Journal of Hazardous Materials 161 (2009) 878–883

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Risk assessment of cadmium-contaminated soil on plant DNA damage using RAPD and physiological indices

Wan Liu[a], Y.S. Yang[b,c,*], P.J. Li[a], Q.X. Zhou[a], L.J. Xie[a], Y.P. Han[a]

[a] KeyLaboratory of Terrestrial Ecological Process, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, PR China
[b] Jilin University, College of Environment & Resources, Changchun 130026, PR China
[c] Cardiff University, School of Earth Sciences, Main Building, Park Place, Cardiff CF10 3YE, UK

ARTICLE INFO

Article history:
Received 24 August 2007
Received in revised form 17 March 2008
Accepted 12 April 2008
Available online 18 April 2008

Keywords:
RAPD
Cadmium (Cd)
Genotoxic effects
Barley seedlings
Physiological indices

ABSTRACT

Impact assessment of contaminants in soil is an important issue in environmental quality study and remediation of contaminated land. A random amplified polymorphic DNA (RAPD) 'fingerprinting' technique was exhibited to detect genotoxin-induced DNA damage of plants from heavy metal contaminated soil. This study compared the effects occurring at molecular and population levels in barley seedlings exposed to cadmium (Cd) contamination in soil. Results indicate that reduction of root growth and increase of total soluble protein level in the root tips of barley seedlings occurred with the ascending Cd concentrations. For the RAPD analyses, nine 10-base pair (bp) random RAPD primers (decamers) with 60–70% GC content were found to produce unique polymorphic band patterns and subsequently were used to produce a total of 129 RAPD fragments of 144–2639 base pair in molecular size in the root tips of control seedlings. Results produced from nine primers indicate that the changes occurring in RAPD profiles of the root tips following Cd treatment included alterations in band intensity as well as gain or loss of bands compared with the control seedlings. New amplified fragments at molecular size from approximately 154 to 2245 bp appeared almost for 10, 20 and 40 mg L^{-1} Cd with 9 primers (one–four new polymerase chain reaction, (PCR) products), and the number of missing bands enhanced with the increasing Cd concentration for nine primers. These results suggest that genomic template stability reflecting changes in RAPD profiles were significantly affected and it compared favourably with the traditional indices such as growth and soluble protein level at the above Cd concentrations. The DNA polymorphisms detected by RAPD can be applied as a suitable biomarker assay for detection of the genotoxic effects of Cd stress in soil on plants. As a tool in risk assessment the RAPD assay can be used in characterisation of Cd hazard in soil.

1. Introduction

Along with the rapid development of modern industry, soil contaminated by heavy metals, e.g. Cd and Cr, is commonly identified in nowadays world. Cd has been released into the environment largely found in the water and soil through anthropogenic activities, such as mining deposits, aerial fallout from smelters and industrial processes etc. Cd has a capability not only to cause morbidity or/and mortality in the exposed organisms, but may potentially result in higher order changes such as alterations to population dynamics and change to biological diversity at both intra- and inter-species levels [1–4]. Such changes may initiate direct and adverse ecological consequences. Genotoxicity of Cd is directly related to its effect on structure and function of DNA, which may be determined using a number of laboratory methods [5,6]. However, there have been few direct experimental demonstrations of the wider relationships between DNA effects and their subsequent consequences at higher levels of biological organization in soil [7–11]. To address this issue, it is necessary to develop reliable and reproducible genotoxicity assays that can then be used in conjunction with traditional assays for detecting any impairment of population parameters (e.g. growth, reproduction, and viability of offspring).

In the soil genotoxicity study, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis. Classical polymerase chain reaction (PCR) assays, simply requiring a target DNA sequence and two synthetic oligonucleotide primers complementary to opposite strands of the target DNA, have been used to detect mutations and DNA damage. Indeed, mutations may affect the annealing of the primers whereas DNA damage may interfere with the DNA polymerase activity, thus altering the number of newly synthesized amplicons [12]. PCR-based protocols have also been used to detect DNA damage and

* Corresponding author.
E-mail address: yangy6@cf.ac.uk (Y.S. Yang).

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doi:10.1016/j.jhazmat.2008.04.038

repair in specific genes and, under appropriate conditions, quantitative data can be obtained [10,13,14].

Despite the advantages of using traditional PCR assays for detecting DNA alterations, there are some potential difficulties: (1) the nucleotide sequence flanking upstream and downstream the target DNA needs to be determined; (2) the size of PCR products plays a crucial role in the detection of DNA damage because the amplification of short fragments (e.g. less than 300 bp) may not be possibly inhibited at all even in the case of extensive DNA damage; (3) it is conceivable that any reduction in the intensity of PCR amplicons could be due to a partial inhibition of PCR reaction caused by factors other than DNA damage such as residual phenol remaining after DNA extraction [15].

Such disadvantages can be eliminated by using the random amplified polymorphic DNA (RAPD) 'fingerprinting' technique, which is based on the amplification of genomic DNA with 10 bp primers of arbitrary nucleotide sequence which anneals to multiple regions of the genomic DNA [9,16]. The PCR reactions thus generate many amplicons of variable lengths (e.g. between 100 and 4000 bp for RAPD) which can be separated by gel electrophoresis to obtain DNA fingerprint. RAPD assays were successfully used to detect genetic instability in DNA alterations in animals, bacteria and plants induced by low doses of pollutants, mainly in the aquatic systems [7,9,12,15,17,18,19]; while a few of the research on DNA alterations in plants induced by soil contamination stress have been reported [6,11,18,20]. The objectives of this study were to detect DNA damage induced by Cd-contaminated soil using the RAPD technique, to compare changes in RAPD profiles with root growth and total soluble protein level in the root tips of barley seedlings measured under laboratory conditions, and to evaluate the usefulness of the RAPD assay in risk assessment of contaminated soil.

2. Materials and methods

2.1. Plant material, growth and total soluble protein level test

We designed experiments to study the effect of soil contamination by Cd, at various concentrations, on plant (barley, *Hordeum vulgare*). Dry mature seeds of barley (cultivar Kepin No. 7 from Nanjing University) were soaked in distilled water at 4 °C for three days and germinated to radicle lengths of 2 mm in a petri dish (diameter 12 cm) containing three pieces of filter paper at 15 °C under dark condition, so simulate the soil condition. Uniformly 20 germinated seeds were selected and transferred to Petri pots (diameter 9.5 cm and height 14 cm) containing soil of quartz sands at sizes of 1.2–2.2 mm (about 40%) and 0.6–1.2 mm (60%), saturated with 400 mL of distilled water or test solution. The growth inhibition test was performed with the above barley plantlets exposed to 0, 10, 20, and 40 mg L^{-1} Cd, respectively (in the form of $CdCl_2$ $2H_2O$ with purity 99.5%) for 10 days. The test solution in each container was checked into target concentrations of Cd as above every other day. Petri pots were incubated in a growth chamber at 21 ± 1 °C and a 16–8 h day–night photoperiod with a light intensity approximately of 8000 lux. Each treatment was replicated three times.

After the 10 days of incubation, the root length was measured using a ruler, and total soluble protein level of root tips in barley seedlings was measured. Inhibitory rate (IR, %) of the above indices was calculated by the following formula:

$$\mathrm{IR} = \left(1 - \frac{x}{y}\right) \times 100 \quad (1)$$

where x and y are the average values detected in the control and each sample treated, respectively.

2.2. DNA isolation, RAPD procedures and estimate of genomic template stability

Approximately 1.5 cm root tips of 20 seedlings were collected, ground in liquid nitrogen, and total DNA from the root tips was extracted using CTAB protocol in previous study [11]. DNA concentration of each sample was quantified fluorimetrically by a Biophotometer. The condition of DNA amplification was optimized following procedure of Conte et al. [17] with some modifications. PCRs were performed in reaction mixtures of 25 μL containing approximately 80 ng of genomic DNA, 1.7 μM primer, 200 μM dNTPs (50 μM each) and 1× reaction buffer. Sequences (5′ → 3′) from primers 1–9 were presented as follows: CTGGCGAACT; TCCGATGCTG; CTGCGCTGGA; CTGAGGTCTC; CTGGGGCTGA; TCATCCGAGG; TC TCCGCCCT; AAAGTGCGGC; ACCTTTGCGG; respectively. Reactions were hot-started at 94 °C for 5 min before the addition of 2.2 U of Taq DNA polymerase. Amplification was carried out in a thermocycler (Little Genius, China) with heated lid for 35 cycles (30 s at 94 °C, 60 s at 38 °C, then 60 s at 72 °C), followed by a final 10 min extension at 74 °C. Control PCRs lacking genomic DNA were run with every set of samples. PCR products were resolved electrophoretically in a 1.4% agarose gel (12 cm × 12 cm × 0.5 cm) run on 0.5× TBE buffer at 0 °C at 100 V for about 3 h and visualized after ethidium bromide staining using the Bio Image Analyzer system (Vendor). Chemicals were ordered from TaKaRa Biotechnology Ltd. (PR China).

Genomic template stability (GTS, %) was calculated as following:

$$\mathrm{GTS} = \left(1 - \frac{a}{n}\right) \times 100 \quad (2)$$

where a is the average number of polymorphic bands detected in each treated sample and n the number of total bands in the control. Polymorphism in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison to control. The average was calculated for each experimental group exposed to different Cd treatments. To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%).

2.3. Statistical analysis

The statistical analyses were carried out using the package software SPSS 10.0 for Windows. Changes in total soluble protein level and root growth were tested statistically by performing one-way analysis of variance (ANOVA). The least significant differences (LSD) test was used to reveal statistical difference.

3. Results

3.1. Effect of Cd on root growth and total soluble protein level

This test was performed to evaluate the inhibitory effect of Cd-contaminated soil on root growth of barley seedlings at various concentrations used in this experiment. Data (Table 1) obtained suggest that root lengths were substantially inhibited with the increase of 10, 20, and 40 mg L^{-1} Cd concentration after the 10-day exposure ($P < 0.05$, <0.01, <0.01, respectively) compared with the control plantlets, indicating a dose-dependent response. These results confirmed that Cd is indeed a toxic agent for barley plants as described previously by Singh and Tewari [21] and Enan [20].

The results obtained for the total soluble protein level in the root tips of barley seedlings in response to Cd pollutant are also shown in Table 1. Total soluble protein levels in seedlings increased slightly ($P > 0.05$) at 10 mg L^{-1} Cd and increased significantly ($P < 0.05$ and $P < 0.01$) along with the increase of Cd concentration in comparison

Table 1
Effects of Cd stress on root length and total soluble level in the root tips of barley seedlings after 8-day exposure

Cd concentration ($mg\,L^{-1}$)	Root in barley seedlings		Total soluble protein of root tips	
	Root length (cm)	Inhibitory rate (%)	Soluble protein level mg/g (FW)	Increase rate (%)
0	10.5 ± 0.5	0	61.8 ± 2.1	0
10	8.9 ± 0.3 [a]	15.2	67.1 ± 1.9	8.6
20	7.4 ± 0.3[b]	29.5	75.7 ± 4.6[a]	22.5
40	6.5 ± 0.4[b]	38.1	85.1 ± 2.7[b]	37.7

[a] $P<0.05$. .
[b] $P<0.01$.

with the control plantlets at 10 days after Cd exposure. There was a positive correlation between Cd concentration and total soluble protein content in barley seedlings, with a correlation coefficient of $r=0.9928$.

3.2. Effect of Cd contamination on RAPD profiles

In total, seventeen 10-mer priming oligonucleotides were used to analyze the PCR products and only nine gave specific and stable results (Fig. 1; Tables 2 and 3). In all cases, RAPD patterns generated by the Cd-exposed plantlets were clearly different from those obtained using control DNA. The results obtained from nine primers are presented in Fig. 1. The principal events observed following the Cd exposure were a variation in band intensity, loss of normal bands and appearance of new bands compared with the normal control seedlings. For instance, extra bands appeared almost for 10, 20 and 40 $mg\,L^{-1}$ Cd respectively, with nine primers (one to four new PCR products; Fig. 1; Tables 2 and 3). Extra bands of molecular size from approximately 154–2245 bp appeared (primer 2, and 8 in Fig. 1; Table 3). Intensity of the new RAPD bands varied with the increase of Cd concentration (e.g. primer 1, and 5 in Fig. 1). The number of disappearing RAPD bands increased with the increasing Cd concentration for nine primers, and bands of molecular size from 144–2639 bp were shown to disappear (primer 3, and 1 in Fig. 1 and Table 3). Furthermore, the decrease in band intensity was particularly obvious for barley exposed to 10, 20 and 40 $mg\,L^{-1}$ Cd for nine primers used in this experiment, whereas an increase in band intensity occurred mainly for 10 and 20 $mg\,L^{-1}$ Cd for primer 1, 2, 4, 6 and 9 (Table 2).

Nine primers gave a total of 129 RAPD fragments ranging from 144–2639 bp in molecular size in the control seedlings (primer 3, and 1, respectively in Fig. 1 and Table 3). Different polymorphic bands were detected at each concentration of Cd for different primers. Value of polymorphisms is $P\%=37.9\%$, 52.7% and 53.4% for 10, 20 and 40 $mg\,L^{-1}$ Cd, respectively. In all cases, polymorphisms were due to the loss and/or gain of PCR fragments in the treated plantlets compared with control. Table 2 also indicates that changed bands observed in RAPD profiles (e.g. disappearance, appearance of bands, decrease and increase in band intensity in comparison to control) in the Cd-contaminated barley seedlings increased dramatically after exposure to Cd but decreased slightly at 40 $mg\,L^{-1}$ Cd of exposure (Table 2). In addition, further experiments confirmed that the variation in band intensities in Fig. 1 was stable and not a consequence of either a change in concentration of template DNA within a certain range or a change in PCR reagent concentration.

Table 2
Changes of total bands in control and of polymorphic bands and varied bands in the root tips of Cd-contaminated barley seedlings for 10 days

No. of primers	Cd concentration ($mg\,L^{-1}$)												
	0	10				20				40			
		a	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*
Primer 1	16	3	3	4	2	2	5	4	1	2	6	4	1
Primer 2	15	2	4	2	1	1	13	1	2	1	14	1	0
Primer 3	21	4	5	7	0	4	7	6	0	3	8	6	0
Primer 4	17	2	1	3	2	1	5	3	1	1	6	3	0
Primer 5	10	2	1	2	0	2	1	2	0	2	1	2	0
Primer 6	7	3	2	1	2	3	2	1	2	2	2	1	1
Primer 7	17	2	4	5	0	4	4	5	0	3	4	5	0
Primes 8	9	3	3	3	0	3	3	3	0	4	3	3	0
Primer 9	17	1	4	4	2	2	6	4	2	1	6	4	1
Total bands	129	22	27	31	9	22	46	29	8	19	50	29	3
a+*b*	–	49	–	–	–	68	–	–	–	69	–	–	–
a+*b*+*c*+*d*	–	89	–	–	–	105	–	–	–	101	–	–	–

Note: *a* indicates appearance of new bands, *b* disappearance of normal bands, *c* decrease in band intensities, and *d* increase in band intensities. *a*+*b* denotes polymorphic bands, and *a*+*b*+*c*+*d*, varied band.

3.3. Comparison of RAPD profiles, root growth and soluble protein content

Results indicate that the general tendency of root growth and GTS of the root tips of barley seedlings was a progressive reduction with ascending Cd concentration in the soil media. In contrast, soluble protein content substantially increased after the exposure to Cd.

In Fig. 2, the genomic template stability, a qualitative measure reflecting change in RAPD patterns, was used to compare the changes of RAPD profiles with modifications in root growth and soluble protein content of root tips in barley seedlings. Following exposure to ascending Cd concentration in the sand media for 10 days, soluble protein content substantially increased. However, root growth and GTS of the root tips in barley seedlings reduced gradually, but GTS stabilized after 20 $mg\,L^{-1}$ Cd of exposure. The plateau effect was ascribed to multiple changes in RAPD profiles (e.g. appearance of new bands, disappearance of normal bands), which tend to counterbalance each other. In other words, the disappeared band was compensated by the low frequency of newly appearing bands at 20 or 40 $mg\,L^{-1}$ Cd concentration for nine primers applied in this experiment.

4. Discussion

In earlier studies, RAPD profiles generated from marine macroalgae exposed to benzo(a)pyrene revealed appearing and disappearing bands in comparison to control RAPD patterns [19]. Changes in DNA fingerprint analyses observed reflected DNA alterations from single base changes to complex chromosomal rearrangements [9]. Similarly, in this experiment, DNA damage induced by Cd soil exposure of 10 days was reflected by changes in RAPD profiles: decrease and increase in band intensity, disappearance of bands, and appearance of new PCR products occurred in the profiles. Moreover, the frequency of band loss was shown to enhance with ascending Cd doses (Tables 2 and 3). The disappearance of PCR products mainly affected the high molecular weight bands

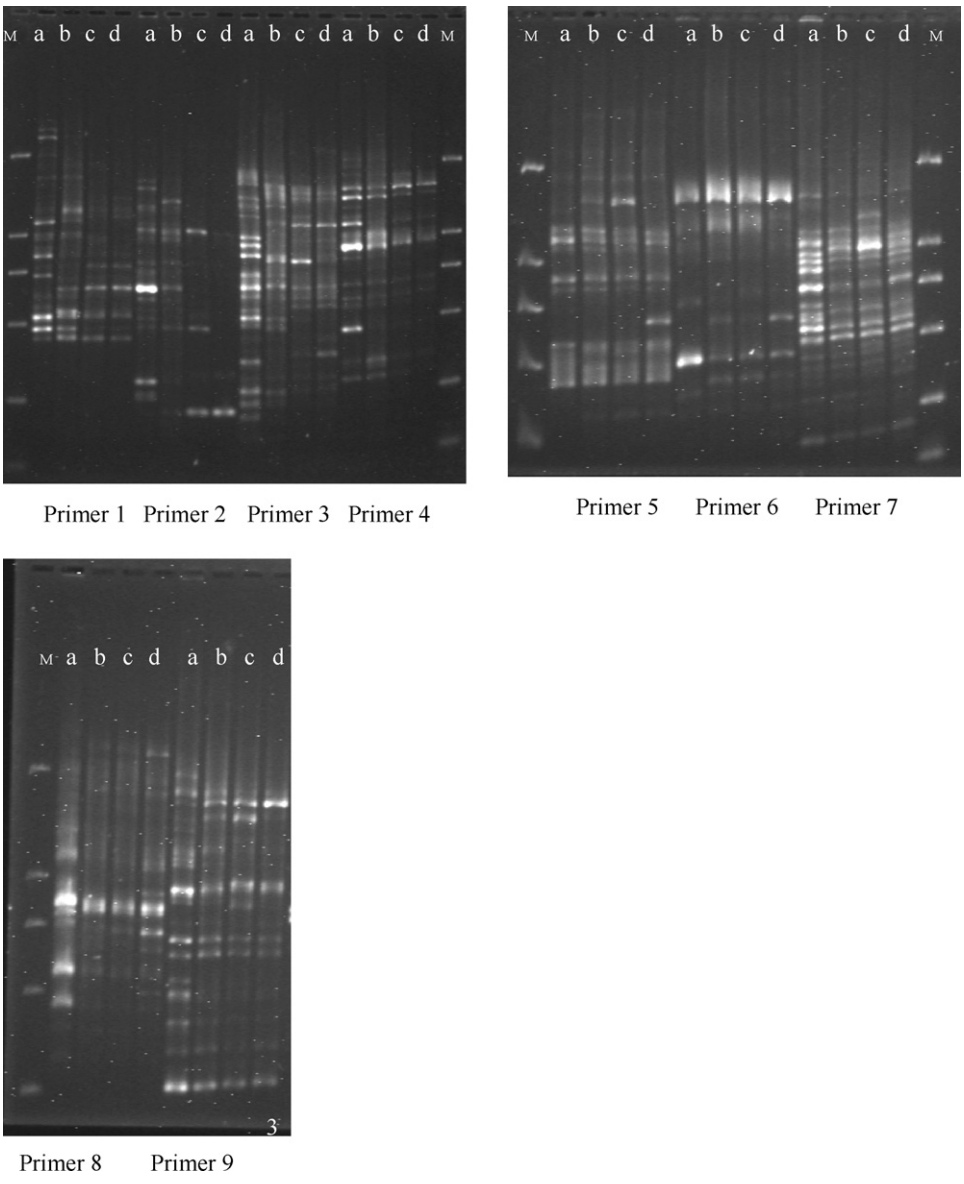

Fig. 1. RAPD profiles of genomic DNA from root tips of barley seedlings exposed to Cd for 10 days. Lane *a* = control; *b* = 10 mg L^{-1} Cd; *c* = 20 mg L^{-1} Cd and *d* = 40 mg L^{-1} Cd, respectively. M, DNA molecular size marker (2000, 1000, 750, 500, 250 and 100 bp from top to bottom).

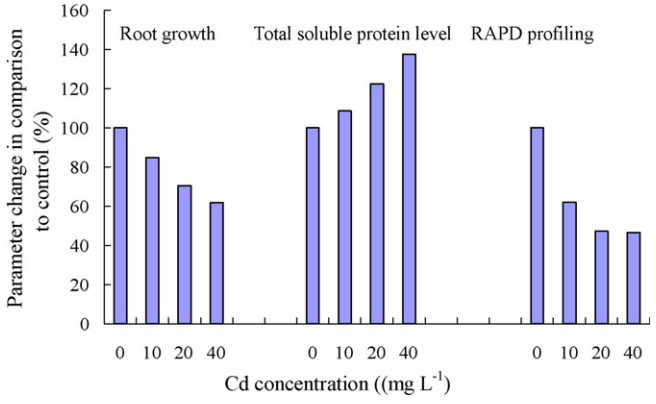

Fig. 2. Comparison of root growth, soluble protein level and genomic DNA template stability in root tips of barley seedlings exposed to various Cd levels.

(i.e., greater than 1 kb) for primers 1–4 and primers 8–9 because the odds of obtaining DNA damage increased with the length of the amplified fragment. However, some smaller amplicons in this experiment were even much more affected for primers 2–3 and primers 5–6 (Fig. 1; Table 3), thus suggesting nonrandom interaction between DNA and Cd contamination in soil. Modifications of the RAPD patterns are likely due to one or a combination of the following events: (1) changes in oligonucleotide priming sites mainly due to genomic rearrangements and less likely to DNA damage and point mutation in the primer binding sites because the binding site is only 10 base long whereas genomic rearrangements occur in much longer fragments (e.g. several kb); (2) important structural changes owning to diverse types of DNA damage and less probably to mutations because the constraint induced by DNA damage such as strand breaks and DNA adduct etc. is more important than a point mutation; (3) interactions of DNA polymerase in barley seedlings with damaged DNA. These events can affect polymerization of DNA in the PCR reaction as reported by Atienzar et al. [19] and Atienzar and Jha [12].

Table 3
Molecular sizes (base pair, bp) of appearance and disappearance of bands for the nine primers in the root tips of Cd-contaminated barley seedlings for 10 days using Software quantity one 4.2.3

No. of primers	Cd concentration (mg L^{-1})		
	10	20	40
Primer1			
+	489; 607; 655	607; 655	607; 655
–	2065; 2449; 2639	1089; 1686; 2065; 2449; 2639	1089; 1686; 1876; 2065; 2449; 2639
Primer 2			
+	154; 1334	154	154
–	198; 242; 1516; 1633	198; 242; 468; 489; 556; 607; 701; 765; 944; 1011; 1251; 1516; 1633	198; 242; 428; 468; 489; 556; 607; 701; 765; 944; 1011; 1251; 1516; 1633
Primer 3			
+	772; 1055; 1422; 1532	772; 1055; 1422; 1532	772; 1055; 1422;
–	144; 299; 500; 883; 1149	144; 299; 428; 468; 681; 883; 1278	144; 299; 428; 468; 681; 883; 1149; 1278
Primer 4			
+	289; 743	743	743
–	1149;	794; 1149; 1251; 1856; 1979	530; 794; 1149; 1251; 856; 1979
Primer 5			
+	213; 1414	213; 1414	213; 533
–	361	361	361
Primer 6			
+	308; 537; 904	308; 537; 904	308; 537;
–	273; 645	273; 645	273; 645
Primer 7			
+	582; 954	431; 582; 954; 1256	431; 582; 954
–	541; 563; 600; 904	541; 563; 600; 904	541; 563; 600; 904
Primer 8			
+	650; 906; 2245	650; 906; 2245	491; 650; 906; 2245
–	803; 956; 1160	803; 956; 1160	803; 956; 1160
Primer 9			
+	1498	860; 1498	1498
–	866; 1189; 1391.1 1981;	866; 1123; 1189; 1201; 1391; 1981;	866; 1123; 1189; 1201; 1391; 1981

+ Indicates appearance of a new band, – disappearance of a normal band.

Previous studies have also shown that DNA fingerprinting offers a useful biomarker assay in soil toxicology study, changes in RAPD profiles induced by pollutants can be regarded as changes in genomic DNA template stability and this genotoxic effect can be directly compared with alterations in other parameters [11,19]. In this experiment, genomic DNA template stability was more sensitive than soluble protein level in the root tips and was of at least equal sensitivity to root growth (Fig. 2; Table 1). Labra et al. [22] reported that RAPD or amplified fragment length polymorphism was more sensitive than classic genotoxic tests since RAPD analysis was capable of detecting temporary DNA changes that may not finally manifest themselves as mutations. The present results of Cd soil exposure in barley seedlings are consistent with these earlier studies on other organisms.

The measure of molecular and population parameters present several advantages. First of all, in soil toxicology, it is fundamental to accumulate data at different levels of biological organization in order to fully understand the effect of a contaminant on organisms. Secondly, the measure of some parameters at the population level facilitates the interpretation of the results at the molecular level [7]. For example, an obvious reduction in growth of *Daphnia magna* correlated with a significant inhibition in DNA replication, suggesting that the extent of DNA damage may be important in the majority of the cells. In this experiment, it seems that for Cd concentrations used, DNA replication was significantly reduced due to a higher level of DNA damage. On the other hand, as root growth and total soluble protein content in the range of 10–40 mg L^{-1} Cd displayed a negative tendency compared to the control (Fig. 2), it can be assumed that DNA damages were repaired to some extent and that DNA replication was not totally inhibited.

Recently, RAPD assay allowed detection of low doses of pollutants mentioned as above. The most significant advantages of the RAPD technique lie in its rapid speed, no radioactivity or enzymatic degradation of PCR products, applicability to any organisms, and potential to detect a wide range of DNA damage and mutations including point mutations and large rearrangements [6,9,20]. However, RAPD is only a qualitative method. Effect of each category of DNA damage (e.g. strand breakage, modified bases, abasic sites, oxidized bases, and bulky adducts) on RAPD profiles can only be speculated unless amplicons are analyzed (e.g. sequencing) and more specific methods such as the comet assay and ^{32}P-post-labelling assay are needed to obtain quantitative data [23].

5. Conclusions

The RAPD method has successfully been used as a sensitive means of detecting Cd-induced DNA damage and showed potential as a reliable assay for soil genotoxicity. The results indicate that inhibition of root growth and increase of total soluble protein level in the root tips occurred with the increasing Cd concentrations. From RAPD, nine 10 bp RAPD primers were found to produce unique polymorphic band patterns which were used to produce a total of 129 RAPD fragments of 144–2639 bp in molecular size in the root tips of control. The change occurring in RAPD profiles of the root tips following Cd treatment presents alterations in band intensity, gain or loss of bands compared with control. New amplified fragments of 154–2245 bp in molecular size appeared for 10, 20 and 40 mg L^{-1} Cd. The number of missing bands enhanced with the increasing Cd concentration and the genomic template stability reflecting changes in RAPD profiles were significantly affected.

This study suggests that the RAPD analysis used in conjunction with other biomarkers from higher levels of biological organization, such as growth parameter, can be a powerful tool for detection of genotoxic effects of metal contamination in soils.

Acknowledgements

This work was supported by Knowledge Innovation Project (kzcx1-yw-06-03), 973 Project (2004CB418506), NSFC (20377043), the Royal Society BP International Incoming Fellowship (UK) and MoE Changjiang Scholars Program (China).

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