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POTENTIAL OF *Alternanthera bettzickiana* (REGEL) G. NICHOLSON FOR REMEDIATION OF CADMIUM-CONTAMINATED SOIL USING CITRIC ACID

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The *Alternanthera bettzickiana* (Regel) G. Nicholson belongs to *Amaranthaceae* family and has great potential to decontaminate cadmium-affected soils. Organic acids including citric acids play a vital role in cadmium uptake and its detoxification. Thus, an experiment was performed to investigate the role of *Alternanthera bettzickiana* in phytoremediation of soils contaminated with Cd in the presence or absence of citric acids. Soils treated with different level of Cd (0, 25, 50, 100 mg kg^{-1}) concentrations and citric acid (0, 250 and 500 µM) were used to grow plants. The results indicated that more Cd was accumulated in aerial parts of the plant as compared to underground parts. Biomass, photosynthetic pigment, and plant growth enhanced with the increasing level of Cd concentration in soil but it decreased at the highest Cd concentration in the growth medium. With the maximum applied dose of CA (500 µM), the plant height was significantly increased by 141, 107 and 90% at Cd level 25, 50 and 100 mg kg^{-1}, respectively. The activities of superoxide dismutase (SOD) and peroxidase (POD) enhanced with lower metal level (Cd 25, 50 mg kg^{-1}), while reduced at the maximum Cd concentration (100 mg kg^{-1}). With the maximum applied dose of (Cd 50+CA500), superoxide dismutase (SOD) in leaves and POD in leaves was significantly increased by 63 and 75%, respectively. It is concluded that *Alternanthera bettzickiana* has maximum ability to remediate moderately contaminated soils. Moreover, phytoremediation potential was further enhanced by the citric acid application. Enhancement of phytoremediation potential in *Alternanthera bettzickiana* was associated with citric acid-induced modulation in oxidative stress and antioxidant potential.
Keywords: *Alternanthera bettzickiana*, antioxidant enzymes, Cd toxicity, heavy metal, phytoremediation, pollution

INTRODUCTION

Heavy metal pollution is a serious environmental concern, as it poses a major threat to terrestrial plants, animals, and humans via the food chain. Rising problems of heavy metal in the edible parts of plants is a severe risk to plants, animals and the humans (Wang *et al*., 2017). Among various heavy metals, cadmium (Cd) has high mobility in the food chain and thus greater harmful impacts on human health. Plants uptake cadmium through roots followed by translocation to other parts of the plant (Liu *et al.*, 2010). In plants, greater accumulation of Cd reduces plant growth and yield by affecting different biochemical and morpho-physiological processes which may include inhibition of photosynthesis, reduction in water and nutrient uptake, enhancement in reactive oxygen species (ROS) and lipid peroxidation that leads to reduction in plant growth and yield (Wang *et al.*, 2009). However, the effects of Cd toxicity on plants vary with the type of species. Some plants species can accumulate more Cd from the soil with slight adverse effects. Such plant species can be used to remediate the cadmium-polluted soils. The use of indigenous plants is one of the best methods for phytoremediation of soils, contaminated with heavy metals, on account of their wider applicability, effectiveness and sustainable benefits (Kamran *et al*., 2014). Moreover, the local species has better adaptation to the local ecosystem conditions thus making them a first priority compared to exotic plant species (Kamran *et al*., 2014; Habiba *et al*., 2015).

In the current study, *A. bettzickiana* native to South America was chosen and this belongs to class Alternanthera (Amaranthaceae). The leaves are reddish or green. The leaves and shoots of this plant are similar to vegetables like spinach which is cooked by mixing with other vegetables, in soups or alone as single vegetable that is served as staple food like others (Quattrocchi *et al.*, 2012). Since *A. bettzickiana* can grow on Cd-contaminated soils, it might have a certain physiological mechanism to tolerate excessive Cd. In the present study, the physiological bases of Cd tolerance were investigated in *A. bettzickiana*. Moreover, its potential to remediate Cd contaminated soils was explored.

It is widely accepted that organic acids e.g. citric acid (CA), augment the uptake of heavy metals in plants (Turgut *et al.*, 2004). Desorption of metals from soil to its solution and their uptake by plants is achieved by using various chelating agents like citric acid (CA) which is frequently used for this purpose (Afshan *et al*., 2015; Sinhal *et al*., 2010; Pessel *et al*., 2001). Moreover, it has been found that citric acid is helpful for phytoextraction and mobilization of cadmium (Sinhal *et al.*, 2010). The use of CA might diminish the environmental problems. In this context, the present study was designed to assess the extent to which citric acid helped in enhancing phytoremediation potential of *A. bettzickiana* plants. Plant growth and biomass, Chlorophyll contents, activities of antioxidant enzymes, reactive oxygen species and Cd concentration were also studied under Cd stress with and without CA application.

MATERIALS AND METHODS

***Soil sample collection and analysis*:** The soil used in this study was collected from the GC University Faisalabad (GCUF). The collected soil was ground with a wooden roller, sieved through 2 mm sieve and thoroughly mixed. Standard procedures were followed for the determination of soil characteristics such as particle size by sodium adsorption (Bouyoucos, 1962). Mainly the methods depicted in US Salinity Laboratory Staff (1954) or Page *et al*. (1982) were employed unless mentioned otherwise. Electrical conductivity (EC) and pH was determined by using EC meter and pH meter after calibration. Trace elements were determined using methods of Soltanpour (1985) and Amacher (1996). Organic carbon by Walkley-Black method (Jackson, 1962) and calcium carbonate according to Moodie *et al*. (1959). The soil characteristics are presented in Table 1.

***Pot experiment*:** This pot study was conducted at GCU Faisalabad in the ambient environment. The experimental design was CRD (complete randomized design) with three replicates. The appropriate Cd levels (0, 25, 50 and 100 mg kg^{-1}) were mixed with soil. All plastic pots were filled with soil, each carrying 3 kg of soil. Then the soil in each pot was saturated from top to bottom with tap water. The preferred moisture level was maintained at 70% for two weeks. Four seedlings of *Alternanthera bettzickiana* of similar size were grown in one pot. CA (0, 250 and 500 µM) was applied four times with the seven days of interval. All the treatments had three replicates. After 15 days, the required amount of NPK fertilizers were applied in the form of urea, DAP and K_2SO_4 (applied as 2.19 g, 1.36 g and 2.40 g per pot, respectively). Plants were treated with citric acid solution regularly after seven days of interval. In the case of controlled plants, they were irrigated with tap water as and when required.

***Harvesting*:** The plants were harvested after 3 months of vegetation period. At harvesting, stem, leaves, and roots were separated, washed with tap water. To remove any deposition these samples were washed with 2% HCl solution and finally with distilled water. Roots and leaves were dried in an oven at 70°C for 48 hours to get constant root and leaf dry weight.

***Chlorophyll contents*:** The method by Lichtenthaler (1987) was used to determine chlorophyll contents. The leaves were taken after eight weeks of Cd stress and measurements were done spectrophotometrically. The following pigments *chlorophyll a, chlorophyll b* and carotenoids were then calculated as mg/g of fresh weight.

***Electrolyte leakage*:** The electrolyte leakage was determined with EC meter. The EL was calculated by using the formula (Dionisio-Sese and Tobita, 1998).

$$EL = (EC_1/EC_2) \times 100$$

***Determination of antioxidant enzymes*:** SOD (superoxide dismutase) and POD (peroxidase) in roots and leaves were also determined by using Spectrophotometer and method described by (Zhang, 2009).

***Cadmium (Cd) concentration*:** The method described in Rehman *et al*. (2015) was employed for the determination of Cd in dry samples using acid digestion following by reading on atomic absorption spectrophotometer.

***Statistical analyses*:** The data were then subjected to statistical analyses using one-way ANOVA at a probability level of 5.0% (SPSS Statistics, Version 21.0. IBM Corp.). The comparison of means was made by Tukey's HSD post hoc test.

Table 1. Soil physico-chemical properties.

Texture	Clay Loam
Sand (%)	21.00
Silt (%)	16.00
Clay (%)	63.00
EC_e (dS m^{-1})	2.79
pH (1: 2.5 soil to water ratio)	7.86
Organic matter (%)	0.54
SAR ($mmol_c^{-1}$) 1/2	6.27
HCO_3^- (mmol L^{-1})	3.33
Available P (mg kg^{-1})	2.21
SO_4^{-2} (mmol L^{-1})	6.38
Cl^- (mmol L^{-1})	2.29
K^+ (mmol L^{-1})	0.04
Na^{2+} (mmol L^{-1})	3.52
Ca^{+2} + Mg^{+2} (mmol L^{-1})	3.61
Available Zn^{+2} (mg kg^{-1})	0.76
Available Cu^{+2} (mg kg^{-1})	0.21
Available Cd^{+2} (mg kg^{-1})	0.43

RESULTS

***Morpho-physiological parameters*:** In order to see the effect of CA on *A. bettzickiana* under Cd stress, growth parameters were measured. The leaf area, plant height, root dry weight, and shoot dry weight were measured as given in Figure 1. All the growth parameters significantly increased ($P<0.05$) with increasing concentration of CA. At the maximum level of CA (500 µM), the plant height increased by 52, 141, 107, 90% and leaf area increased by 28, 79, 67, 54% at Cd levels 0, 25,

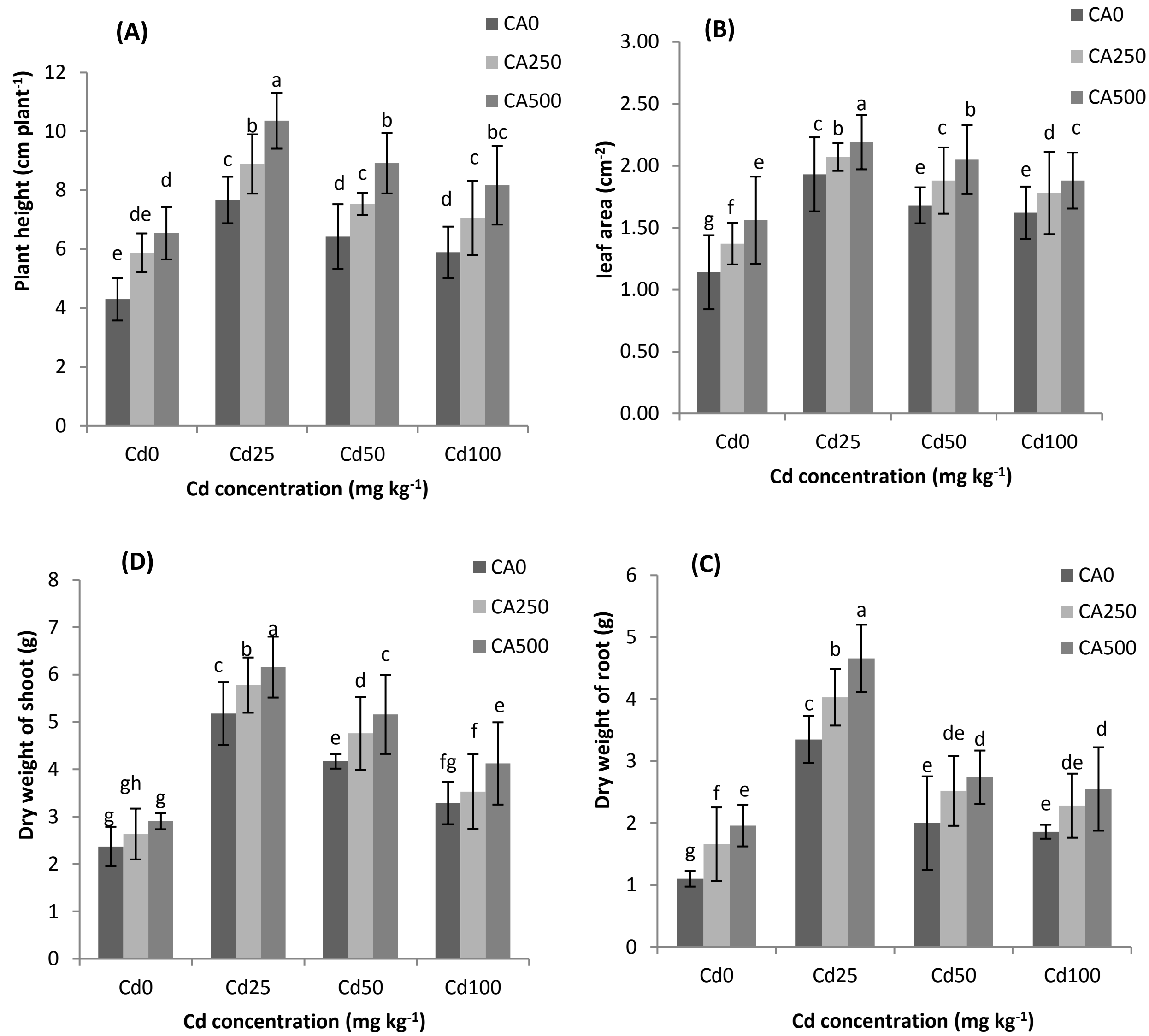

Figure 1. Effect of different levels of Cd and citric acid on the growth of seedlings of *A. bettzickiana* (Regel) G. Nicholson grown in soil: (A) plant height, (B) leaf area, (C) dry weight of root and (D) dry weight of shoot. The values are means of 3 replications with standard deviations. The significance is determined at $P<0.05$ and denoted by different letters.

50, 100 mg kg^{-1}, respectively. Furthermore, root dry weight increased by 78, 323, 149, 132% and shoot dry weight increased by 63, 247, 190, 132% at Cd levels 0, 25, 50, 100 mg kg^{-1}, respectively at CA level 500 µM (Fig. 1).

***Chlorophyll contents and carotenoids*:** The *Chlorophyll a*, *chlorophyll b* and carotenoids of the *A. bettzickiana* under Cd stress and various levels of CA are shown in Figure 2. All the chlorophyll contents significantly increased with increasing concentration of CA. At maximum level of CA (500 µM), *Chl a* increased by 26, 90, 50, 44% and *chl b* increased by 31, 80, 51, 42% at Cd levels 0, 25, 50, 100 mg kg^{-1}, respectively. Furthermore, carotenoids increased by 60, 19, 6, 28% at Cd levels 0, 25, 50, 100 mg kg^{-1}, respectively at CA level 500 µM (Fig. 2).

***Antioxidant enzymes and reactive oxygen species*:** The activities of the superoxide dismutase and peroxidase and electrolyte leakage from roots were measured and are shown in Figure 3. All the antioxidant enzymes significantly increased with increasing concentration of CA. At the maximum level of CA (500 µM), SOD in root increased by 38, 77, 107, 48% at Cd levels 0, 25, 50, 100 mg kg^{-1}. Furthermore, POD in root increased by 42, 68, 86, 69% at Cd levels 0, 25, 50, 100 mg kg^{-1}, respectively at CA level 500 µM (Fig. 3). The EL was significantly decreased ($P<0.05$) with increasing concentration of CA. At the maximum level of CA (500 µM), EL in root decreased by 7, 6, 10 and 5% at Cd levels 0, 25, 50, 100 mg kg^{-1}.

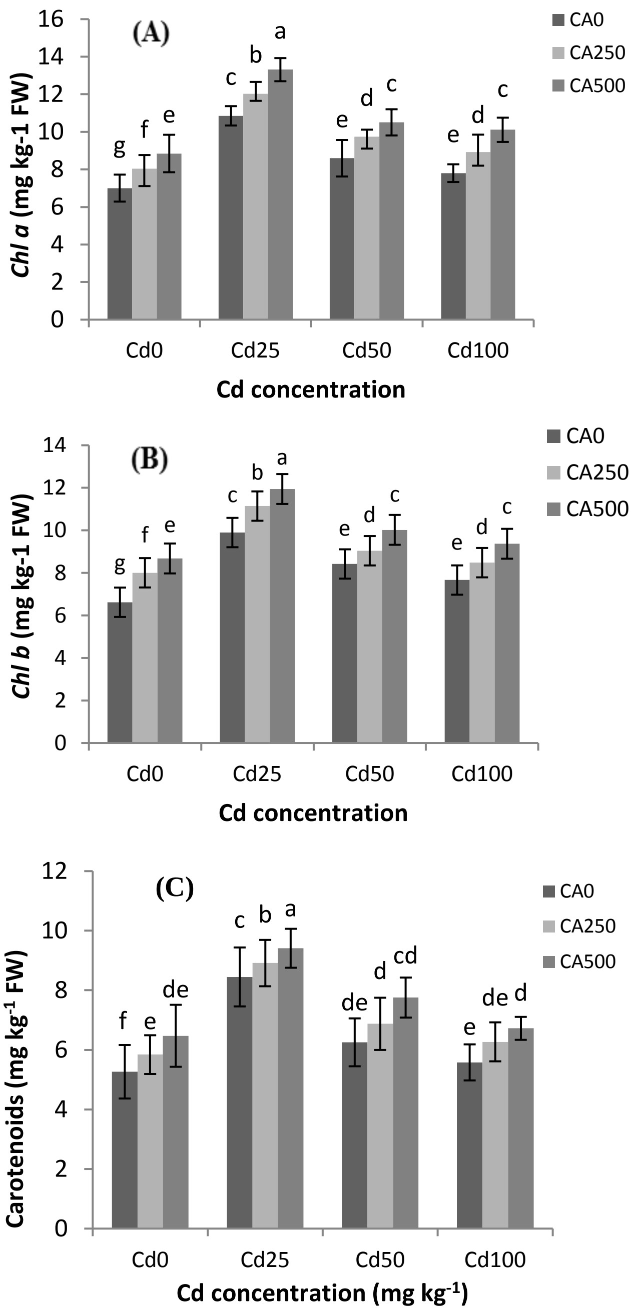

Figure 2. Effect of different levels of Cd and citric acid on plant pigments of *A. bettzickiana* (Regel) G. Nicholson seedlings grown in soil: (A) *chlorophyll a*, (B) *chloropyhll b* and (C) carotenoids. The values are means of 3 replications with standard deviations. The significance is determined at *P<0.05* and denoted by different letters.

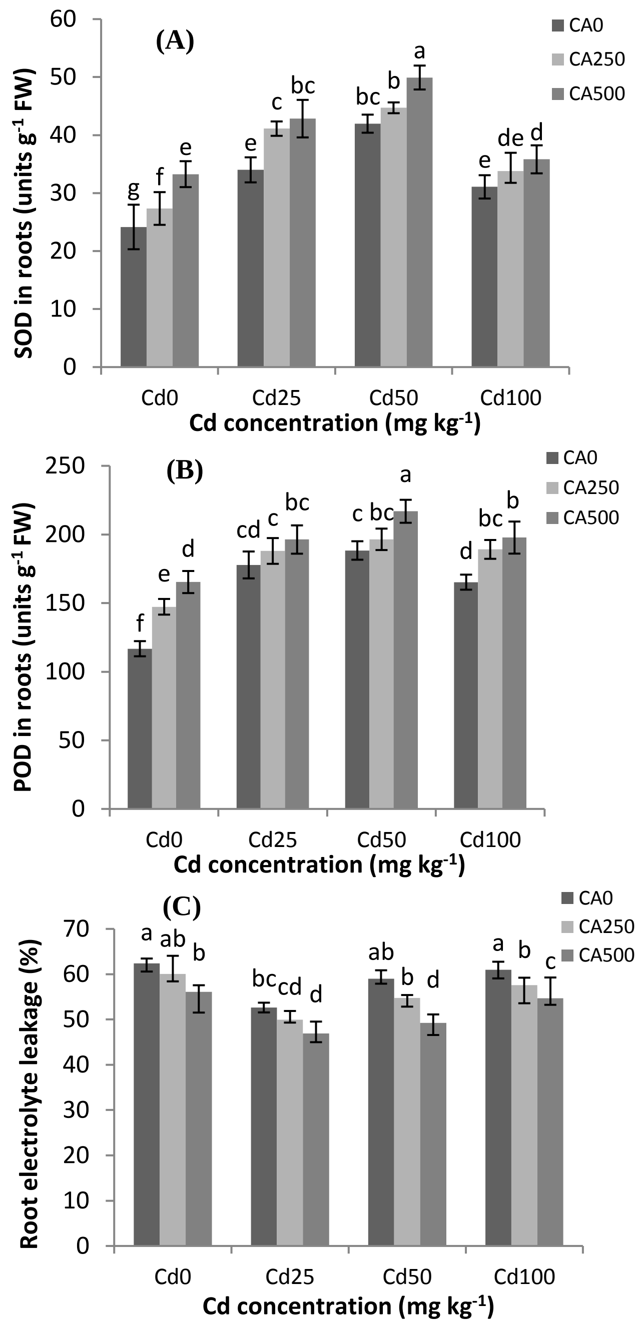

Figure 3. Effect of different levels of Cd and citric acid on plant biochemical parameters of seedlings of *A. bettzickiana* (Regel) G. Nicholson grown in soil: (A) SOD root, (B) POD root and (C) root electrolyte leakage. The values are means of 3 replications with standard deviations. The significance is determined at *P<0.05* and denoted by different letters.

Cd concentration: The Cd concentrations (shoots, roots) were significantly increased (*P<0.05*) with increasing concentration of CA. At the maximum level of CA (500 µM), Cd concentration in shoot increased by 17, 25 and 41% at Cd concentration of 100, 50 and 25 mg kg^{-1}, respectively when compared to control. On the hand, the maximum level of CA (500 µM), Cd concentration in root were increased by 15, 17 and 17% at Cd concentration of 100, 50 and 25 mg kg^{-1}, respectively when compared to control (Fig. 4).

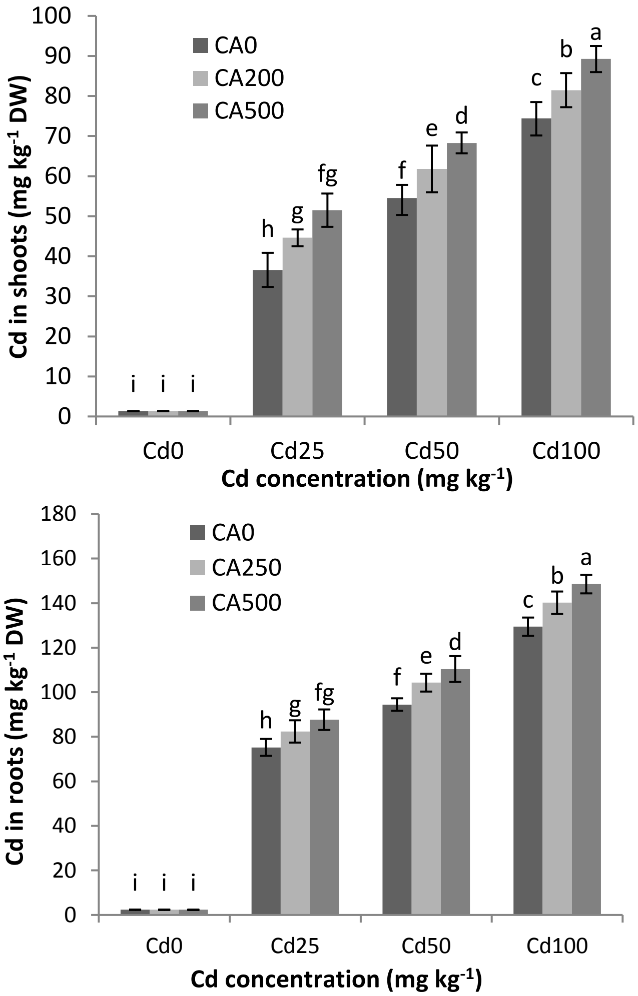

Figure 4. Effect of different levels of Cd and citric acid on Cd concentrations in (A) shoot and (B) root *A. bettzickiana* (Regel) G. Nicholson seedlings grown in soil. The values are means of 3 replications with standard deviations. The significance is determined at $P<0.05$ and denoted by different letters.

DISCUSSION

***Plant growth*:** The growth of *A. bettzickiana* decreased considerably due to increasing Cd concentration (Fig. 1). Reduction in growth due to excessive Cd in the growth medium might have been due to alteration in metabolism associated with photosynthesis. Application of citric acid substantially recovers growth, biomass of the plant grown under Cd stress. With the maximum level of citric acid (Cd25+CA500) dry weight of root and dry weight of shoot increased significantly by 323 and 247%, respectively. The plant parameters were elevated by the addition of CA 250 µM as compared to Cd treatment alone. The application of citric acid has previously been shown to improve plant growth and biomass under metal toxicity (Najeeb *et al.,* 2009). The accomplishment of phyto-extraction can be decided by the percentage of metals in roots, metal uptake and biomass of plant (McGrath, 1998). Jabeen *et al*. (2016) and Najeeb *et al*. (2011) attributed the increased plant biomass to increased plant uptake of mineral nutrients. On the other hand, Sun *et al*. (2007) and Khan *et al*. (2015) termed this increase to the efficient uptake and assimilation of sulfate. Muhammad *et al.* (2009) related this increase in growth and plant uptake of metal to the synthesis of phytochelatin while Ghani (2011) described this as the capability of the plant to detoxify Cd. Present growth medium is similar to some of earlier studies (Gill *et al.*, 2011).

***Photosynthetic pigments*:** Reduction in growth due to excessive Cd in the growth medium might have been due to alteration in metabolism associated with photosynthesis. Photosynthetic pigments harness the solar energy and help in the conversion of solar energy into biochemical energy in thylakoid reaction. However, Cd toxicity disrupts the biosynthesis of chlorophyll. In the present study, *chl a*, *chl b* and carotenoids of plants have been decreased by an increase in the concentration of Cd (Fig. 2). It was previously reported that Cd prevents photosynthetic pigment (Meng *et al.,* 2009). The reduction in carotenoids and chlorophyll ultimately affected the photosynthetic parameters which directly or indirectly resulted in plant growth reduction. Citric acids significantly enhanced photosynthetic parameters of a plant grown under Cd stress (Fig. 2). This decrease might be due to deformation of the ultrastructure of chloroplast which resulted in inflated thylakoids and an abnormal shape (Mishra *et al.,* 2006; Najeeb *et al.,* 2011; Park *et al*., 2012).

***Reactive oxygen species and antioxidant enzymes*:** In this experiment, oxidative stress generated by Cd highlighted the boosting in electrolyte leakage and antioxidant enzymes SOD, POD (Fig. 3). In this study, it was revealed that activity of antioxidant enzymes reduced at 250 µM of CA but enhanced significantly at 500 µM of CA. POD in root and shoot increased at CA level (Cd50+CA500) significantly 75 and 86%, respectively. It showed that overexpression of antioxidant enzymes acts as a protective tool of plants for its life and functions with excess metal uptake capacity (Muhammad *et al.*, 2009). It was suggested that the decline of antioxidant activity under high metal stress might be due to excess severity of ROS (Mishra *et al.,* 2006). Application of CA highly boosted up the activities of antioxidant enzymes in plant parts as that of Cd treatment alone. It has been concluded in various researches that application of CA decreases oxidative stress and increase the activity of antioxidant enzymes (Najeeb *et al.*, 2009; Najeeb *et al.*, 2011; Meng *et al.*, 2009). It was found that the application of citric acid disrupts electrolyte leakage and oxidative stress by boosting up the antioxidant enzymes activities. At the maximum level of CA500 electrolyte leakage was decreased by -6% significantly

***Metal uptake*:** The concentration of Cd and its uptake by *A. Bettzickaiana* was increased in plant parts as levels of Cd increased (Fig. 4). Interestingly, the application of CA further increased the Cd uptake. Lu *et al*. (2013) found an increase in Cd concentrations with the use of citric acid in *Sedum alfredii* plants while similar results were found by Najeeb *et al.* (2009, 2011) in *J. effuses* plants. However, this result was opposite in the maize plant (Anwer *et al.*, 2012). Increase in Cd uptake might be due to ATPase in roots plasma member due to the application of CA. These results are similar to findings of (Najeeb *et al.*, 2009; Park *et al.,* 2012).

***Conclusion*:** Based on these results, it is concluded that the plant, *A. Bettzickaiana*, has potential of remediating Cd-contaminated soils. Moreover, the application of to the *A. Bettzickaiana* plants growing on Cd-contaminated soils further increased its remediating potential by improving plant growth parameters and antioxidant enzyme activities. Further studies are suggested where other metals or mixed metal concentrations may be used to check its adaptability under various ecological conditions.

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