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Evaluating Wastewater-Induced Plant Genotoxicity Using Randomly Amplified Polymorphic DNA

K. M. Swaileh, R. Hussein, A. Ezzughayyar

Department of Biology and Biochemistry, Birzeit University, P. O. Box 14, West Bank, Palestinian Authority

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ABSTRACT: Wastewater often contains genotoxic substances that can resist different stages of the treatment process. In the present study, randomly amplified polymorphic DNA technology was applied to evaluate the genotoxic effects of wastewater (treated and raw) irrigation on oat plants (*Avena sativa*). RAPD profiles obtained showed that both treated and raw wastewater (RWW) were having genotoxic effects on oat plants. This was apparent by the appearance/disappearance of bands in the treatments compared with the control plants. From the 15 primers used, 186 bands were obtained with an average of 12.4 bands per primer. Irrigating plants with RWW caused 51 new bands to appear and 19 to disappear. Treated wastewater (TWW) caused only 16 new bands and the loss of 17 bands. This makes TWW less genotoxic than RWW. The Euclidean distances shown on the dendrogram, revealed the presence of two clusters according to dissimilarity values. One cluster contained the control plants and those irrigated with TWW, whereas the second contained the plants irrigated with RWW. Similarity indices calculated between the treatments and the control plants showed that the control and the plants irrigated with TWW had a similarity index of 0.87, the control and plants irrigated with RWW 0.73 and between the treatments 0.75.
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Keywords: genotoxicity; RAPD; *Avena sativa*; wastewater

INTRODUCTION

Increasing demand for the limited water supplies in arid and semiarid regions leads to the use of treated wastewater (TWW) for irrigation purposes. Wastewater (especially of industrial origin) contains thousands of chemicals, like: polychlorinated biphenyl (PCBs), polycyclic aromatic hydrocarbons (PAHs), *N*-nitroso compounds, aromatic amines, and heavy metals (White and Rasmussen, 1998). These chemicals might contaminate valuable resources, like water and soil and food, causing environmental and health threats. Besides, several studies have revealed that TWW often still contains mutagens (genotoxic substances) that can not only injure the integrity of the genome of organisms but also negatively affect the expression of DNA directly or indirectly (Shugart and Theodorakis, 1994; Filipic and Toman, 1996; Grisolia et al., 2005). This leads to an increase in different types of tumors and subsequently an alteration in the genetic variability of the exposed populations over time (Zhiyi and Haowen, 2004). These facts make it imperative to establish methods to evaluate and monitor genotoxicity caused by these chemicals in wastewater.

Recently, molecular geneticists have provided a number of new, fast, and reliable methods for genotoxicity measurements. One of these is the Random Amplified Polymorphism DNA (RAPD) technology. This technique, developed by Williams et al. (1990) and Welsh and McClelland (1990), is a powerful technique that involves the

Correspondence to: Dr. K. M. Swaileh; e-mail: kswaileh@birzeit.edu

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amplification of random segments of genomic DNA using Polymerase Chain Reaction (PCR). Since RAPD is simple, fast, and sensitive, it is now being used in many different fields, such as phylogeny, taxonomy, genotoxicity, and epidemiology (Marillia and Scoles, 1996; Fancelli et al., 1998). The results of RAPD allow the evaluation of how environmental pollutants modify the structure of DNA in living organisms (Conte et al., 1998; Zhiyi and Haowen, 2004).

Higher plants provide a useful genetic system for screening and monitoring environmental pollutants (Sandermann, 1994). They are good indicators of cytogenetic and mutagenic effects which can be applied both indoors and outdoors. Therefore, mutagenic activity of wastewater and other pollutants has been tested with different plant systems such as *Allium cepa*, *Arabidopsis thaliana*, *Hordeum vulgaris*, *Vicia faba*, and *Zea mays* (Cabrera and Rodriguez, 1999; Nielsen and Rank, 2004; Grisolia et al., 2005; Ma et al., 2005; Abdel Migid et al., 2007). With these systems, chromosome aberration assays, mutation assays, and search for chlorophyll-deficient mutants and chlorophyll spot mutation were performed.

The Wastewater Treatment Plant at Birzeit University Campus uses the activated sludge process; a biological process requiring a considerable amount of energy and generating large amounts of organic sludge. This treatment plant is unique as it treats chemical and domestic wastewater. Thousands of chemicals originating from university laboratories reach this treatment plant. Treated water resulting from this plant is used to irrigate garden plants within the University Campus.

The present aims to evaluate the genotoxic effect of influent wastewater reaching the University Treatment Plant and evaluate the efficiency of the treatment process in removing genotoxicants from effluent wastewater. The study uses RAPD technology to evaluate the genotoxic effects and the plant used was oat (*Avena sativa*) irrigated with treated and raw wastewater (RWW).

MATERIALS AND METHODS

Plant Material

Oat for physiology (*Avena sativa var.,* Carolina Co., Burlington, NC) plants were used in this study. Oat seeds were surface-bleached in 5% sodium hypochlorite (NaOCl, Sigma, St. Louis, MO) for 10 min. Then, seeds were washed with sterile double distilled water. Thereafter, seeds were imbibed in tap water for about 3 h before seeding in plastic beakers containing acid-washed sand. Beakers were divided into three treatments (control), treated wastewater (TWW), and raw wastewater (RWW). Each treatment was run in triplicate with 5 seeds/beaker. Seeds were allowed to germinate and grow at room temperature while irrigated with tap water. Treated and raw wastewaters were collected on daily basis from the storage tank and equalization tank of the treatment plant, respectively. After 2 weeks, shoots from plants reaching about 10 cm length were taken from each treatment group and pooled for DNA extraction. To calculate germination percentages, the same experimental design was repeated and data from both experiments were used in the calculations and statistical analysis.

DNA Extraction

Shoots were thoroughly washed with double distilled water. Thereafter, DNA was extracted from the shoots using The MasterPure^{TM} Plant Leaf DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) following the manufacturer's procedure as follows: about 0.5 g of fresh plant leaves was ground in a 1.5 mL microcentrifuge tube containing 300 μL of Plant DNA Extraction Solution. The ground tissue was incubated at 70°C for 30 min, transferred to ice and chilled for 10 min. Cellular debris was collected by centrifugation in a microcentrifuge for 5 min at 10,600 $\times$ g. The supernatant was transferred into a clean microcentrifuge tube and the centrifugation step was repeated to remove any residual debris. Thereafter, the supernatant was transferred into another microcentrifuge tube. Then, an equal volume of isopropanol was added to the clarified supernatant and mixed thoroughly by inversion and DNA was collected by centrifugation in a microcentrifuge for 5 min at 10,600 $\times$ g. The supernatant was removed and the pelleted DNA was completely resuspended in 100 μL Cleanup Solution. After that, 100 μL of isopropanol were added to the resuspended DNA and mixed thoroughly by inversion. At the end, DNA was pelleted again by centrifugation in a microcentrifuge for 5 min at 10,600 $\times$ g. The DNA pellet was washed with 70% ethanol, after which ethanol was removed carefully and discarded. Then, the DNA pellet was resuspended in 50 μL of TE buffer. Finally, DNA yield was calculated by spectrophotometry (Spectronic 601) at 260 nm. The index of DNA purity (OD 260/280) was found to be 1.85.

RAPD Analysis

Fifteen decamer primers (Table I) purchased from Invitrogen, Carlsbad, CA, were used in this study. The amplification reaction volume was 50 μL and contained 3 mM $MgCl_2$, 0.3 mM dNTPs, 1.4 μM of the primer, and 1.25 U *Taq* DNA polymerase (Promega, San Luis Obispo, CA). Two hundred nanograms of template DNA were used in each PCR.

The amplification mixture was overlaid with mineral oil (Promega, San Luis Obispo, CA) and subjected to the following PCR cycling program: initial denaturation for 5 min at 95°C, followed by 40 cycles of 1 min 95°C, 1 min 35°C, 2 min 72°C in a Hybaid Omni-Gene thermocycler. Amplification products were resolved on 1.5% agarose.

TABLE I. Primers used in this study and the total number of bands and polymorphic bands generated by RAPD analysis of oat (*Avena sativa*)

RAPD Primer	Sequence 5′→3′	G-C Content (%)	Total Bands	Polymorphic Bands
1	AGTGCTACGT	50	10	9
2	CTCTCCGCCA	70	11	3
3	CAGGCCCTTC	70	7	3
4	GGTCCCTGAC	70	6	1
5	AGGGAACGAG	60	9	6
6	GGGTAACGCC	70	8	3
7	CTGATGCTAC	50	13	10
8	CGGTGGGGAA	70	18	8
9	GAGAGGCACC	70	10	4
10	GTTGCCAGCC	70	13	3
11	TGCCGAGCTG	70	14	3
12	GGTGCGGGAA	70	14	9
13	CCCGTCAGCA	70	15	9
14	AAGAGCCCGT	60	20	9
15	CAATCGCCGT	60	18	6
Total			186	86

Gels were prepared and run in TAE buffer. Then, DNA was stained with ethidium bromide (Sigma, St. Louis, MO) and detected using UV light. Polaroid Camera (Gelcam) was used for gel documentation using instant black and white FUJIFILM (FP-3000B).

Before running the randomly amplified polymorphic DNA (RAPD) analysis, the consistency of the method was detected using DNA from different control plants and three different primers (Fig. 1). This is important to check the repeatability of the method and the integrity of the DNA.

Band Scoring and Data Analysis

Each change observed in RAPD profiles (appearance/disappearance of bands compared was control) was scored. The presence or absence of a band was determined by making a binary matrix (1 for band presence and 0 for absence) for each sample analyzed (Padmesh et al., 1999). Numerical analysis based on banding patterns obtained from treatments (Treated and Raw wastewater) was compared with the control via hierarchical cluster analysis. A dendrogram was created by the between groups linkage method using squared Euclidean distance measurement (Enan, 2006). All calculations were done and graphs were plotted using the computer statistical program "SYSTAT for windows version 11" (Systat software Inc., 2004). Finally, genotoxicity judgments were made on the basis of the distance between the specimens.

Similarity Index

DNA fingerprint similarities between treatments and the control, and between the treatments were calculated according to Nei and Li (1979) as:

$$S_{xy} = 2n_{xy}/n_x + n_y$$

where, S_{xy}, is the similarity index between two organisms x and y; n_{xy}, is the number of common bands between two organisms x and y; n_x, is the total number of bands of organism x; n_y, is the total number of bands of organism y.

RESULTS AND DISCUSSION

The germination of oat seeds was variable between the three treatments. Table II shows the germination percentages of oat seeds irrigated with tap water, TWW, and RWW. The statistical analysis (ANOVA) indicated that the

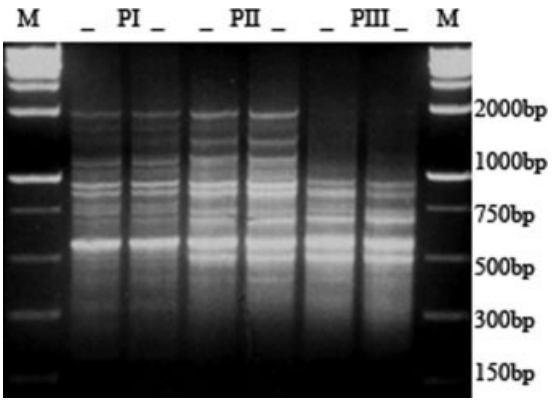

Fig. 1. Reproducibility of RAPD fingerprinting generated from different control oat plants (*Avena sativa*) using three different primers. M, stands for the PCR marker. PI–III, are three different primers.

TABLE II. Experimental design and germination rates of oat seeds (*Avena sativa*) irrigated with tap water (control), treated wastewater (TWW), and raw wastewater (RWW)

	Germination			
Treatment	Experiment 1	Experiment 2	Total	%
Control	5, 4, 5	5, 5, 5	29/30	97
TWW	4, 4, 3	4, 3, 3	21/30	70
RWW	2, 3, 1	3, 2, 1	14/30	47

Number of seeds in each triplicate is 5.

germination rates of the three treatments were statistically different ($p < 0.001$). Post Hoc pairwise test showed that the germination rates of the control groups were significantly higher than those irrigated with TWW and RWW ($p < 0.005$). In addition, the germination rates of seeds irrigated with TWW were significantly higher than those irrigated with RWW ($p < 0.013$).

The DNA purity index was found to be 1.85 indicating a high degree of DNA purity. RAPD consistency (Fig. 1) showed that the method was consistent and the DNA of oat plants used gives the same banding pattern with the same primer. This indicates also that the integrity of the DNA isolated was good.

From RAPD analysis, a total of 186 bands were obtained using the 15 decamer primers. Each primer generated between 7 and 20 bands with an average of 12.4 bands per primer. Padmesh et al. (1999) used 10 decamer primers to assess genetic variability in *Andrographis paniculata* and obtained an average of 6.1 bands per primer. Yoon and Kim (2001) used 20 decamer primers to study genetic similarity and diversity between two populations of Korean catfish (*Silurus asotus*) and obtained 8.2–13.6 bands per primer. From the total number of bands (186) obtained in this study, 86 (46%) were found to be polymorphic (different) (Table I). Other studies obtained similar percentage of polymorphic bands, 48% (Enan, 2006) and 56.4%–59.6% (Yoon and Kim, 2001). However, some other studies (Padmesh et al., 1999) obtained much higher percentage (83.56%) of polymorphic bands.

The RAPD profiles of control and the treatments generated from the 15 primers are shown in Figure 2. RAPD profiles of the control plants and treatments showed differences in the banding patterns. This is clearly exhibited by the appearance/disappearance of some bands when treatments and control are compared. These differences were observed in all RAPD profiles. However, some primers resulted in alteration of a few amplification products (Fig. 2, p4), while others gave more complicated patterns of band gains or losses (Fig. 2, p14). All bands generated ranged between 150 bp and 2000 bp. This range is in accordance with other studies. Enan (2006) analyzed the genotoxic effects of heavy metals on kidney bean (*Phaseolus vulgaris*) using RAPD analysis. His RAPD profiles obtained from six

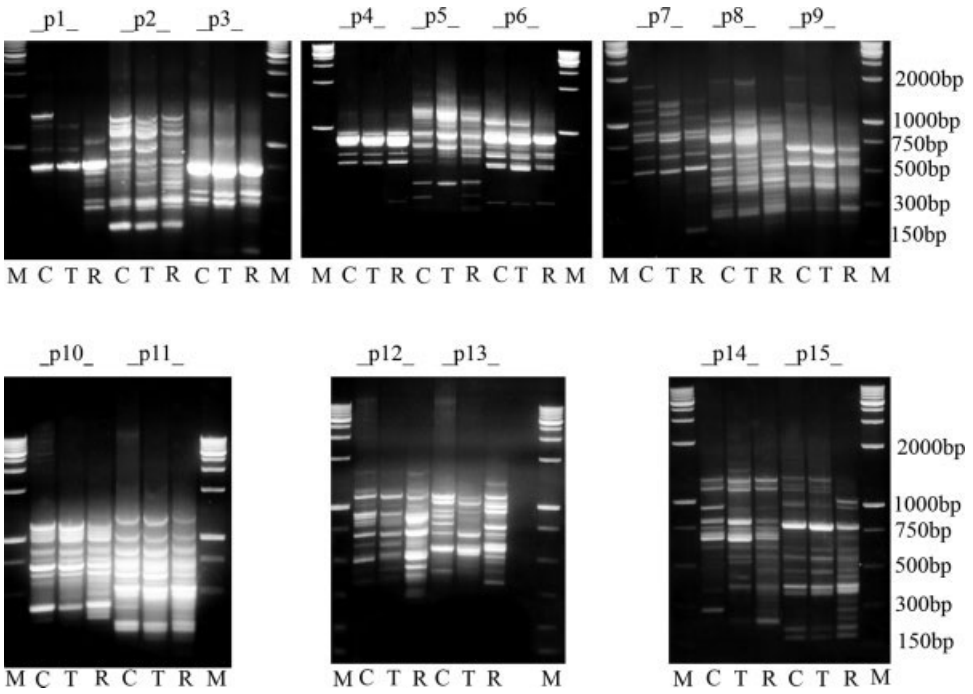

Fig. 2. The RAPD fingerprinting derived from oat plants (*Avena sativa*) irrigated with wastewater (treated 'T' and raw 'R') and those irrigated with tap water (control 'C'). M, stands for the PCR marker. P1–15, are the primers used in this study.

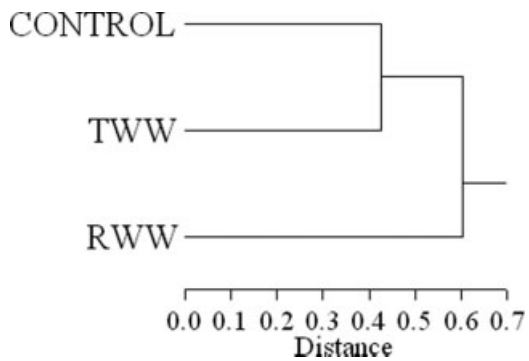

Fig. 3. Dendrogram obtained from RAPD profiles using Euclidean distance constructed with tap water-irrigated control oat plants (*Avena sativa*) and those irrigated with wastewater. TWW: treated wastewater, RWW: raw wastewater.

primers exhibited bands between 200 bp and 1600 bp in length. Yoon and Kim (2001) obtained bands ranging between 190 bp and 1350 bp from 20 decamers used to detect differences between two populations of Korean catfish (*Silurus asotus*).

Compared with the control plants, those irrigated with TWW recorded the emergence of 16 new bands and the disappearance of 17 bands. However, plants irrigated with raw wastewater yielded 51 new band and 19 bands disappeared. This makes the total number of bands that disappeared because of both treatments 36 and that for the bands that emerged 67. The results illustrate that raw wastewater was more genotoxic than TWW. In total, the number of bands that emerged was almost twice as much as those who disappeared. Enan (2006) found that 22 new fragments appeared and 43 disappeared as a result of using 350 mg^{-1} heavy metals to irrigate kidney beans. Less band appearance/disappearance was observed when using 150 mg^{-1}. The disappearance of bands may attribute to the presence of DNA photoproducts (like pyrimidine dimmers, 6–4 photoproducts), which can act to block or reduce (bypass event) the polymerization of DNA in the PCR reactions (Donahue et al., 1994; Nelson et al., 1996). However, new fragments can be amplified because some sites become accessible to the primer after structural changes in the DNA take place (Pietrasanata et al., 2000; Enan, 2006). This could be due to point mutations and/or large rearrangements of the DNA. A single point mutation within the primer site can generate significant changes in RAPD patterns (Williams et al., 1990).

The cluster analysis method is considered one of the most effective methods in numerical computation regarding band scoring and analysis of RAPD fingerprinting. It can calculate the distances between every pair of entities and then summarize the community data sets (Gauch, 1995). In the present study, cluster analysis was applied to construct dissimilarity values and estimate the level of DNA polymorphism between the control plants and those irrigated with treated or raw wastewater. The Euclidean distances shown on the dendrogram (Fig. 3) between control plants and those irrigated with TWW and raw wastewater were 0.428 and 0.605, respectively. This indicates that even TWW can induce some genotoxic effects in the plants. However, raw wastewater produced more genotoxic effects. This is clearly demonstrated by the dendrogram obtained where the control plants and those irrigated with TWW are joined together in a cluster at a shorter distance (0.428) than that for the plants irrigated with raw wastewater (0.605). These results indicate that the treatment process in the University treatment plant removes some, but not all, genotoxic chemicals from influent wastewater. This reduces the DNA damage caused by pollutants in wastewater used to irrigate plants.

Similarity indices, which measure the proportion of shared fragments in the amplification profile, were calculated and are shown in Figure 4. Oat plants irrigated with tap water and those irrigated with TWW showed genetic similarity reaching 87%. Plants irrigated with raw wastewater showed clearly less genetic similarity (73%) to the control ones. In addition, plants irrigated with TWW showed 75% similarity to those irrigated with raw wastewater. This is in accordance with the results obtained from the cluster analysis, where the control and treated water-irrigated plants grouped in one cluster and the plants irrigated with raw wastewater were grouped in a separate cluster joined at a larger distance.

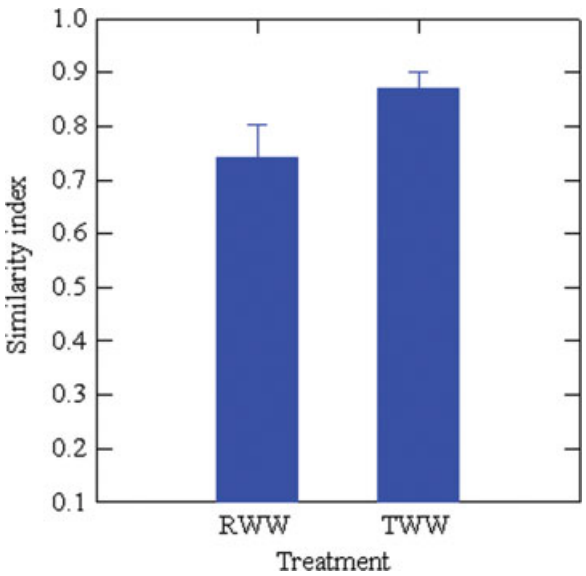

Fig. 4. Genetic similarity between oat plants (*Avena sativa*) irrigated with raw wastewater (RWW) and treated wastewater (TWW) and those irrigated with tap water (control). Error bars represent ± standard errors of the mean values from 15 primers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

These results indicate that both treated and raw wastewaters generated from Birzeit University Wastewater Treatment Plant are genotoxic. The results demonstrated clearly that raw wastewater is much more genotoxic than TWW. Therefore, the treatment process in this treatment plant removes some, but not all, genotoxic substances from wastewater. This makes reusing TWW from this treatment plant for irrigation purpose not a recommended practice.

As a result, treated effluents from this treatment plant and other plants treating industrial or mixed wastewater are not good enough to be used in irrigation of plants. If this TWW is going to be used for irrigation of fodder plants, then the efficiency of the treatment process should be further improved to eliminate more genotoxins. In addition, wastewater from laboratories can be separated from other domestic wastewater reaching the treatment plant from other University facilities.

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