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Effect of Gamma Irradiation on Viability, Pathogenicity, Reproductivity of *Heterorhabditis bacteriophora* and *H. indica* and detection of DNA Damage

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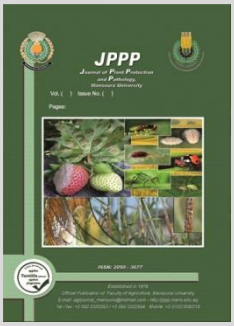

ABSTRACT

Entomopathogenic nematodes (EPNs) are a promising alternative method for insect pest control. The idea of the work is to investigate the effect of gamma irradiation on EPNs pathogenicity under laboratory conditions aiming to enhance their lethal effect against insect pests. *Heterorhabditis bacteriophora* (Hb) as well as *H. indica* (Hi) were irradiated directly in nematode suspension and indirectly within the insect cadavers by gamma irradiation dosages of 0, 100, 250, 500, 750 and 1000 Gy. The mortality percentage of nematodes were recorded, the extracted nematodes from the irradiated insect hosts were used to investigate their pathogenicity and reproductivity against *Galleria mellonella* larvae. The damage of DNA was analyzed using the comet assay and the LC50 of the two nematode species was recorded. The data demonstrated that, the low dosage (100 Gy) enhanced the pathogenicity of EPNs and didn't affect the initial populations (Pi), the final population of emerged IJs (Pf) and the rate of reproduction (Rr%). Moreover, *H. bacteriophora* was more tolerant to gamma irradiation than *H. indica* which recorded more DNA damage %, DNA in tail, tail length, tail moment and olive tail moment in the all different irradiated dosages. In conclusion, low dosages of gamma irradiation can be used to enhance the pathogenicity and reproductivity of entomopathogenic nematodes in order to use as a biocontrol agent.

Keywords: Gamma irradiation, *Heterorhabditis bacteriophora*, *H. indica*, *Galleria mellonella*, DNA Damage, comet assay

INTRODUCTION

Biocontrol agents by entomopathogenic nematodes (EPNs) are a satisfaction alternative control method to insect chemical pesticides to avoid the hazard effect of chemical residues on environment, the lethal effect on human and the non-target organisms and without inducing resistant races of insect hosts (Rojht *et al.* 2009; Shapiro-Ilan *et al.* 2015). *Heterorhabditis* is one of the most prevalent and effective genera of EPNs in the field application (Lacey and Shapiro-Ilan, 2008; Shapiro-Ilan *et al*., 2014). In this respect, heterorhabditids nematodes have a specific symbiosis bacterium which could prolong the life cycle, encourage persistence of nematodes in soils, and have critical role in insect killing (Lewis and Clarke, 2012).

Efficacy of EPNs as a biocontrol agent may be affected by some environmental conditions such as temperature, desiccation, and radiation (Shapiro-Ilan *et al.*, 2012). Previous research focused on the effect of ultraviolet rays on nematode viability and virulence (Mason and Wright, 1997; Fujiie and Yokoyama, 1998). These two parameters are essential to evaluate the potential of EPNs as a biocontrol agent (Shapiro-Ilan *et al.* 2015). Reasonably, it is very important to investigate the effect of Gamma irradiation on nematode survival and virulence efficiency.

Single cell gel electrophoresis (SCGE) or Comet assay technique has become one of the accurate methods that used for measurement of DNA damage due to its simplicity, reliability, sensitivity, low cost, and versatility (Fabbrizi *et al.* 2021; Cordelli *et al*., 2021). For the first time, comet assay was able to detect the fraction of radiobiologically within an irradiated population of cells; it was using to asses that the rejoining rate of DNA breaks were relatively homogenous (Olive, 2009). This study aimed to investigate the effect of gamma radiation on *Heterorhabditis bacteriophora*, and *Heterorhabditis indica* survival, virulence, and reproduction within their insect host *Galleria mellonella*, and to detect the effect of gamma radiation of DNA damage by using comet assay

MATERIALS and METHODS

Materials

1- Entomopathogenic Nematode Culture:

The EPNs used in this research were, *Heterorhabditis bacteriophora* (Hb) and *Heterorhabditis indica* (Hi). These nematodes were obtained from Nematology laboratory, Faculty of Agriculture, Cairo University, Giza. The nematodes were reared on the fifth instar larvae of the greater wax moth (*Galleria mellonella* L.). Insect larvae were placed in a 12-cm-diam Petri dish lined with a moistened paper tissue and exposed to about 100 IJ3s/insect larva at 25 ºC. After 48 hr. Dead insect larvae were transferred to spongy trap dishes (consisted of 10 cm length x5cm width x3mm thickness) in a petri dish and kept in a plastic bag. The amount of water in wetted sponge was calculated approximately as 10 ml of water /1gm of sponge. All sponge traps were maintained at 25±2 ˚C according to the method made by Kassab and Taha

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(2016). After 6-10 days, the IJs were collected and used prior 2 weeks of collecting.

2- The Greater wax moth, *G. mellonella* Culture:

G. mellonella larvae were obtained from beehives, Faculty of Agriculture, Ain Shams, Shoubra El-Kheima, Qalubia and reared on artificial feeding media in plastic jars at 25 ±2 oC, in the laboratory and then eggs were gently removed and incubated in other rearing jars provided with the hatching medium (Kassab and Taha 2016).

Methods

1- Irradiation technique

a- For EPN suspension:

The third infective juveniles (IJs) suspension of Hb and Hi were irradiated separately by different Gamma irradiation doses which were: 0, 100, 250, 500, and 1000, Gy. by using Gamma Cell Irradiation Unit (cobalt 60 source), in the National Centre for Radiation Research and Technology (NCRRT). The dose rate was 1000Gy/75min.

b- For infected insect larvae:

Infected *G. mellonella* larvae of each nematode species *H. bacteriophora*, *H. indica* (after three days of infection) were exposed to the previous gamma irradiation levels in a plastic Eppendorf tube, each dose was replicated five times. After exposure, the infected irradiated insect larvae were transferred to spongy trap dishes separately and they maintained at 25±2 ˚C (Kassab and Taha 2016). After 10 days, the new emerged IJs were collected and used in the following experiment to assay the virulence of the newly emerged irradiated nematode juveniles.

3.Experimental methods:

Viability (survival) bioassay:

Five ml of nematode suspension containing approximately 1,000 IJs of each nematode species were placed in a 50-cm plastic tube separately. The nematodes were exposed to the previous irradiation doses, each dose was replicated 5 times. The nematode suspension was transferred immediately after exposure into 6-cm-diam Petri dish. One day after, the living (movable) and dead (immovable and straight) nematodes were counted and the corrected mortality percentage were calculated using the Abbott's formula Abbott (1925) as follows: Corrected Mortality (%) = $\left\{\frac{T-C}{100-C} x100\right\}$. Where, T is mortality percentage in the treatment and C is mortality percentage in the control. Values of LC50 were calculated using a software sigma plot (11.0) program (Iwasa *et al.*, 2003).

Virulence (pathogenicity and reproduction) bioassay:

Unirradiated infected insect larvae were placed in a 12-cm-diam Petri dish lined with a moistened paper tissue and exposed to about 400 IJ3s/replicate (extracted from the previous irradiated insect cadavers) at 25 ºC. After 48 hr., the 5 replicates were dissected and the initial populations (Pi) were recorded, the other five replicates were transferred separately to spongy trap dish, and maintained at 25±2 ˚C. After 10 days, the final population of emerged IJs (Pf) were counted and the rate of reproduction (Rr%) was calculated as follows: Rr%= (Pi/Pf)*100. Nematode pathogenicity was measured by LC50 of tested insect.

4. **Effect of Gamma Irradiation on DNA damage by alkaline comet assay:**

Extraction

Suspension of *H. bacteriophora* and *H. indica* was placed in a petri dish with Sörensen buffer (50 mM sodium phosphate, pH 6.8, 0.1 mM ethylene diamine tetra acetic acid (EDTA), 0.5% dimethyl sulfoxide (DMSO) kept on ice. The nematode was repeatedly dipped in the cold Sörensen buffer. The suspension with released nuclei was filtered through a 30 µm disposable filter (Partec, Műnster, Germany) to remove most of the debris and centrifuged at 550 g for 5 min at 4ºC, the protocol described by Imanikia *et al.*, (2016).

Preparation of alkaline Comet assay

Microscope slides were coated with 0.5% normal melting agarose and dried at room temperature. Forty µl of the nuclei suspension was mixed with 40 µl of 0.1% low melting agarose, spread on the slide surface and subjected to gel formation for at least 10–15 min on a cooling plate at 4°C. Lysis was carried out in 2.5 M NaCl, 10 mM Na_2EDTA (pH8), 10 mM Tris-HCl (pH8), 1% N-lauroylsarcosine sodium salt, 1% TritonX-100, 10% DMSO for 15 min at 4°C in the dark. Electrophoresis was performed in prepared TAE buffer (pH8) at 0.5, 1, 2 and 5 V/cm for 10 min for nematode nuclei. The slides were dehydrated in 70% and 96% ethanol for 5 min and dried at room temperature. The slides were covered with solution of the fluorescent dye acridine orange (10 µg/ml). Visualization of the stained comets was carried out using a fluorescence microscope (Zeiss Jenamed-2) coupled with a digital camera (Samsung Digimax V50). Three independent experiments were performed, and 50 comets were analyzed per point in each experiment. Damage was detected according to the fragments intensity which migrated during electrophoresis (Parrella *et al.* 2015).

4. Statistical analysis:

The data of all experiments were statistically analyzed using analysis of variance procedure proposed by Snedecor and Cochran (1989). The differences between means were compared using Duncan's Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Results

In regard to the effect of irradiated juveniles of *Heterorhabditis* on their, viability the nematode corrected mortality percentage recorded significantly differences in all the irradiation levels in the two species of nematodes and generally it was high in *H. indica*, than these of *H. bacteriophora* (Fig. 1).

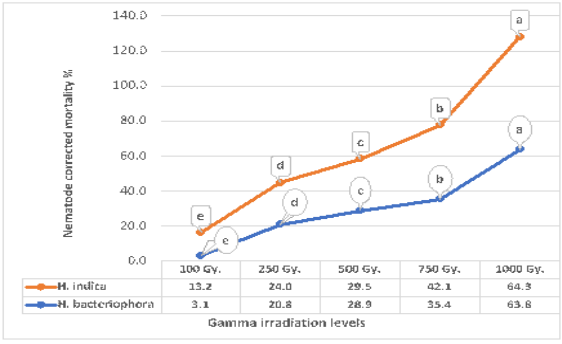

Figure 1. Effect of gamma radiation dosages on nematode corrected mortality percentage of IJs of *Heterorhabditis bacteriophora*, and *H. indica*. Different letters indicate that mean values of treatments are significantly different at P < 0.5.

Data in probit demonstrated that, the LC_{50} in the case of *H. bacteriophora* (Fig. 2) and in *H. indica* (Fig. 3) was 870 Gy which mean that, the lethal effect of gamma irradiation on nematode viability is similar in both cases.

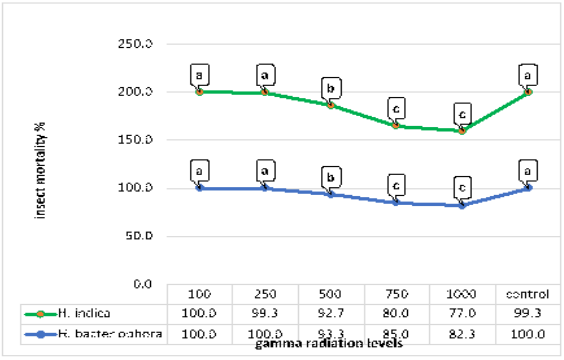

Figure 2. Probit regression line of the effect of gamma irradiation on *Heterorhabditis bacteriophora*

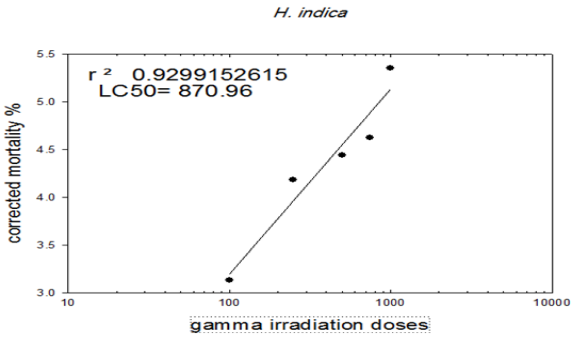

Figure 3. Probit regression line of the effect of gamma irradiation on *Heterorhabditis indica*

In the connection to evaluate the effect of Gamma irradiation on nematode pathogenicity, Gamma irradiation has a high effect on both *H. bacteriophora*, and *H. indica* pathogenicity against *G. mellonella* as shown in Fig. 4. Generally, *H. bacteriophora* was less affected to gamma irradiation than *H. indica*. Moreover, irradiation didn't affect the pathogenicity of *H. bacteriophora*, and *H. indica* with the dosages of 100 and 250 Gy, while it was decreased with the high ones.

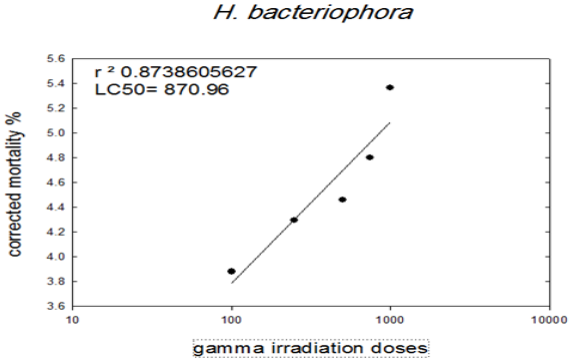

Figure 4. Effect of gamma radiation dosages on *Heterorhabditis bacteriophora,*and *H. indica* pathogenicity against *Galleria mellonella*. Different letters indicate that mean values of treatments are significantly different at P < 0.5.

On their reproduction, data in Table (1) demonstrated that, there was no significant difference between the initial population (Pi) and (Pi%) in the control and the first irradiation level (100Gy), in the investigated Hb and Hi nematode, while there were a significant difference in the rest levels of irradiation (250, 500, 750 and 1000 Gy).

Table 1. Effect of gamma irradiation dosages on the initial population (Pi), (Pi %), final population (Pf) of *Heterorhabditis bacteriophora* and *H. indica* and rate of reproduction (Rr) in *Galleria mellonella* larvae.

Dosages	*H. bacteriophora*				*H. indica*			
	Pi	Pi %	Pf	Rr	Pi	Pi %	Pf	Rr
0	156.7 a ±4.0	39.2 a ±1.0	30933.3 a ±3330.7	224.7 ab ±50.3	151.3 a ±5.5	37.8 a ±1.4	30800.0 b ±400.0	238.4 abc ±31.4
100 Gy	153.3 a ±6.0	38.3 a ±1.5	35066.7 a ±7046.5	212.2 b ±1.7	150.0 a ±8.2	37.5 a ±2.0	36000.0 a ±4000.0	208.6 bc ±17.4
250 Gy	132.7 b ±5.0	33.2 b ±1.3	32533.3 a ±1404.8	305.5 a ±34	119.7 b ±5.5	29.9 b ±1.4	31200.0 b ±1058.3	299.8 a ±53.8
500 Gy	102.3 c ±8.6	25.6 c ±2.2	31066.7 a ±832.7	217.5 b ±18.4	94.0 c ±11.1	23.5 c ±2.8	27866.7 b ±2837.8	218.7 bc ±31.0
750 Gy	83.7 d ±6.7	20.9 d ±1.7	18133.3 b ±1006.6	229.8 ab ±84.9	80.7 d ±2.1	20.2 d ±0.5	17600.0 c ±2116.6	181.3 c ±58.2
1000 Gy	54.3 e ±11.0	13.6 e ±2.7	11866.7 c ±1803.7	233.2 ab ±23.7	49.0 e ±5.6	12.3 e ±1.4	8666.7 d ±1803.7	257.8 ab ±14.2

Means followed by the same letter (s) within a column are not significantly different at 5% level of significance, while different letters had a statistically significant differences.

On the other hand, there were no significant differences between the final population (Pf) in first three irradiation dosages (100, 250 and 750 Gy) in the case of *H. bacteriophora*, while in the case of *H. indica*, all the irradiation dosages are significantly different. In contrast, the rate of reproduction in the two species of nematodes was higher in the treated low dosages than the control.

Regarding to DNA damage, comet assay was performed on the EPNs, *H. bacteriophora* (Fig. 5), and *H. indica* (Fig. 6). Most accurate parameter of DNA damage was DNA percentage in tail because it reflects the total tail intensity while comet total intensity being not reliable on the tail length. Meanwhile, DNA %, DNA in tail, tail length, tail moment and olive tail moment were presented in Figures (5 and 6). Generally, the damage in DNA parameters were higher in *H. bacteriophora* than in *H. indica.*

In case of *H. bacteriophora*, Figure (5) indicated that, the lowest percentage of DNA damage was 10.6 % which recorded with 100 Gy and the highest one was 19.6% exhibited with 1000 Gy. The lowest tail length was 5.15 pixel appeared with 1000 Gy and the highest was 6.38 pixel which observed with 250 Gy. For the percentage of DNA in the tail of comet, the lowest percentage was 8.89 % which detected with 750 Gy and the highest percentage was 13.45 % observed with 1000 Gy. However, the lowest tail moment was 0.42 appeared with 750 Gy and the highest value (0.71) was recorded with 500 Gy. For the olive tail moment, the lowest value (1.17) was recorded with 750 Gy while the highest one (1.67) was exhibited with 1000 Gy.

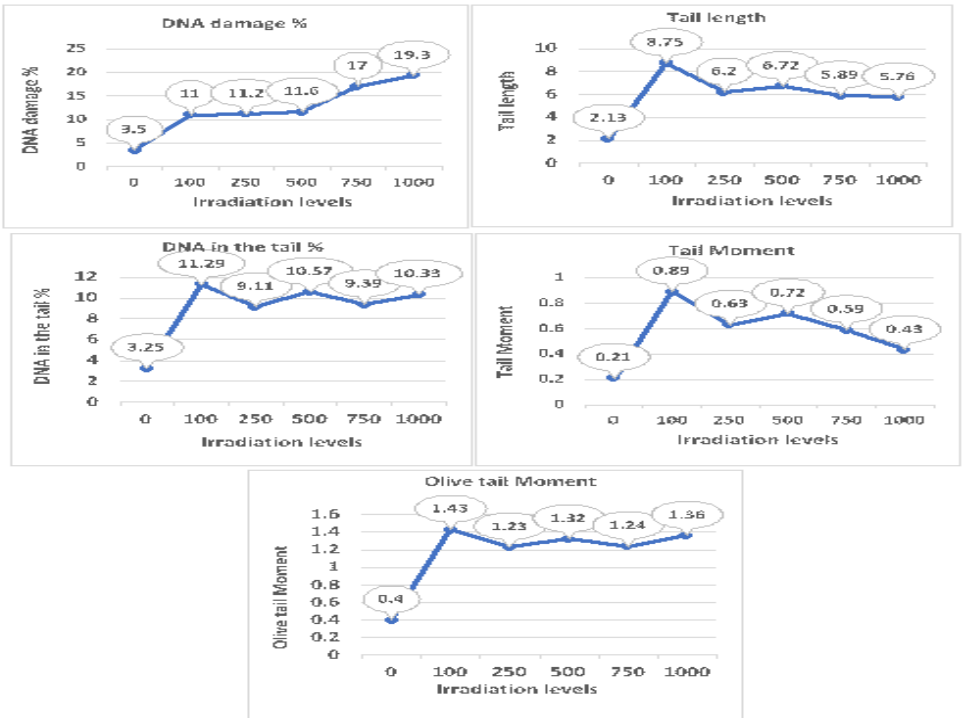

Figure 5. Effect of 5 gamma irradiation dosages on DNA damage in *Heterorhabditis bacteriophora* nematode by comet assay.

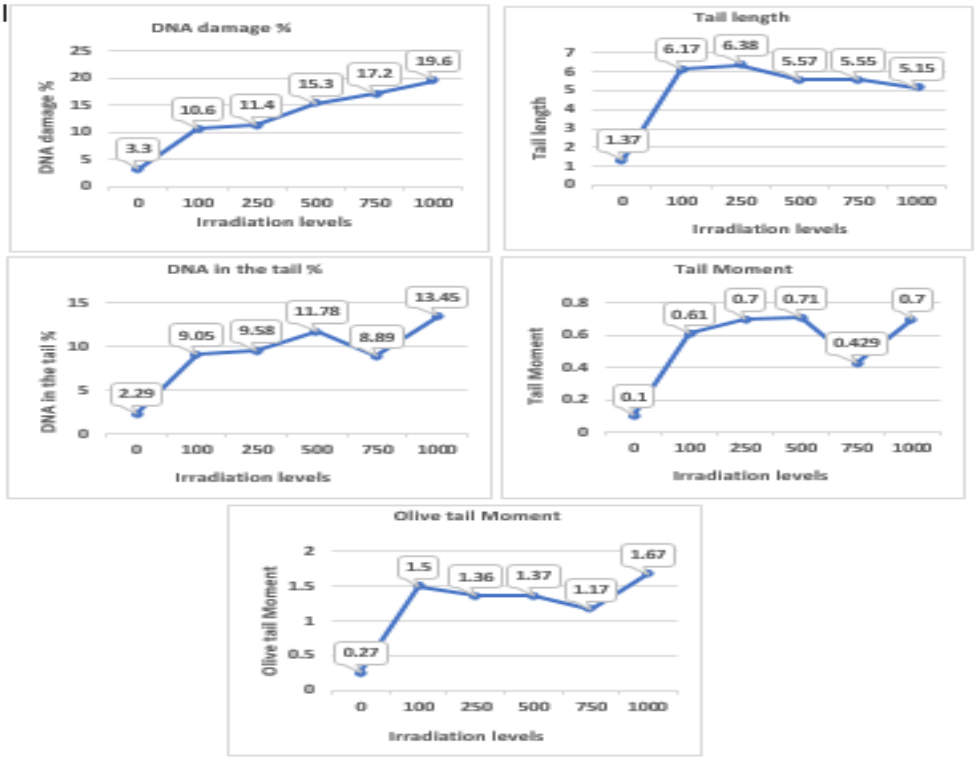

Figure 6. Effect of 5 gamma irradiation dosages on DNA damage in *H. indica* nematode by comet assay.

On the other hand, *H. indica* Figure (6) revealed that, by using 100 Gy the lowest percentage of DNA, damage recorded (11 %) and the highest percentage was detected with 1000 Gy (19.3 %). On the opposite the lowest tail length was observed with 1000 Gy (5.76 pixel) and the highest tail length was 8.75 pixel with 100 Gy. For the percentage of DNA in the tail of comet, the lowest percentage 9.11 % was detected with 250 Gy and the highest percentage was 10.57 observed with 500 Gy. Moreover, the lowest tail moment was 0.43 appeared with 1000 Gy and the highest one 0.89 was detected with 100 Gy. As with olive tail moment, the lowest value was 1.23 recorded with 250 Gy while the highest value was 1.43exhibited with 100 Gy.

Discussion

Regarding nematode pathogenicity, our obtained data are supported by Youssef (2006); Sayed (2008) and Salem *et al.* (2014) who found that, the low doses of gamma irradiated *Steinernema. scapterisci* or *H. bacteriophora* were more

efficient in killing *Callosobruchus maculatus* larvae faster than the unirradiated nematodes. This efficiency may be attributed to the damage of the antioxidant contents in the insect larval body which could cause the faster larval death (Sayed *et al.,* 2015). The total protein was decreased in the irradiated IJs of *S. scapterisci* and *H. bacteriophora* and in turn, decreased in the homogenecity of the *G. mellonella* body tissue (Sayed, 2011) and of *Agrotis ipsilon* body tissue (Hassan *et al.,* 2016). These reductions of protein may be due to the breakdown proteases effect on the total protein and the effect of the extracellular enzymes which secreted by the nematode symbiotic bacteria i.e *Xenorhabdus* for steinernematids and *Photorhabdus* for herterorhabditids (Poinar, 1979) in the insect body. This proved that, why the pathogenicity was affected by irradiation (Bowen *et al*., 2000, 2003; Marokhazi *et al*., 2007).

Regarding to the rate of reproduction of irradiated nematode, Ebrahimi, *et al*. (2014) and Gupta (2001) indicated that, insect host killing may be affected by the decrease in the lysozyme and phenoloxidase activities which observed in the treated insect larvae that means the suppression in the immune system responses against any pathogen. This illustrated why they die faster, and the nematode can propagate more than do in the unirradiated larvae. Wu *et al.* (1992); Wu and Lam (1997) detected that, the lactate dehydrogenase increased in the insect larval haemolymph treated with irradiated *S. scapterisci and H. bacteriophora*. This component is always related to the cell damage and lyses. This is the more rapid and easier entering and killing effect of EPNs occurred this is congruent finding with our data.

Influences of symbiotic bacteria on pathogenicity of irradiated nematode were explained by Bashandy and El-sinary (2002), who attributed the more efficient of gamma irradiated nematodes to the symbiotic bacterial toxicity via enhancement with the low dose. Cotter *et al.* (2004) reported that, Phenoloxidase is an important factor in the immunity in insect with a critical role in synthesis of melanin, and responsible for some vital process such as recognition, of any foreign particles and encapsulation and coagulation it in the haemolymph. On the contrary, the effect of irradiated nematodes may cause morphological destructive response to some hemocytes in *G. mellonella* larvae and become more susceptible to nematodes infection (Salem *et al.* 2020).

Referring to the viability of irradiated IJs of Hb and Hi all previous researches used low doses of gamma irradiation, and this lead to little effect on nematodes. The irradiation of EPNs inside their larval insect host caused more tolerance to the side effect of gamma irradiation on nematodes when exposed directly, and the following of this technique helped us to increase the irradiation doses and get the benefits of irradiation with little lethal effect on IJs. This notion is logically accepted in this study.

The present results of comet analysis of DNA are important in determining the level of gamma irradiation dosages required to apply with *H. bacteriophora* and *H. indica*. The obtained data show that the damage in DNA parameters were increased proportionally with irradiation doses. These data agreed with those obtained by Galea *et al.* (2016); Hassan *et al.* (2016) and Enciso *et al.* (2018) who studied the effect of gamma irradiation on *Heterorhabditis* and *Steinernema* species. They found that, the efficiency was enhanced against *G. mellonella*. (Sayed, 2011) and *Spodoptera littoralis* (Sayed and Shairra, 2017).

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تأثير أشعة جاما على حيوية وامراضية وتكاثر نيماتودا الحشرات *Heterorhabditis bacteriophora,* وتلف ال DNA *Heterorhabditis indica*

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تعتبر النيماتودا الممرضة للحشرات من أهم بدائل المكافحة الكيماوية والتى يمكن استخدامها فى مكافحة الحشرات الضارة واستخدامها فى برامج المكافحة المتكاملة، ومن منطلق اليقين بأهميتها فى التطبيق الحقلى بأمان كانت فكرة البحث بتعريض نيماتودا الحشرات *Heterorhabditis bacteriophora*, *Heterorhabditis indica* بطريقة مباشرة فى المعلق النيماتودى وبطريقة غير مباشرة داخل عائلها الحشرى وهو يرقات دودة الشمع الكبيرة *Galleria mellonella* لاشعة جاما بتركيزات مختلفة وهى 0, 100, 250, 500, and 1000 Gy. بغرض رفع كفاءتها فى الاستخدام الحقلى. تم تسجيل نسب الموت فى النيماتودا المعرضة للاشعاع تعريض مباشر لتقدير تأثير التركيزات المختلفة من الاشعاع على حيوية الاطوار المعدية للنيماتودا، كما تم استخلاص الاطوار المعدية المشععة بطريقة غير مباشرة من يرقات ديدان الشمع واختبار كفاءتها فى اصابة ديدان شمع أخرى وتم تشريح جزء من المكررات لتحديد تأثير الاشعاع بالجرعات المستخدمة على القدرة الامراضية للنيماتودا والاحتفاظ بالجزء الاخر لاستخلاص الاطوار المعدية الجديدة منها لتحديد مدى تأثر القدرة التكاثرية بالاشعاع، كما تم حساب ال LC50 من الاشعاع باستخدام برنامج سيجما وأخيرا تم عمل تحليل المذنب comet المتبع لتقدير تأثير الاشعاع الواقع على ال DNA داخل خلايا النيماتودا وكانت النتائج كالتالى: بشكل عام كانت النيماتواد *H. bacteriophora* أكثر تحملا لتأثير الاشعاع من ال *H. indica* كما أنه *H. bacteriophora*, اعتمادا على النتائج تم تحديد الجرعة 100 Gy. كجرعة محفزة للقدرة الامراضية والتكاثرية للنيماتودا بأقل تأثير على ال DNA داخل اليرقات المشععة. بناءا على النتائج سابقة الذكر فانه ممن الممكن استخدام الاشعاع بجرعات لا تتعدى 100 Gy. بغرض رفع كفاءة النيماتودا الممرضة للحشرات *H. bacteriophora, H. indica* دون أى ضرر على كفاءتها أو قدرتها الامراضية لاستخدامها فى برامج مكافحة الحشرات الضارة.