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GROWTH AND ANTIOXIDATIVE RESPONSES TO EXCESS CADMIUM IN KENAF (*Hibiscus cannabinus L*).

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ABSTRACT

In order to study the mechanisms responsible for cadmium (Cd) detoxification in Kenaf (*Hibiscus cannabinus L*), the growth and antioxidative responses of the plant grown on a Cd polluted soil were investigated. Kenaf seeds were grown in artificially contaminated soils containing increasing concentrations of Cd as $CdCl_2$ (0, 150, 300, 450 and 600 mg Cd kg^{-1}) for 14 weeks in a screenhouse. Growth parameters (plant height and stem girth), Cd concentration, oxidative lipid peroxidation, glutathione (GSH) content, activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were measured using established techniques. Cd inhibits growth as shown by decrease in plant height and stem girth at all concentrations used compared with control. Plant Cd concentrations increased with soil Cd concentrations, with maximum Cd uptake being 73.37±8.82 mg kg^{-1} dry weight at 600 mg Cd kg^{-1}. The content of malondialdehyde (indication of lipid peroxidation) showed a significant ($p<0.05$) increase in Kenaf shoots at 150 and 300 mg Cd kg^{-1} level of treatment, while the content of non-enzymatic antioxidant, GSH, showed a significant ($p< 0.05$) depletion at all treatment levels compared with control. Activity of SOD was increased while the activities of CAT and APX were inhibited at all Cd concentrations used. Results show that Cd induces oxidative stress in Kenaf and that both enzymatic and non-enzymatic antioxidants play significant roles in Cd detoxification in Kenaf.

KEYWORDS: Antioxidant enzymes, cadmium, kenaf, non-enzymic antioxidants, oxidative stress.

INTRODUCTION

Cadmium (Cd) is an extremely toxic metal without any metabolic or physiologic significance and its presence in the environment is of concern in terms of health for man and biosphere. It enters the environment mainly from industrial processes and phosphate fertilizers and is then transferred to the food chain [1]. Studies in different plant species have revealed that Cd causes various phytotoxic symptoms including chlorosis, growth inhibition, water imbalance, phosphorus and nitrogen deficiency and accelerated senescence [2-4]. However, the mechanisms involved in the toxicity of cadmium are not completely understood yet. Some authors have reported that cadmium produces alterations in the functionality of membranes by inducing lipid peroxidation [3]. Also disturbances in chloroplast metabolism by inhibiting chlorophyll biosynthesis and reducing the activity of enzymes involved in CO_2 fixation have all been mentioned as possible mechanisms [3, 5, 6].

Cd causes oxidative stress either by inducing oxygen free radical production or by decreasing enzymatic and non-enzymatic antioxidants [3, 7, 8]. Oxygen free radicals which includes superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) are amongst the most reactive compounds known to be produced during heavy metal stress. These reactive compounds can lead to multifold effects such as membrane peroxidation, loss of ions, protein cleavage, inactivation and damage, and even DNA strand breakage [9, 10]. To control the level of reactive oxygen species (ROS) and protect cells under stress conditions plant tissues have evolved antioxidant defense systems comprising of enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and the non-enzymic constituents, α-tocopherol, ascorbate and reduced glutathione (GSH) which remove, neutralize and scavenge ROS [9- 11].

Phytoremediation offers a great potential as a method for removing metals, especially Cd from contaminated soil [12, 13]. For efficient remediation of Cd contaminated soil, plants that accumulate high Cd concentrations and also have a high biomass must be used. Kenaf (*Hibiscus cannabinus L.*) is such a plant which exhibits a relatively high Cd concentration and biomass [14]. Previous studies in our laboratory have also indicated that Kenaf

can accumulate lead to a reasonable extent from lead contaminated soil (unpublished result). However the mechanisms responsible for Cd accumulation and detoxification in kenaf remain unknown. To better understand the mechanisms of cadmium detoxification by kenaf, this study therefore examined the physiological and biochemical responses of kenaf under high level of Cd stress.

MATERIALS AND METHODS

Soil Sampling, Preparation and Planting

The screenhouse experiment was carried out in the University of Agriculture, Abeokuta (UNAAB), (Latitude 7^0 9' N and longitude 30^0 21' E) Ogun State, Nigeria. Top soil (0-15 cm) was collected from UNAAB Teaching and Research Farm, mixed thoroughly by a mechanical mixer and passed through 4 mm sieve to remove fibre and non soil particulate in the sample. The chemical and physical properties of the soil were determined prior to planting. Ten-litre plastic pots were filled with 10kg soil that passed through a 4 mm sieve. Five Cd levels, applied as Cadmium chloride (0, 150, 300, 450 and 600 mg Cd kg^{-1} soil) were introduced and the pots arranged in Randomized Complete Block Design (RCBD), with 5 replicates per treatment. The soil in the pots were thoroughly mixed for even distribution of the contaminant and watered to field capacity. Three seeds of kenaf (Cuba 108) obtained from the Institute of Agricultural Research and Training (IART), Ibadan, Nigeria were planted and thinned to one plant per pot two weeks after germination. 60 kg N/ha of N.P.K. (20:10:10) fertilizer was applied third week after planting. The plants were protected against insects by spraying with Nuvacron at sixth week after planting and this continue at two weeks interval until when harvested.

Soil properties analysis

Soil pH was determined using a glass electrode pH meter (Rent Model 720) in distilled water according to Thomas [15]. Soil organic carbon was determined by the chromic acid digestion method of Walkley and Black as reported by Sparks [16]. The total N concentration was determined by Macro-kjeldahl method according to Bremner [17], available P was determined by Bray-I method as described by Kuo [18]. Exchangeable Ca, Mg, K and Na were extracted with neutral normal ammonium acetate buffer according to Helmke and Sparks [19]. K and Na were determined using Flame Photometer (Gallenkamp Model FH 500) and exchangeable Ca and Mg by Atomic Absorption Spectrophotometry (AAS).

Cadmium accumulation

Harvested plants were thoroughly washed in distilled water and the leaves and stems (shoot) removed and then oven dried at 80°C for 48 hours. About 2 g of dried plant materials was powdered and digested in 10 mL of HNO_3 at 100°C until the solution turned clear. The final volume was adjusted to 25 ml with double distilled water. Cadmium concentration was estimated using an atomic absorption spectrophotometer.

Growth analysis

The effect of Cd on growth of Kenaf was studied in terms of plant height and stem girth. Plant height and stem girth were measured using a metre rule and vernier caliper respectively starting from sixth week after planting at two weeks interval until harvest.

Plant extract preparation

The extraction was carried out according to the procedure of Rani et al. [20]. Briefly, kenaf shoots were thoroughly washed and homogenized in 50 % ethanol (1:2, w/v) in a pre-chilled mortar and pestle. The homogenates were then centrifuged at 5000 g for 10 minutes. The supernatant obtained for each sample was used within 72 hours for lipid peroxidation, protein content, and various enzymatic as well as non-enzymatic antioxidants assays.

Biochemical assays

The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as standard by method of Beuge and Aust [21]. Catalase (EC 1.11.1.6) activity was measured following the procedure of Clairborne [22], in which the disappearance of H_2O_2 was monitored spectrophotometrically at 240nm. Superoxide dismutase (EC 1.15.1.1) activity was assayed according to the procedure of Das et al. [23]. The activity of ascorbate peroxidase (EC 1.11.1.11) was determined according to the procedure of Nakano and Asada [24] by estimating the rate of ascorbate oxidation (extinction coefficient 2.8 mM^{-1} cm^{-1}). GSH concentration was determined in samples according to the method of Boyne and Ellman [25]. Protein content of the extract was determined according to the method of Bradford [26] using bovine serum albumin as standard. All chemicals used in the enzymatic and non-enzymatic activity determinations were of analytical purity and were obtained from Sigma Aldrich Chemicals.

Statistical analysis

The values were presented as mean ± SD. Differences between group means were estimated using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) and Duncan test for multiple comparisons. Results were considered statistically significant when $p< 0.05$. All the statistics were carried out in SAS (The SAS system for windows, v8; SAS Institute Inc., Cary, NC).

RESULTS

The soils chemical and physical properties prior to planting are shown in Table 1. The pH of the soil was slightly acidic [27]. The soil was low in nutrient when compared to the nutrient ratings for soil fertility classes in Nigeria

and the Cd content of the soil was within the range (0.01-2.4 mg kg^{-1}) of Cd in agricultural soil [28].

TABLE 1 - Chemical and physical properties of the soil before planting

Parameters	soil
pH (H_2O)	6.30
Sand (g/kg)	755.00
Clay (g/kg)	75.00
Silt (g/kg)	170.00
Texture	Sandy loam
Exch. Ca ($cmolkg^{-1}$)	1.38
Exch.Mg ($cmolkg^{-1}$)	1.10
Exch.K ($cmolkg^{-1}$)	0.18
Exch.Na ($cmolkg^{-1}$)	0.12
Organic matter (g/kg)	16.30
Available P. (mg/kg)	7.50
Total N. (g/kg)	1.20
Cd (mg/kg)	0.07

Cadmium concentration and growth parameters

Table 2 shows the cadmium concentrations in kenaf shoots after harvesting. Kenaf shoots showed a dose-dependent accumulation of Cd. This could be seen in the significant positive correlation between Cd treatment in soil and Cd concentration in the shoot of kenaf (r = 0.982, p = 0.001, in Table 3). Treated plants accumulate up to 73.37 mg Cd kg^{-1} DW at 600 mg Cd kg^{-1} soil level of treatment. Figures 1 and 2 show the plant height and stem girth respectively as affected by Cd concentrations from sixth week after planting (6WAP) to fourteenth week after planting (14WAP). Plant height and stem girth increased from 6WAP to 14WAP at every concentration level. However, compared with control, plant height and stem girth decreased as cadmium concentration increased. At 150 mg Cd kg^{-1} level of treatment, the decrease in plant height was not statistically significant ($p<0.05$) compared with control, but at other Cd treatment level, the reduction in plant height was significant ($p<0.05$) when compared with control.

Lipid peroxidation

Malondialdehyde (MDA) content was measured as an indication of lipid peroxidation. Cd treatment significantly ($p<0.05$) increased the content of MDA in the shoots of kenaf at 150 and 300 mg Cd kg^{-1} soil level of treatment, with the increase being about 156 and 209% respectively. However, treatment at 450 mg Cd kg^{-1} soil level significantly ($p<0.05$) reduce the MDA content by about 16%. At 600 mg Cd kg^{-1} soil treatment level, the MDA content was statistically similar ($p<0.05$) to the observed value for control (Table 2).

TABLE 2 - Effect of Cd at different concentrations on Cd content, lipid peroxidation (LPO), GSH content as well as SOD, CAT and APX activities in kenaf shoot (*Hibiscus cannabinus*)

Treatments (mg Cd kg^{-1})	Cd content in leaves (mg kg^{-1} DW)	LPO (µmol MDA g^{-1} FW)	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)	GSH (µmol g^{-1} FW)
0	0.02 ± 0.002^{e}	0.73 ± 0.05^{c}	0.036 ± 0.005^{b}	1.47 ± 0.03^{a}	0.70 ± 0.03^{d}	14.50 ± 0.47^{a}
150	19.63 ± 0.20^{d}	1.87 ± 0.01^{b}	0.068 ± 0.008^{a}	0.88 ± 0.03^{b}	2.19 ± 0.15^{c}	10.83 ± 0.57^{b}
300	33.79 ± 2.08^{c}	2.26 ± 0.17^{a}	0.066 ± 0.011^{a}	0.72 ± 0.14^{c}	4.33 ± 0.67^{a}	8.54 ± 0.31^{d}
450	42.63 ± 0.11^{b}	0.61 ± 0.05^{d}	0.074 ± 0.002^{a}	0.78 ± 0.03^{c}	2.67 ± 0.15^{b}	9.07 ± 0.09^{c}
600	73.37 ± 8.82^{a}	0.71 ± 0.04^{c}	0.072 ± 0.008^{a}	0.38 ± 0.03^{d}	2.74 ± 0.20^{b}	9.16 ± 0.34^{c}

Values are means ± SD of five replicates. Different superscript in the same column indicate significant differences at $p<0.05$ (DMRT).

TABLE 3 - Relationship between Cd treatment in soil and biochemical parameters.

		Cd Treatment	Cd in shoot	MDA	SOD	CAT	APX	GSH
Cd Treatment	Pearson Correlation	1	.982(**)	-.267	.794	-.910(*)	.554	-.807
	Sig. (2-tailed)		.003	.664	.109	.032	.332	.099
	N		5	5	5	5	5	5
Cd in shoot	Pearson Correlation		1	-.217	.763	-.937(*)	.536	-.771
	Sig. (2-tailed)			.726	.134	.019	.352	.127
	N			5	5	5	5	5
MDA	Pearson Correlation			1	.173	-.112	.588	-.296
	Sig. (2-tailed)				.781	.858	.298	.629
	N				5	5	5	5
SOD	Pearson Correlation				1	-.893(*)	.700	-.924(*)
	Sig. (2-tailed)					.041	.188	.025
	N					5	5	5
CAT	Pearson Correlation					1	-.721	.899(*)
	Sig. (2-tailed)						.169	.038
	N						5	5
APX	Pearson Correlation						1	-.902(*)
	Sig. (2-tailed)							.036
	N							5
GSH	Pearson Correlation							1
	Sig. (2-tailed)							
	N							

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

Enzymic and non-enzymic antioxidants level

The changes in enzymic and non-enzymic antioxidants are shown in Table 2. SOD activity showed a significant increase at all treatment level compared with control. However, the increase at all the cadmium concentrations was not dose-dependent ($r = 0.794$, $p = 0.109$, Table 3). The maximum increase occurs at 450 mg Cd kg^{-1} soil treatment level and it is about 106% of the control. CAT activity showed a steady but significant ($p<0.05$) decrease at 150 mg Cd kg^{-1} soil level of treatment when compared with control. The steady decrease was sustained in the 300 and 450 mg Cd kg^{-1} soil treatment level. At 600 mg Cd kg^{-1} soil level of treatment, there was a sharp decrease amounting to 74% of the control value. This showed that there is a significant negative correlation between Cd treatment in soil and CAT activity in the shoot of kenaf ($r = 0.910$, $p = 0.032$, Table 3). APX activity showed a significant ($p<0.05$) increase at all cadmium treatment levels compared with control. The increases are 212, 519, 281 and 291% of the control at 150, 300, 450 and 600 mg Cd kg^{-1} soil treatment levels respectively. The level of GSH indicated a significant ($p< 0.05$) depletion at all Cd concentrations in comparison to the control, although the depletions are not dose-dependent. The maximum decrease of 41% occurs at the 300 mg Cd kg^{-1} soil level of treatment. We also analyzed our data to see whether any correlation existed among the biochemical parameters investigated. As shown in Table 3, we observed that a significant negative correlation existed between (1) Cd concentration in the shoot and CAT activity (2) SOD and CAT activity (3) SOD activity and GSH level and (4) APX activity and GSH level, while a significant positive correlation was observed between CAT activity and GSH level.

DISCUSSION

The aim of this study was to evaluate the mechanisms responsible for Cd detoxification in kenaf. This was done by investigating the growth and antioxidative response of the plant grown in a Cd polluted soil. In this study, the Cd concentrations used (up to 600 mg kg^{-1}) are higher than those normally observed in most polluted soils, but we chose these concentrations after preliminary work in our laboratory have confirmed them to be suitable. Increasing Cd concentrations significantly enhanced Cd concentration in the shoot of kenaf in a dose dependent manner. This is expected because accumulation of Cd from soil depends on Cd concentration in the soil and its bioavailability, modulated by other factors such as presence of organic matter, pH, redox potential, temperature and concentrations of other elements [3].

Cd produces a significant inhibition in the growth of kenaf as seen in the reduction of plant height and stem girth of treated plants in comparison to control (Figures 1 and 2, respectively). Several workers have reported growth inhibition in Cd-exposed plants [3, 8, 29, 30]. Reasons adduced for this growth inhibition includes inhibition of cell division and elongation rate of cells [29], as well as degradation of chlorophyll or the inhibition of its biosynthesis [3, 8, 30].

Lipid peroxidation is one of the main manifestations of oxidative damage which plays important role in the toxicity of many xenobiotics. Intoxication with metals induces oxidative stress because they are involved in several different types of ROS generating mechanisms. Cd has been found to produce oxidative stress [3, 31], but in contrast with other heavy metals such as Cu, it does not seem to act directly on the production of ROS via Fenton and / or Haber Weiss reactions. However, it enhances prooxidant status by reducing the GSH pool and / or blocking the photosynthetic electron transport chain [3], as well as activating calcium-dependent systems and affecting iron-mediated processes [32]. In this study, there was a significant increase in MDA level at the 150 and 300 mg Cd kg^{-1} soil level of treatment compared with control (Table 2) which in accordance with many authors [30, 33, 34] is an indication of

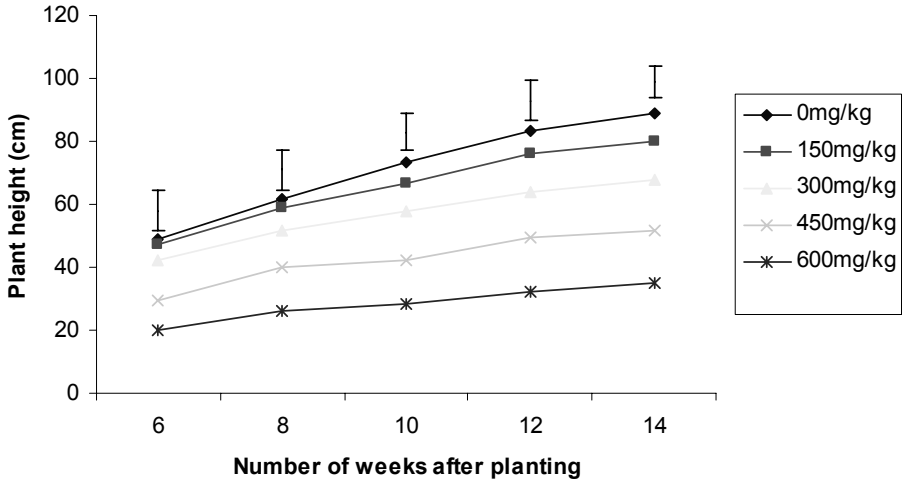

FIGURE 1 - Plant height of kenaf as affected by cadmium concentration

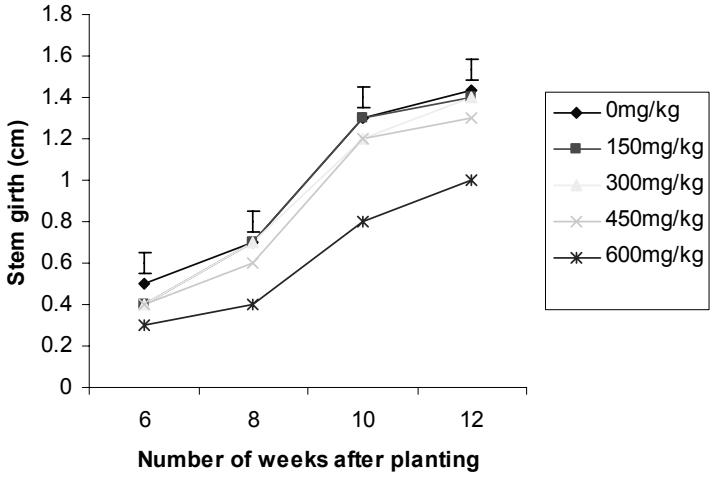

FIGURE 2 - Stem girth of kenaf as affected by cadmium concentration

oxidative stress. However, the MDA content became less in the 450 and 600 mg Cd kg^{-1} levels of treatment. This may presumably be due to scavenging mechanisms [31].

SOD is the first enzyme in the ROS detoxification process. It converts superoxide radicals to H_2O_2 at very fast rate [9]. In this study, we observed an enhanced SOD activity at all treatment levels compared with control (Table 2). This is consistent with previous reports in which other plant species were treated with Cd [2, 11, 30, 33]. The increase in SOD activity observed may be attributed to increased production of active oxygen species and/or increased expression of gene encoding SOD [35].

As a result of the action of SOD, H_2O_2 concentration is expected to increase in the cell. The control of this buildup is essential to prevent oxidative damage to membranes and proteins. The enzymes CAT and APX under most circumstances are responsible for this detoxification. CAT activity of kenaf shoots was reduced by increasing concentrations of Cd in the soil in this study. Several researches using different plant species have reported that CAT activity often decreased following exposure to elevated Cd concentration as observed [8, 30, 36]. This observed decrease in CAT activities may be as a result of enzyme inhibition, since Cd is known to bind to the thiol group of this enzyme thereby inactivating it [3, 37, 38]. Also in this study, there is an increase in the APX activities at all the Cd levels used compared with control. This observation is similar to result obtained by Gratao et al. [9] in green barley seedlings, Markovska et al. [34] in *Brassica juncea* plants and Romero-Puertas et al. [39] in leaves of pea. Report have shown that the equilibrium of SOD, APX, and CAT activities is essential in order to determine the steady-state level of $O_2^{\bullet-}$ and H_2O_2 [40]. Therefore the increase in the activity of APX observed may be a compensatory mechanism for the inhibition of CAT earlier noticed [9, 41-43].

The fact that GSH is involved in defense reactions against oxidative stress as an antioxidant is widely acknowledged. GSH is the predominant non-protein thiol in fungal, plant and animal tissues and it possess strong antioxidative properties and consequently able to counteract oxidative stress [3, 44]. In this study, we observed a significant depletion in the level of GSH at all Cd treatments used compared with control. Several researches have reported Cd-induced depletion of GSH in several plant species [45, 46]. The depletion of GSH observed might be caused by an increased rate of phytochelatin (PC) synthesis induced by Cd ions [47]. The decline might also be attributed to a decreased glutathione reductase (GR) activity [46], an increased utilization for ascorbate synthesis or for a direct utilization with Cd [48].

It can be concluded from the present study that Cd concentrations as used, strongly inhibit growth in kenaf as shown by decrease in stem height and girth. Also, exposure to Cd for 14 weeks is associated with induction of oxidative stress evidenced by increase in MDA production as well as with marked alterations of enzymatic (SOD, CAT, APX) and non enzymatic (GSH) antioxidant components.

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