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Cadmium accumulation and its effects on growth and biochemical parameters in *Tagetes erecta* L

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Abstract
Tagetes erecta L. was raised in pots containing soil treated with various concentrations of Cd $(NO_3)_2$ (6, 12, 18, 24 and 30mg/kg). At maturity plants were separated into root, stem, leaves and inflorescence and cadmium accumulated in each part was quantified. The effect of cadmium accumulation on growth was analyzed by the measurement of various parameters like root and shoot length, fresh and dry weight of root and shoot and total leaf area per plant. Moreover effect of cadmium accumulation on biochemical parameters was checked by quantitative estimation of various biochemical parameters like chlorophyll, total protein, free amino acids, total sugar, reducing sugar and starch. Results showed that there is no remarkable negative effect of accumulation of lead on the morphological growth of the plant. Biochemical analysis showed that amount of total protein continuously decreased whereas that of free amino acids continuously increased with increasing concentrations of cadmium. Amount of chlorophyll, total sugar, reducing sugar and starch continuously increased till mid-level i.e., Cd-18mg/kg and then continuously decreased at higher concentrations. Results of quantitative estimation of Cd in root, stem, leaves and inflorescence showed that roots accumulated highest amount of Cd followed by stem and leaves, whereas inflorescence contained least amount of Cd.

Keywords: Cadmium (Cd), Heavy metal, *Tagetes erecta* L., Accumulation, Phytoremediation

Introduction
Heavy metal pollution is an important environmental problem in the world. These are a unique class of toxicants since they cannot be broken down to non-toxic forms easily. Soil pollution by heavy metals has reasonably increased in last few decades due to discharge of wastewater and waste from anthropogenic sources (Ghosh and Singh, 2005) [14]. Metals like Pb, Hg, Cd, Ar, and Cr have no biological function and are toxic to life even at very low concentration (Salt *et al*., 1995) [25].

Among many heavy metals Cadmium (Cd) is non -essential and harmful heavy metal pollutant which is usually released in soil (Wagner, 1993) [30]. Cd is widely used in the electro plating, nickel-cadmium batteries, pigment plastic stabilizer and its common sources of release include metal industry, waste incineration and combustion of fossil fuels (Costa *et al*., 1994) [10]. The application of sewage sludge, city waste, and Cd-containing fertilizers causes the increase of Cd content in soils (Williams and David, 1973) [31].

Although Cd is not essential for plant growth, but it is readily taken up by roots and accumulated in plant tissues at high levels (Prasad, 1995) [23]. Many experiments described that cadmium (Cd) is a non-essential toxic heavy metal which produces physiological and morphological alterations in plants (Chaoui *et al*., 1997) [7]. Cadmium (Cd), being a highly toxic metal pollutant of soils, inhibits root and shoot growth and yield production, affects nutrient uptake and homeostasis, and is frequently accumulated by agriculturally important crops and then enters the food chain with a significant potential to impair animal and human health (di Toppi and Gabrielli, 1999) [12]. Excess of cadmium causes a number of toxic symptoms to the plants, viz. growth retardation, inhibition of photosynthesis, induction and inhibition of enzymes, altered stomatal action, efflux of cations and generation of free radicals (Chen and kao, 1995) [8]. Cadmium has been shown to affect various aspects of metabolism in different plant systems (Shah and Dubey, 1997) [26].

The objectives of this study were to assess and study –

1. The accumulation potential of *Tagetes erecta* L. to Cadmium (Cd).
2. Effect of Cd accumulation on morphological growth parameters like root and shoot length, total leaf area and fresh and dry weight of root and shoot of *Tagetes erecta* L.
3. Changes in biochemical parameters like total chlorophyll, protein, amino acids, carbohydrate, total sugar, reducible sugar and starch contents due to accumulation of Cadmium (Cd).

2. Materials and Methods

2.1 Pot Culture Experiments

The pot culture experiments were conducted in the Botanical garden, Gujarat University, Ahmedabad. Plants were grown in 6 groups of pots. Each group contained 21 pots and each pot was filled with 6kg air-dried soil. 1 group was filled with untreated soil (i.e. control) whereas the other 5 groups were treated with different concentrations of $Cd(NO_3)_2$, i.e. 6, 12, 18, 24 and 30mg/kg respectively. Healthy seeds were sown. Plants were irrigated regularly. At maturity plants were analyzed for following parameters.

2.2 Morphological and Growth Analysis

At maturity, various morphological parameters such as root length, shoot length, total leaf area and fresh and dry weight of root and shoot per plant were determined for every sample to check the effect of Cd accumulation on above mentioned parameters. Root and shoot length were measured with meter rule. Fresh weight of root and shoot were measured with analytical balance. Plants were dried in hot air oven at 80° C for 48 hours or until the constant dry weight was attained. The total leaf area was measured by leaf area meter. Average of five replicates was calculated and standard deviation (S.D.) was found out. Results were expressed as mean±S.D.

2.3 Biochemical Analysis

Effects of Cd accumulation on some biochemical parameters such as total and reducing sugar, starch, total protein, free amino acids and chlorophyll were analyzed. The biochemical methods for estimation of above mentioned parameters are as follows. Three replicates were prepared and analyzed from each treatment to analyze each parameter. Results were expressed as mean ± Standard Deviation (S.D.).

2.3.1 Estimation of chlorophyll (Arnon, 1949) [6]

1g of fresh leaves were ground in a mortar and pestle with 20 ml of 80% acetone. The homogenate was filtered through whatman no. 42 filter paper. The filtrate was saved. The residue was reextracted with 5ml of 80% acetone each time, until it become colourless. All the filtrates were pooled and final volume was made 50ml by adding 80% acetone. This filtrate was utilized for chlorophyll determination. Absorbance was read at 645nm and 663nm in spectrophotometer. The chlorophyll a and chlorophyll b contents were measured by using the formula given by Arnon (1949) [6].

$$\text{Chlorophyll a (mg/g)} = \frac{12.7\,A663 - 2.69\,A645}{1000 \times W} \times V \;\&$$

$$\text{Chlorophyll b (mg/g)} = \frac{22.9\,A645 - 4.68\,A663}{1000 \times W} \times V$$

Wherer, V = Volume of extract in ml, W = fresh weight of the leaf sample in gram.

2.3.2 Estimation of protein (Lowry *et al.*, 1951) [18]

Fresh tissue weighing 0.5 g was macerated in 10ml cold distilled water followed by 20ml of 10% trichloroacetic acid in mortar and pastle. Homogenate was then centrifuged at 600 rpm for 30 min and the supernatant was discarded. 10 ml of 0.1 N NaOH was added to the pellet and it was centrifuged for 30 min. The supernatant was used for the estimation of protein. To 1 ml of the extract, 5 ml of copper reagent 'C' was added (Reagent C: mixture of reagents A and B in 50:1 ratio; Reagent A: 2% Na_2CO_3 in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4$ in 1% sodium potassium tartrate). The tubes were shaken well and incubated for 10 min at room temperature, 0.5 ml of properly diluted Folin-Ciocalteau reagent was added to the solution and mixed thoroughly. The absorbance was read at 660 nm in a spectrophotometer against an appropriate blank. Bovin serum albumin was used as the standard.

2.3.3 Estimation of Total Free Amino Acids (Lee & Takahashi, 1966) [17]

Sample was prepared by extraction with 80% ethanol. Repeated homogenization and centrifugation were done and supernatants were mixed and used as a sample to determine free amino acids. Total free amino acids were estimated using 2% ninhydrin reagent. This reagent was prepared by mixing the following constituents (A, B and C) in the ratio of 5: 12: 2; (A) 1% ninhydrin in 0.5 M citrate buffer (pH 5.5); (B) pure glycerol; (C) 0.5 M citrate buffer (pH 5.5). To 0.2 ml of extract 3.8 ml of ninhydrin reagent was added. The contents were heated in boiling water bath for 12 min and cooled to room temperature. The optical density of purplish blue coloured resultant solution was measured at 570 nm. Glycine was used as the standard.

2.3.4 Estimation of Total Sugar (Nelson, 1944) [20]

Plant extract was prepared using 80% ethanol. To 1ml of alcoholic aliquot, 1ml 1N H_2SO_4 was added and heated at 49° C in water bath for 30 minutes for hydrolysis of the mixture. 2-3 drops of methyl red indicator were added followed by addition of 1N NaOH drop wise for neutralization (colour change: pink to yellow). Then 1ml Nelson Somogyi's reagent was added to it and the test tube was kept in boiling water bath for 20 minutes. After cooling off the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water. O.D. was noted at 540nm. Blank was prepared in the same manner. Glucose was used as the standard.

2.3.5 Estimation of Reducing Sugar (Nelson, 1944) [20]

Plant extract was prepared using 80% ethanol. To 1ml of this alcoholic extract, 1ml Nelson Somogyi's reagent was added and kept in boiling waterbath for 20min. After cooling off the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water. Optical density was noted at 540nm. Blank was prepared in the same manner. Glucose was used as standard.

2.3.6 Estimation of Starch (Chinoy, 1939) [9]

Sample was prepared by extraction with 80% ethanol. Repeated homogenization and centrifugation were done and residue left at all stages were mixed and used as a sample to determine starch content. The residue was dissolved in 20ml 0.7% KOH and boiled for 40 minutes for gelatinization. It was allowed to cool down and then centrifuged. Supernatant was used for further analysis. 1ml aliquot (Supernatant) was added with 0.5ml 20% acetic acid; 1ml citrate buffer (0.05M, pH 5.0) and 1ml I2KI and incubated at room temperature for 10minutes. Optical density was measured at 600nm. Blank was prepared in the same manner. Starch was used as standard.

2.4 Estimation of Cadmium (Cd) Content (AOAC, 1990) [4]

At maturity plants were uprooted from the pots. Plants grown in each concentration were separated into root, stem, leaves and inflorescence and were dried in hot air oven at 80°C for

48 hours. Each dried plant part of each concentration was powdered thoroughly and used for analysis.

1g dry powder of each sample was weighed into a conical flask and 10ml concentrated HNO_3 was added. The mixture was boiled at a constant temperature for about 45mins. After cooling, 5ml of 70% $HClO_4$ was added and the mixture was further boiled until the release of dense white fumes. After cooling, 20ml distilled water was added and heated until a clear solution was obtained. At room temperature, the mixture was filtered through Whatman no. 44 filter paper and transferred quantitatively to a 50ml volumetric flask by adding de-ionized and double-distilled water. Samples were analyzed through Atomic Absorption Spectrophotometer.

2.5 Statistical Analysis

One way ANOVA was conducted to compare the means of different treatments at $p<0.05$ level of significance.

3. Results and Discussion

Accumulation of Cd in roots, stem, leaves and inflorescence are depicted in Table 1.

Table 1: Accumulation of Cd in root, stem, leaf and inflorescence of *Tagetes erecta* L.

Cd in Plant Part/ Treatment	Cd in Root mg/g	Cd in Stem mg/g	Cd in Leaf mg/g	Cd in Inflorescence mg/g
Control	1.80*±0.05	1.23*±0.11	1.17*±0.06	0.42*±0.10
6 mg/kg	77.41*±6.67	27.90*±2.08	22.25*±2.13	4.08*±0.51
12 mg/kg	80.65*±5.49	32.17*±2.75	25.21*±2.11	5.14*±0.62
18 mg/kg	83.92*±7.33	34.35*±3.06	30.33*±2.85	6.50*±0.57
24 mg/kg	91.34*±9.81	37.51*±3.00	36.60*±3.14	9.39*±0.89
30 mg/kg	100.83*±12.05	41.02*±2.83	38.68*±3.38	9.52*±0.93

Values are mean ±S.D. (n=3)

* indicate probability level of significant difference at $p<0.05$. Replicate (n) = 3

Cadmium content in plant parts gradually increased by increasing cadmium concentrations in soil. The results related to the uptake of Cd in this study suggest that roots of *T. erecta* are efficient barriers to Cd translocation to the above ground plant parts. Roots were storing highest amount of Cd followed by stem, leaves and inflorescence respectively. Our results are in confirmation with that of Thamayanthi *et al*., (2013) [28] who also observed higher accumulation of Cd into the roots of *T. erecta* followed by stem and leaves respectively. Cd accumulation in stem and inactivation in root cells are probably related to its binding in cell walls, compartmentalization in vacuoles and complexation with metal binding proteins and peptides, especially phytochelatin and metallothioneins (Gupta and Goldsbrough, 1991) [15]. These processes are strategies employed by plants, at least in part, to face unavoidable stress conditions. Accumulation potential of *Tagetes erecta* L. was found very high at lower concentrations of Cd whereas at higher concentrations it decreases.

The growth parameters as affected by cadmium treatments are shown in Table 2., which shows that different concentrations of Cd did not adversely affect the growth of *T. erecta* in terms of root length, shoot length, fresh and dry weight of root and shoot and leaf area but some minute non-significant differences between treatment plants and control plants have been noticed. Although Cd at higher concentrations was found to have reduced level of all the morphological parameters studied. The similar findings were also noticed by Turgut *et al*., (2004) [4] where they found that increasing concentration posed a severe phytotoxicity as evidenced by stunted growth in *Helianthus annuus*.

Table 2: Effect of Cd accumulation on Morphological Parameters.

Parameter /Treatment	Root Length (cm)	Shoot Length (cm)	Fresh Weight Root (g)	Fresh weight Shoot(g)	Dry weight Root (g)	Dry Weight Shoot (g)	Leaf Area (cm^2)
Control	13.0*±0.78	31.0*±1.42	3.53*±0.62	11.30*±0.87	0.673*±0.05	2.050*±0.20	87.13*±3.48
6 mg/kg	12.9*±1.09	31.0*±1.03	3.41*±0.76	11.33*±1.21	0.650*±0.08	1.989*±0.16	87.25*±4.31
12 mg/kg	12.9*±0.91	30.8*±1.31	3.32*±0.59	11.00*±0.90	0.629*±0.03	1.970*±0.31	87.10*±3.39
18 mg/kg	12.7*±1.02	30.6*±0.89	3.19*±0.70	10.86*±0.57	0.610*±0.07	1.958*±0.09	86.98*±4.40
24 mg/kg	12.6*±0.62	30.3*±1.11	3.05*±0.40	10.75*±0.68	0.583*±0.01	1.924*±0.20	86.85*±3.51
30 mg/kg	12.6*±0.65	30.1*±1.08	2.85*±0.55	10.52*±0.92	0.540*±0.05	1.911*±0.27	86.79*±3.72

Values are mean ±S.D (n=5)

* indicate probability level of significant difference at $p<0.05$. Replicate (n) = 5

The reduction in the growth in *Tagetes erecta* could also be attributed to the suppression of the elongation growth rate of cells, because of an irreversible inhibition performed by Cd on the proton pump responsible for the process (Aidid and Okamote, 1993) [2]. The most common effect of Cd toxicity in plants is stunted growth, leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways (Arduini *et al*., 1996) [5].

Table 3. shows the results of changes in biochemical parameters in *T.* erecta due to Cd accumulation. Our results indicate that the exposition of *Tagetes ereta* to different concentrations of Cd results in an increase in sugar content at lower concentration whereas at higher concentrations their decrease was observed. Our results corroborate with the findings of Ahmad *et al*. (2006) [1] who found that an increase in soluble sugars at low concentrations of salt stress and decrease at higher concentrations in *Pisum sativum*. Same type of results were observed by John *et al*. (2008) [16] who found soluble sugar content increased at lower concentration whereas decreased at higher concentrations of Cd in *Lemna polyrrhiza* L.

The decrease in protein content as observed at increasing concentrations of Cd in *T. erecta* may be because of enhanced protein degradation process as a result of increased protease activity (Palma *et al*., 2002) [22] which is found to increase under stress conditions.

Table 3: Effect of Cd accumulation on Biochemical Parameters.

Parameter /Treatment	Total Protein (mg/g)	Free Amino Acids (mg/g)	Total Sugar (mg/g)	Reducing Sugar (mg/g)	Starch (mg/g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Control	7.99*±0.75	5.99*±0.34	6.67*±0.52	4.90*±0.45	3.06*±0.19	0.761*±0.02	0.207*±0.01	0.968*±0.03
6 mg/kg	6.42*±0.35	6.17*±0.19	9.71*±0.62	5.81*±0.25	7.00*±0.50	0.805*±0.03	0.243*±0.02	1.048*±0.05
12 mg/kg	5.12*±0.42	6.89*±0.27	11.30*±1.66	6.96*±0.40	8.91*±0.61	0.854*±0.05	0.285*±0.01	1.139*±0.06
18 mg/kg	4.31*±0.26	7.67*±0.30	14.25*±1.30	7.25*±0.51	10.87*±1.07	0.899*±0.04	0.287*±0.03	1.186*±0.07
24 mg/kg	3.58*±0.19	8.42*±0.22	13.31*±1.17	7.04*±0.36	9.98*±1.12	0.739*±0.04	0.207*±0.02	0.946*±0.06
30 mg/kg	3.34*+0.27	8.90*±0.17	7.58*±0.47	5.77*±0.21	5.24*±0.34	0.612*±0.03	0.176*±0.03	0.788*±0.06

Values are mean ±S.D (n=3)

* indicate probability level of significant difference at $p<0.05$. Replicate (n) = 3

It is also likely that these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content. Radha *et al*., (2006) [24] reported that protein contents decreased with an increase in cadmium supply to the varieties of sugarcane. Similar trend has been discussed by *Hydrilla verticillata***.** Our studies coincide with Costa and Spitz (1997) [11] who also reported a decrease in soluble protein content under heavy metal stress in *Lupinus albus.* The reduction in protein content in plants exposed to Cd^{2+} stress is believed due to cadmium bound with three families of peptides forming high molecular weight Cd^{+2} binding complexes such as (g- glutamic acid-cysteine)n- glycin [(g-glu-cys)n-Gly]; so the free peptides decreased and consequently protein synthesis inhibited (Winfried, 1995) [32].

Several types of pigments are present in plants such as chlorophylls, xanthophylls, carotenoids etc. Among these, chlorophylls are most abundant and important pigments in higher plants. These are responsible for photosynthesis as they capture light. In several cases, heavy metals are known to reduce the productivity by reducing the rate of photosynthesis. Our results are in accordance with that of John *et al*., 2008 [16], who found that lower concentrations of Cd marginally increased the chlorophyll (chl *a*, chl *b* and total chlorophyll) in *Lemna polyrrhiza*. At higher concentrations of Cd, Chlorophyll (chl *a*, chl *b* and total chlorophyll) started decreasing. The decline in the pigment composition in plants exposed to Cd^{2+} stress is believed due to inhibition of important enzymes, such as aminolevulinic acid dehydratase (ALA dehydratases) (Padmaja *et al*. 1990) [21].

In the present investigation Cd treated plants contained higher amounts of free amino acids as has been observed in different parts of plants under heavy metal stress (Alia and Saradhi. 1991) [3]. Narwal and Singh, (1993) [19] also observed an increase in amino acid content in Maize treated with Cd. Excess cellular concentrations of cadmium either inhibit the utilization of amino acids or promote protein hydrolysis, thus affecting the normal balance of cellular proteins (Tandon and Srivastava, 2004) [27].

4. Conclusion

The study suggests that due to Cd accumulation in *Tagetes erecta* L., there was a very less negative effect on its growth parameters. Biochemical parameters were affected upto certain extent, but the plant showed a very good capability to accumulate Cd. So *Tagetes erecta* L. can be effectively used for the phytoremediation of the soils contaminated with cadmium.

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