*International Research Journal of Environment Sciences*______________________________ ISSN 2319–1414
Vol. **4(1),** 52-58, January **(2015)** *Int. Res. J. Environment Sci.*

Phytoremediation of Commonly used Metals (Cu and Zn) from Soil by *Calendula officinalis* (I)

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Available online at: www.isca.in, www.isca.me
Received 16th December 2014, revised 9th January 2015, accepted 21st January 2015

Abstract

Phytoremediation is an emerging technology for removing heavy metals contamination from polluted sites. It is cost effective and has exquisite advantages and long term applicability. The present study suggests about the accumulation ability, mobility and plant growth response on exposure to heavy metals in soil. Experiments were carried out to find the accumulation ability of Cu and Zn in the Calendula officinalis. The selected metals were mixed at various concentrations ranging from 50, 100, 150, 200 and 250 mg/Kg for Cu and 150, 300, 450 and 600 mg/Kg for Zn separately in soil. The results showed that plants grown in Cu supplemented soil had shorter shoot and root length as compared to plants grown in control condition. This may be due to accumulation of Cu in shoot and root of Calendula officinalis. In comparison to Cu concentration accumulation, effect of Zn (150, 300, 450 and 600 mg/Kg) in soil reduced the length of shoots and roots of Calendula officinalis and accumulation potential of Zn was more. The results revealed that as the concentration of both metals (Cu and Zn) were increased in the soil; the overall length of Calendula officinalis decreased due to potential accumulation ability in shoot and root.

Keywords: Phytoremediation, *calendula officinalis*, copper, zinc.

Introduction

Metals contaminate the agriculture use of soils by atmospheric deposition or by dumping of sewage sludge. This is resulted as a risk of either leaching of metals into the groundwater or too much accumulation in the top layer of soil[1-2]. The amount of Metal concentrations in soil ranges from less than 1 mg/kg to high as 100,000 mg/kg, even if it occurs due to the geological origin of the soil or may be as a result of human activity[3]. Overindulgence concentrations of some heavy metals in soils such as Cd, Cr, Cu, Ni, and Zn have caused the interruption of natural aquatic and terrestrial ecosystems[4-5]. High amount of Cu and Zn in plants can caused some trouble. At concentrations on those needed for optimal growth; Cu were shown to slow down the growth and to hinder with important cellular processes such as photosynthesis and respiration[6-7]. Plants grown in the presence of excessive levels of Cu usually show decreased biomass and chlorotic symptoms. Zn concentration in excess amount can also affect the production of NADPH in plant. In inclusion, production of free radicals will increase to plants.

Activity of RUBP carboxylase enzyme and Photo system II decreases by accumulation of zinc toxicant. Further, Zinc toxicity also diminishes ATP synthesis and chloroplasts activity with this photosynthesis will also started to decrease. The amount that causes toxicities is more than 300 ppm[8-11].

Copper (Cu) is the micronutrient naturally found in the soil. The major copper contamination sources of soils are pigs and poultry manures, pesticides and metal finishing and microelectronics byproducts. Also, Zinc (Zn) is a vital trace element for plants and animals, but is toxic when present at high levels. Zinc is willingly absorbed by clay minerals, carbonates, or hydrous oxides. It was found that the maximum percent of the total Zn in contaminated soils and sediments was related with Fe and Mn oxides[12-14]. Human is at danger from copper polluted soils through dermal contact, ingestion, consumption of food grown in polluted soil and by inhalation of dust or vapors and causes sensitive effects. Zinc also influences human beings by causing chronic diseases such as gastrointestinal, heart, respiratory, brain and kidney damage and effects on skin and mucous membranes. It also causes DNA damage and carcinogenic effects due to its mutagenic capability.

Phytoremediation is projected to a cost-effective alternate for the treatment of contaminated soils. From this the top soil would be preserved and the amount of hazardous materials reduced drastically[15].

In present study, *Calendula officinalis* (pot marigold) which are a popular ornamental plant with medicinal value taken on account to resolve metal eradicating potential. It belongs to genus *Calendula* of the family Asteraceae. *Calendula officianalis* are a short-lived aromatic herbaceous perennial, growing to 80 cm tall, with almost branched lax or erect stems. The objective of this study was to determine the accumulation ability of heavy metal (copper and zinc) by *Calendula officinalis* (ornamental plant).

Material and Methods

Soil collection and characterization: Soil was collected from the farm site of Rajmata Vijyaraje Scindia Krashi Vishwa Vidhayala, Gwalior (Madhya Pradesh), India; at 10 cm to 20 cm deep surface by using soil sampler. Soil was dried, sieved through 2 mm screens and made ready to determine physical and chemical properties.

Physical Properties: Determination of pH: The pH of the soil is an important factor of determining the growth rate of plant because pH regulates nutrients availability in plant. 20 grams weighed soil was placed into 100 ml polyethylene beakers and 40 ml water was added to make a saturated paste. Then it was shaken for 2 hrs or was stirred well with glass rod and electrode was immersed in the suspension. pH was reading when reading was stabilized on pH meter [16].

Determination of moisture content: 100 grams of soil was weighed with water contents and after that the soil sample was dried in the hot air oven and after proper drying the soil sample was weighed again and finally the moisture content of soil was calculated as mentioned below. Moisture content was determined by the ratio of the weight of water in the soil to the weight of dry soil and expressed in % [17].

$$\text{Percentage of soil moisture} = \frac{\text{Initial weight of soil – the oven dried weight soil}}{\text{Initial weight of soil}} \times 100$$

Electrical Conductivity Measurement: The same soil suspension prepared for determination of pH was used for conductivity determination. After soil pH was recorded allow the soil suspension in the beaker to settle for maximum of ½ hours.

The conductivity cell was rinsed and filled with standard KCl solution to read the standard conductivity. Then the cell was rinsed and filled with soil extract (reading on the salt bridge), corrected to 25°C, and reading was recorded[16].

Specific Gravity Measurement: Specific gravity of soil was find out by using Pycno Meter. With the help of weighing machines firstly the weight of empty pycnometer was measured, W1.Then the weight of pycnometer with half soil filled pycnometer was measured, W2. Then remaining portions of pycnometer was filled with water, mixed it well with soil and was weighed again, W3. Whole mixture of soil and water was removed and washed and then it was fully filled with water and then was weighed, W4. All values were put in formula[17].

$$\text{Specific gravity} = \frac{W2\ \ W1}{(W2-W1)-(W3-W4)}$$

Bulk Density Measurement: To measure bulk density, a cylinder with 1000 cm^3s was weighed W1, then cylinder was filled with soil and again it was weighed; W2 [17]. The bulk density was calculated as follows:

$$\text{Bulk Density} = \frac{W2-W1}{\text{Volume of cylinder}}$$

Particle size: 40 g air-dry soil (2-mm) was weighed and 60-mL dissolving solution [$(NaPO_3)_{13}$ + (Na_2CO_3)] was added to this soil. A beaker was taken and covered with a watch glass, and left for overnight. Then Stir the suspension at high speed for 3 minutes using the stirrer. Stirring paddles was washed into a cup, and allow standing for 1 minute. Quantitatively suspension was move to a 1-L calibrated a cylinder (hydrometer jar), and bring to volume with water. Then suspension was poured through a 50 µl sieves to determine the sand clay and slit[17].

Chemical properties: Determination of available phosphorous: The Olsen method was used[18] to determine the concentration of phosphorous. 2.5 g air dried soil was weighed into a 150 ml Erlenmeyer flask. To it a little of the Darco G 60 or equivalent grade and 50 ml of Olsen's reagent (soil to solution ratio of 1:20) was added and shaken on the reciprocating shaker for 30 min. Without soil blanks were running. The sample was filtered through Whatman number 40 or 42 filter paper into a clean and dry beaker and before pouring suspension into funnel flask was shaken immediately. Then 5 ml aliquots of the extract from 25 ml volumetric flasks was taken and acidified with 2.5 M H_2SO_4 of pH 5.0 and 20 ml distilled water and 4 ml of reagent B was added. OD was taken on spectrophotometer after 10 minutes and value was calculated against the standard curve[16].

Determination of Nitrogen: 5g of soil was taken in a digestion tube and little water was added into it. Then 20 ml. of 0.32% $KMnO_4$ solution was taken and added to sample and fit the tube in the distillation unit. 20 ml of 2.5% NaOH solution was added through the distyl-em-dosing pump. Then 20 ml. of 2.5% of boric acid was pipette out in a conical flask and clip the receiving end of the distyl-em in it. From the tube distil ammonia gas was collected in the received acid. 5 drops of mixed indicators were added and was titrated with 0.02N H_2SO_4. Blank correction (without soil) was to be made for final calculations[16].

Determination of Potassium: 5 gm soil was placed in a 150 ml flask and 25 ml of neutral N ammonium acetate was added. After shaking for 5 minutes filter the solution to Whatman no. 1 filter paper. First few ml. of the filtrate was rejected. A solution was mixed and concentration of K was estimated by the flame photometer[16].

Determination of available metal (Zinc and copper) in soil: Sieving of the soil was completed with the help of 0.5 mm sieves and was dried for 24 hours after drying it was made cool at room temperature. After drying soil was digested with HNO_3

and distilled water. The reading were taken with the help of Atomic Absorption Spectrophotometer (AAS) and the value of Zn and Cu was calculated by running standards of Cu and Zn[16].

Experimental Pots Preparation, Sowing and Cultivation of Plants: Equal quantity of soil (1kg) was taken in a cylindrical polythene bag (25cm diameter and 14 cm length) and various concentration of Cu salt as a (Copper sulphate) (50, 100, 150, 200 and 250 mg/kg) and Zn metal (zinc sulphate hydrated salt) (150, 300, 450 and 600 mg/kg) was added in the experimental polythene bags. A triplicate of each respective metal salt concentration was prepared. These metals were properly mixed with soil. 10-15 seeds of *Calendula officinalis* plant were sown with each metal treated and control soil pots. Each ions concentration had been prepared with triplicates. These pots were kept in green houses properly, regularly irrigated and observed daily.

Plant harvesting and analysis: Plants harvesting took place early in the morning between 8.0 to 9.0 AM; after 75 days of sowing. The collected plant samples were placed in plastic bags, tagged and carried to the laboratory carefully. The overall length of plants was measured using measuring scale and accumulation ability of Cu and Zn ions in shoot and root of *Calendula officinalis* were detected by Atomic Absorption Spectrophotometer. In this method wave length 327.4 nms, slit 0.2 nm and flame nitrous oxide-acetylene was used for detection of Cu from shoot and root of plant and detection of Zn wave length was 213.9 nm, slit and flame was similar with Cu was used.

Statistical analysis: All the observations were taken in triplicates and mean value and standard error was calculated for each concentration. The significant results were calculated by student's t test and one way ANOVA at 5% level of significance.

Results and Discussion

Soil analysis: Soil was manually collected from Rajmata Vijyaraje Scindia Krashi Vishwa Vidyalay, Gwalior, India. It was alluvial type soil. The various physical properties of soil were examined and mention in Table 1 of this section. pH of soil influences the solubility and mobility of Cu and Zn ions. At pH above 7.0 of soil most Cu and Zn ions got reduced and the solubility of these ions was greater than the salt which was initially added to the soil. In this study pH of the soil was 7.3. Therefore, this pH value was suitable for Cu and Zn ions uptake by plants. The various chemical properties of soil were determined the values were shown in table-1.

Growth and Accumulation ability of *Calendula officinalis* plant upon Cu and Zn exposure: The growth of *Calendula officianlis* plant on exposure to various concentrations of heavy metals was observed. It was found that the *Calendula officinalis* plant grew well at the low concentrations of the respective Cu and Zn metal and uptake by shoot and root parts of plants were determined by using of Atomic Absorption Spectrophotometer.

Toxic effect of Zn on Calendula officinalis on shoots and roots growth: The effect of Zn on a shoot and root of Calendula officinalis is shown in table-3. There were not significant decreases in length of shoots and roots of Calendula officinalis plants with increasing Zn concentration in the soil. The consequence of Zn was not found very toxic. At 150, 300, 450 and 600 mg/Kg of Zn the shoot length in given table values with the difference in 6.2%, 15.5%, 23.1%, and 28.4 % decrease, with respect to control. The shoot length of plant was 75 % significant. The effect of Zn on root length of plant was different as compared to the effect on shoot length. At various Zn concentrations the length of root in (table-3) decreased with the differences in 14.8%, 18.2%, 27.3% and 35.6% with respect to control. It was showing linear decreases root length upto 600 mg/Kg as compared to control and data was 70 % significant. The length of roots gradually decreased at control and various concentrations of Zn (150, 300, 450 and 600 mg/Kg). The significant values were represented by the symbol '#' and non significant values were represented by the symbol '*' in the given table.

Toxic effect of Cu on *Calendula officinalis* on shoots and roots growth: The effect of Cu on a shoot and root of *Calendula officinalis* was shown in table-4. There was decreasing length of shoots and roots of *Calendula officinalis* plants with increasing Cu concentration in the soil. The effect of Cu the shoot length of plant was not as toxic. The shoot length of plant at Cu concentration was decreasing from compared to control. At 50, 100, 150, 200 and 250 mg/Kg of Cu the length difference in shoot length of given table value with the difference of 14.7%, 19.6%, 28.7%, 31.6% and 38.8% respectively, with respect to control. The shoot length was 60 % significant. Plant was able to tolerate up to 150 mg/Kg of Cu concentration on soil. It was shown linearly decreasing to shoot length up to 250 mg/Kg. The length of shoots was gradually decreased from various concentration of Cu as compared to control a shoot length and the effect of Cu on root length of plant was different as measure up to the result on shoot length. At 50, 100, 150, 200 and 250 mg/Kg of Cu the length difference in root length of given table value with the difference of 5.8%, 13.2%, 13.2%, 20.6% and 29% respectively, with respect to control and data was 70 % significant. It was shown linear decreasing root length up to 250 mg/Kg as compared to control. The length of root gradually decreased from control and various concentrations of Cu (50, 100, 150, 200 and 250). The significant values were represented by the symbol '#' and non significant values were represented by the symbol '*' in the given table.

Uptake of Zn in shoot and root of *Calendula officinalis*: The metal uptake by a shoot and root parts of plants was determined by using of Atomic Absorption Spectrophotometer. The accumulation ability of Zn in shoots and roots of *Calendula officinalis* was shown in Table 5. It was shown that the Zn in a shoot was found accumulate in the experimental pot of 600 mg/Kg. The doses of 150, 300, 450 and 600 mg/Kg of Zn increased the accumulation ability in plant shoot in given table values with the difference of 12%, 17%, 17.6% and 21.8% respectively, with respect to the accumulation ability of control. The uptake value of Zn in a shoot was increased to compare to control and the accumulation ability of root having also increased gradually at control and various concentration of Zn (150, 300, 450 and 600). The accumulation ability in plant roots in given table value of the difference of 18.3%, 26.2%, 29.4% and 30.5% respectively, with respect to the accumulation ability of control. It was shown, increasing accumulation in plant roots up to 600 mg/Kg as compared to control and shown that *Calendula officinalis* was much more efficient to tolerate Zn. The accumulation ability of metal (Zn) by a shoot and root of *Calendula officinalis* was significant 78.5 % and 70 % respectively. The significant values were represented by the symbol '#' and non significant values were represented by the symbol '*' in the given table.

Uptake of Cu in shoot and root of *Calendula officinalis*: The accumulation ability of Cu on shoots and roots of *Calendula officinalis* was shown in table-6. It was shown that the maximum concentration of Cu of plant shoots was accumulating in the experimental pot of 250 mg/Kg. The uptake value of Cu in a shoot was increased to compare to control at the various concentrations 50, 100, 150, 200 and 250 mg/Kg of Cu ion increased the accumulation ability in plant shoot in given table value with the difference of 13.3%, 19.3%, 22.6%, 23.3% and 25% respectively, with respect to the accumulation ability of control. Similarly the accumulation ability of Cu in plant root having also increased at various concentration of Cu ion (50, 100, 150, 200 and 250) with some variations on 15%, 15.8%, 19.3%, 20.5% and 25.6% as compared to control. It was shown non linear graphs, increasing accumulation in plant roots up to 250 mg/Kg as compared to control. It was observed that Cu was toxic to *Calendula officinalis* with increasing its concentration. The accumulation ability of metal (Cu) by a shoot and root of *Calendula officinalis* was significant 75% and 72% respectively. The significant values were represented by the symbol '#' and non significant values were represented by the symbol '*' in the given table.

So, *Calendula officinalis* have no potentiality to uptake and store Cu ion as copper sulphate causes lethal effects on plant, but Zn ion have potentiality to uptake and store Zn as a zinc sulphate. It is good phytoremediation for contaminated soil.

Table-1
Physical and Chemical properties of soil

S. No.	Parameters	Results
1.	pH of the soil	7.3
2.	Electrical Conductivity	0.54 ds/m
3.	Moisture Content	17.64 %
4.	Particle Size	0.5 mm
5.	Specific Gravity	2.17
6.	Bulk Density	1.6 g/cm^3
7.	Phosphorous Content	268.0 Kg/ha
8.	Nitrogen Content	104 Kg/ha
9.	Potassium Content	246.4 Kg/ha
10.	Available metal (Copper and Zinc) in soil	0.18 ppm and 0.34 ppm

Table-2
Length of shoot and root of *Calendula officinalis* upon exposure to different concentrations of Zn

Sample	Treatments of Zn	Mean of shoot length growth±SE	t-value	Mean of root length growth±SE	t-value
1	Control	43.3±1.2	0	12.1±0.2	0
2	150	40.6±1.3	1.49*	10.3±0.7	2.37*
3	300	36.6±0.8	4.48#	9.9±0.3	6.51#
4	450	33.3±0.8	6.72#	8.8±0.2	11.77#
5	600	31±0.5	9.25#	7.8±0.2	16.65#

*sign shows non significant at 5% by student t-test and # sign shows significant at 5% by Student t-test (above the value of $t_{0.05}$2.132 at degree of freedom of 4).

Table-3
Analysis of variance (ANOVA) for the length of shoot of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	406.2	5	81.2	7.903
Within sample	123.3	12	10.2	
Total	529.5	17		

Table-4
Analysis of variance (ANOVA) for the length of root of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	16.1	5	3.2	6.164
Within sample	6.2	12	0.5	
Total	22.3	17		

Table-5
Length of shoot and root of *Calendula officinalis* upon exposure to different concentrations of Cu

Sample	Treatments of Cu	Mean of shoot length growth±SE	t-value	Mean of root length growth±SE	t-value
1	Control	37.3±3.5	0	12.1±0.2	0
2	50	31.8±1.2	1.25*	11.4±0.3	1.47*
3	100	30±1.6	4.49#	10.5±0.3	1.96*
4	150	26.6±0.7	2.32*	10.5±0.6	2.96#
5	200	25.5±0.7	6.06#	9.6±0.3	3.27#
6	250	22.8±1.5	8.61#	8.6±0.3	3.74#

*sign shows non significant at 5% by student t-test and # sign shows significant at 5% by Student t-test (above the value of $t_{0.05}$2.132 at degree of freedom of 4).

Table-6
Analysis of variance (ANOVA) for the length of shoot of *Calendula officinalis* upon exposure to different concentration of Cu. D.F represents the degree of freedom

Source of variation	Sum of square	D.F	Mean of square	F- ratio
Between sample	94.4	4	23.5	15.56
Within sample	15.2	10	1.5	
Total	109.6	14		

Table-7
Analysis of variance (ANOVA) for the length of root of *Calendula officinalis* upon exposure to different concentration of Cu. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	88.3	4	22.1	49.075
Within sample	4.5	10	0.4	
Total	92.8	14		

Table-8
Concentration of Zn detection by Atomic Absorption Spectrophotometer in shoot and root of *Calendula officinalis*

Sample	Dose/Treatment in mg/kg	Average metal uptake by shoot (in ppm)±SE	t-value	Average metal uptake by root (in ppm)±SE	t-value
1	Control	5.2±0.5	0	6.7±0.5	0
2	150	7.9±0.5	3.44#	8.1±0.5	1.88*
3	300	8.9±0.5	4.8#	10.8±0.2	7.43#
4	450	10.8±1.2	4.2#	12.02±0.2	15.07#
5	600	12.5±0.3	11.34#	13.3±0.3	10.63#

*sign shows non significant at 5% by student t-test and # sign shows significant at 5% by Student t-test (above the value of $t_{0.05}$2.132 at degree of freedom of 4).

Table-9
Analysis of variance (ANOVA) for the of shoot of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	31.4	4	7.8	16.92
Within sample	4.6	10	0.4	
Total	36.0	14		

Table-10
Analysis of variance (ANOVA) for the of root of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F	Mean of square	F-ratio
Between sample	309.3	4	77.3	25.21
Within sample	30.6	10	3.06	
Total	340.9	14		

Table-11
Concentration of Cu detection by Atomic Absorption Spectrophotometer in shoot and root of *Calendula officinalis*

Sample	Dose/Treatment in mg/kg	Average metal uptake by shoot (in ppm)±SE	t-value	Average metal uptake by root (in ppm)±SE	t-value
1	Control	0.3±0.04	0	0.2±0.07	0
2	50	0.4±0.05	1.65*	0.3±0.05	8.87#
3	100	0.7±0.1	3.352#	0.9±0.2	2.68*
4	150	0.8±0.05	6.46#	1.2±0.08	8.04#
5	200	1.3±0.1	5.814#	1.3±0.04	12.56#
6	250	1.8±0.03	7.54#	1.6±0.09	12.3#

*sign shows non significant at 5% by student t-test and # sign shows significant at 5% by Student t-test (above the value of $t_{0.05}$2.132 at degree of freedom of 4).

Table-12
Analysis of variance (ANOVA) for the of shoot of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	4.9	5	0.9	18.76
Within sample	0.6	12	0.05	
Total	5.5	17		

Table-13
Analysis of variance (ANOVA) for the of root of *Calendula officinalis* upon exposure to different concentration of Zn. D.F represents the degree of freedom

Source of variation	Sum of square	D.F.	Mean of square	F-ratio
Between sample	5.04	5	1.008	21.3636
Within sample	0.56	12	0.04	
Total	5.60	17		

Conclusion

The study was carried out to the copper accumulation ability from soil to *Calendula officinalis* at all concentrations i.e. 50, 100, 150, 200 and 250 mg/Kg badly affected on germination period and shoots and roots length. As compared to Cu; Zn accumulation ability from soil to *Calendula officinalis* at all concentrations i.e.150, 300, 450 and 600 mg/Kg tolerate on shoots and roots length, but germination period was affected. All values were significant at 5 % level on the basis of one way ANOVA. As the metals concentration on soil increased the accumulation ability was also increased. Higher the concentration of metals in soil; uptake into shoots and roots of *Calendula officinalis* was also higher. Hence it is suggested that *Calendula officinalis* is able to tolerate Cu and Zn much more efficiently. So, *Calendula officinalis* is able to grow on Cu and Zn contaminated soil.

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