toxicity. A study supports the results of present study (Dey et al., 2014). They studied that due to copper toxicity the number of leaves reduced or slowed down with the less arrival of new leaves and stems. As a whole the growth was slowed. The height of treated Phlox drummondii when compared with control, reduction in plant height of treated plants was noticed over control. The inhibitory action of excessive copper on shoot length might be due to harmful effect of copper on the process of photosynthesis, protein synthesis, reduction in cell division, and respiration. These obviously cause hindrance in normal growth (Kupper et al., 1998). Vegetative response like shoot length mostly used to indicate resistance of metal to plants (Karataglis, 1980). A decline in the height of plant with simultaneous decrease of root growth was previously reported in Prunus cerasifera, Pisum sativum, Vicia faba, Sesbania drummondii and Camellia sinensis due to copper toxicity (Lombardi and Sebastiani, 2005; Souguir et al., 2008; Israr et al., 2011; Sen et al., 2013; Dey et al., 2014).

According to measurement of leaf area is an important parameter in plant studies as it may provide information about photosynthetic efficiency, fertilizers, and irrigation response, evapotranspiration and plant growth (Blanco and Folegatti, 2005). On the whole leaf area decreased with the increase of copper concentration but when it comes to leaf area per plant it was increased throughout the experiment whereas there was less leaf area of treated plants over control plants. A study supports present study in which they reported that leaf area of Withania somnifera decreased with the increase of copper in soil (Singh et al., 2014). A decline in leaf area of Sesuvium portulacastrum L. was observed upon increasing copper concentration in soil (Kalaikandhan et al., 2014). Relative water content of control was less as compared to treated plants of Phlox drummondii. Change in relative water content has been recommended as a sign of phytotoxicity after it was found in Brassica juncea and Pteris vittata as a result of metal stress (Heidari and Sarani, 2011). A group researcher conducted an experiment for the effects of high concentration of copper on production of corn in the field (Syuhada et al., 2014). According to this study relative water content was increased as the concentration of copper (0, 0.2, 1.5 and 3 ppm) increased in corn plants. Like present study this study also showed that relative water content of control plants was less as compared to copper treated plants and this trend was significant. These results indicate that this trend could be because plants treated with copper can perform their metabolism functions well than that of control plants.

General responses of higher plants to metal (e.g. copper) toxicity include biomass reduction and growth inhibition (Ouariti et al., 1997). In excess copper concentration reduction of biomass might be a result of low protein formation which in turn results in inhibition of photosynthesis or it may also hamper carbohydrate translocation (Manivasagaperumal et al., 2011). Excessive amount of copper in the root surroundings generally results in slow or retarded production of root and shoot biomass along with a reduced photosynthetic activity (Shahbaz et al., 2010). In present study as Phlox drummondii were exposed to elevated level of copper the biomass mass reduction was coincided with decreased pigment contents (chlorophyll a, b, and total along with carotenoids; Figure 1) which may have resulted in reduced photosynthetic activity and dark respiration rate (Shahbaz et al., 2010). A study reported that a progressive reduction in dry weight content of above ground biomass with an increase in copper level was observed in Sesuvium portulacastrum L (Kalaikandhan et al. (2014).

They also observed a significant decline of dry matter content of below ground biomass with the increasing copper levels in Sesuvium portulacastrum L. Copper largely accumulate in roots compared to shoots thus it may hinder root elongation and damage root cell membrane or root epidermal cells. A significant reduction of chlorophyll content could be associated to uptake of copper in high concentration. Like present study this is also shown in many other studies (Patsikka et al., 2002; Nagajyoti et al., 2010). A group researcher investigated accumulation of sunflower plants by copper and other metals (Rivelli et al., 2012). Concentration of copper given to sunflower plants was 400mg/kg. Excessive copper cause a high amount of copper accumulation in roots which was in higher amount compared to roots.

5. Conclusion

This study demonstrated that copper (like other heavy metals) has toxic effects when given in excess amount. Chlorophyll (a, b and total) and carotenoid content were decreased. The growth of all treated Phlox drummondii was slow as compared to control plants. Overall, the plant was tolerant to copper because no major morphological changes appeared from copper toxicity and Phlox drummondii grew well in given concentrations of copper hence it can be grown in soil having high copper concentration. As copper concentration was higher in roots than shoots so Phlox drummondii has great capacity to be used for phytostabilization remediation of copper contaminated soils. It could not only be used as hyperaccumulator for contaminated soil but can enhance the beauty of environment at the same time.

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