

Changes in growth and antioxidant enzymes activities during cadmium stress in the mangrove plant *Kandelia candel* (L.) Druce

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Abstract. Changes in growth and antioxidant enzymes activities during cadmium (Cd) stress in *Kandelia candel* (L.) Druce seedlings were investigated. The seedlings were grown in green house condition for three months in nutrient solution with 0, 1, 5, 10, 15, 20, 25 ppm CdCl₂. Growth parameters, guaiacol peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT), activities were analyzed in leaves and roots collected after three month exposure. This study demonstrates that under high concentration of Cd stress, the total biomass, the average seedlings stem height and leaf number of the *K. candel* decreased by 41.57%, 30.54% and 42.68%, respectively compare to control (CK). The activity of antioxidant enzymes POD, SOD and CAT in leaves were significant increase, this was noticed especially at the lower concentration. In root, this effect was more evident and they responded to low Cd stimulation more rapidly. However at the highest concentration of Cd exposure, the activity of POD, SOD and CAT were decreased both in leaf and root. The reductions in the activities of antioxidative enzymes in *K. candel* at the high concentration of Cd treatments indicate that Cd tolerance characteristics of this plant cannot be attributed to antioxidative defense. This result indicated that the higher concentration of Cd might affect the antioxidant enzyme system of *K. candel*. That may be inhibited the plant growth and biomass.

Key words: cadmium stress, growth, antioxidative enzymes, *Kandelia candel*.

Introduction. Cadmium (Cd) is an inhibiting toxin for living organisms, including mangrove wetland species, which concentrate heavy metals in their tissues, becoming highly contaminated in the process (MacFarlane et al 2007; Rahman et al 2009). Mangrove ecosystems can act as sinks for heavy metals, which can become pollution sources to plants (Yim & Tam 1999). Some mangrove plants appear to possess a great tolerance to high levels of heavy metal pollution (Peters et al 1997), but in excessive heavy metal contamination, mangrove plants may initiate a variety of subcellular responses, i.e. metabolic reactions, which can cause damage at the cellular level or lead to wider phytotoxic responses (Vangronsveld & Clijsters 1994; Zhang et al 2007). In plants, exposure to Cd causes inhibition of growth and even plant death owing to its influence on photosynthesis, respiration, water and nutrient uptake (Baszynski et al 1980; Sanità di Toppi & Gabbriellini 1999). Toxic levels of heavy metal affect a variety of processes in plants (Maksymiec 1997; Siedlecka et al 2001). One of the major consequences is the enhanced production of reactive oxygen species (ROS), which damage cell membranes, nucleic acids and chloroplast pigments (Fang & Kao 2000; Tewari et al 2002; Hana et al 2008). Mangrove plant tissues equipped with antioxidant systems as a mechanism to avoid oxidative stress. This system is composed of a series of enzymes and reductants such as SOD, POD, CAT and glutathione reductase (Xiang & Oliver 1998; Srivastava et al 2004; Zhang et al 2007). Superoxide dismutase is the most important of them. SOD can eliminate oxygen free radical and prevent membrane lipid from being peroxidated, peroxidase (POD) and catalase (CAT) could erase the excessive

H₂O₂ and oxygen free radicals. CAT dismutates H₂O₂ into H₂O and O₂, which is found in peroxisomes, cytosol and mitochondria (McKersie & Leshem 1994). POD decomposes H₂O₂ by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Blikhina et al 2003). Antioxidant enzymes such as SOD, POD and CAT play a vital role in scavenging reactive oxygen species (ROS) produced under oxidative stress thereby protects potential cell injury against tissue dysfunction.

The Cd presence cause oxidative damage to the biomolecules such as lipids, proteins and nucleic acids (Kanazawa et al 2000). Very little information is available on mangrove plant growth, physiological and biochemical changes under Cd metal stress. Actually, mangrove plants are growing in a complex environment including heavy metal pollution. It is necessary to study mangrove plants and heavy metal effects for the purpose of improving mangrove ecosystem. This study investigated the Cd effects on changes in growth and SOD, CAT, and POD antioxidant enzymes activities in leaves and roots of *K. candel*.

Materials and Methods. The changes of growth and antioxidant enzymes activity were determined in mangrove seedlings of *Kandelia candel* (L.) exposed to 7 concentrations of Cd. The experiment had five replicates for each treatment, with a completely randomized block design.

The propagules of *K. candel* were collected from plants grown at the Jiulongjiang mangrove forest stand (24°24' N, 117°23' E), Xiamen, Fujian, China. The region is subtropical with most of the annual rainfall (1284 mm) derived from summer typhoons. The average annual temperature range of estuarine waters is from 14.8 to 27.8 °C, with salinities adjacent to the mangroves ranging from 12 to 26 psu. After removal of the bracts, only complete, undamaged propagules with testa intact and no emergent hypocotyls or radicles were selected for planting. Propagules chosen for germination were those collected in the most abundant weight class, 18.0-19.55 g fresh weights. Propagules were planted in plastic pots containing filled with washed sand. Three plastic pots were placed inside a plastic container (30 cm long × 40 cm wide × 30 cm high). Four propagules were randomly planted in a plastic pot for germination and growth. All the pots were placed in the green house. The propagules were kept in a greenhouse under natural lighting with a temperature of 28 ± 5 °C. A quantity of two liters tap water was irrigated to each pot 2 times each week. The water level of each container was adjusted daily with tap water (free-NaCl) to compensate for the amount of water lost by evaporation. Propagules started to germinate within one month and growth continued therefore. After three weeks, the young seedlings were adapted to Hoagland's nutrient solution (Hoagland & Arnon 1950). The solutions were changed every seven day to prevent depletion of metals, nutrients and oxygen.

Two-months old *K. candel* seedlings were put into individual plastic containers holding 1000 ml of Hoagland's solution prepared with the addition of Cd (as CdCl₂) treatment in seven levels: 0, 1, 5, 10, 15, 20, and 25 ppm. The concentrations of metal ion were maintained by adding tap water up to the mark in the plastic container in order to correct the evaporation loss. Plants were exposed to Cd for 12 weeks under greenhouse conditions. Control (CK) plants were irrigated with 1000 ml of Hoagland's solution without CdCl₂. In three months cultivation under Cd stress, the symptoms of heavy metal toxicity appeared in the seedling.

During the period of treating heavy metal of Cd, the growth parameters of mangrove plants in each pot were as monitored monthly by counting the leaf number and measuring the stem height, and length of the largest leaf in each individual plant (Chen et al 1995). Plants were divided into roots, hypocotyls, stem and leaves and dry mass was determined on plant organs oven-dried at 80 °C to constant mass. Biomass data were corrected for fresh leaves and roots sampled for biochemical analysis. To measure the effect of Cd stress on antioxidative enzymes (SOD, POD and CAT activities) in leaves and roots of mangrove plant seedlings, leaves and roots were harvested after exposing to the heavy metals. At the end of three months cultivation of seedlings, the plants were harvested and washed thoroughly with tap water and then with deionized water. Then leaves and roots were separated from the plant for the extraction of enzymes. 0.5 g

freshly collected leaves and roots were weighed, and homogenized in a chilled mortar and pestle in 5 ml 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 4% polyvinylpyrrolidone (PVP) and SiO₂ to neutralize the interference effects of phenol in mangrove plant tissues. The homogenate was centrifuged at 19000 × g for 20 minutes at 4 °C and the supernatant was used for the enzymatic assays.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to the photo-chemical nitroblue tetrazolium (NBT) method (Beyer & Fridovich 1987). The assay was based on the ability of SOD to inhibit reduction of NBT to formazan by superoxide. The 3 ml assay mixture containing 50 mM phosphate buffer (pH 7.8), 130 mM methionine, 0.75 mM NBT, 0.1 mM EDTA-Na₂, 0.02 mM riboflavin, and 50 µL enzyme extract was placed under cool fluorescent light at light intensity 175 µmol s⁻¹ m⁻² for 20 min. In addition, riboflavin was added as the last component. Another two tubes without enzyme extract were used as the controls, one incubated in dark and the other control tube was placed under light, the same as the sample tubes. With the dark control tube as the blank, the absorbance at 560 nm was measured by a UV-vis spectrophotometer (Model UV-1206, Shimadzu, Japan). The increase in absorbance of the light control (without the enzyme extract) was taken as 100% and the enzyme activity of the sample was calculated by determining the percentage inhibition per minute and 50% of inhibition was taken as equivalent to 1 unit of SOD activity. The SOD activity in leaf and root was then normalized to total protein content of the respective tissue.

For the measurement of peroxidase (POD; donor H₂O₂ – oxidoreductase, EC 1.11.1.7) activity, 2.9 ml of 0.05 mol/L phosphate buffer + 1.0ml (0.05mol/L) guaiacol, pH 7.8, was mixed with 0.1 ml of enzyme extract and allowed to stand at room temperature for 3 minutes. One microliter (1.0 ml) of 2% hydrogen peroxide was added to activate the reaction. The absorbance at 470 nm was measured at every 30s intervals for 5 min, and an increase of 0.01 absorbance units per minute was equated to one unit of peroxidase activity. The activities of SOD and POD were expressed as units per mg of protein (Umg⁻¹ protein). Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Catalase (CAT, H₂O₂: H₂O₂ oxidoreductase, EC 1.11.1.6) activity was measured adapting the method of Beer & Sizer (1952), Zhang et al (2007), with minor modifications. The reaction mixture (1.5 ml) consisted of 100 mmol/l phosphate buffer (pH 7.0), 0.1 mmol/l EDTA, 20 mmol/l H₂O₂ and 50 µl enzyme extract. The reaction was started by addition of the extract. The decrease of H₂O₂ was monitored at 240 nm and quantified by its molar extinction coefficient (36 mol/l cm) and the results were expressed as CAT units per min and mg of protein.

Data analysis was accomplished using two statistical programs, namely, Microsoft Excel 2003 package and SPSS 13.0, Chicago, IL, USA. A one way analysis of variance (ANOVA) followed by Duncan's post-hoc test was employed to examine any statistical differences between different treatments of Cd stress in terms of growth and antioxidative enzymes (SOD, POD and CAT) activities. The differences were considered statistically significant when *P*- value was less than 0.05.

Results and Discussion

Changes in growth and biomass during cadmium stress in *K. candel* (L.) Druce.

The stress effect of Cd on *K. candel* growth was assessed by stem height and leaf number. Cd treatments inhibited stem elongation, the difference between CK and Cd treatment was significant (Table 1). High heavy metal concentrations of 25 ppm caused significant reduction in stem height and leaf number of plants, indicating metal toxicity on the growth of *K. candel*. It was observed that old leaves in the lower position of the plant started to turn yellow and shed off while the young leaves still survived in these metal-treated plants. At the end of the experiment, the stem of plants receiving strong heavy metal stress, from the top to the lower parts, were found to become deep brown in color. As shown in Table 1, no significant difference in leaf number and plant height was found between 1 ppm Cd treatment and control. However, increasing Cd concentration (5~25 ppm) in the medium induced a significant decline (*P* < 0.05). The deleterious

effect of Cd became more severe with increasing Cd level and extended time of exposure. For example, at the 90 d of 25 ppm Cd exposure, the average plant stem height, and leaf number of the *K. candel* decreased by 30.54% and 42.87%, respectively, while, the leaf area per plant was significantly smaller at higher Cd concentration (data not shown) indicating that irreversible damage to tissue formation was induced under higher Cd level. Wong et al 1988 reported that when plants absorbed and accumulated heavy metals, the vessels became constricted and pits had some unknown materials deposited which blocked the vascular system and retarded the water transportation. The effect of Cd treatments on root was obvious. The development of root system worsened with less root hair. On the 90th day the poisoned root system turned to black-brown. Zheng et al 1994 also found the similar result in their study regarding the influence of Cd on the germination, growth of *K. candel* seedlings cultivated in sand and in red loamy soil.

Table 1

Growth parameter changes under Cd supply after of 90 days cultivation

Treatment (ppm)	Stem height (cm)	Leaf number (n)	Total biomass DW (g/pot)
CK	20.06 ± 0.66(a)	8.13 ± 0.70(a)	17.63 ± 2.61
1.0	19.13 ± 0.58(ab)	7.93 ± 0.50(ab)	16.53 ± 2.47
5.0	18.00 ± 0.62(abc)	7.50 ± 0.50(ab)	15.40 ± 1.33
10	16.80 ± 0.26(abcd)	7.06 ± 0.60(abc)	14.00 ± 1.07
15	16.20 ± 0.29(bcd)	6.23 ± 0.56(bcd)	12.90 ± 1.75
15	14.90 ± 0.90(cd)	5.56 ± 0.50(cd)	11.30 ± 1.86
25	13.93 ± 0.60(d)	4.66 ± 1.19(d)	10.03 ± 1.02

Note: Mean values in the same column with different letters are significantly different at $P < 0.05$ level. The values were the means of 5 replicates.

Plant biomass of root, hypocotyls, stems and leaves decreased with increasing Cd level in the nutrient solution for *K. candel* seedlings after 90 d Cd exposure. For example, at the highest Cd concentration (25 ppm), the root, hypocotyls, stem, leaf dry weight was decreased by 62.17%, 31.58%, 50%, 56.31% compared to the control (Table 2). The stem biomass decreased with the increasing of Cd concentration in the nutrient solutions. The reduction in leaf biomass due to Cd treatment was more obvious than that in stem biomass (Table 2), suggesting that growth parameters related to leaves were more sensitive than those associated with stems. The differences between fresh and Cd on growth inhibition were significant in the present study. The Cd stress resulted in a significant decrease in plant biomass. As a consequence, the final dry matter values of roots ranged from 4.23 to 1.6 g/pot and that of leaves ranged from 2.06 to 0.90 g/pot harvested on Cd treated plants. The presence of excessive amount of Cd in environment causes many toxic symptoms in plants, such as reduction of growth, especially root growth (Weigel & Jäger 1980), disturbances in mineral nutrition and carbohydrate metabolism (Moya et al 1993), and may therefore strongly reduce biomass production. The reduction of biomass by Cd toxicity could be the direct consequence of the inhibition of chlorophyll synthesis (Padmaja et al 1990) and photosynthesis (Dong et al 2005). Some studies reported a marked reduction in photosynthetic rate for different plant species under exposure to Cd stress (Dong et al 2005). The Cd may interfere with nutrient uptake by affecting the permeability of plasma membranes. Abdel-Sabou et al (1988) showed an antagonistic effect of Cd on Zn uptake. According to Jalil et al (1994), Cd addition decreased the concentration of some nutrition like K, Zn and Mn in wheat root and shoot. Zhang et al (2002) reported that Cd inhibits P, K and Mn translocation to shoots, leading to more retention in roots. The water content of leaves decreased by

about 10% compared to control after 90 days cultivation in 25 ppm of Cd stress. Prasad (1995) found the similar result in Cd stress on bean plants.

Table 2

Biomass (DW) of different parts of *K. candel* seedlings under Cd supply after of 90 days cultivation

Treatment (ppm)	Root	Hypocotyls	Stem	Leaf
CK	4.23±0.30	10.13±1.80	1.2±0.10	2.06±0.10
1	3.96±0.55	9.53±1.95	1.13±0.11	1.9±0.20
5	3.53±0.23	9.06±0.66	1.06±0.25	1.73±0.15
10	3.06±0.152	8.5±0.51	0.93±0.11	1.5±0.10
15	2.8±0.20	8.0±0.87	0.8±0.11	1.3±0.10
20	2±0.26	7.53±1.31	0.7±0.10	1.06±0.35
25	1.6±0.52	6.93±0.45	0.6±0.20	0.9±0.40

Effect of cadmium stress on antioxidant enzymes activities in *K. candel*. The Cd effects on SOD enzyme activities were assayed after three months of exposure. SOD activity in leaves increase in the treatment with 10 ppm Cd concentration but sharply decreased at treatment with high Cd concentrations in 25 ppm but there was no significant difference between highest Cd concentration and control samples (Fig. 1a). SOD activity in roots of *K. candel* peaked at 10 ppm while at the highest Cd concentration roots showed slightly lower enzyme activity values. There was significant difference between highest Cd concentration and control samples (Fig. 1b). Therefore roots seem to respond more rapidly to Cd to enhance antioxidant enzymes reaction to metal concentrations. SOD can remove free radical O_2^- , decrease peroxidation of membrane lipids, and maintain cell membrane stability (Zhang et al 2007). SOD is one of the ubiquitous enzymes in plants that play a key role in cellular defense mechanisms against reactive oxygen species (ROS). Its activity modulates the relative amounts of free O_2^- and H_2O_2 , the two Haber-Weiss reaction substrates, and decreases the risk of OH radical formation, which is highly reactive and may cause severe damage to membranes, protein and DNA (Peng 1999; Zhang et al 2007). Increase in SOD activity was observed in leaves and roots at 10 ppm (Figs 1a, b), but at the highest concentration of Cd treatment SOD activity in roots and leaves both were decreased rapidly. The decline in SOD activity as from treatment 15 to 25 ppm indicated that the oxygen scavenging function of SOD was damaged. SOD activity in roots of *K. candel* peaked at higher metal concentrations and the response of *K. candel* was strong, suggesting that this increase in SOD has better protection against oxidant damage (Bowler et al 1992; Takemura et al 2000). On the other hand, at the high concentration of Cd treatment, SOD could not protect the oxidant damage.

The Cd effects on POD enzyme activities were examined, Cd stress resulted in POD activity increase in leaves and roots (Figs 2a, b). The response of POD activity to Cd stress varied with expose of different Cd level. At 10 ppm Cd, POD activity in both leaves and roots of *K. candel* were much higher than the control. At 10 ppm, the POD activity in roots and leaves of *K. candel* reached 1395.95, and 1215.01 U mg^{-1} protein respectively. POD activities in *K. candel* were quite high in both leaves and roots when exposed to heavy metal stress. For this reason *K. candel* eliminates H_2O_2 produced by peroxidation of membrane lipids more efficiently at the treatment of 10 ppm.

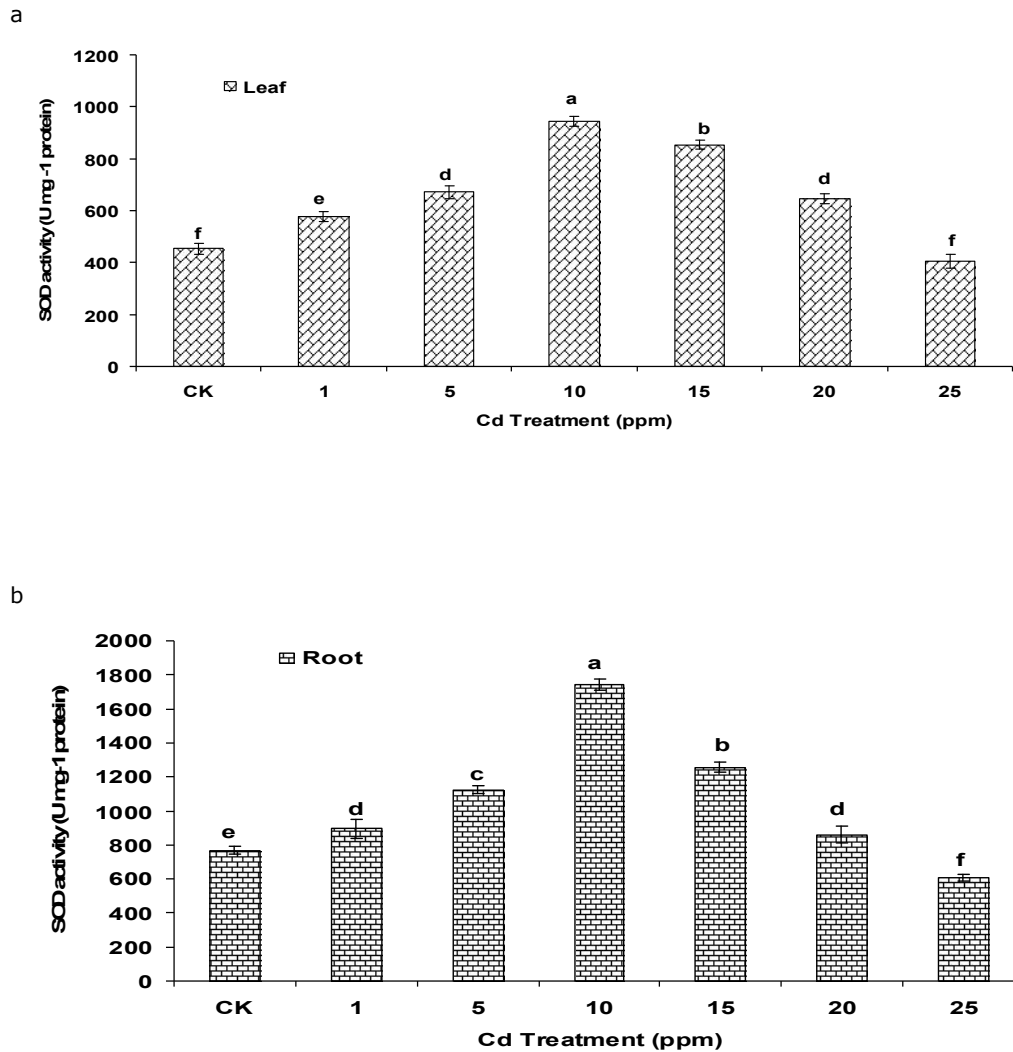


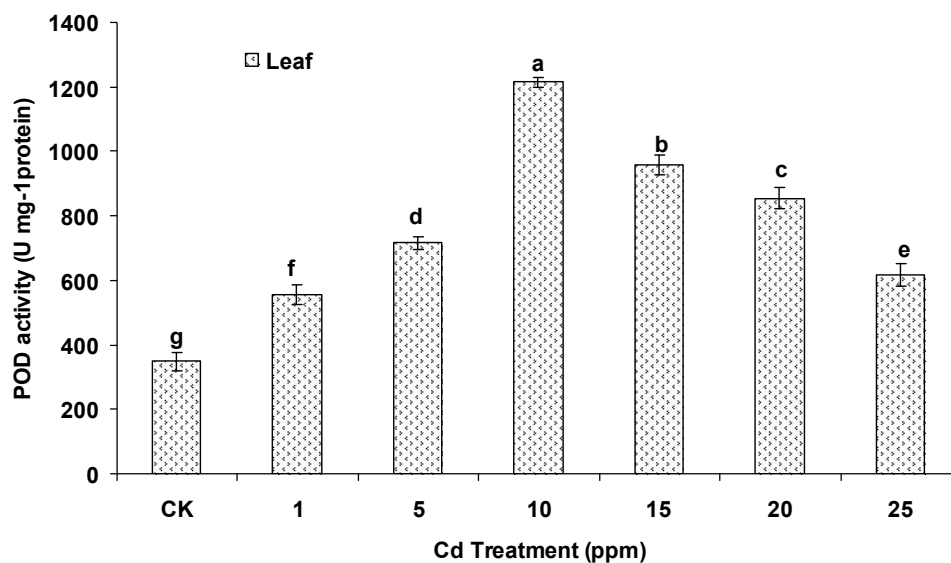
Fig. 1. Effect of Cd on SOD activity in leaves **(a)** and roots **(b)** of *K. candel*. (Mean \pm S.D.). One way ANOVA, $P < 0.05$. Different superscripts on bars showed significant ($P < 0.05$) difference between the means according to Duncan's test).

POD is widely distributed in the plants and is one of the key enzymes involved in the elimination of active oxygen species (AOS). POD catalyses H_2O_2 -dependent oxidation of substrate. Previous studies in other plants have reported increase, decrease and no changes in POD activity in response to heavy metal exposure (Shaw et al 1995; Schützendübel et al 2001, 2002).

The effect of Cd stress on the membrane protection enzyme system was evaluated and the activity of CAT increased with the treatment of 0 to 10 ppm Cd and then decreased with the higher concentration of Cd in roots and leaves (Figs 3a, b). CAT can eliminate H_2O_2 and play a key role in the elimination of O_2^- . In our experiments, the activity of antioxidant enzymes endured significant changes. However, Cd differently affected leaves and roots and their response depended on the concentration. In leaves, an increase in CAT, POD, SOD activities was noticed especially after three months exposure at the lower Cd concentrations. In roots this effect was more evident and they responded to low Cd stimulation more rapidly. This reaction may be explained by the fact that roots are the first organs coming in contact with Cd. Moreover, the different enzyme activities in leaves might also be due to the lower Cd concentration in the aerial than in the hypogea part. Previous studies in other plant species have reported the similar results (Arduini et al 2004). In higher concentrations of Cd, the decrease in the activity of POD, SOD, and CAT indicating that the higher concentration of Cd in *K. candel*

antioxidant enzyme systems affected and might be damaged the cell membrane, protein, and DNA. That might have inhibited the plant growth and biomass.

a



b

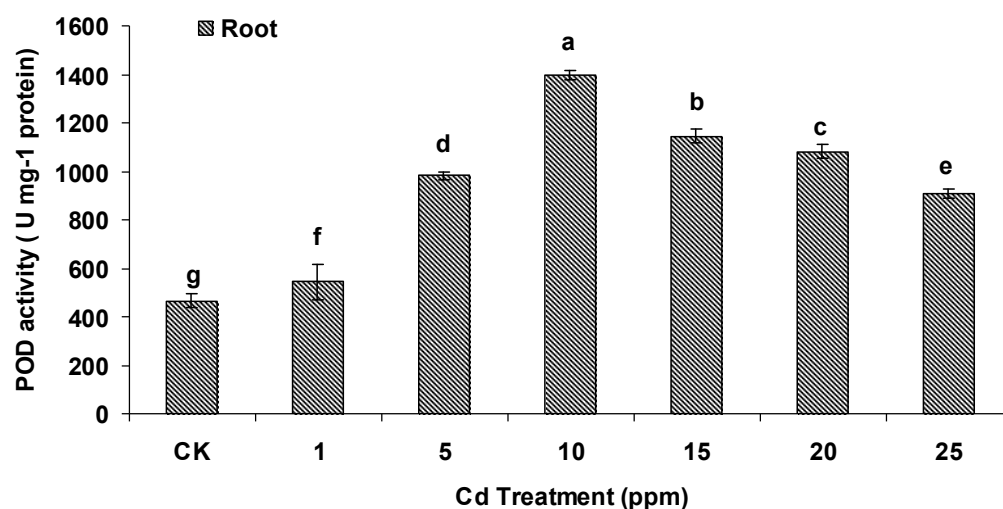
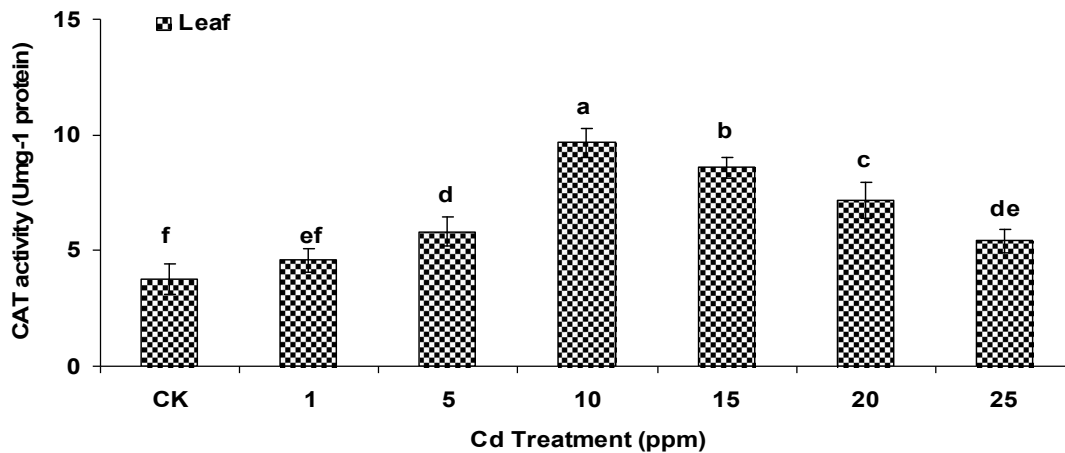


Fig. 2. Effect of Cd on POD activity in leaves **(a)** and roots **(b)** of *K. candel*. (Mean \pm S.D.). One way ANOVA, $P < 0.05$. Different superscripts on bars showed significant ($P < 0.05$) difference between the means according to Duncan's test).

In leaves of heavy metal-stressed plants, SOD, POD and CAT activities fluctuated in different stress levels compared to the control. In comparison with the control, the dynamic tendency of SOD, CAT, and POD activities in roots of heavy metal-stressed plants all ascended and then declined. The increase in enzyme activities demonstrated that *K. candel* is tolerant to Cd in certain concentration.

a



b

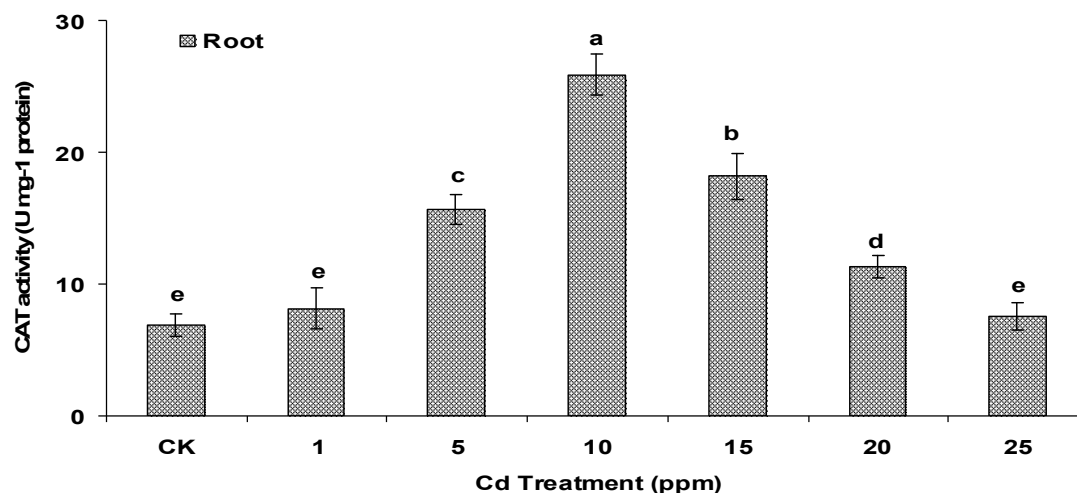


Fig. 3. Effect of Cd on CAT activity in leaves (a) and roots (b) of *K. candell*. (Mean \pm S.D.). One way ANOVA, $P < 0.05$. Different superscripts on bars showed significant ($P < 0.05$) difference between the means according to Duncan's test).

Conclusions. At higher Cd level, e.g., 5 to 25 ppm as used in this study, a significant decrease in plant stem height, leaf number and corresponding decrease in biomass of *K. candell* plants seedlings was observed. The Cd led to the change of SOD, POD, and CAT activities at different concentrations. The activity of antioxidant enzymes in leaves of *K. candell* seedlings indicates that enzymes engaged in antioxidant defense in certain level especially in low concentration of Cd treatments. However at the highest amount of Cd exposure, the activity of POD, SOD and CAT were decreased both in leaf and root. The reductions in the activities of antioxidative enzymes in *K. candell* at the high concentration of Cd treatments indicate that Cd tolerance characteristics of this plant cannot be attributed to antioxidative defense. This result indicated that the higher concentration of Cd might affect the antioxidant enzyme system of *K. candell*. That may be inhibited the plant growth and biomass.

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