Global NEST Journal, Vol 18, No 1, pp 223-232, 2016

DETECTION OF PHYSIOLOGICAL AND GENOTOXIC DAMAGES REFLECTING TOXICITY IN *KALANCHOE* CLONES

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Received: 27/01/2014
Accepted: 24/02/2016
Available online: 13/04/2016

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ABSTRACT

In order to make assessments in understanding of physiological and genotoxic effects of imposing cadmium (Cd) on photosynthetic pigment contents along with the changes occurring in genetic material of *Kalanchoe* plants were used in relation to various Cd-treatments. Young plantlets were originated from a single host plant as clones, and developed *in vitro.* Developed clones were grown in standard pots with daily watering of Hoagland solution (20 ml) containing different concentrations of cadmium chloride for two months. Cd concentrations of the collected samples were measured by employing ICP-OES and RAPD-PCR technique was applied for detecting the genotoxic effects of Cd. After two month of experimental period, the comparisons between unexposed and exposed *Kalanchoe* clone groups revealed reductions in photosynthetic pigment contents, especially at the highest level of Cd exposure and a genomic instability when application of Cd concentration increases. RAPD-PCR analyses demonstrated the distinguishable banding pattern in number and band intensities between Cd-treated and control groups. In addition, progressive Cd accumulations in leaves, stems and roots of plant samples were observed when the application of exposure level increased.

Keywords: RAPD-PCR, heavy metal pollution, photosynthetic pigments, genotoxicity

1. Introduction

Heavy metal pollution is one of the most serious problems in human history and it has gained more impetus in recent decades due to the heavy reliance of heavy metal utilizations in industries. Heavy metals accumulated in soil, water and air could persist for many years and therefore they create potential risks for ecosystems (Duruibe *et al.*, 2007; Lone *et al.*, 2008; Wang *et al.*, 2012).

Recently, Cd utilization in much of the modern world has been progressively increased. Consequently, Cd-related toxicity has become a serious problem for the environment. Cd could be taken up by plants

although it is not an essential for plant life (Xu *et al.*, 2012; Zhao *et al.*, 2014). Prolonged exposure or accumulation of Cd by plants could cause the various toxicity-related consequences such as reduced photosynthesis (Mobin and Khan, 2007), generation of reactive oxygen species (ROS) (Schutzendubel *et al.*, 2001), inhibited respiration (Seregin and Ivanov, 2001) and cell proliferation (Liu *et al.*, 2003; Deckert, 2005), diminished water balance (Sandalio *et al.*, 2001; Garinova *et al.*, 2007; Wan *et al.*, 2011), and disturbed carbohydrate (Lorenc-Plucinska and Stobrawa, 2005) and nutrient uptake metabolisms (Wu and Zhang, 2002; Zhang *et al.*, 2002) resulting in visible symptoms such as chlorosis, necrosis, root blackening, stunting and general reductions in biomass production (Sandalio *et al.*, 2001). Although plants have elaborative mechanisms to eliminate or alleviate the environmental stress factors to some point, excessive levels of heavy metals could cause the substantial cellular stress subsequently giving damages to the various cellular components such as DNA, proteins and membranes (Zhang *et al.*, 2005; Bertin and Averbeck, 2006; Liu *et al.*, 2009). For example, Cd could induce the generation of ROS, whose subsequent accumulation leads the oxidative stress finally causing to enzyme inhibition, lipid peroxidation, DNA/RNA damage and/or protein oxidation (Mittler, 2002).

Kalanchoe daigremontiana is a succulent CAM plant but is not a halophyte one (Bartha and Fodorpataki 2007). Although it is native to Madagascar, it is distributed in many parts of tropical and subtropical Africa, Asia, North America and South Africa as well as found in Bangladesh (Nahar *et al.*, 2008).

In this research, possible physiological and genotoxic effects of Cd were investigated in *Kalanchoe* plants grown under different levels of Cd exposures. For detecting genotoxicity, the RAPD-PCR (Randomly Amplified Polymorphic DNA) technique was employed and photosynthetic pigment contents were evaluated in order to make assessments for the effects of Cd toxicity. Using RAPD-PCR in assessing of genotoxic effects of heavy metals is commonly used technique, which enables to detect the DNA band features such as band intensity and gain or loss in relation to toxicant exposures (Atienzar & Jha, 2006; Swaileh *et al.*, 2008; Kekec *et al.*, 2010). Therefore, this study results will pave the way for understanding of alterations in genetic material in relation to heavy metal exposure such as Cd as well as in changes of metabolic processes relatedly.

2. Materials and methods

2.1. In vitro development of Kalanchoe plantlets

Kalanchoe plantlets (clones) were obtained from the leaves of a single host plant and grown on wet filter papers in glass dishes in growth chamber under 4000-4200 lux of irradiance with day (14)/night (10) regimens, respectively, 23 ± 2°C temperature and 50-55% relative humidity. Plantlets were daily watered with 20ml of full strength Hoagland solution for 14 days (Hoagland and Arnon, 1950). Following *in vitro* development, 90 young plantlets were transferred into standard plastic pots (Gardol®) containing sterilized compost (161.5 g). Appearing the second mature leaves, each experimental group (5 plantlets) was daily watered with 20 ml of Hoagland solution containing 0, 50, 100, 200 and 400 µM of Cd (in $CdCl_2$ form) for 60 days. For control and experimental groups, pH value was kept as 5.6. At the end of 60-days experimental period, plantlets were harvested for RAPD and the content of photosynthetic pigment analyzes.

2.2. Determination of the contents of photosynthetic pigments

Photosynthetic pigments were extracted in 15 ml of 80% acetone (v/v) using 0.5 g of leaf materials from each sample and then extraction was centrifuged at 3000 g and +4 °C for 10 min. Wavelengths used in chlorophyll *a* and *b,* and carotenoid measurements include 645, 663 and 470 nm, respectively. Chlorophyll *a* and *b*, and carotenoids concentrations were calculated according to relevant literature (Arnon, 1949) as followed below;

$$C_a = [12.7 \times D_{663} - 2.69 \times D_{645}] \times V / 1000$$
$$C_b = [22.9 \times D_{645} - 4.68 \times D_{663}] \times V / 1000$$

$$C_{Total} = [20.2 \times D_{645} + 8.02 \times D_{663}] \times V / 1000$$
$$C_{x+c} = 1000\ D_{470} - 1.90\ C_a - 63.14\ C_b / 214 \times V / 1000$$

Where C_a is the chlorophyll *a* concentration (mg ml^{-1}), C_b is the chlorophyll *b* concentration (mg ml^{-1}), C_{Total} is the total chlorophyll concentration (mg ml^{-1}), C_{x+c} is the carotenoids concentration (mg ml^{-1}), D_{663}, D_{645} and D_{470} are the optical density at 663, 645 and 470 nm, respectively, and V is the volume of the supernatant obtained after the centrifugation (ml).

2.3. Analysis of Cd accumulation in plant samples

Root, stem and leaf samples taken from the plants were oven-dried at 80 °C for 24 h. Then, the samples were milled in a micro-hammer cutter and fed through a 1.5-mm sieve. Later, samples were weighed as 0.5 g and transferred into Teflon vessels. Following the addition of 8 ml 65% HNO_3, the samples were mineralized in microwave oven (Berghof - MWS2) at 145 °C for 5 min, 165 °C for 5 min and 175 °C for 20 min. After cooling of the samples, they were filtered by Whattman papers and made up to 50 ml with ultra-pure water and stored in falcon tubes. Then, standard solution was prepared and Cd concentrations were measured using Inductively Coupled Plasma Optical Emission Spectroscopy (PerkinElmer-Optima 7000 DV).

2.4. Analysis of genotoxic effects

RAPD analysis was performed to analyze the genotoxic effects of Cd in plant samples. DNA isolation was carried out using DNeasy Plant DNA Extraction Mini Kit (Qiagen) according to supplier's instructions. PCR mix was prepared for 25 µl, containing 1x PCR buffer $(NH_4)_2SO_4$, 0.2 mM from each dNTP (2 mM dNTP mix), 25 picomoles of primer OPA08 5' CCACAGCAGT 3' (QIAGEN Operon RAPD® 10mer Kits), 20-200 ng of genomic DNA, and 0.5 units of Taq DNA polymerase, and filled up with sterile de-ionized water to final volume. Tubes containing all reaction components, except template DNA, were included as controls for each reaction. Amplication was performed using Techne Endurance TC-512 Gradient Thermal Cycler at 95 °C for 3 min. followed by 45 cycles of 1 min. at 94 °C, 1 min. at 37 °C, 2 min. at 72 °C, and 5 min. at 72 °C. Amplification products were analyzed and estimated using 100bp DNA ladder/marker (Gene Ruler ™ 100 bp DNA Ladder, ready-to-use, MBI Fermentas) in 2% agarose gel, stained with ethidium bromide. Results were documented using GelDoc 2000 (BioRAD) gel documentation system. Clearly observed bands were scored to create the genetic profiles of plant samples. Based on the band intensity, and loss or gains, genomic instability and DNA variations were estimated.

3. Results

3.1. Photosynthetic pigments in Kalanchoe plantlets exposed to Cd at different levels

Considerable reductions were observed on photosynthetic pigment contents of *Kalanchoe* plants after 60 days of Cd exposure at different levels in comparison with control group (0, 50, 100, 200 and 400 µM) (Fig. 1). The reduction rates were ~40.57% for chlorophyll *a* (from 0.212 mg ml^{-1} to 0.126 mg ml^{-1}) (Fig. 1A), ~37.63% for chlorophyll *b* (from 0.093 mg ml^{-1} to 0.058 mg ml^{-1}) (Fig. 1B), ~20.58% for chlorophyll *a*/*b* (from 2.293 mg ml^{-1} to 1.821 mg ml^{-1}) (Fig. 1D), ~36.27% for total chlorophyll (from 0.284 mg ml^{-1} to 0.181 mg ml^{-1}) (Fig. 1C)and ~37.66% for carotenoids (from 0.077 mg ml^{-1} to 0.048 mg ml^{-1}) (Fig. 1E) under severe Cd stress (400 µM Cd). Slight increases in the contents of chlorophyll *a*, chlorophyll *b*, chlorophyll *a*/*b*, total chlorophyll and carotenoids were noticed at the level of 100 µM Cd treatment in comparisons with the levels of 50, 200 and 400 µM Cd treatments. Overall, it was revealed that the chlorophyll *a*, *b*, *a*/*b*, total chlorophyll and carotenoid contents of *Kalanchoe* plants were substantially decreased when different levels of Cd were applied.

3.2. Accumulation of Cd in different parts of the plant grown in different levels of Cd exposure

Dramatic increases in Cd concentrations were detected in the leaves (from 0.629 mg ml^{-1} to 3.164 mg ml^{-1}) (Fig. 2A), stems (from 0.460 mg ml^{-1} to 2.890 mg ml^{-1}) (Fig. 2B) and roots (from 1.327 mg ml^{-1} to

5.178 mg ml^{-1}) (Fig. 2C) of *Kalanchoe* plants when increasing levels of Cd were applied (Fig. 2). The rates of increases at the level of 400 µM treatments were found to be ~5.03 fold in leaves, ~6.28 fold in stems and ~3.90 fold in roots of *Kalanchoe* plants. The data reveals that the accumulation of Cd was mainly seen in all parts of the plant and was progressive when increasing amount of exogenous Cd was applied.

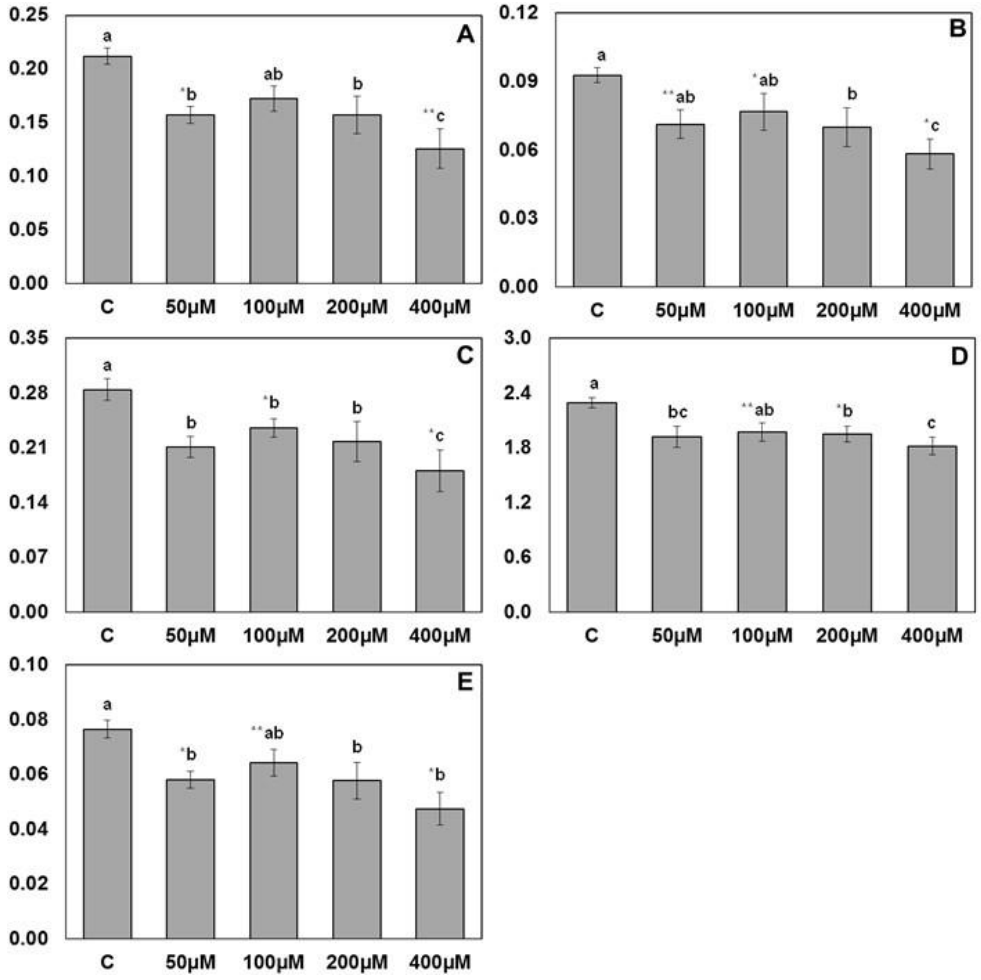

Figure 1. Pigment concentrations (mg ml^{-1}) of controls (C) and Cd-treated (50, 100, 200 and 400 µM) *Kalanchoe* leaves. A) Chlorophyll *a*, B) Chlorophyll *b*, C) Total Chlorophyll, D) Chloropyll *a/b*, e) Carotenoids. The mean difference is significant at 0.01(**) and 0.05(*) levels by the Tukey HSD and multivariate analysis of variance (MANOVA)

Different application levels of Cd (up to 800 µM) were used in researches related with Cd toxicity (Yang *et al.*, 2004; Nedjimi and Daoud, 2009; Goncalves *et al.*, 2009; Meng *et al.*, 2009). By previous studies performed by Wagner (1993), and Sanita di Toppi and Gabrielli (1999), Cd concentrations (in mM) were found to be between 0.04-0.32 in unpolluted and 0.32-1 in moderate level of polluted soils, respectively. Between 0.2-0.8 mg kg^{-1} DW of Cd was reported as normal ranges whereas between/over 5-30 mg kg^{-1} DW of Cd was accepted as toxic levels for plants (Ross 1994; Kabata-Pendias and Pendias, 2001). When taken into consideration of above-mentioned literatures, Cd concentrations used in this study were chosen in 0-400 µM range.

3.3. *Detection of genotoxicity in leaf tips of Kalanchoe plantlets exposed to Cd at different levels*

Genotoxic effect of different Cd concentrations on *Kalanchoe* genome were evaluated by comparing RAPD-PCR band profiles. RAPD-PCR analysis was carried out using DNA taken from leaves of *Kalanchoe* plants exposed to Cd at different levels (0, 50, 100, 200 and 400 µM) for 60-day experimental period.

Distinguishable banding patterns in number and band intensities including increase or decrease in band intensities and loss or gain of bands were produced using primer designated as OPA08 (CCACAGCAGT, QIAGEN Operon RAPD® 10 mer Kits) between exposed and unexposed (control) groups (Table 1). The representative RAPD-PCR band profiles are shown in Fig. 3 as well as Table 2. Distinctive RAPD-PCR band patterns due to alterations (i.e. increase or decrease in band intensities and the loss or the gain of bands) were obtained based on comparisons performed between DNAs of Cd-exposed and unexposed plantlets.

Table 1. Number and intensity changes of RAPD-PCR profiles (a, b, c, and d) detected with primer OPA-08 in Cd-exposed *Kalanchoe* plantlets in comparison to the unexposed *Kalanchoe* plantlets.

Cd concentrations (µM)	Total band numbers	a	b	c	d
Control	3	-	-	-	-
50 µM	5	2	-	-	2
100 µM	3	-	-	1	-
200 µM	6	3	-	-	2
400 µM	4	1	-	-	1

a: indicates the number of new bands, **b:** indicates the number of disappearing normal bands, **c:** indicates the number of normal bands having a decrease in band intensity and **d:** indicates the number of normal bands having an increase in band intensity. **-:** indicates none detected bands.

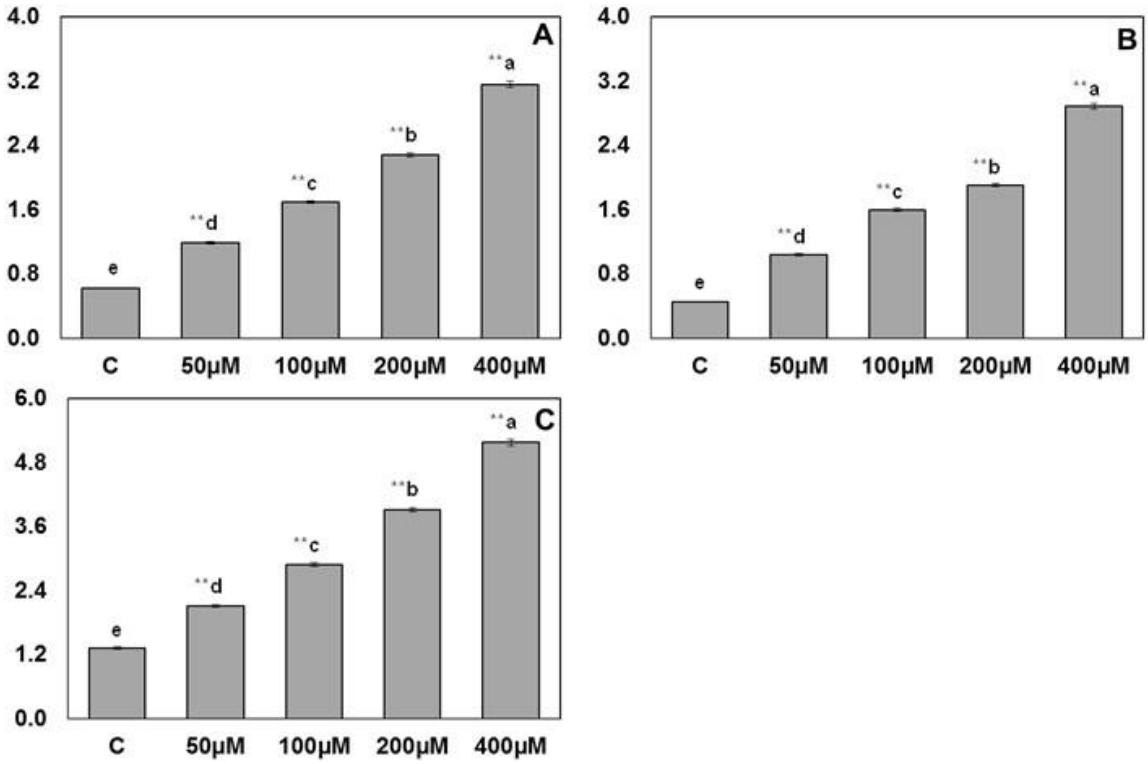

Figure 2. Concentrations of Cd (mg kg^{-1} DW) in controls (C) and leaf, stem and root samples of *Kalanchoe* plants grown in different levels of Cd (0, 50, 100, 200 and 400 µM) for two months.
A) Leaf, B) Stem and C) Root. The mean difference is significant at 0.01(**) level by the Tukey HSD and multivariate analysis of variance (MANOVA)

The molecular sizes of obtained bands using OPA08 demonstrated a range of 123-822 bp. Decreases and increases in band intensities were found to be existed at all level of Cd treatments. Decrease in band intensity was indicated at the level of 100 µM of Cd-treatment whereas increases were found at the levels of 50, 200 and 400 µM Cd-treatments with the molecular sizes of 402 and 648 bp (Table 2 and Fig. 3). Extra bands appeared at the levels of 50, 200 and 400 µM of Cd treatments with molecular sizes of 286, 312, 348, 712 and 822 bp whereas there was no normal band disappearance (Table 2 and Fig. 3).

Table 2. Molecular sizes (bp) of appearing and disappearing bands, and changes in band intensities

Cd concentrations (µM)	RAPD bands			
	a	b	c	d
50 µM	312, 286	-	-	648, 402
100 µM	-	-	123	-
200 µM	822, 712, 286	-	-	648, 402
400 µM	348	-	-	402

a: indicates appearance of new bands, **b:** indicates disappearance of normal bands, **c:** indicates decrease in band intensities and **d:** indicates increase in band intensities. **-:** indicates none detected bands.

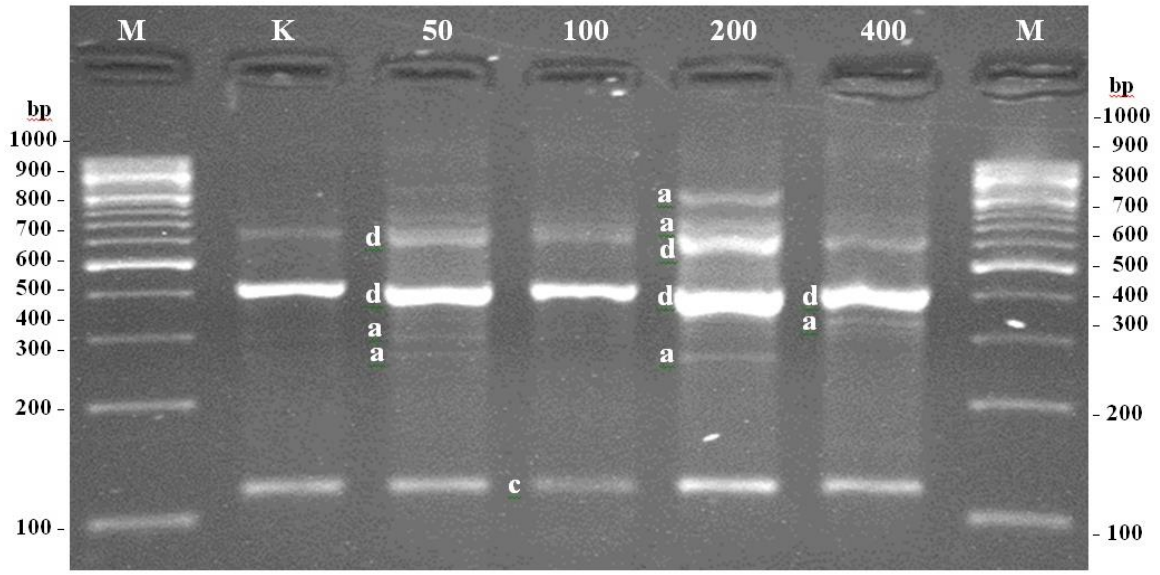

Figure 3. RAPD-PCR band profiles of *Kalanchoe* plantlets exposed to different Cd concentrations at the levels of 50, 100, 200 and 400 µM. **a:** appearance of new bands, **b:** disappearance of normal bands, **c:** decrease in band intensities, **d:** increase in band intensities. **M:** 100 bp DNA Ladder, **K:** Control

4. Discussion

In this work, Cd was found to have suppressive effects on photosynthetic pigment concentrations in *Kalanchoe* plantlets. Cd over normal limits could produce cellular responses other than regular and prolonged exposure of Cd appears to be having negative effects on different cellular structures (Bertin and Averbeck, 2006; Liu *et al.*, 2009). Cd-related oxidative stress in cells induces occurrences of ROS, which consequently leads to the enzyme inactivation, DNA damage and lipid peroxidation (Sandalio *et al.*, 2001; Mittler, 2002; Gichner, 2003; Sun *et al.*, 2007). In our study, reductions were observed in the pigment concentrations of Cd-exposed *Kalanchoe* leaves at the levels of 50, 100, 200 and 400 µM; however, a slight deviation was noticed at the level of 100 µM in comparisons with the levels of 50 and 200 µM. Taking into account the physiological parameters, it could be said that the plant tries to take necessary adjustments to compensate negative effects of Cd in some degree by carrying out some modifications but after a certain point, Cd toxicity becomes inevitable for the plant.

The results from RAPD-PCR profile analysis showed that the appearance of extra bands and increases in band intensities at the levels of 50, 200 and 400 µM Cd. A decrease in band intensity was also observed at the level of 100 µM Cd. The reasons could be related with Cd-derived DNA damage(s) causing the genomic instabilities. Overall changes at genomic DNA level in relation to Cd-exposure indicate that abiotic environmental factors, including heavy metals could have influential effects on appearance of new individual characteristics in plants. By previous researches it was demonstrated that Cd toxicity could

cause various damages at DNA level, including base modifications and oxidations, single and double strand breaks, DNA-protein cross links, abasic sites, bulky adducts and DNA lesions such as 8-hydroxyguanine in organisms (Bisova *et al.*, 1993; Hsiao and Stapleton, 2004). The occurrence of these various DNA mutations and lesions could also generate the substantial structural changes in genetic material thereby affecting the PCR kinetics (Bowditch *et al.*, 1993). Thus, appearances and disappearances in RAPD band profiles could have been arisen from the availability or absence of priming site/s in relation to these structural modifications in DNA sequences (Atienzar *et al.*, 1999).

DNA fingerprinting was reported to be an important bio-indicator used in evaluation of toxicants' effects (Atienzar *et al.*, 1999; Becerril *et al.*, 1999; Grayson *et al.*, 1999). The pollutant-induced changes in RAPD profiles were used to comparatively analyze the alterations in genomic DNA stability and genotoxic effects with other parameters (Atienzar *et al.*, 2000; Liu *et al.*, 2005). In this work, the levels of photosynthetic pigments, including chlorophyll *a*, *b*, *a*/*b*, total chlorophyll and carotenoids in leaf tips of *Kalanchoe* were demonstrated to be used as a biomarker in detection of Cd effects. Similar studies were also performed by others (Hou *et al.*, 2007; Maurya *et al.*, 2008; Mishra *et al.*, 2008). Also, it was revealed that Cd was progressively accumulated in all parts of *Kalanchoe* plants when increasing amount of exogenous Cd was applied. Similar results from previous studies were also reported (Salt *et al.*, 1995; Hart *et al.*, 1998; Uraguchi *et al.*, 2009; Prasad *et al.*, 2001). The reductions in photosynthetic pigment contents in *Kalanchoe* plants exhibited an inverse relationship with the level of Cd applied (except the level of 100 µM) and our findings were in agreement with previous the studies (Hou *et al.*, 2007; Maurya *et al.*, 2008; Mishra *et al.*, 2008). Alterations in both photosynthetic pigment levels and RAPD-PCR profiles of *Kalanchoe* plants were well correlated. It seemed that extent of DNA damage could be serious in majority of leaf tip cells in *Kalanchoe* plants. Recently, similar observations were shown by similar investigations. For example, the reduced photosynthetic pigment concentrations and RAPD profile changes in *Brassica rapa* and watercress (*Nasturtium officinale*) plants were reported in response to lead (Pb) and arsenic (As) treatments, respectively (Cenkci *et al.*, 2010; Ozturk *et al.*, 2010). Aly (2012) reported the significant decreases in photosynthetic pigment contents and genetic alterations in Egyptian clover and Sudan grass in relation to Cd-exposure. In another study, accumulation of heavy metals, including Cd, Pb and Zn in *Hibiscus rosa-sinensis* were found to influence the photosynthetic pigment contents as well as to induce the DNA changes (Bhaduri and Fulekar, 2014). All these studies indicate that there could be a positive correlation between genomic template stability and photosynthetic pigment content parameters in relation to heavy metal treatments.

5. Conclusions

In the present research, induced genomic alterations and changes in some physiological parameters were detected in *Kalanchoe* plants grown in the presence of Cd. Reductions in photosynthetic pigments, and genomic instabilities were detected after applications of 50 µM, 100 µM, 200 µM and 400 µM Cd concentrations by the comparisons done between unexposed and exposed *Kalanchoe* clone groups. The obtained results suggest that the data from RAPD-PCR analysis along with the data from and physiological parameters can be used together for estimation of Cd pollution.

Acknowledgement

This study was funded by Marmara University, Commission of Scientific Research Project under grant FEN-C-YLP-040712-0283.

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