GENETIC VARIATION IN *ULVA LACTUCA* (CHLOROPHYCEAE) MARINE ALGAE UNDER CADMIUM STRESS

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ABSTRACT

Seaweeds have been successfully used as a potential bioindicator for heavy metals pollution in marine ecosystems. Cadmium (Cd) genotoxicity in *Ulva lactuca* marine green algae has been evaluated under different Cd (0, 2.5, 5 and 10 mg/L) concentrations for 4 days, using 20 RAPD primers. Our data revealed that, UBC159 RAPD primer yielded the highest polymorphic bands (PB) at the above Cd applied concentrations. In this regards, estimated PB were recorded to be 99 (71.7%), 45 (32.6%) and 32 (23.2%) at 2.5, 5 and 10 mg/L Cd, respectively. Moreover, Genomic template stability (GTS%) as a qualitative measurement reflect DNA changes induced by Cd treatment was displayed by RAPD marker. Where, Cd stress caused GTS% reduction. In this respect, estimated GTS% values increased as Cd applied concentration increased. Thereby, RAPD marker could be used as a powerful tool to evaluate Cd-genotoxicity in algae and other plants.

Keywords: *Ulva lactuca*, RAPD, Cd stress, genotoxicity, DNA variation

No of Tables: 3 **No of Figures: 2** **No of References: 23**

INTRODUCTION

Cadmium (Cd) is an important environmental pollutant and a potent toxicant to bacteria, algae, and fungi, where algae were the most sensitive (Trevors *et al*., 1986). Their danger comes from their easily absorption and fast accumulation in low organisms and then transported to human through food chain (World, 1992; Pinto *et al*., 2004). Since 2013, Jamers *et al*. reported that, Cd is considered by the US Environmental Protection Agency (EPA) as one of the three contaminants of greatest threat to the environment (along with mercury and lead). Cadmium like other heavy metals can stimulate reactive oxygen species (ROS) induction causing DNA damage, direct or indirect interaction with DNA or DNA destabilization (Fusconi *et al*., 2007; Azimi *et al*., 2013). Since 2003, Jin *et al*. reported that Cd caused high DNA genomic instability due to their role directly inhibition of mismatch DNA repair linked to their impact as a mutagen agent. Their toxicity has been investigated at morphological, physiological, biochemical and molecular levels in different plants. In this respect, previous researches investigated Cd genotoxicity based on growth parameters, total soluble protein and DNA changes as revealed by RAPD marker in barley (*Hordeum vulgare*) (Liu *et al*., 2005) and in rice (*Oryza sativa* L.) (Liu *et al*., 2007). Similar study has been achieved in okra (*Abelmoschus esculantus* L.) (Aydın *et al*., 2013) and wheat wheat (*Triticum aestivum* L.) (Azimi *et al*., 2013). Recently, Al-Khateeb and Al-Qwasemeh (2014) reported comparative assessment of Cd, Cu and Zn impact on growth parameters and genome stability in *Solanum nigrum* L. (wild relative for tomato) using ISSR marker. Indeed, Ghiani *et al*. (2014) applied RAPD marker to investigate combined toxicity and genotoxicty effects of cadmium (Cd) and arsenic (As) on white clover. More recently, Saleh (2015) applied Random amplified microsatellite polymorphism (RAMP) marker to screening DNA changes induced in *U. lactuca*, 5 days after exposure to Pb, Cu, Cd and Zn heavy metals. Molecular markers have been successfully employed and became recently the most efficient tool in plant breeding programs. They were more potent compared to the other methods such as morphological, physiological and biochemical ones in many investigations. Out of these techniques, RAPD marker has been extensively employed to monitoring DNA alterations induced by heavy metals stress (Liu *et al*., 2005; 2007; Azimi *et al*., 2013; Ghiani *et al*., 2014). Jiang *et al.* (2013) reported Cd-toxicity on *Ulva* spp. algae at physiological and biochemical levels. However, very little is known about Cd impact on DNA genetic variation of *U. lactuca* algae grown in Cd metal solution. Thereby, the current study was conducted to investigate Cd-genotoxicity in *U. lactuca* under different Cd concentrations. These alterations have been manifested by variants bands and genomic template stability in comparative study between treated algae and their respective control as revealed by RAPD marker system. This

investigation provides a first report regarding Cd-genotoxicity in *U. lactuca* under different Cd concentrations, at genomic DNA level.

MATERIALS & METHODS

Algal samples for *U. lactuca* marine algae were collected along the Syrian coast of the Mediterranean Sea. Collection of samples was carried out from 34°37'734"N longitude, 38°29'766"E latitude at 4 km North Lattakia - Syria. Only individual with the similar size was harvested by hand with disposable gloves, biomass was washed with seawater where the algae were collected and then transported within a flask with 5 L seawater. *U. lactuca* algae were evaluated under different concentration of Cd ion under Cd $(NO_3)_2$ forma salt. Upon algae arrival to laboratory, they washed twice with autoclaved artificial seawater ASW (500 mM NaCl, 10 mM KCl, 30 mM $MgSO_4$, 10 mM $CaCl_2$ and 10 mM Tris-HCl at pH 7.8) medium as previously described by Unal *et al*. (2010). Then, they divided to a fresh flask with a fresh ASW previously described solution and kept under controlled laboratory conditions (Temperature of 20˚C, photoperiod of 12/12 h dark/light and illumination of 3195 Lux (~ 52.7 µmol photons $m^{-2}s^{-1}$) for 3 days before heavy metals stress application. The mentioned ASW was considered as a control. Cadmium stress was applied by adding Cd (under their nitrate forms) to achieved 0, 2.5, 5 and 10 mg/L as final concentration for each treatment with three replicates/treatment. Experiment was carried out in flask with 300 ml ASW with or without Cd metal. The same previous described controlled conditions were maintained during the experiment stress application. Four days later, algae were harvested for molecular study. Algal genomic DNA was isolated from (bulk of 3 replicates/ treatment) tissues for both of the control and stressed algae by a CTAB (cetyltrimethylammonium bromide) protocolas previously described by Doyle and Doyle (1987) with minor modifications. DNA concentration was quantified by DNA Fluorimeter at 260/280 nm and adjusted to final concentration of 10 ng/µL. DNA was stored at -80 °C until needed. RAPD technique was performed as previously described by Williams *et al*. (1990). Nine RAPD primers belonging to Operon Technologies Inc., USA, ten primers RPi-C01 to RPi-C10, cat number 610692700101730; designed by Merck, India and three primers from the University of British Columbia were employed to monitoring Cd genotoxicity in *U. lactuca* (Table 1).

Table 1. Selected RAPD primers tested in this study

Primer name	Primer Sequence 5' to 3'	Primer name	Primer Sequence 5' to 3'
OPA02	TGCCGAGCTG	RPi-C03	AAGCGACCTG
OPB17	AGGGAACGAG	RPi-C04	AATCGCGCTG
OPC13	AAGCCTCGTC	RPi-C05	AATCGGGCTG
OPD08	GTGTGCCCCA	RPi-C06	ACACACGCTG
OPG05	CTGAGACGGA	RPi-C07	ACATCGCCCA
OPK17	CCCAGCTGTG	RPi-C08	ACCACCCACC
OPQ01	GGGACGATGG	RPi-C09	ACCGCCTATG
OPR12	ACAGGTGCGT	RPi-C10	ACGATGAGCG
OPY10	CAAACGTGGG	UBC132	AGGGATCTCC
RPi-C01	AAAGCTGCGG	UBC159	GAGCCCGTAG
RPi-C02	AACGCGTCGG	UBC702	GGGAGAAGGG

PCR amplification reaction was carried out in 25 µL reaction volume containing 1x PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 25 pmol primer, 1.5 unit of *Taq* DNA polymerase and 50 ng template DNA. PCR amplification was performed in a *T-gradient* thermal cycler (Bio-Rad; T-Gradient). It was programmed to 42 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step for 1 min at 94°C, an annealing step for 2 min at 35°C, and an extension step at 72°C for 2 min, followed by extension cycle for 7 min at 72°C in the final cycle. Then PCR products were separated on a 1.5% ethidium bromide-stained agarose gel (Bio-Rad) in 0.5x TBE buffer. Electrophoresis was performed for 2 h at 85 V and visualised with a UV transillumінator. A VC 100bp Plus DNA Ladder (Vivantis) ladder standard was used to estimate molecular weight of amplification products.

RAPD data analysis was performed by comparing the PCR products profile for control with treated Cd samples at the mentioned Cd applied concentrations.

Genomic template stability value was calculated as previously described by Atienzar *et al*. (2002) according to the following formulate:

$$GTS\% = (1 - a/n) * 100$$

Where (a) was RAPD polymorphic profiles detected in each samples treated and (n) the number of total bands in the control. Polymorphism observed in RAPD profiles included disappearance of a normal band and induction of a new band in comparison to the control RAPD profiles.

RESULTS& DISCUSSION

Twenty-two RAPD primers were employed to monitoring Cd-genotoxicity in *U. lactuca* green algae exposed to different Cd (0, 2.5, 5 and 10 mg/L) concentrations. DNA alteration induced by different Cd

concentration yielded by RPi-C03, RPi-C10, UBC159, OPB17 and OPY10 RAPD primers, was illustrated in Fig. 1.

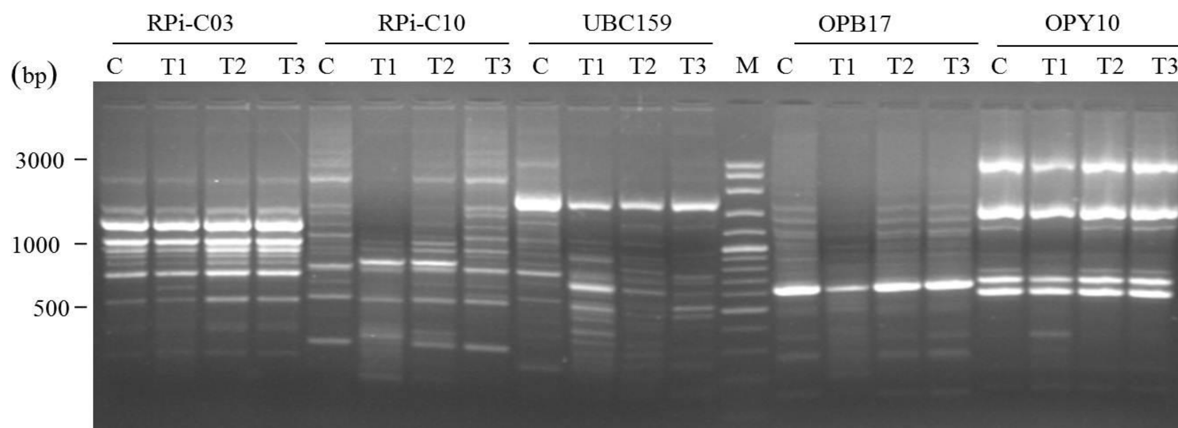

Fig. 1. RAPD profile produced by RPi-C03, RPi-C10, UBC159, OPB17 and OPY10 primers into *U. lactuca*, revealed DNA alterations under different Cd concentrations. M: A VC 100bp Plus DNA Ladder (Vivantis) ladder standard. C: control; T1: 2.5 mg/L, T2: 5 mg/Land T3: 10 mg/L Cd.

Out of the 22 tested RAPD primers, OPQ01 and RPi-C04 gave monomorphic bands between treated algae and their respective control with 6 and 10 common bands yielded by these primers, respectively. Thereby, only 20 RAPD primers were used in this study to detect DNA changes under applied Cd concentrations (Table 2).

Table 2. DNA change patterns under different Cd concentrations (mg/L) as revealed by RAPD marker

Primer	Event	Cd concentrations (mg/L) 0	2.5	5	10
OPA02	-	7	5 (400, 500, 700, 900 & 1000)	ND	ND
	+		2 (350 & 400)	ND	ND
OPB17	-	10	4 (400, 450, 850 & 950)	1 (450)	1 (450)
	+		ND	ND	ND
OPC13	-	6	1 (700)	ND	ND
	+		2 (1200 & 3000)	ND	ND
OPD08	-	6	ND	1 (800)	ND
	+		1 (700)	2 (700 & 900)	1 (700)
OPG05	-	6	2 (1100 & 2500)	3 (500, 1100 & 2500)	1 (500)
	+		3 (150, 200 & 300)	3 (150, 250 & 450)	3 (150, 300 & 450)
OPK17	-	5	2 (500 & 1100)	2 (500 & 900)	1 (900)
	+		3 (400, 450 & 600)	ND	ND
OPR12	-	9	5 (300, 500, 550, 700& 900)	ND	ND
	+		2 (200 & 800)	ND	2 (750 & 800)
OPY10	-	6	2 (700 & 1200)	ND	ND
	+		1 (400)	ND	ND
RPi-C01	-	3	ND	ND	ND
	+		2 (300 & 750)	ND	ND

RPi-C02	-	5	ND	ND	ND
	+		2 (300 & 400)	ND	ND
RPi-C03	-	10	ND	ND	ND
	+		1 (600)	1 (400)	1 (400)
RPi-C05	-	7	ND	ND	ND
	+		1 (300)	1 (300)	1 (300)
RPi-C06	-	4	ND	ND	ND
	+		6 (300, 500, 700, 900, 1350 & 2500)	6 (300, 700, 900, 1350, 1400 & 2500)	4 (300, 700, 900 & 1350)
RPi-C07	-	5	ND	ND	ND
	+		2 (200 & 1500)	1 (1500)	2 (700 & 1500)
RPi-C08	-	7	5 (650, 900, 1200, 1850 & 2500)	2 (900 & 1850)	1 (1850)
	+		5 (350, 500, 550, 600 & 650)	5 (350, 500, 550, 600 & 650)	2 (550 & 600)
RPi-C09	-	5	1 (250)	2 (250 & 600)	1 (600)
	+		5 (300, 400, 450, 700 & 900)	1 (700)	2 (700 & 900)
RPi-C10	-	13	9 (350, 600, 1100, 1200, 1350, 1500, 2000, 2500 & 3000)	ND	ND
	+		3 (200, 380 &800)	2 (400 & 800)	1 (1100)
UBC132	-	9	4 (550, 650, 700 & 1850)	ND	ND
	+		3 (250, 300 & 400)	1 (1850)	ND
UBC159	-	8	4 (300, 550, 1350 & 2500)	3 (300, 550 & 2500)	2 (300 & 550)
	+		6 (350, 400, 450, 500, 600 & 1500)	3 (450, 500 & 900)	3 (450, 500 & 600)
UBC702	-	7	3 (300, 450 & 550)	3 (300, 450 & 550)	2 (450 & 550)
	+		2 (350 & 500)	2 (350 & 500)	1 (500)
Total		**138**	**99**	**45**	**32**

ND: No differences

Where, observed polymorphic bands were varied according to the Cd applied concentrations. In this respect, their estimated values were recorded to be 99, 45 and 32 polymorphic bands at 2.5, 5 and 10 mg/L Cd, respectively (Table 2). From data presented in Table 2, it worth noting that OPA02, OPC13, OPR12, OPY10, RPi-C01 and RPi-C02 RAPD primers failed to produce polymorphism in DNA pattern at all Cd tested concentrations.

DNA alteration profile yielded by Cd treatment as expressed by disappeared bands and appearance of new induced bands compared to their respective control, were presented in Fig. 2.

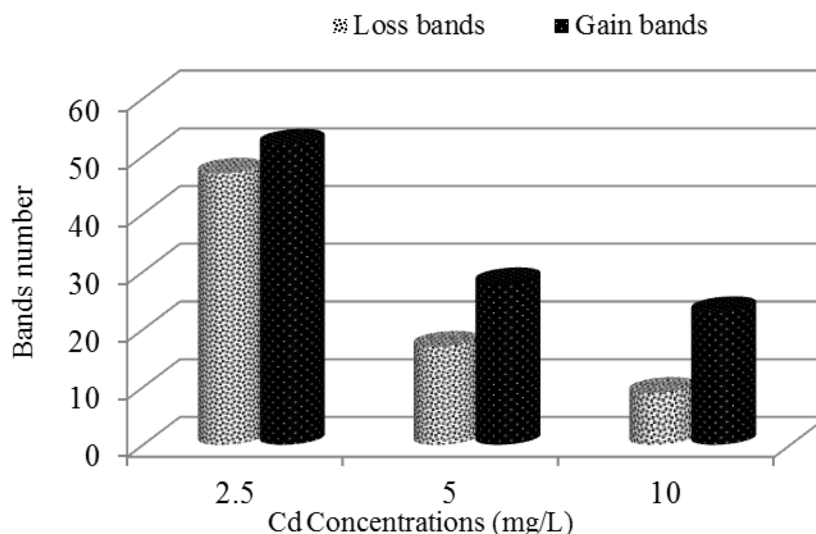

Fig. 2. Number of loss and gain bands induced by different Cd concentrations in *U. lactuca*, 4 days after treatment

Genomic template stability (GTS%) was also calculated as previously reported in many investigations. Our data mentioned that cd stress caused GTS% reduction (Table 3). Where the highest estimated GTS% value (72.8%) was recorded at 10 mg/L Cd; while the lowest one (45.4%) was recorded at the lowest Cd tested concentration (2.5 mg/L).

Table 3. Genomic template stability (%) under different Cd concentrations (mg/L) as revealed by RAPD marker

	Cd concentrations (mg/L)			
Primer name	**0**	**2.5**	**5**	**10**
OPA02	100.0	100.0	100.0	71.4
OPB17	100.0	60.0	90.0	90.0
OPC13	100.0	50.0	100.0	100.0
OPD08	100.0	83.3	50.0	83.3
OPG05	100.0	16.7	0.0	33.3
OPK17	100.0	0.0	60.0	80.0
OPR12	100.0	22.2	100.0	77.8
OPY10	100.0	50.0	100.0	100.0
RPi-C01	100.0	33.3	100.0	100.0
RPi-C02	100.0	60.0	100.0	100.0
RPi-C03	100.0	90.0	90.0	90.0
RPi-C05	100.0	85.7	85.7	85.7
RPi-C06	100.0	50.0	50.0	0.0
RPi-C07	100.0	60.0	80.0	60.0
RPi-C08	100.0	42.9	0.0	57.1
RPi-C09	100.0	20.0	40.0	40.0

RPi-C10	100.0	7.7	92.3	92.3
UBC132	100.0	22.2	88.9	100.0
UBC159	100.0	25.0	25.0	37.5
UBC702	100.0	28.6	28.6	57.1
Average	**100.0**	**45.4**	**69.0**	**72.8**

Genotoxicity of Cd in *U. lactuca* algae was expressed as DNA change patterns in treated algae comparing to their reference control. Many researches mentioned that Cd ion could be directly interacting with DNA leading to DNA alteration patterns according to the applied Cd concentrations (Fusconi *et al*., 2007; Liu *et al*., 2007; Aydın *et al*., 2013).

Data presented herein revealed that UBC159 primer gave the highest polymorphic DNA profile with 10, 6 and 5 polymorphic bands at 2.5, 5 and 10 mg/L, respectively. The observed polymorphic DNA patterns induced by Cd-genotoxicity were monitored by disappearance of normal bands presented in their respective control and by appearance of new bands in treated algae. Based upon data obtained herein, it was found that polymorphic DNA pattern decreased as Cd concentrations increased. In this regards, estimated total polymorphic bands were recorded to be 99, 45 and 32 polymorphic bands that represent polymorphism level of 71.7%, 32.6% and 23.2% at 2.5, 5 and 10 mg/L Cd, respectively. Our findings were in accordance with Aydın *et al*. (2013) who reported that polymorphic bands decreased (29.1, 21 and 25.5%) as Cd concentrations increased from 30 to 120 mg/L in okra (*A. esculantus* L.) using 9 RAPD primers.

Other studies however revealed an inverse tendency. In this respect, Aly (2012) reported that Cd (25, 50 and 100 µM for 21 days) induced 20, 26 and 33 polymorphic bands in Sudan grass and 22, 29 and 33 polymorphic bands in Egyptian clover using 8 RAPD primers, under the above Cd concentrations, respectively. Whereas, Azimi *et al*. (2013) studied DNA variation induced by different Cd concentrations (0 to 120 mg/L) on wheat (*T. aestivum* L.) using 15 RAPD primers. The latter study mentioned that DNA instability at >5 mg/L Cd; compared to the other researches which mentioned that DNA changes was detected at levels above 30 mg/L. The previous investigation reported that polymorphic bands induced by different Cd concentrations in wheat (*T. aestivum* L.) increased as Cd concentration increased from 5 to 120 mg/L using 15 RAPD primers. Whereas, Liu *et al*. (2012) reported the appearance of 20 - 23 mutated loci between untreated and 0.125–3.0 mg/L Cd-treated *Arabidopsis* plants, respectively after 26 days Cd exposure using 12 RAPD primers.

Over all, Cd treatment induced DNA alteration patterns as manifested by disappeared bands and appearance of new induced bands (Fig. 2). In this respect, this value was recorded to be 47, 17 & 9 disappeared bands at 2.5, 5 and 10 mg/L Cd, respectively. Whereas, for the new

induced bands, it was recorded to be 52, 28 & 23 bands at 2.5, 5 and 10 mg/L Cd, respectively. Evidently, loss and gain bands decreased as applied Cd concentrations increased (Fig. 2).

Our data were in coherent with Aydın *et al*. (2013) who investigated Cd genotoxicity in okra after 21 days exposure different Cd concentrations using RAPD marker. Where, the disappeared bands number was found to be 17, 13 & 15 combined with gain bands number of 8, 5 & 7 at 30, 60 and 120 mg/L Cd, respectively. However, Aly (2012) reported an inverse tendency regarding loss and gain bands followed Cd treatment in Egyptian clover and Sudan grass plantlets exposed to different Cd (0, 25, 50 and 100 µM) concentrations during 21 days. In this regards, the loss bands value was recorded to be 17, 21 & 25 and 16, 18 & 21 in Egyptian clover and Sudan grass under 25, 50 and 100 µM Cd, respectively. Whereas, the gain bands number was recorded to be 5, 8 & 8 and 4, 8 & 12 in Egyptian clover and Sudan grass under 25, 50 and 100 µM Cd, respectively.

More recently Saleh (2015) reported the appearance of 41 (34.8%), 42 (35.4%), 48 (40.2%) and 49 (40.7%) variant bands in *U. lactuca*,5 days after exposure to Pb, Cu, Cd and Zn heavy metals, respectively using RAMP marker.

Other investigation however, revealed disappearance of only one fragment with appearance of 12 new bands have been observed in *Urtica dioica* plants under heavy metals treatment compared to their respective control using 7 RAPD primers (Gjorgieva *et al*., 2013). The latter investigation showed that 14 bands in total were yielded, of which 13 bands (92.86%) were polymorphic. Several researches demonstrated that gain or loss bands observed under Cd stress could be related to DNA mutation, while DNA damage could be responsible for loss bands (Atienzar *et al*., 2002; Atienzar & Jha 2006; Liu *et al*., 2005; 2007; Aydın *et al*., 2013). The previous investigations proposed that heavy metal stress induced an alteration in some site of oligonucleotides priming that lead to new bands induction or disappearance of normal ones. Whereas, Ghiani *et al*. (2014) applied RAPD marker to investigate combined toxic and genotoxic effects of Cd and As on white clover. The latter study revealed 130 and 152 reproducible bands as total bands for shoot and root of which 3.52% and 4.62% were polymorphic compared to their respective control. Otherwise, GTS% as a qualitative measurement for monitoring Cd-genotoxicity was estimated (Table 3). This parameter has been extensively employed in many studies to detect DNA changes (mutation or damage) induced by different chemical pollutants (Liu *et al*., 2005; Aly, 2012; Ghiani *et al*., 2014). In this regards, an increase in estimated GTS% value was observed in Cd treatment. Where, GTS% were recorded to be 45.4%, 69% and 72.8% with 2.5, 5 and 10 mg/L Cd, respectively. From Table 3, this investigation suggest that GTS% increased as Cd concentration increased. Our findings were in accordance with Aydın *et al*. (2013) who investigated Cd genotoxicity in okra after 21 days exposure to 0, 30, 60 and 120 mg/L Cd using RAPD marker. The latter study demonstrated that, Cd

treatment caused DNA damages reflected in GTS% values reduction. Where, higher GTS% values were recorded at higher Cd concentration. In this regards, GTS% value was found to be 59, 76.4 and 72.5% at 30, 60 and 120 mg/L Cd in okra using 9 RAPD primers. Whereas, Aly (2012) successfully applied 8 RAPD primers to detect DNA changes in Egyptian clover and Sudan grass plantlets exposed to different Cd (0, 25, 50 and 100 µM) concentrations during 21 days. The latter study demonstrated that, these plants could be used as biomarker to monitoring Cd genotoxicity effects. The previous investigation demonstrated decrease of estimated GTS% as Cd concentrations increased. In this respect, GTS% value was found to be 52.2, 50.2 and 48.3% in Sudan grass; whereas, it was 48.3, 35.7 and 25.5% in Egyptian clover using RAPD marker. Previously, Liu *et al*. (2005) reported that Cd stress reduced GTS% in barley using RAPD marker. Indeed, Cenkci *et al*. (2010) reported that Pb ion induced GTS% reduction in *Brassica rapa* L. Recently, Saleh (2015) investigated DNA changes induced in *U. lactuca*, 5 days after exposure to Pb, Cu, Cd and Zn heavy metals using RAMP marker. The latter study revealed that estimated GTS% value was found to be 65.215, 64.630, 59.835 and 59.250% for Pb, Cu, Cd and Zn treatment, respectively. It is well documented that RAPD or AFLP markers were more sensitive than classic genotoxic tests, even RAPD marker could be considered as a powerful tool for temporary DNA changes screening that might not consequently manifest themselves as mutations (Labra *et al*., 2003). Our data were comparable with the other mentioned investigations, because algae were out of the higher annual plants (wheat, okra, *Urtica dioica,* Egyptian and white colver…). So, they tend to develop different protective mechanisms against the Cd toxic effect when they exposed to different Cd concentrations. Otherwise, in algae, biomass was directly interacted with Cd ions in aqueous solution. While, presence of root system in other plants and their absence in algae, lead the higher plant to develop different other mechanisms to minimize Cd toxicity. As known, Cd is a toxic element even at very low concentrations. In our case study, algae were in better physiological state (vigor) at lower Cd concentration (2.5 mg/L Cd) compared to the other two applied concentrations. This study could suggest that algae tend to graduate death state at 5 and 10 mg/L Cd. This phenomenon could explain the graduate decrease in observed polymorphic bands number.

CONCLUSION

Comparative assessment between control and Cd-treated *U. lactuca* algae genome stability using RAPD marker revealed that this technique could be applied for monitoring Cd-genotoxicity in algae. Thereby, the current study suggested that RAPD marker could be serving as a powerful tool to evaluate Cd-genotoxicity. The current study suggested also the Cd-genotoxicity even at very low Cd concentration (2.5 mg/L). RAPD marker

could be used as powerful technique to detect Cd-genotixicity in algae.

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