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Evaluation of cytotoxic effects of atrazine and glyphosate herbicides on *Biomphalaria glabrata* snails

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Abstract *Biomphalaria glabrata* snails were used as bio-indicators for freshwater, atrazine, and glyphosate herbicides. SDS–PAGE protein profiles of *B. glabrata* snails, isozyme analysis (α- and β-esterase, peroxidase and catalase) and RAPD-PCR were used to detect the genotoxicity caused by the two tested herbicides. SDS–PAGE protein profiles showed bands with different molecular weights (53–220 and 1.82–12.5 KDa). The low molecular weight bands appeared in all treated groups except those exposed to 5 ppm glyphosate for 3 weeks. Isozyme analysis revealed three zones of activity (loci), in both α- and β-esterase. On the other hand, peroxidase and catalase showed one locus with different band intensities. Of six arbitrary 10-mer primers, only three primers gave detectable amplifications. A 500 bp band was generated by OPA-10 in all treated groups except in the control and in 5 and 10 ppm atrazine-exposed groups for 2 weeks. A diagnostic band with 400 bp was observed only in 10 ppm atrazine treated group for 3 weeks. Our results indicated that those atrazine and glyphosate herbicides may be considered to be highly genotoxicant agents.

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Introduction

Herbicides, specifically made for weed control, composed of a heterogeneous family of chemical products. They can purposely or indirectly reach aquatic ecosystems, through soil surface run off or from areas where they are applied (Tanguy et al., 2005).

Biomphalaria glabrata snails are vulnerable to various aquatic pollutants from agricultural or industrial wastes. Those pollutants include herbicides such as atrazine, diurons, isoproturon, simazine, alachlor, metolachlor, and recently glyphosate (Tanguy et al., 2005).

The pollutants, especially herbicides, can disturb the aquatic organisms at the cellular, physiological and molecular levels (Fournier et al., 2000). Although, studies regarding the biological effects of herbicides have increased over the last years, the results on the genotoxicity of these products are often incomplete and sometimes contradictory (Siu et al., 2008). The genotoxic effects of pollutants can be monitored using vivo tests. Micronuclei test and comet assay are the most widely utilized tests in genotoxic evaluations, mainly because of the sensitivity of both in detecting DNA damage and their rapid performance (Dimitrov et al., 2006).

In Egypt, the two herbicides, atrazine and glyphosate are usually used. They are utilized under the commercial names Atrazex and Herfosat, respectively. The glyphosate-based herbicides are most often applied and their utilization increased in the period of 2000–2004 (Battaglin et al., 2005).

Isozyme analysis may be used as a bio-marker assay for molecular changes occurred in organisms exposed to the environmental stress such as accumulation of pollutants (Zadory and Muller, 1981). De Wolf et al. (2003) demonstrated that toxic compounds may interfere with the genetic constitution of populations, either directly through mutagenic activity, or indirectly via population-mediated processes.

The RAPD-PCR technique is considered to be a useful tool to assess toxicological population genetic effects of environmental chemical stress, particularly since this technique is relatively inexpensive and yields information on a large number of loci without having to obtain sequence data (Vernon et al., 1995). Conte et al. (1998) showed that RAPD-PCR can be used to evaluate how the environmental pollutants modify the structure of DNA in living organisms. These modifications in DNA structure based on band intensity gain/loss and band numbers are compared with the control (Atienzar and Jha, 2006).

The aim of this work was to evaluate the genotoxic effects in *B. glabrata* snails exposed to herbicides atrazine and glyphosate using SDS–PAGE protein profiles, isozyme assay, and RAPDs.

Materials and methods

Snails

Adult *B. glabrata* snails with shell 8-10 mm diameter were from the Schistosomiasis Biological Supply Center (SBSC), Theodor Bilharz Research Institute, Giza, Egypt. Prior to the toxicity experiments, the snails were acclimated for 4 weeks to the laboratory conditions (pH = 7.4 ± 0.2 and $T = 27 \pm 2$ °C) in plastic containers with dechlorinated tap water under constant aeration.

Toxicity experiments

Serial dilutions were prepared from atrazine and glyphosate to determine the sub-lethal concentration. The snails were fed on dried lettuce leaves, *B. glabrata* snails were exposed to sub-lethal concentration for 3 weeks and their feeding continued during the toxicity tests.

The snails were separated into five groups with 15 snails each. The 1st and 2nd groups were exposed to 5 and 0.5 ppm of glyphosate for 3 weeks, respectively. The 3rd and 5th groups were exposed to 10 and 5 ppm of atrazine for 3 weeks, respectively. One negative control group (5th group), exposed only to dechlorinated water was used. The contaminated water was renewed every three days.

Protein and isozyme analysis

Total protein contents were extracted from 0.02 g of either the exposed or the control snail tissues using 200 µl of 0.1 M Tris-buffer (pH = 7.5) containing EDTA (0.01 M), KCl (0.01 M), $MgCl_2$ (0.1 M) and 4% polyvinyl pyrrolidine (PVP). Tissue homogenates were centrifuged at 8050*g* for 20 min at 4 °C (Sigma 3K18 cooling centrifuge). The protein extract was then transferred into a fresh eppendorff tube and used directly for electrophoresis or kept at −20 °C until use (Gottlieb, 1981). SDS–PAGE analysis was performed on 12% polyacrylamide gel according to Laemmli (1970). 25 µl of the protein extract was mixed with equal volume of SDS-sample buffer and heated in a boiling water bath for 3 min. 25 µl of the boiled mixture was loaded onto gels. Electrophoresis was carried out at a constant current of 25 mA/gel.

Four isozymes α-esterase (α-Est), β-esterase (β-Est), peroxidase (Per) and catalase (Cat) were examined on 10% acrylamide separating gel (Laemmli, 1970), on a native without SDS. 25 µl aliquots of the total protein extract were electrophoresed on 10% vertical slab polyacrylamide gels using 15 mA/gel at low temperature for approximately 2 h. All the electrophoresis

analyses were done using Cole-Parmer; model SE 400, vertical slab electrophoresis apparatus. Native gels were stained for the desired enzymes according to the protocols described by Soltis et al. (1983). After gel staining, bands were observed and the relative mobility (Rf) of each band was calculated. Bands having very close values of electrophoretic mobility (Rf) were grouped as members of a distinct zone (locus, Pasteur et al., 1988).

DNA analysis

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was used to study the impact of sub-lethal concentrations of the two herbicides (glyphosate and atrazine) on genotoxicity of *B. glabrata* snails. DNA was extracted from the whole snail of control and exposed groups using genomic DNA purification kit (Ferments, Germany). Six 10-mer random primers (OPA-10, OPD-10, OPK-18, OPS-02, OPAJ-05 and OPAV-18 (Metabion, Germany) were used for RAPD analysis. DNA templates were adjusted to 250 ng per 5 µl. The amplification reaction was performed in 16 µl reaction volume and in sterile 0.2 ml PCR tubes. The following PCR program was used: 1 cycle of 94 °C for 3 min, followed by 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 1 min, the last three steps were repeated for 36 cycles and a final extension step at 72 °C for 10 min. Amplification was carried out in a Bio-Rad gene cycler (Bio-Rad, USA) and PCR fragments were

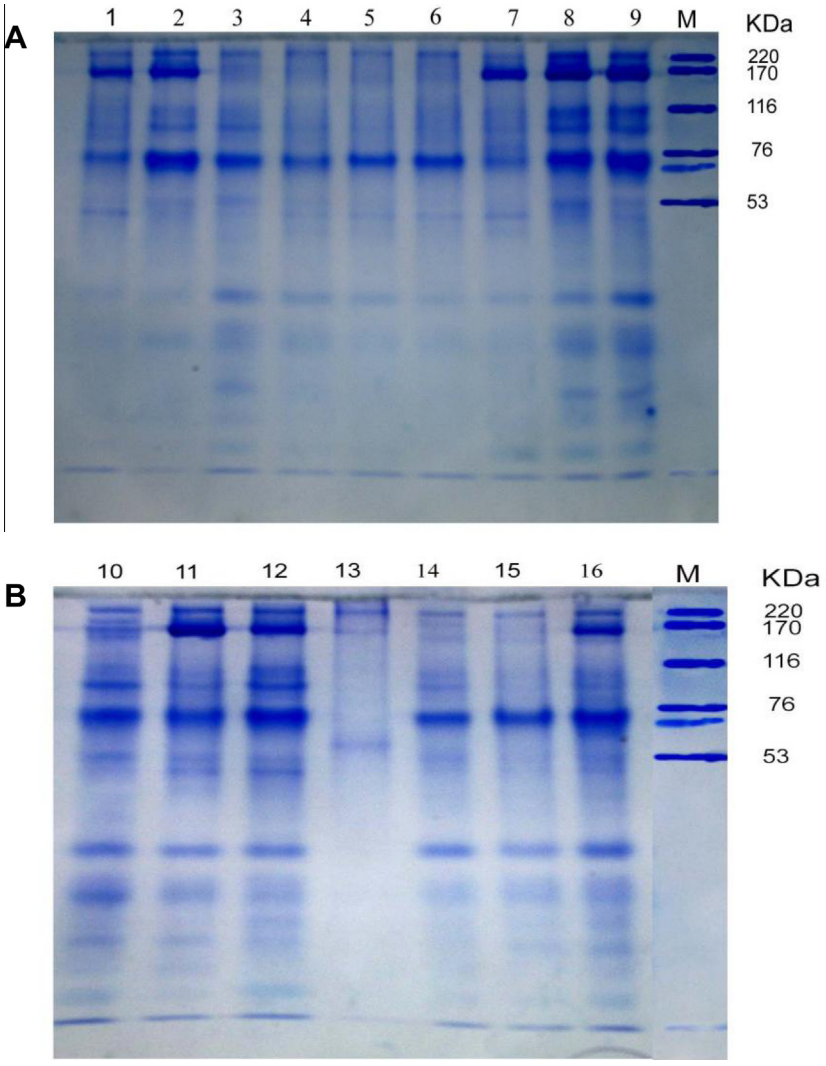

Fig. 1 (A and B) SDS–PAGE illustrating protein bands of control and treated snails with either glyphosate or atrazine during 3 weeks. A: lane 1: protein extract from control snails after zero day, lane 2: control snail after 1 week, lane 3:5 ppm glyphosate-exposed groups after 1 week, lane 4: 0.5 ppm glyphosate-exposed groups after 1 week, lane 5: 10 ppm atrazine-exposed groups after 1 week, lane 6: 5 ppm atrazine-exposed groups after 1 week, lane 7: control snails after 2 weeks, lane 8: 5 ppm glyphosate-exposed groups after 2 weeks, lane 9: 0.5 ppm glyphosate-exposed groups after 2 weeks. B: lane 10: 10 ppm atrazine-exposed groups after 2 weeks, lane 11: 5 ppm atrazine-exposed groups after 2 weeks, lane 12: control snails after 3 weeks, lane 13: 5 ppm glyphosate-exposed groups after 3 weeks, lane 14: 0.5 ppm glyphosate-exposed groups after 3 weeks, lane15: 10 ppm atrazine-exposed groups after 3 weeks, lane 16: 5 ppm atrazine-exposed groups after 3 weeks. (C) α-Esterase zymogram of control and either glyphosate or atrazine-exposed snails, indicating 3 zones of activity. Lanes 1, 2, 7 and 12: control snails after zero day, one, two, and three weeks respectively. Lanes 3, 4, 5 and 6:5 and 0.5 ppm glyphosate and 10 and 5 ppm atrazine exposed for 1 week. Lanes 8, 9, 10 and 11: same treatments after 2 weeks. Lanes 13, 14, 15 and 16: same treatments after 3 weeks. (D) Peroxidase zymogram of control and either glyphosate or atrazine-exposed snails indicating one zone of activity.

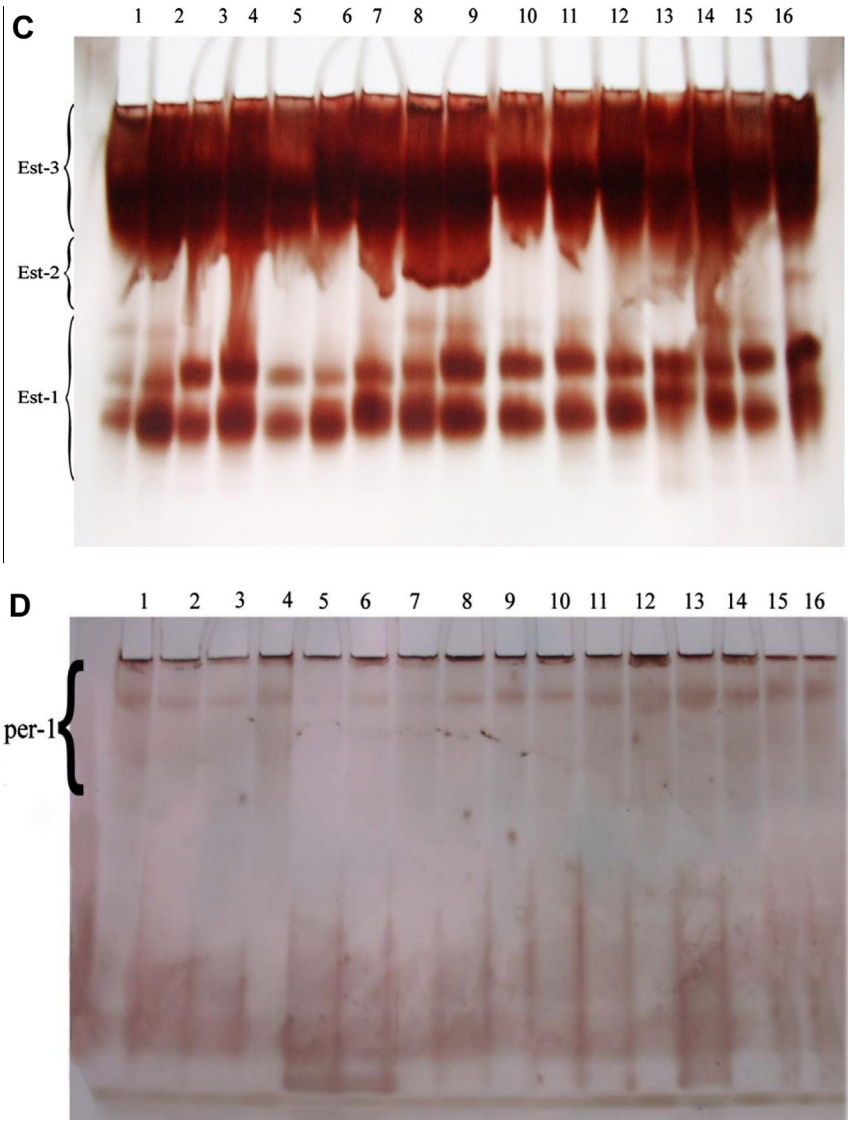

Fig. 1 (*continued*)

run on 1.5% agarose gels and visualized using UV Transilluminator (Bio-Rad, USA). The molecular weight (bp) of each band was compared with those of the standard DNA molecular markers (Fermentes, Germany).

Statistical and data analysis

In case of SDS protein analysis, the protein bands produced by each sample were counted and their relative mobilities (Rfs) were compared with those of the standard protein molecular marker. The intensities of the protein bands of each sample (lane) protein profile were measured using Gel-Pro Analyzer version 4.0 for windows (Media Cybernetics, Inc.).

Results

SDS–PAGE protein analysis

Three bands (220, 76, and 12.5 KDa) were common in control and in all treated groups, while in 5 ppm glyphosate-exposed snails, the last two bands (76 and 12.5 KDa) disappeared and 60 KDa band appeared (Table 2 and Fig. 1B, lane 13). Protein bands 116, 113, 104, 53, 9, 3.68, 2.53, and 1.82 KDa appeared in 5 ppm glyphosate-exposed snails only after 1 week (Fig. 1A and lane 3), while appearance of 45 KDa band was characteristic to the remaining groups (Table 2 and Fig. 1A, lanes 4–6). In addition, the band 205 KDa was found only in both concentrations (5 and 10 ppm) of atrazine-exposed snails. Meanwhile, l0 ppm atrazine-exposed snails exhibited an extra-band 166 KDa after 2 weeks of treatment. After 3 weeks, 166, 76, 12.5, and 9 KDa appeared in all groups except 5 ppm

Table 1 Primer sequences and their G/C content used for RAPD-PCR.

Primer	Sequence	G/C content (%)
OPA-10	5′-GTGATCCCAG-3′	60
OPD-10	5′-GGTCTACACC-3′	60
OPK-18	5′-CCTAGTCGAG-3′	60
OPS-02	5′-CCTCTGACTG -3′	60
OPAJ-05	5′-CAGCGTTGCC-3′	70
OPAV-18	5′-TTGCTCACGG-3′	60

glyphosate-exposed snails. 5 ppm atrazine-exposed snails were characteristic by the presence of low molecular weight bands of 5.8 and 2.53 KDa (Table 2 and Fig. 1B). Band intensity analysis revealed that there were differences in band intensity among all treated groups. For example, 220 KDa band was observed in all groups, but its intensity varied between groups during the 3 weeks of exposure. As mentioned above, the 76 and 12.5 KDa bands were observed in most groups but showed high intensity in lanes 2, 8, 9, 10, 12 and 16.

Isozyme analysis

Esterase isozyme revealed three loci (α-Est-1, α-Est-2 and α-Est-3), two loci showed strong activity while the third one had faint activity (Fig. 1C). α-Est-1 was represented with three bands or three co-dominant alleles, except for both concentrations 10 and 5 ppm of atrazine after 1 week of exposure, 5 ppm glyphosate and 5 ppm atrazine after 3 weeks of exposure (Fig. 1C, lanes 13 and 16) which exhibited two bands in that locus. Monoallelic locus represented in α-Est-2, whereas there is one band observed only in 5 ppm glyphosate and 5 ppm atrazine exposed snails after 3 weeks (lanes 13 and 16). The α-Est-3 locus was monomorphic, which specified by one band that is very intense at the last group, 5 ppm atrazine after 3 weeks (lane 16). Similarly, β-esterase showed three loci β-Est1, β-Est2, and β-Est3 with the same band patterns have been observed in case of α-esterase.

The peroxidase enzyme showed only one locus (Per-1) which was polymorphic (Fig. 1D) where one band was observed in each lane, except lanes 12 and 13 which represent control and 5 ppm glyphosate groups after 3 weeks, showed two bands (with Rf values of 0.111 and 0.129). In contrast, catalase was monomorphic, only band (observed in control after 1 week, 5 ppm glyphosate, 10 ppm atrazine exposed snails after 1 week of exposure and also observed after 3 weeks in both the control group and 5 ppm glyphosate-exposed snails) appeared with varied intensities between groups.

RAPD analysis

Genotoxicity of long-term exposure to glyphosate and atrazine herbicides was conducted by RAPD-PCR using six arbitrary 10-mer primers OPA-10, OPD-10, OPK-18, OPS-02, OPAJ-05 and OPAV-18 (Table 1). The snail groups exposed to three herbicide concentrations (5 ppm glyphosate, 5 ppm, and 10 ppm atrazine), which showed considerable changes in the protein pattern compared with the control, were selected for DNA extraction and RAPD-PCR analysis. Only one amplified fragment of 500 bp could be generated by OPA-10 primer using DNA extracted from herbicide-treated snails after 1 and 3 weeks of exposure to 5 ppm glyphosate (lanes 2 and 7) and 5, 10 ppm atrazine (Fig. 2A). The 500 bp band also appeared in case of snails treated with 5 ppm glyphosate after 2 week exposure (lane 4). There was no PCR product obtained with the control group or with both concentrations of atrazine after 2 weeks of exposure (Fig. 2A).

OPAJ-05 amplified two PCR products of 850 and 650 bp (Fig. 2B) in case of the control group, 5 ppm glyphosate (lanes 1 and 2) and 10 ppm atrazine-exposed snails after one and 3 weeks of exposure (lanes 3 and 8). 300 bp was characteristic to 10 ppm atrazine-exposed snails after one and 3 weeks of

Table 2 Molecular weights of protein bands of *B. glabrata* after 1 week (lanes 1–6), 2 weeks (lanes 7–11) and 3 weeks of herbicide treatments (lanes 12–16).

Band No.	Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Marker (KDa)	Control zero	Control (1)	5 ppm Glyphosate	0.5 ppm Glyphosate	10 ppm Atrazine	5 ppm Atrazine	Control (2)	5 ppm Glyphosate	0.5 ppm Glyphosate	10 ppm Atrazine	5 ppm Atrazine	Control (3)	5 ppm Glyphosate	0.5 ppm Glyphosate	10 ppm Atrazine	5 ppm Atrazine
1	220	220	220	220	220	220	220	220	220	220	220	220	220	220	220	220	220
2	–	–	–	–	–	–	–	–	–	–	205	205	–	–	205	–	–
3	170	170	170	–	–	–	–	170	170	170	170	170	170	170	–	–	170
4	–	–	–	–	–	–	–	–	–	–	166	–	–	–	166	166	166
5	116	116	116	116	–	–	–	116	116	116	116	–	116	–	116	–	116
6	–	113	113	113	–	–	–	113	113	113	113	113	113	–	113	–	113
7	–	104	104	104	–	–	–	–	–	–	–	–	–	–	–	–	–
8	76	76	76	76	76	76	76	76	76	76	76	76	76	–	76	76	76
9	–	–	–	–	–	–	–	–	–	–	–	–	–	60	–	–	–
10	53	–	53	53	–	–	–	53	53	53	53	53	53	–	53	–	53
11	–	45	–	–	45	45	45	45	45	45	45	45	45	–	45	–	45
12		12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	–	12.5	12.5	12.5
13	–	–	–	9	–	–	–	–	9	9	9	9	9	–	9	9	9
14	–	–	6.75	6.75	6.75	–	–	–	6.75	6.75	6.75	6.75	6.75	–	–	6.75	6.75
15	–	–	–	–	–	–	–	–	–	–	–	–	5.8	–	–	–	5.8
16	–	–	–	3.68	–	–	–	–	3.98	3.98	3.98	3.98	3.68	–	–	3.68	3.68
17	–	–	–	2.53	–	–	–	–	2.53	2.53	2.53	2.53	–	–	–	–	2.53
18	–	–	–	1.82	–	–	–	1.82	1.82	1.82	1.82	1.82	1.82	–	1.82	–	1.82

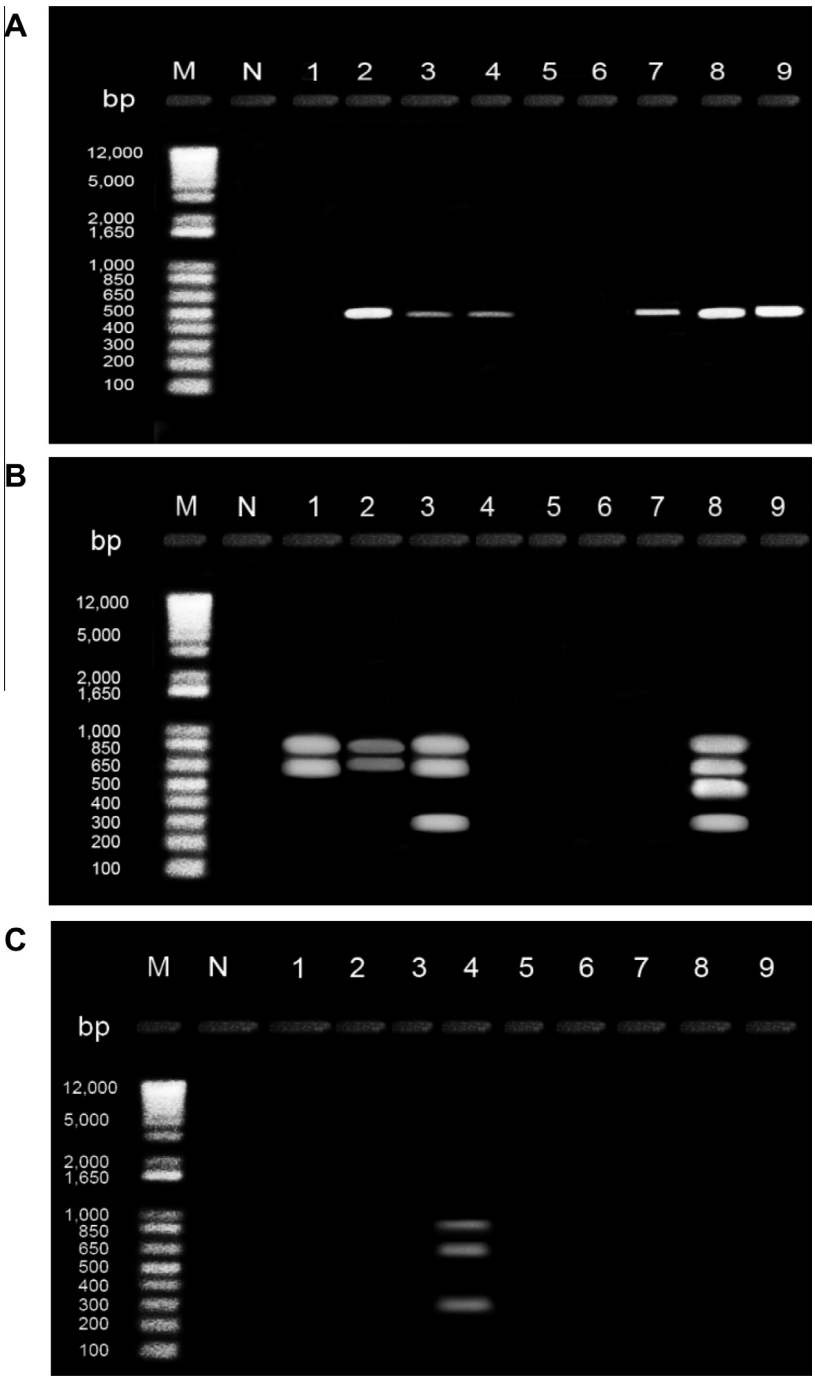

Fig. 2 RAPD profiles of different groups of *B. glabrata* genomic DNA represents PCR products with primer (A) OPA-10, (B) OPAJ-05 and (C) OPAV-18. Lane M: DNA marker (bp). Lane N: negative control without DNA template. Lane 1: DNA extracted from control snails. Lanes 2 and 3: DNA extract from 5 ppm glyphosate and 10 ppm atrazine-exposed groups after 1 week of exposure respectively. Lanes 4–6: DNA extracted from 5 ppm glyphosate, 10 and 5 ppm atrazine-exposed groups after 2 weeks of exposure. Lanes 7–9: DNA extracted from 5 ppm glyphosate, 10 and 5 ppm atrazine-exposed groups after 3 weeks of exposure.

exposure (lanes 3 and 8). A unique 400 bp PCR product with the same herbicide concentration after 3 weeks was observed (lane 8). OPAV-18 primer amplified 850, 650, and 300 bp PCR products of snails exposed to 5 ppm glyphosate for 2 weeks (Fig. 2C, lane 4). OPD-10, OPK-18, and OPS-02 primers generated no PCR products with the DNAs of all herbicide-treated groups.

Discussion

The SDS–PAGE results of this study were in accordance with Fryer et al. (1989) and Richard and Renwrantz (1991), where *B. glabrata* snail responded to pollution with herbicide gain and/or loss of some bands and variation in their intensities. In our study, the band 205 KDa, which is present in both

concentrations of atrazine-exposed snails, might be similar to group (I) molecules (G1M, 200 KDa on SDS–PAGE gels) that appeared in *B. glabrata* following infection by *Echinostome paraensei* (Adema et al., 1999). In addition, the band 60 KDa, which is characteristic to 5 ppm glyphosate-exposed snails after 3 weeks of exposure, in the current study, is similar to 65 KDa appeared following of snails' infection with the same previous parasite (Adema et al., 1999).

Presence of low molecular weight bands (1.82–12.5 KDa) may be due to damage of protein–complex structure with high molecular weights as a result of Reactive Oxygen Species (ROS) release under the effect of herbicide exposure (Charlet et al., 1996; Hoffmann et al., 1996; Hubert et al., 1996; Ganz and Lehrer, 1998).

In this study, disappearance of band in Est-3 in most of snails exposed to the herbicide concentrations may be because herbicides perhaps act as activators of the gene that control the suppression or switching off this locus which agreed with Narang and Narang (1975). Hydrolytic activity of esterases or what is called detoxifying mechanism is crucial for protecting genetic material against toxic substances (Argentine and James, 1995). In the present study, perhaps similar mechanisms of the function of esterases could be postulated.

Catalase activity was restricted only to the treated groups for 1 and 2 weeks and this may be due to variations of ROS release from hemocytes during this period. Therefore, catalase is responsible for antioxidant mechanisms where catalase reduces hydrogen peroxide content (Kristoff et al., 2008). Therefore, it can be postulated that, the catalase, and peroxidase enzymes may be selected as molecular markers to study the herbicide effects due to their role in detoxifying H_2O_2 generated under normal and stress conditions.

In the RAPD-PCR analysis, the major observation in RAPD profiles was the appearance of single band with 500 bp in all groups except control and both concentrations of atrazine-exposed groups for 2 weeks, with primer (OPA-10). This band varied in its intensity and the differences in RAPD-PCR band intensity are due to primer mismatch or relative sequence abundance (Williams et al., 1990). On the contrary, Lee et al. (2007) found same intensities for band patterns for most of 10-mer primers when studying DNA damage induced by arsenite.

Furthermore, a unique band of 400 bp may be considered as a diagnostic marker which can attribute to 3 weeks of exposure to 10 ppm atrazine with the same primer (Hassan and Alam, 2007). This is consistent with the recent study of the appearance of a new diagnostic band which may be due to long time of exposure (3 weeks) to a high concentration of atrazine (Abbas and Ali, 2007).

Modifications of the RAPD patterns in treated groups can be due to changes in primer binding sites, structural changes due to DNA damage under the effect of herbicides exposure. These effects in DNA, probably due to an increase of free radical activity or free radical life span in organisms following exposure to herbicides and could deteriorate antioxidant defensive system by Reactive Oxygen Species (Lai and Singh, 2004; Lee et al., 2004; Guier et al., 2006).

Finally, the results of the present study indicated that the investigated herbicides may be highly genotoxicants because of their toxic effects on DNA and proteins. These subsequent effects probably arise from DNA damage due to oxidative stress of these herbicides.

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