COMET ASSAY PARAMETERS OF *SPODOPTERA LITTORALIS* (BOISD.) LARVAE RESISTANCE SYSTEM CELLS AS AFFECTED BY DIFFERENT COMPOUNDS EXPOSED TO GAMMA IRRADIATION

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Abstract

The Comet assay, called the single cell gel electrophoresis (SCGE) assay or microgel electrophoresis (MGE) assay, primarily measures DNA strand breakage in single cells according to the cell type in vitro and in vivo experiments. The main objective of this study is to assess the sensitivity of genotoxic to be considered in cotton leafworm, *Spodoptera littoralis* (Boisd.) larvae by causing depletion in cells responsible for functions of the resistance system of this pest to the pesticides as affected by three compounds; *Bacillus thuringiensis,* Kurs. (Bactericide); *Metarhizium anisopltae,* Metsch. (Fungicide) and chitosan (biopolymer) exposed to gamma irradiation at doses of 15, 30 and 60 Gy to increase purpose of its activity on genotoxicity parameters of the cotton leaf worm, *S. littoralis* (Boisd.) treated as 4th instars larvae by LC_{50}'s of compounds mentioned. *B. thuringiensis* exposed to gamma doses of 15, 30 & 60 Gy had potentiating efficacy on *S. littoralis* than *B. thuringiensis* used alone without exposing to gamma doses. While, *M. anisopltae* and chitosan had nearest results among tested compounds singly and the same compound exposed to gamma doses used. Different comet assay parameters; Cell numbers, comet length (µ), head diameter (µ), % DNA in head, tail length (µ), % DNA in tail and % tail moment were affected by direct effect of compounds used on *S. littoralis* larvae lead to death the most of cells and its DNA degradation or another effect by division increasing in DNA lead to increase the malformation cells or % DNA as well as examined nuclei numbers compared to normal *S. littoralis* larvae. Also, % DNA in examined nuclei had destruction grades according to the treatments used that ranged from 0-3 grades. In addition, the resistance system cells of *S. littoralis* that investigated by fluorescence microscopy had clearly injurious appeared in disruption; lost its roundness and cell wall; swelling or shrinking in the most investigated cells. It can be concluded that chitosan exposed to different gamma doses was the most genotoxicity compound affect on resistance system cells of *S. littoralis* larvae, followed by *B. thuringiensis* and *M. anisopltae* exposed to gamma doses.

Key words: gamma irradiation, *B. thuringiensis, M. anisopltae,* chitosan*, S. littoralis,* genotoxicity.

INTRODUCTION

The single-cell gel electrophoresis assay (comet assay) is widely used for the detection of DNA damage and repair in a variety of cells in vitro and in vivo (Olive and Banath, 2006). DNA damage detected by the alkaline version of the comet assay includes single-and double-strand breaks and alkali-labile (e.g. apurinic) sites. The assay has the advantage of being a rapid, sensitive and relatively inexpensive method. It is not only commonly used in genotoxicity testing but it also has widespread applications in environmental biomonitoring and human population monitoring (Amaeze *et al.*, 2015).

The "comet assay" was designed by Ostling and Johanson (1984). Its name was derived from the shape of the nuclear DNA after some steps of manipulation. The comet assay can be applied to all cells, tissues and organs as long as it is possible to generate single cells. The comet assay in its various modifications definitely is a valuable tool for the assessment of DNA damage and is now used in many fields of research. Thus, the release of DNA from a highly supercoiled DNA–protein complex is actually measured in the comet assay and there is a lot of evidence that the comet tail consists of DNA loops and not of DNA fragments (Collins *et al.*, 1997).

Undoubtedly, among more than 1300 pest species recorded on cotton, the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) is considered as the most important cotton pest that is found almost everywhere cotton is grown in Egypt (Hosny and Isshak, 1967). The control strategy was based mainly on uses of synthetic insecticides but most are toxic to all animals including human beings, and persistent in environment, numbers of them have carcinogenic and mutagenic effects on human, domestic animals, birds and predators. In order to avoid the insecticidal hazards, there is a great need to develop alternative control agents with new mode of action. Among these agents is gamma irradiation as a genetic control method. Genetic pest suppression is unique among biological methods in it involves the release of genetically modified insects to control the same species. The irradiated biocide, Dipel-2x with doses of 5, 10, 20, 40 & 80 Gy activated its insecticidal efficiency against *P. gossypiella* newly hatched larvae and eggs of 1-4 day old in laboratory and field experiments during seasons of 2004 and 2005 (Amer, 2006). In addition, Amer, *et al.* (2011) mentioned that gamma doses used (100, 200 & 300 Gy). had antagonism effect in biover efficiency and sub lethal doses becomes higher than untreated biover against 4th instars larvae of *S.littoralis*. Moreover, Amer, *et al.* (2012) reported that biocide compound, Protecto (*B.thuringiensis,* Kurs.) applied on *P. gossypiella*, *S. littoralis* and *A. craccivora* to assess the insecticidal activity in three combinations

(Protecto +150 Gy, Protecto +250 Gy and Protecto +350 Gy). Data showed that LC_{50}'s on subjected insects treated with Protecto and exposed to gamma doses were lower than untreated Protecto.

Also, *M. anisopliae* was studied on *S. littoralis* susceptibility or *M. anisopliae* combined with gamma irradiation by Gabarty, *et al.* (2013).

Chitosan compound might be used as alternatives pesticides because it might possess insecticidal activity and non toxic effect on vertebrates and humans (Badawy, *et al.* (2005). The insecticidal activity of chitosan was reported against *S. littoralis* by Rabea, *et al.* (2003). Also, El-Gendy, *et al.* (2014) evaluate the toxicity and biochemical effects of chitosan against *Bactrocera zonata* (Saund.). Chitosan effect on female and male adults after 24 and 48 hours. Also, it caused inhibition in AChE and ATPase.

The aim of the present study is evaluate the combined effects of the compounds, *Bacillus thuringiensis,* Kurs. (Bactericide); *Metarhizium anisopltae,* Metsch. (Fungicide) and biopolymer, chitosan exposed to gamma irradiation at doses of 15, 30 and 60 Gy on resistance system cells of the cotton leafworm, *Spdoptera littoralis* (Boisd.) treated as 4^{th} instars larvae.

MATERIALS AND METHODS

A. Compounds.

1- Bactericide: *Bacillus thuringiensis* subsp. *kurstaki* :(Biotect) 9.4% WP (32000 IU/mg), produced by Organic for biotechnology company. Dose rate: 300 gm/feddan (2400 IU/ml).

2- Fungicide: *Metarhizium anisopltae* (Metsch.): (Bio Magic) 1.75% WP ($1x10^8$ CFU/S/gm). Manufacturer Company: M/S. T. Stanes Company Limit- India. Import Company: Gaara Establishment, Import & Export. Dose rate: 10gm/ L Water ($1x10^6$ CFU/S/ml).

4- Chitosan (Biopolymer): Chitocare 2.5%, product of Egypt Chemical Company (E.C.C.). Rate dose: 1L/feddan for crop or vegetable fields.

All the compounds used exposed to gamma irradiation doses of 15, 30, & 60 Gy. All irradiations were done by a Se^{137} Hendy Cell Research, delivered at a dose rate 0.75/rad/sec. The radiation was done at National Center for Radiation Research and Technology.

B. The pest.

Laboratory strain of the cotton leafworm, *S. littoralis* fourth instars larvae was reared at Cotton leaf worm Department, Plant Protection Research Institute, Agriculture Research Center on castor oil leaves, *Ricinus communis* (L.). Rearing of insects was conducted following the technique described by El-Defrawi *et al*. (1964). Rearing conditions were adjusted at 27±1°C and 65-75% RH.

C. Toxicity of gamma irradiation doses and different compounds on *S. littoralis*.

Twenty five of *S. littoralis* fourth instar larvae with castor oil leaves Petri-dishes exposed to gamma irradiation doses of 15, 30 & 60 Gy. Four replicates for each gamma doses used and the control was done. The larval mortality percent was recorded daily after treatments.

Dipping technique was used at the present work. The castor oil leaves dipping in tested compound concentrations of 50, 25, 12.5, 6.25 & 3.125 gm/L of *B. thuringiensis* (Biotect), *B. thuringiensis* +15 Gy, *B. thuringiensis* +30 Gy and *B. thuringiensis* + 60 Gy. Concentrations of 30, 15, 7.5, 3.75 & 1.875 gm/L of *M. anisopltae* (Bio magic), *M. anisopltae* +15 Gy, *M. anisopltae* +30 Gy and *M. anisopltae* +60 Gy. Concentrations of 50, 25, 12.5, 6.25 &3.125 ml/L of Chitosan (Chitocare), Chitosan + 15 Gy, Chitosan + 30 Gy and Chitosan +60 Gy. The control was done by castor oil leaves dipping in water only. Four replicates/ concentration/ tested bio-agent and left the leaves until water evaporated and put in glass jars (11x22 cm). Each jar was prepared by 25 fourth instar larvae of *S. littoralis* after larvae starving about 4 hours and maintained under 26±1°C. Then the numbers of alive and dead larvae were counted at three days after treatment.

LC_{50}; LC_{90} and slope values were assessed according to Finney (1971) by using Ldp line software (www.Ehabbakr software/Ldp line). The efficiency of different insecticides could be measured by using Sun 's equation (1950) as follows:

Toxicity index = LC_{50} (LC_{90}) of the compound A/ LC_{50} (LC_{90}) of the compound B*100

Where A: is the most effective compound.

B: is the other tested compound.

Fourth instar larvae of *S. littoralis* were treated by LC_{50}'s of each compound or the same compound exposed to gamma irradiation doses that were used. Ten days later of treatment, the larvae kept by freezing at -18°C until comet analysis used.

Comet assay was done at Animal Health Research Institute, A.R.C.

D. Single cell gel electrophoresis (Comet).

The alkaline comet assay was conducted as described by Amaeze, *et al.* (2015). Comets were analyzed using a Leica fluorescence microscope (Leica DMLB 020-519-010 LB30T). DNA damage was scored using the Comet IV capture system (version 4.11; Perceptive Instruments, UK). Fifty cell nucleotides were assessed per slide, and each sample was analyzed in duplicate. All samples were measured blind. The tail intensity (% tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage induced, that is a meaningful end-point to assess genotoxicity.

The technique of single cell gel electrophoresis (comet) regarding preparation of the base slides, cell isolation, electrophoresis of micro-gel slides and buffers preparation were performed according the protocol mentioned by Singh, *et al.* (1988). The protocol includes:

1. Preparation of base slides.

1.1. Prepare 1% (500 mg per 50 ml PBS) and 0.5% LMPA (250 mg per 50 ml PBS) and 1.0% NMA (500 mg per 50 ml in Milli Q water). Microwave or heat until near boiling and the agarose dissolves. For LMPA, aliquot 5 ml specimens into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37ºC dry/water bath to cool and stabilize the temperature.

1.2. Dip the slides in methanol and burn them over a blue flame to remove the machine oil and dust.

1.3. While NMA agarose is hot, dip conventional slides up to one-third the frosted area and gently remove. Wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed at 50^{o}C for quicker drying. Store the slides at room temperature until needed; avoid high humidity conditions. We generally prepare slides the day before use.

2. Cell isolation / treatment.

Cut larvae specimens into large pieces; add fresh solution of aspirate, mince into finer pieces, remove and settle it down mix 5 µl of the cell suspension with 75 µl LMPA, and process accordingly. The volume of the cell suspension to mix with 75 µl of LMPA must be 10 µl or less, while the optimal cell number is 10,000 cells per slide**.**

3. Electrophoresis of micro gel slides.

3.1. After at least 2 hour at 4ºC, gently remove slides from the lysing Solution. Place slides side by side on the horizontal gel box near one end, sliding them as close together as possible.

3.2. Fill the buffer reservoirs with freshly made pH>13 Electrophoresis Buffer until the liquid level completely covers the slides (avoid bubbles over the agarose).

3.3. Let slides sit in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage

3.4. Turn on power supply to 24 volts (~0.74 V/cm) and adjust the current to 300 milli amperes by raising or lowering the buffer level. Depending on the purpose of the study and on the extent of migration in control specimens, electrophoreses the slides for 30 minutes.

3.5. Turn off the power. Gently lift the slides from the buffer and place on a drain tray. Drop wise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Drain slides and repeat two more times.

3.6. Slides may be stained with 80µL Ethidium Bromide, leave for 5 min and then dipped in chilled distilled water to remove excess stain. The cover slip is then placed over it and the slides are scored immediately or dried before staining as in step 7.

3.7. Drain slides; keep them for 20 min in cold 100% ethanol or cold 100% methanol for dehydration. Dry the slides by air and place them in an oven at 50^{o}C for 30 min. Store in a dry area.

3.8. When convenient, rehydrate the slides with chilled distilled water for 30 min and stain with Et-Br as in step 6 and cover with a fresh cover slip. Before viewing slides, blot away excess liquid on the back and edges. After scoring, remove cover slip, rinse in 100% alcohol to remove stain, let dry, and store for archival purposes if needed.

4. Evaluation of DNA Damage.

4.1. For visualization of DNA damage, the observations by made of EtBr-stained DNA using a 40X objective on a fluorescent microscope.

4.2. Although any image analysis system may be suitable for the quantization of SCGE data, we use comet score to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per specimen.

4.3. Compare the amount of migration per cell, the number of cells with increased migration, the extent of migration among damaged cells and viability.

Finally, the program calculates the number of cells, comet length (µ), head diameter (µ), % DNA in head, tail length (µ), % DNA in tail, tail moment (% DNA in tail/ % DNA in head).

Grading of DNA damage: There are five grades of DNA destruction according to Singh *et al.* (1988)

$$\textbf{\% DNA damage} = \frac{\text{Tail length}}{\text{Comet length}} \times 100$$

Grade 0: NO damage, normal cell, tail length less than 5 %
Grade 1: Slightly damaged, tail length 5 – 20 %
Grade 2: Moderately damaged, tail length 20 – 40 %
Grade 3: Heavily damaged, tail length 40 – 92 %
Grade 4: Totally damaged, tail length higher than 95 %

All genotoxicity parameters of *S. littoralis* were analyzed using Costat statistical program software, 1990 and Duncan's multiple range test (Duncan, 1955) at 5% probability level to compare the differences among time means.

RESULTS AND DISCUSSION

A. Efficacy of gamma irradiation and different compounds on *S. littoralis*.

Toxicity of gamma irradiation and different compounds singly or in combination were investigated previously by Amer, *et al.* (2015) as following

Table 1. Effect of gamma irradiation on larval mortality of *S. littoralis* treated as 4th instar larvae.

Gamma doses (Gy)	% Larval mortality after			
	4-day	6-day	8-day	10-day
Control	0^{c}	0^{c}	0^{d}	0^{c}
15	20^{b}	30^{b}	42^{c}	60^{b}
30	23^{b}	32^{ab}	52^{b}	63^{b}
60	28^{a}	35^{a}	58^{a}	69^{a}
$LSD_{0.05}$	3.26	2.46	4.15	3.87

Larval mortality rates depended on gamma doses. Dose of 60 Gy had the highest larval mortality, followed by 30 and 15 Gy, respectively (Table 1).

The present results, in general are in agreement with Amer (2006), Amer, *et al.* (2011) and Amer, *et al.* (2012).

$LC_{50'}$ s of tested compounds; *B. thuringiensis*, *M. anisopliae* and chitosan singly or combined with gamma irradiation doses of 15, 30 and 60 Gy described in Table (2).

Table (2) showed that LC_{50} of *Bt* was 1133 $x10^6$ IU/L against 4th instar larvae of *S. littoralis*. On the other hand, when *Bt* was exposed to gamma irradiation, it showed potentiating efficacy, where LC_{50} was decreased to 810.2 $x10^6$ IU/L (*Bt* + 15 Gy), 337.9$x10^6$ IU/L (*Bt* + 30 Gy) and 163.9 $x10^6$ IU/L (*Bt* + 60 Gy) at 3 days post larval treatments. Also, *Bt* + 60 Gy was considered the most efficacious compound against 4th instar larvae, followed by *Bt* + 30 Gy, *Bt* + 15 Gy and then *Bt* non-irradiated that had the least efficacy compared to the same compounds exposed to gamma radiation.

Table 2. Different compounds exposed to gamma doses against *S. littoralis* larvae at 3- day post treatments.

Treatments	LC_{50} 95%Confidence limits	LC_{90} 95%Confidence limits	Slope	Toxicity index	
				LC_{50}	LC_{90}
	(IU/L)				
B. thuringiensis	1133 $x10^6$ 965.1 $x10^6$ ± 1551$x10^6$	2719$x10^6$ 1934 $x10^6$±4497$x10^6$	1.33	14.5	23.8
B. thuringiensis + 15 Gy	810.2 $x10^6$ 581.8 $x10^6$±1257$x10^6$	2247$x10^6$ 1639$x10^6$±3165$x10^6$	1.76	20.2	28.8
B. thuringiensis + 30 Gy	337.9 $x10^6$ 136.9 $x10^6$±643.8 $x10^6$	1682$x10^6$ 877.1$x10^6$±2669$x10^6$	1.88	48.5	38.5
B. thuringiensis + 60 Gy	163.9 $x10^6$ 29.7 $x10^6$±484.2 $x10^6$	647.7 $x10^6$ 391.7 $x10^6$±1748$x10^6$	1.98	100	100
	(CFU/S /L)				
M. anisopliae	62.23 $x10^8$ 32.55$x10^8$±90.38$x10^8$	950.40 $x10^8$ 340.2$x10^8$±1192.3$x10^8$	0.5	96.8	99.7
M. anisopliae +15 Gy	62.1 $x10^8$ 32.56$x10^8$±90.42$x10^8$	950.3 $x10^8$ 340.2$x10^8$±1191.3$x10^8$	0.52	97.0	99.7
M. anisopliae +30 Gy	61.41 $x10^8$ 30.42$x10^8$±89.58$x10^8$	949.4 $x10^8$ 320.9$x10^8$±1189.4$x10^8$	0.56	98.0	99.8
M. anisopliae +60 Gy	60.22 $x10^8$ 30.12$x10^8$±87.87$x10^8$	947.3 $x10^8$ 310.4$x10^8$±1177.7$x10^8$	0.62	100	100
	(ml/L)				
Chitosan	24.41 18.88 ±40.28	50.81 32.21 ±80.56	3.1	77.1	81.9
Chitosan +15 Gy	21.22 15.46±35.38	47.98 30.35±73.87	3.1	88.7	86.7
Chitosan +30 Gy	20.45 13.25±33.54	43.68 25.45±73.75	3.2	92.0	95.2
Chitosan +60 Gy	18.82 10.89±30.98	41.59 21.98±70.70	3.2	100	100

Obtained results were confirmed previously by Amer (2006) and Amer *et al.* (2012).

The fungus, *M. anisopliae* effected on 4th instar larvae (LC_{50}: 62.23 $x10^8$ CFU/S/L), but when *M. anisopliae* was exposed to gamma doses, it had little increase in its

efficacy compared to *M. anisopliae* when it was applied alone (Table 2). Present results are nearly those of Amer, *et al.* (2011) who reported that gamma irradiation doses of 100, 200 and 300 Gy had antagonism effect on biover efficacy against cotton leaf worm 4th instar larvae and gamma doses used had sub lethal doses higher than untreated biover. In addition, Gabarty, *et al.* (2013) investigated the efficacy of sub sterilizing doses of gamma irradiation or/ and entomopathogenic fungi *B. bassiana* and *M. anisopliae* on the immune enzyme response of *S. littolaris* larvae. It was found that gamma radiation (50,100&150 Gy) and tested entomopathogenic fungi synergistically inhibit the immune system of *S. littolaris* larvae to become susceptible to the treatments.

Biopolimer, chitosan had efficacy on 4th instar larvae and LC_{50} was 24.41 m/L. When chitosan was exposed to gamma doses 15- 60 Gy, its efficacy had medium increase reaching 18.82 m/L after 3- days from treatment, in case of chitosan +60 Gy. El-Gendy, *et al.* (2014) stated that chitosan gave inhibition of Ach.E and ATPase activities of *Bactrocera zonata*.

B. Comet assay parameters.

Fourth instars larvae of *S. littoralis* treated with LC_{50}'s of different compounds and gamma irradiation in singly or in combination. Ten-days later after larvae treatment as described previously for comet assay to investigate the genotoxicity or comet parameters of resistance system cells as a result of different treatments.

Table (3) and figure (1) showed the comet assay parameters of no. cell, comet length or cell length (micron, µ) that contain both head diameter (µ) and tail length (µ). In each head and tail of the cell, it was DNA percentage determined; also, tail moment parameter was calculated.

1. Cell numbers.

Normal resistance system cell numbers of *S. littoralis* larvae was 39 cells. Treatments of 60 Gy, 30 Gy, *M. anisopliae, B. thuringiensis* + 60 Gy, chitosan, chitosan + 15 Gy and chitosan + 30 Gy as well as *M. anisopliae* + 15 Gy causes lost no. of cells that appeared in the slides to become 19, 21, 28, 30, 31, 33 and 35 cells, respectively compared to untreated samples. Contrary was happened in treatments of 15 Gy (76 cell), *M. anisopliae* +60 Gy (56 cell), *B. thuringiensis* + 15 Gy (53 cell), chitosan + 60 Gy (52 cell), *B. thuringiensis* (51 cell), *M. anisopliae* + 30 Gy (49 cell) and *B. thuringiensis* + 30 Gy (43 cell) as a result of DNA division increasing that happened compared to normal no. cell.

2. Comet length.

Comet length (resistance system cell length) that was 16.12 µ in normal *S. littoralis* larvae; each cell contained head and tail; both of them called comet length. Treated *S. littoralis* larvae comet length had decreased to 13.08, 13.64, 13.97, 14.76, 15.71 and 15.93 µ for *M. anisopliae* +60 Gy, *M. anisopliae* +30 Gy, *B. thuringiensis* +

15 Gy, 15 Gy, chitosan and *M. anisopliae* +15 Gy, respectively. Meanwhile, treatments of chitosan + 15 Gy, chitosan + 30 Gy and *M. anisopliae* had values close to control.

3. Head diameter and % DNA.

Comet length or resistance system cell length as described previously consists of head diameter and tail length as in Table (3) and figure (1).

Head diameter of untreated cell samples was 14.46 µ that close to chitosan + 15 Gy and *M. anisopliae* +15 Gy treatment values. The treatments effect on head diameter of *S. littoralis* larvae cell by decreasing the head diameter to 10.02, 11.13, 11.96, 12.19, 12.35, 12.93, 13.46 and 13.6 µ for 60 Gy, *M. anisopliae* +60 Gy, chitosan + 60 Gy, *B. thuringiensis* + 15 Gy, *M. anisopliae* +30 Gy, 15 Gy, chitosan and chitosan + 30 Gy, respectively. The rest treatments causes cell head diameter increased compared to control.

Normal % DNA in cell head was 92.49%. Some treatment causes DNA division increased to reach 95.07 and 93.35% in *M. anisopliae* and *M. anisopliae* +30 Gy; while, treatments of *B. thuringiensis* + 60 Gy and 60 Gy had values close to control. Other treatments decreased % DNA in the cell head as described in table (3) and figure (1).

4. Tail length and % DNA.

Tail length indicated the cell malformation. The normal had tail length 1.662 µ; the tail length was increased in all the treatments except for 60 Gy (1.421 µ), *M. anisopliae* (1.247 µ) and *M. anisopliae* +30 Gy (1.3 µ) that had tail length lower than control.

Normal % DNA in the tail that migration from the head was 7.503 %, this percent decrease just only in *M. anisopliae*; while, treatments of 60 Gy and *B. thuringiensis* + 60 Gy had values close to the control as in Table (3) and figure (1). Other treatments had increased in % DNA in tail that means the highly migration from head to tail as indication to the malformation happened in the cells.

5. Tail moment.

That parameter as a result of tail length and its containing from % DNA migrated from head of *S. littoralis* larvae comet length. When tail moment decreased, it mean sever affected on the resistance system cell genotoxicity as shown in Table (3) and figure (1); the most treatments had increased from tail moment, especially in 30 Gy and chitosan + 60 Gy treatments. Another trend was found in 60 Gy treatments that had value near from the control.

Table (4) described the total number of examined nuclei in the resistance system cells of *S. littoralis* larvae and its different % DNA destruction grades as a result of genotoxicity of different treatments.

Different examined nuclei were 628.6 nuclei in the investigated resistance system cells of untreated *S. littoralis* larvae. The value was decreased in the treatments that

causes damage nuclei in the cells; the treatments were 60 Gy (333.4), 30 Gy (424.8), *M. anisopliae* (472.7), chitosan + 15 Gy (539.6) and *M. anisopliae* + 15 Gy (557.6). While, treatments of *M. anisopliae* + 30 Gy and chitosan + 30 Gy had values close to control. Rest of treatments had increased from no. of nuclei in the cells because of the mutations in the nuclei as affected by genotoxicity of the treatments (Table 4 and figure 1).

Different DNA destruction percent affected by genotoxicity of the treatments on *S. littoralis* larvae resistance system cells as follows:

Grade 0: That grade classified as no of damage in DNA normal cell, the tail length less than 5%. This percent was 62.07% DNA destruction in the normal cells. All the treatments had depressed values compared to control, especially in chitosan treatments combined with gamma doses of 15 or 30 Gy.

Grade 1: Classified as DNA had slightly damage, tail length: 5-20%. This percent was 36.59 % in normal cells. Most of treatment increased % DNA destruction ranged between 37 to 54%. Meanwhile, a few treatments had decreased in % DNA destruction compared to control ranged from 26.09 to 35.96%.

Grade 2: At this classified, DNA percentages had moderately destruction, the tail length 20-40 %. The grade value percent was 1.33 % DNA destruction in the control. All the treatments had a high increasing in % DNA destruction ranging from 9.935 to 38.65 % as in Table (4) and figure (2).

Grade 3: Classified as a heavily % DNA destruction rang: 40-92%. Chitosan + 60 Gy had the highly % DNA destruction (8.399%), followed by chitosan + 30Gy (7.829%), *M. anisopliae* + 15 Gy (5.681%), chitosan (3.991%), *B. thuringiensis* + 30 Gy (3.902%), *M. anisopliae*+ 60 Gy (2.604 %) and chitosan + 15 Gy (1.868%). Meanwhile, other treatments had not % DNA destruction classified as grade 3 as well as control (Table 4 and figure 2).

Table (5), illustrated different genotoxicity as affected by different compounds on *S. littoralis* larvae resistance system cells as classified % DNA grade comparing with the control by increasing or decreasing to make easily investigate the recorded data.

Figures (3, 4, 5 & 6) showed the different comet cell shape of *S. littoralis* larvae in the control and different treatments by using Fluorescent microscope at 40X. Figures appeared the different destruction in the resistance system cells as affected by most treatments, especially in chitosan + 60 Gy, followed by chitosan + 30 Gy that had the most genotoxicity on *S. littoralis* larvae as appeared in the most aforementioned parameters compared to genotoxicity of rest of the treatments used compared to control.

The Comet assay has been applied to species already used in bio-monitoring or toxicity testing and has proven to be a resistance system for screening chemicals and complex mixtures for their genotoxicity. Interestingly, all the studies reported in this

review demonstrate that chemicals may be investigated in vitro and in vivo in numerous organisms: plants, worms, molluscs, fish, amphibians, and mammalians. Furthermore, some concern that environmentally complex mixtures can be tested with the SCGE protocol. The simplest method for measuring DNA damage is to calculate the percentage of damaged cells, also called percentage of comets. In order to determine this parameter, different categories of damaged cells are arbitrarily defined, which is a disadvantage according to Tice (1995). Another frequently used parameter is the tail length.

Table 3. Mean comet assay parameters of *S. littoralis* larvae resistance system cells affected by different compounds with gamma irradiation.

Treatments	Cell no.	Comet length (µ)	Head diameter (µ)	% DNA in head	Tail length (µ)	% DNA in tail	Tail moment
Control	39^{de}	16.12^{abc}	14.46^{abcd}	92.49^{ab}	1.662^{a}	7.503^{cde}	0.081^{ab}
15 Gy	76^{a}	14.76^{abc}	12.93^{abcd}	89.90^{abc}	1.862^{a}	10.09^{bcd}	0.112^{ab}
30 Gy	21^{i}	20.23^{ab}	17.67^{ab}	84.72^{c}	2.554^{a}	15.28^{a}	0.180^{a}
60 Gy	19^{j}	17.55^{abc}	10.02^{d}	92.45^{ab}	1.421^{a}	7.548^{cde}	0.082^{ab}
B. thuringiensis	51^{c}	20.60^{a}	18.63^{a}	88.79^{bc}	2.245^{a}	11.21^{abcd}	0.126^{ab}
***B. thuringiensis* +15 Gy**	53^{bc}	13.97^{c}	12.19^{bcd}	91.56^{ab}	1.773^{a}	8.435^{bcde}	0.092^{ab}
***B. thuringiensis* +30 Gy**	43^{d}	17.81^{abc}	15.92^{abc}	90.95^{ab}	1.884^{a}	9.046^{bcde}	0.099^{ab}
***B. thuringiensis* +60 Gy**	30^{gh}	18.83^{abc}	17.03^{ab}	92.75^{ab}	1.800^{a}	7.251^{cde}	0.078^{ab}
M. anisopltae	28^{h}	16.88^{abc}	15.63^{abcd}	95.07^{a}	1.247^{a}	4.933^{e}	0.052^{b}
***M. anisopltae* + 15 Gy**	35^{ef}	15.93^{abc}	13.99^{abcd}	90.73^{abc}	1.934^{a}	9.271^{bcde}	0.102^{ab}
***M. anisopltae* + 30 Gy**	49^{c}	13.64^{c}	12.35^{bcd}	93.35^{ab}	1.300^{a}	6.652^{de}	0.071^{ab}
***M. anisopltae* + 60 Gy**	56^{b}	13.08^{c}	11.13^{cd}	91.65^{ab}	1.948^{a}	8.346^{bcde}	0.091^{ab}
Chitosan	31^{fgh}	15.71^{abc}	13.46^{abcd}	88.00^{bc}	2.253^{a}	11.99^{abc}	0.136^{ab}
Chitosan+15Gy	33^{fg}	16.35^{abc}	14.02^{abcd}	88.83^{abc}	2.749^{a}	11.17^{abcd}	0.126^{ab}
Chitosan+30 Gy	35^{ef}	16.65^{abc}	13.60^{abcd}	88.88^{abc}	2.990^{a}	11.31^{abcd}	0.127^{ab}
Chitosan+60 Gy	52^{bc}	14.09^{bc}	11.96^{bcd}	87.32^{bc}	2.132^{a}	12.68^{ab}	0.145^{ab}
L.S.D. $_{0.05}$	**4.199**	**5.232**	**4.849**	**5.256**	**2.315**	**4.219**	**0.097**
P.	**0**	**0.150**	**0.046**	**0.045**	**0.977**	**0.0027**	**0.543**
Significant	***	**ns**	*	*	**ns**	**	**ns**
F	**103.4**	**1.537**	**2.029**	**2.037**	**0.374**	**3.218**	**0.929**
S.S	**10090**	**544.8**	**530**	**632.02**	**72.87**	**516.7**	**0.157**
Error mean square	**6.375**	**9.896**	**8.5**	**10.10**	**1.938**	**6.438**	**0.0034**

Fig. 1. Comet assay of *S. littoralis* larvae resistance system cells as affected by different compounds with gamma irradiation.

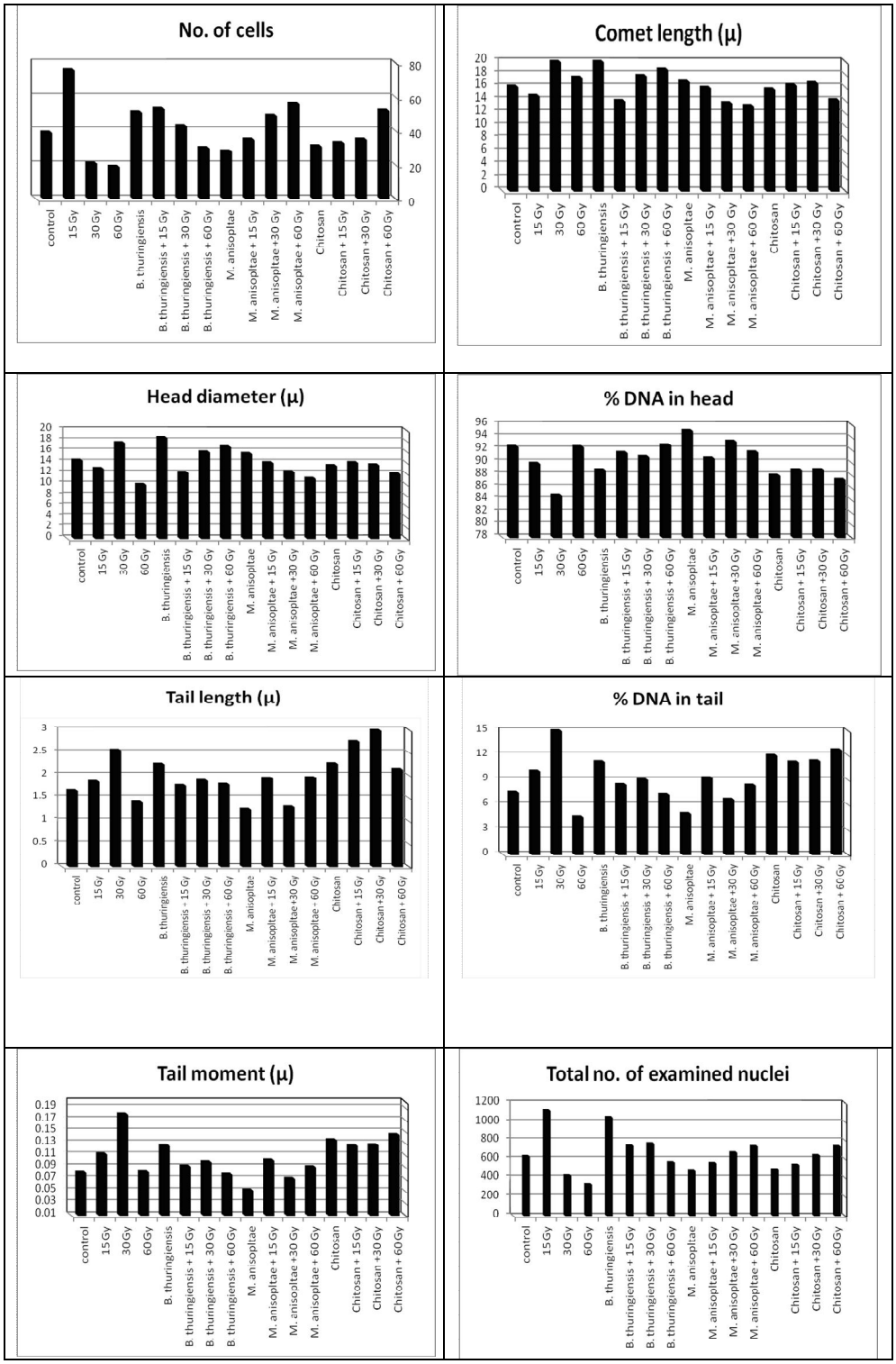


Table 4. Comet assay different grades of *S. littoralis* larvae resistance system cells as affected by different compounds with gamma irradiation.

Treatments	Total no. of examined nuclei	Grade 0		Grade 1		Grade 2		Grade 3	
		No.	%	No.	%	No.	%	No.	%
Control	628.6 d	390.2 ab	62.07 a	230 bcde	36.59 abcde	8.36 f	1.33 d	0	0
15 Gy	1122 a	440.2 a	39.23 abc	403.6 ab	35.96 bcde	248.4 a	22.14 abc	0	0
30 Gy	424.8 h	108.4 fg	25.51 cd	205.9 cde	48.47 abc	72.0 d	16.95 bcd	0	0
60 Gy	333.4 i	190.8 def	57.24 ab	109.4 e	32.83 cde	33.12 ef	9.935 cd	0	0
B. thuringiensis	1050 b	403.9 a	38.44 abc	462.9 a	44.06 abcd	183.9 b	17.51 bcd	0	0
***B. thuringiensis* +15 Gy**	740.5 c	250.9 cde	33.88 abcd	312.1 abcd	42.15 abcde	177.5 bc	23.97 abc	0	0
***B. thuringiensis* +30 Gy**	765.7 c	312.1 bc	40.76 abc	337.3 abc	44.05 abcd	86.4 d	11.28 cd	29.88 bc	3.902 bc
***B. thuringiensis* +60 Gy**	564.8 e	239.4 cde	42.38 abc	268.9 bcde	47.61 abc	56.52 de	10.0 cd	0	0
M. anisopltae	472.7 gh	280.4 cd	59.33 a	138.2 de	29.25 de	54.0 de	11.42 cd	0	0
***M. anisopltae* + 15 Gy**	557.6 e	165.6 ef	29.69 bcd	282.2 abcde	50.61 ab	78.12 d	14.0 bcd	31.68 bc	5.681 abc
***M. anisopltae* + 30 Gy**	668.5 d	269.6 cd	40.33 abc	296.3 abcd	44.32 abcd	69.48 d	10.39 cd	0	0
***M. anisopltae* + 60 Gy**	732.6 c	225.0 cde	30.71 bcd	271.1 bcde	37.00 abcde	217.4 a	29.68 ab	19.08 c	2.604 c
Chitosan	487.1^{fg}	192.2^{def}	39.47^{abc}	127.1^{de}	26.09^{e}	148.3^{c}	30.45_{ab}	19.44^{c}	3.991^{bc}
Chitosan + 15 Gy	539.6 ef	50.04 g	9.273 d	292.4 abcde	54.17 a	187.2 b	34.69 a	10.08 c	1.868 c
Chitosan + 30 Gy	639.6 d	50.04 g	7.823 d	292.4 abcde	45.71 ab	247.2 a	38.65 a	50.08 ab	7.829 ab
Chitosan + 60 Gy	732.9 c	202.7 de	27.65 cd	289.8 abcde	39.54 abcde	171.7 bc	23.43 abc	61.56 a	8.399 a
L.S.D. $_{0.05}$	**55.26**	**83.86**	**24.27**	**157.44**	**14.877**	**29.82**	**15.03**	**23.02**	**3.744**
P.	**0**	**0**	**0.0021**	**0.0048**	**0.0158**	**0**	**0.001**	**0.003**	**0.014**
Significant	***	***	**	**	*	***	***	**	*
F	**118.9**	**15.61**	**3.326**	**2.966**	**2.464**	**57.73**	**3.989**	**5.818**	**4.125**
S.S	**20046**	**6767**	**1744**	**6854**	**5517**	**2886**	**7501**	**8447**	**177.2**
Error mean square	**1104.0**	**2542.3**	**213.0**	**8961.3**	**80.012**	**321.5**	**81.68**	**172.7**	**4.571**

Fig. 2. Comet assay grades (DNA destruction) of *S. littoralis* larvae resistance system cells as affected by different compounds with gamma irradiation.

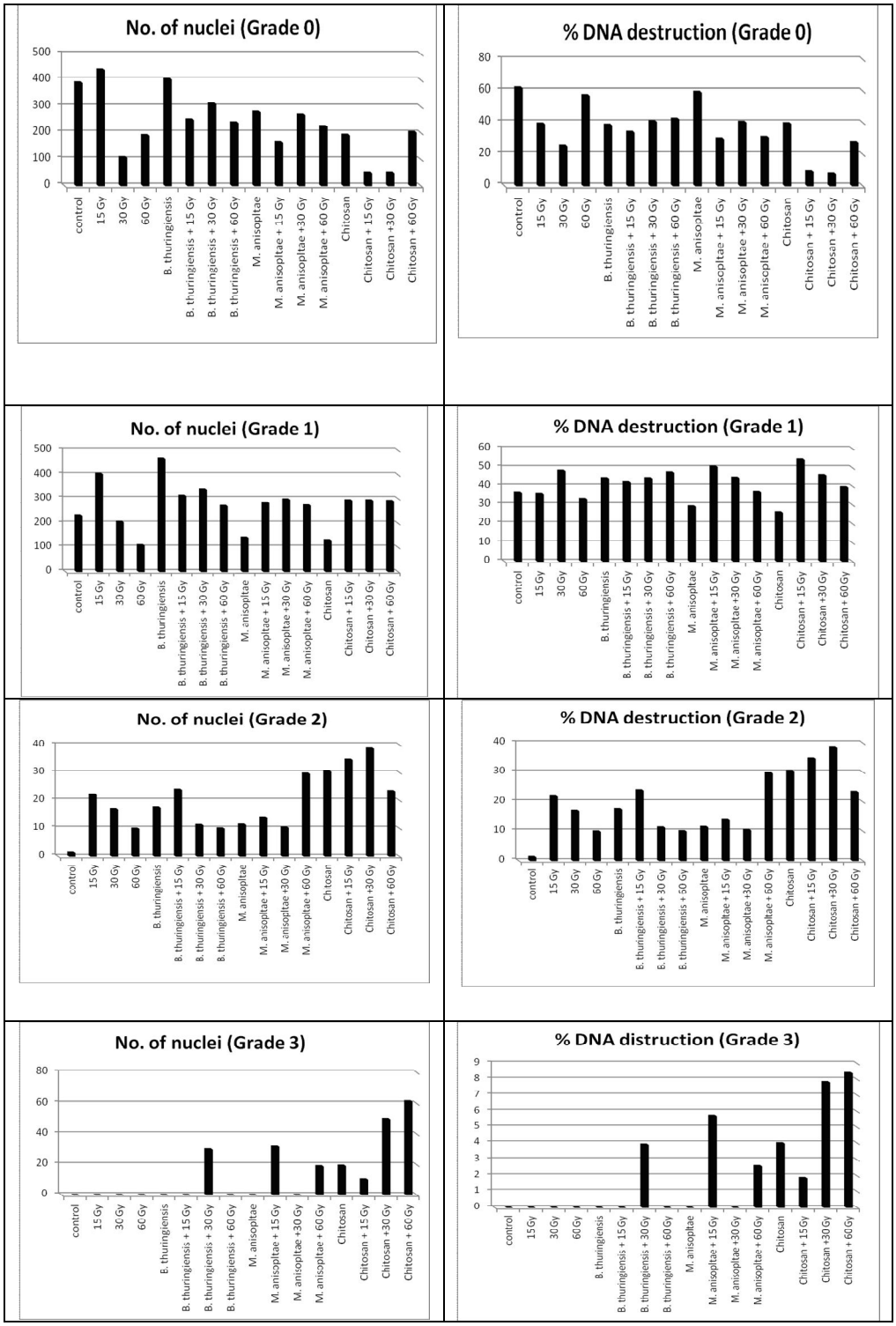


Table 5. Comet assay different grades of *S. littoralis* larvae resistance system cells affected by different compounds with gamma irradiation compared to control.

Treatments	Total no. of examined nuclei	Grade 0		Grade 1		Grade 2		Grade 3	
		No.	%	No.	%	No.	%	No.	%
Control	628.56	390.2	62.07	230.0	36.59	8.36	1.33	0	0
15 Gy	+493.6	+50.0	-22.8	+173	-0.63	+240	+20.8	0	0
30 Gy	-203.8	-281	-36.6	-24.1	+11.9	+63.6	+15.6	0	0
60 Gy	-295.2	-199	-4.83	-120	-3.76	+24.8	+8.61	0	0
B. thuringiensis	+422.3	+13.8	-23.6	+232	+8.37	+175	+16.2	0	0
***B. thuringiensis* +15 Gy**	+111.9	-139	-28.2	+82.1	+5.56	+169	+22.6	0	0
***B. thuringiensis* +30 Gy**	+137.2	-78	-21.3	+107	+7.46	+78.0	+9.95	+29.9	+3.90
***B. thuringiensis* +60 Gy**	-63.72	-150	-19.7	+38.9	+11.0	+48.2	+8.68	0	0
M. anisopltae	-155.9	-109	-2.74	-91.8	-7.34	+45.6	+10.1	0	0
***M. anisopltae* + 15 Gy**	-70.92	-224	-32.4	+52.2	+14.0	+69.8	+12.7	+31.7	+5.68
***M. anisopltae* + 30 Gy**	+39.96	-120	-21.7	+66.2	+7.73	+61.1	+9.06	0	0
***M. anisopltae* + 60 Gy**	+104.0	-165	-31.4	+41.0	+0.41	+209	+28.4	+19.1	+2.60
Chitosan	-141.5	-197	-22.6	-102	-10.5	+139	+29.1	+19.4	+3.99
Chitosan + 15 Gy	-88.92	-340	-52.8	+62.4	+17.6	+178	+33.4	+10.1	+1.87
Chitosan + 30 Gy	11.08	-340	-54.3	+62.4	+9.12	+238	+37.3	+50.1	+7.83
Chitosan + 60 Gy	+104.4	-187	-34.4	+59.8	+2.95	+163	+22.1	+61.6	+8.39

Moreover, since the comet assay allows measurement of an effect on each observed nucleus, we must question whether the average response of all the cells (using classical mean comparison tests) is the best indicator of genotoxic potential or whether distribution comparison tests must be developed to take into account the different responses of the individual nuclei. Another question concerns the fact that small cell samples may not be representative of the total cell population (Tice *et al.*, 1991).

Actually, published studies show that 50 or 100 cells per experimental condition are generally analyzed. Despite the lack of standardization of the method, the comet assay offers a lot of advantages. Of course, the comet assay does not require uptake

of radio labeled DNA precursors (Ostling and Johanson, 1984). Different reviews showed its sensitivity for detecting DNA damage and its rapidity. In individual cells, it allows quantification of different responses from single-strand breakages to apoptosis. Other advantages are the use of extremely small cell samples and that virtually any eukaryote cell population is amenable to analysis (Tice *et al*., 1991).

Fig. 3. Comet assay of *S. littoralis* larvae resistance system cells exposed to gamma irradiation.

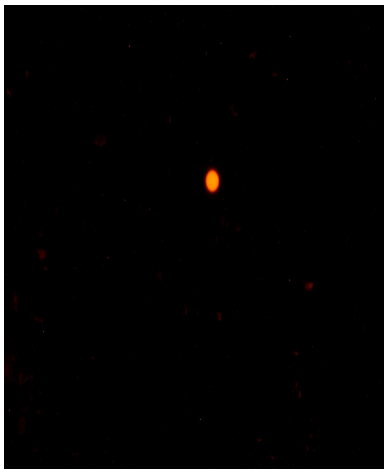
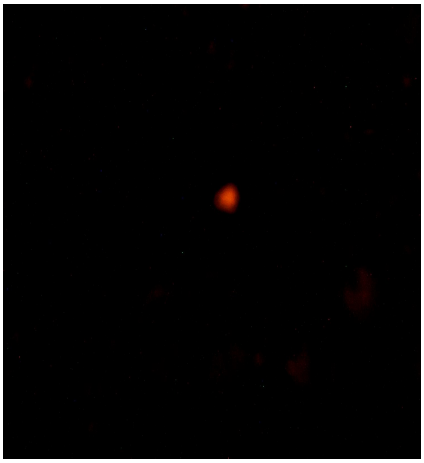
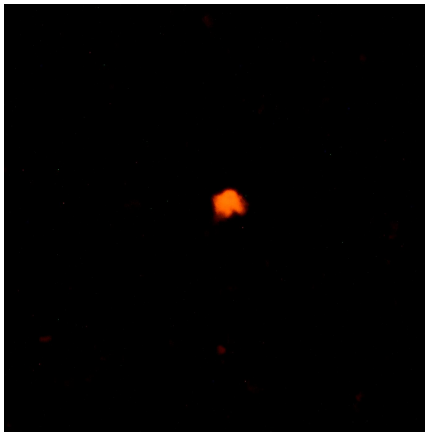
	
Control (40 X) Normal cell, a round cell without a tail, no damage.	15 Gy (40 X) The cell appeared in a disturbance state abnormality, cell wall is not roundness
	
30 Gy (40 X) Most of cell containing and cell wall near from disappearance, the cell tail length was slightly appeared.	60 Gy (40 X) The cell was swelling and the tail clearly appeared and the cell wall was not roundness.

Fig. 4. Comet assay of *S. littoralis* larvae resistance system cells as affected by *B. thuringiensis* exposed to gamma irradiation.

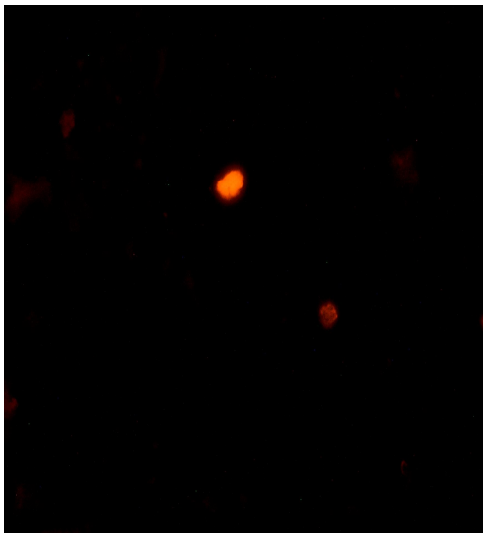
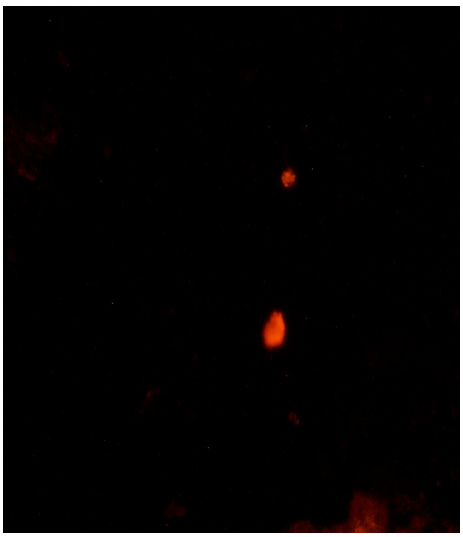
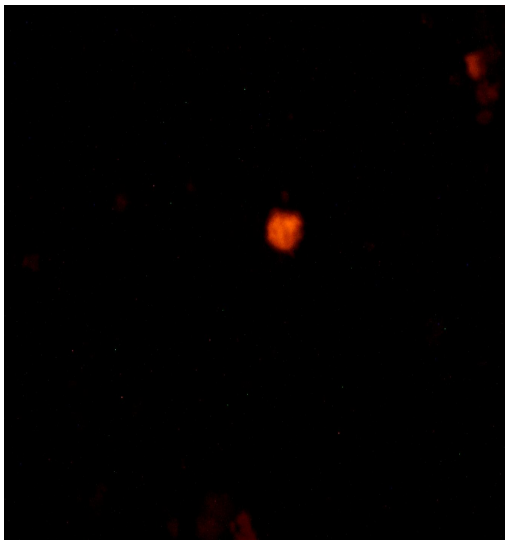
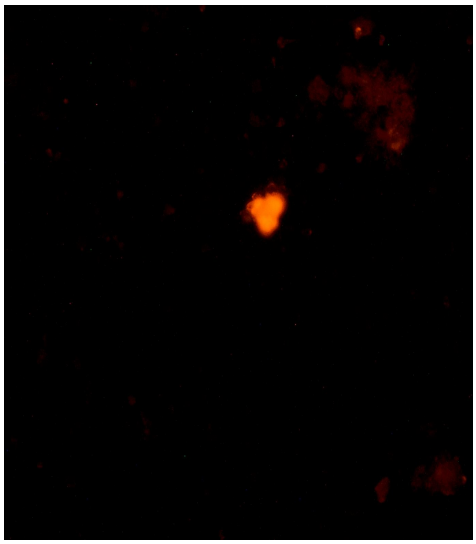
	
B. thuringiensis (40 X) The cell shape was not roundness and had slightly swelling compared to normal cell.	*B. thuringiensis* +15 Gy (40 X) The cell shape was not roundness and the tail slightly appeared.
	
B. thuringiensis +30 Gy (40 X) The cell was swelling; lost the most roundness and the tail appearance.	*B. thuringiensis* +60 Gy (40 X) The cell lost its shape and roundness and clearly swelling with tail appearance.

Fig. 5. Comet assay of *S. littoralis* larvae resistance system cells as affected by *M. anisopltae* exposed to gamma irradiation.

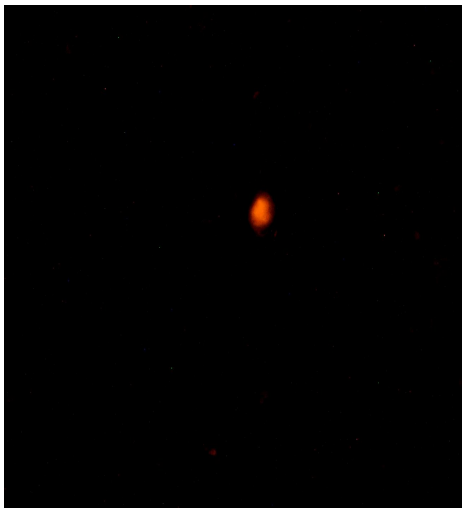
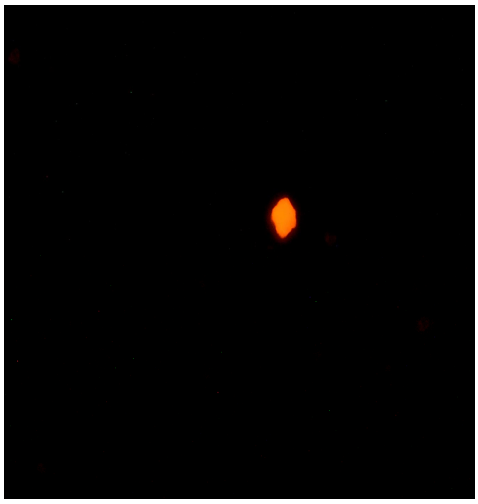
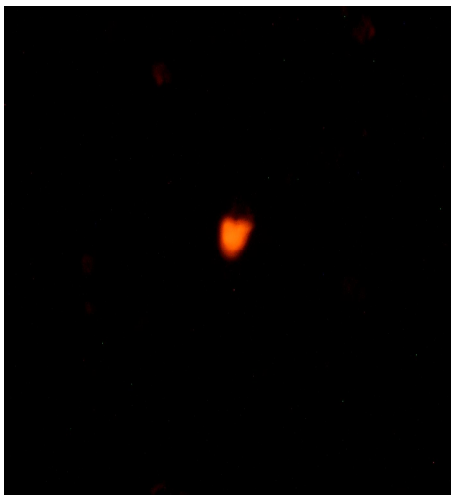
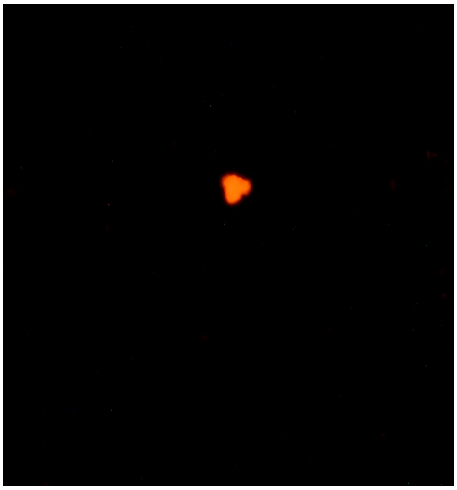
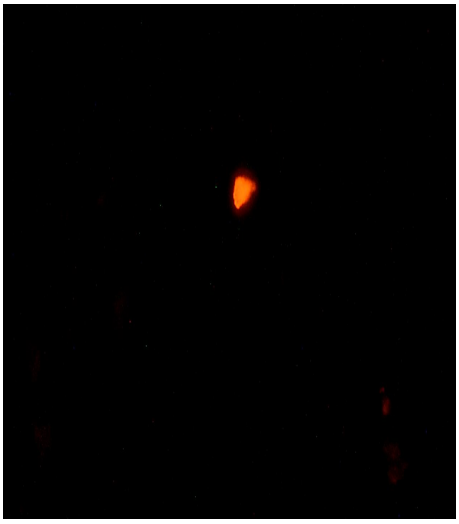
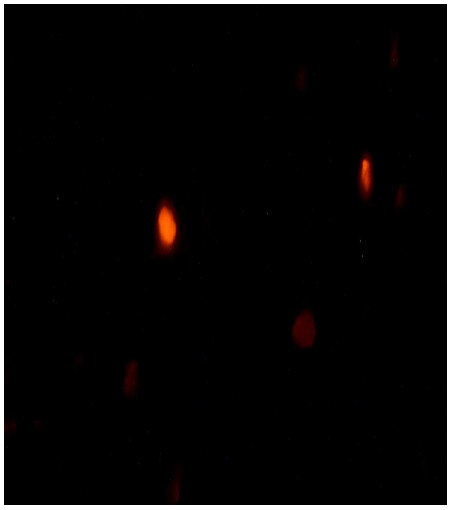
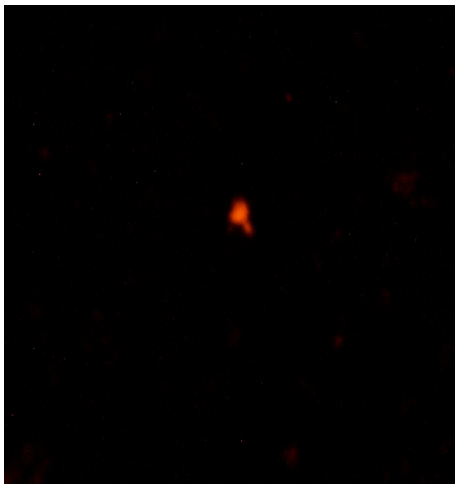
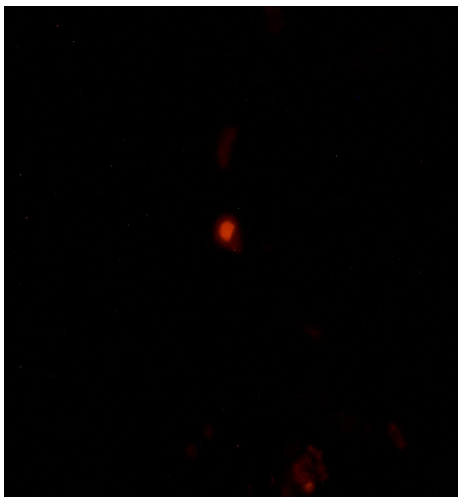
	
M. anisopltae (40 X) The cell lost its roundness and tends to the elongation, the cell wall had disruption and the tail appearance.	*M. anisopltae* +15 Gy (40 X) The cell is swelling; lost its roundness and tends to the elongation; the cell wall is lost its normal shape with appearance the tail.
	
M. anisopltae +30 Gy (40 X) The cell lost the most its characterizations as roundness the cell and tends to triangle shape and disruption with tail appearance.	*M. anisopltae* +60 Gy (40 X) The cell lost the most its characterizations as roundness the cell and tends to triangle shape and disruption with tail appearance.

Fig. 6. Comet assay of *S. littoralis* larvae resistance system cells as affected by Chitosan exposed to gamma irradiation.

	
Chitosan (40 X) The cell has triangle shape, lost its roundness and tail appearance.	Chitosan +15 Gy (40 X) The cell lost the normal roundness and tends to the elongation.
	
Chitosan +30 Gy (40 X) The cell had highly destruction, shrinking, lost its roundness, the tail clearly appearance and destruction in the most cell component.	Chitosan +60 Gy (40 X) The cell completely destruction nearly, shrinking the cells to the smallest size and necrosis.

Furthermore, to see an effect in the comet assay there is no need for cell division, whereas micronuclei can be detected only after mitosis (Belpaeme *et al*., 1996). The most important disadvantage of the assay concerns the necessity for single cell suspensions.

The relationships between DNA structural alterations or genome dis-functioning and effects at a level of organization higher than that of individuals are not straightforward. To predict effects at the population level, genome approaches need to be complemented with phenotypic studies for growth, reproduction and juvenile sensitivity. An integration of molecular and physiological data should optimize the knowledge gained from use of these molecular tools.

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قياسات تحليل الكوميت للخلايا المسئولة عن مقاومة يرقات دودة ورق القطن المعاملة بالمركبات المختلفة المعرضة لآشعة جاما

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يعرف تحليل الكوميت بأنه التفريد الكهربائى للخلايا المنفردة (SCGE) أو التفريد الكهربائى الدقيق (MGE) الذى يقيس أساسا مدى تحطم شريط الحامض النووى طبقا لنوع الخلايا الناتجة فى الإختبار المعملى.

والهدف الرئيسى لهذه الدراسة هو قياس السمية الجينية للخلايا المسئولة عن مقاومة يرقات دودة ورق القطن للمركبات المختلفة وذلك بحدوث إستخلاص للخلايا المسئولة عن مقاومة الآفة للمبيدات والمتأثرة بثلاثة معاملات لمركبات بكتيريا الـــ *B. thuringiensis* وفطر الـــ *M. anisopltae* ومركب بيوبوليمر (الشيتوزان) والذين تم تعريضهم لجرعات آشعة جاما ١٥، ٣٠، ٦٠ جراى وذلك بهدف زيادة كفائتهم السمية الجينية على يرقات العمر الرابع لدودة ورق القطن والتى عوملت بالتركيز النصفى المميت للمركبات السالفة الذكر.

بكتيريا الـــ *B. thuringiensis* والتى عرضت لجرعات آشعة جاما ١٥، ٣٠، ٦٠ جراى حدث لها تقوية وزادت كفائتها الإبادية على دودة ورق القطن عن إستخدام مستحضر البكتيريا منفردا دون التعرض لآشعة جاما. بينما مستحضرات الفطر والشيتوزان أعطيا نتيجة متقاربة فى كفائتهم الإبادية سواء تمت المعاملة بهما منفردين أو معرضين لآشعة جاما.

أظهرت القياسات المختلفة لتحليل الكوميت (الخلايا المسئولة عن مقاومة الآفة) والتى تضمنت عدد الخلايا- طول الكوميت أو طول الخلية (ميكرون)- طول رأس الخلية (ميكرون)- النسبة المئوية للحامض النووى فى رأس الخلية – طول ذيل الخلية (ميكرون)- النسبة المئوية للحامض النووى لذيل الخلية- مدى تشوه الخلية (نسبة الحامض النووى فى الذيل مقسوما على نسبة الحامض النووى فى رأس الخلية) الذين تأثروا جميعا تأثيرا مباشرا عند إستخدام المركبات السالفة الذكر على دودة ورق القطن والذى أدى إلى موت معظم الخلايا وتحطم الحامض النووى بداخلها. كما أدى إستخدام المركبات السابقة إلى زيادة إنقسامات الحامض النووى مما أدى إلى ظهور أعداد أكبرللخلايا المشوهة وزيادة عدد الأنوية والحامض النووى بداخلها مقارنة بالكونترول.

كما أدت المعاملات إلى زيادة درجات النسبة المئوية لتحطم الحامض النووى على حسب المعاملة والتى تتراوح من صفر- ٣ مستويات للتحطم. بالإضافة إلى ظهور الضرر الواضح للخلايا خلال فحصها تحت الميكروسكوب الفلورسنت وقد تمثل الضرر فى ظهور تشويش للخلايا- فقد إستدارة الخلايا- فقد فى جدر الخلايا- إنتفاخ أو إنكماش فى معظم الخلايا التى تم فحصها.

وعلى ذلك يعتبر مركب الشيتوزان والمعرض لجرعات آشعة جاما أكثر تأثيرا من ناحية السمية الجينية على الخلايا المسئولة عن مقاومة يرقات دودة ورق القطن يليه فى ذلك البكتيريا والفطر المعرضين لآشعة جاما.