

HISTOPATHOLOGICAL EFFECTS OF SOME BIO-AGENT COMPOUNDS EXPOSED TO GAMMA IRRADIATION ON THE COTTON LEAF WORM, *Spodoptera littoralis* (BOISD.)

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ABSTRACT

Bio-agent compounds of *Bacillus thuringiensis*, Kurs. (Bactericide); *Metarhizium anisopltiae*, Metsch. (Fungicide) and biopolymer, chitosan exposed to gamma irradiation doses; 15, 30 and 60 Gy to purpose of potentiate the bio-agent efficiency against the cotton leaf worm, *Spodoptera littoralis* (Boisd.) treated as 4th instars larvae by LC₅₀ of tested bio-agent compounds singly or combined with gamma irradiation doses to investigate the histopathological changes in the integument, muscle, fat body and mid gut.

Most of treatments especially gamma doses of 60 Gy singly or combined with bio-agent compounds caused thickening of outer cuticle fibrous layer in the integument of *S. littoralis* larvae. Also, hypodermis layer had swelling and necrosis in gamma treatments and *M. anisopltiae* or chitosan combined with 30 Gy treatments.

Also, most of treatments caused appearance of fissure and breaking down of muscles into small parts.

All the bio-agent treatments caused a noticeable destruction on the fat body cells as vacuolization of the fat cells and destruction of the fat body membranous sheath.

Many deleterious effects in the mid gut of *S. littoralis* as destruction of columnar or hyperphesia cells lining mid gut, losses of brush border with increase of goblet cells.

Dose of 60 Gy is considered the best dose used in the current study to potentiate the bio-agent effects on *S. littoralis* larvae compared with other gamma irradiation doses used.

Keywords: *Spodoptera littoralis*, histopathological, integument, muscle, fat body, mid gut, *Bacillus thuringiensis*, *Metarhizium anisopltae*, chitosan.

INTRODUCTION

It known as use of insecticides eventually created many problems as resistance, environmental pollution and adverse effects on the non-target organisms. Therefore, it is necessary to search for new methods to replace insecticides used or combined them with integrated methods of control. The control of the cotton leaf worm, *Spodoptera littoralis* (Boisd.), by ionizing radiation appears to be one of the possible applications of radiation for field pest control. Several studies were carried out to clarify the possibility of applying irradiation to control many different pests including the cotton leaf worm, *S. littoralis*. This insect appears almost everywhere in Egypt and causes much damage to cotton and other crops. It is considered to be one of the most destructive pests (El- Shall, 1991). Szczepanik and Ignatowicz

(1996) mentioned that degenerative changes in the mid-gut of insect larvae and adults of some stored product pests (*Trogoderma granatum* Ev., *Tnbohum confusum* DuVal and *Plodia mterpunctella* Hubner) are positively correlated with both the gamma dose (0.1, 0.3 and 0.5 kGy) and time elapsed after irradiation exposure. Therefore, a pathological syndrome of irradiation effects on the mid-gut may be used as an efficient method for identification of irradiated insects when the destruction of regenerative nuclei, lack of rush border, and vacuolated epithelial cells are observed within the trans section of the mid gut. Meanwhile, Ghribi, *et al* (2012) throw light on the effect of *Bacillus subtilis* SPB1 bio-surfactant on the third larval instars of the Mediterranean flour moth, *Ephestia kuehniella*, under laboratory conditions. The toxicity of this compound was investigated with emphasis on histopathological effects in the mid-gut of larvae. The tested dose levels showed strong histopathological disturbances in the mid-gut of this pest. The most frequently observed effects were cell vacuolisation, microvilli damage and epithelium cell contents passing into the mid-gut lumen. Also, Farghaly, *et al.* (2014) evaluated three doses (150, 300 and 450Gy) of gamma radiation against full-grown male and female pupae of *Corcera cephalonica* (Stainton). There was positively correlation between gamma radiation dose and cell damage in the full grown larvae of *C.cephalonica* treated as pupae. The larvae resulted from treated males crossed with treated females irradiated with 150 Gy scored the most severe damage in mid gut cells, nucleus, mitochondria and microvilli.

Thus, the purpose of current study was to be investigating the effect of gamma irradiation at doses of 15, 30 & 60 Gy alone or in combination with *Bacillus thuringiensis*, Kurs. (Bactericide); *Metarhizium anisopltiae*, Metsch. (Fungicide) and biopolymer, chitosan on the histopathological structure of the larval integument, muscle, fat body and mid gut tissues of the cotton leaf worm, *S. littoralis* treated as 4th instars larvae.

MATERIALS AND METHODS

A. Insect Rearing Technique.

The culture of the cotton Leaf worm *S. littoralis* was maintained in the laboratory of Cotton Leaf worm Department, Plant Protection Research Institute, Agriculture Research Center. Larvae were fed on fresh castor oil plant leaves, *Ricinus communies* under laboratory conditions of 27 ± 2 °C and 65% R.H. (El-Defrawi *et al.* (1964).

B. Bio-agents.

- 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 anisopltiae* (Metsch.); trade name (Bio Magic) 1.75% WP (1×10^8 CFU'S/gm). Manufacturer Company: M/S. T. Stanes Company Limit- India. Import Company: Gaara Establishment, Import & Export. Dose rate: 10 gm/ L Water (1×10^6 CFU'S/ml).

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

All the bio-agent used exposed to gamma irradiation doses of 15, 30, & 60 Gy. All irradiations were done by a Cesium¹³⁷ Hedy Cell Research, National Center for Radiation Research and Technology, delivered at a dose rate 0.75/rad/sec.

C- Toxicity of gamma irradiation doses and Bio-agent compounds on *S. littoralis*.

Twenty five of *S. littoralis* fourth instars larvae with castor oil leaves in petri-dishes exposed to gamma irradiation doses of 15, 30 & 60 Gy. Four replicates for each gamma doses used and the control was done.

Dipping technique was used at the present work. The castor oil leaves dipping in tested bio-agent compound concentrations of 16×10^8 , 8×10^8 , 4×10^8 , 2×10^8 & 1×10^8 IU/L of *B. thuringiensis* (Biotect), *B. thuringiensis* +15 Gy, *B. thuringiensis* +30 Gy and *B. thuringiensis* + 60 Gy. Concentrations of 30×10^8 , 15×10^8 , 7.5×10^8 , 3.75×10^8 & 1.875×10^8 CFU'S/L of *M. anisopltiae* (Bio magic), *M. anisopltiae* +15 Gy, *M. anisopltiae* +30 Gy and *M. anisopltiae* +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, then put in glass jars (11x22 cm). Each jar was prepared by 25 fourth instars larvae of cotton leaf worm after larvae starving about 4 hours and maintained under $26 \pm 1^\circ\text{C}$. Then the numbers of alive and dead larvae were counted three days after treatment.

LC₅₀ values were assessed according to Finney (1971) by using Ldp line software (www.Ehabbakr software/Ldp line).

D- Pre-Histology:

Fourth instars larvae of *S. littoralis* treated with LC₅₀'s of the bio-agent compounds singly or exposed to gamma doses of (15, 30 & 60 Gy). Also, *S. littoralis* exposed as 4th instars larvae to gamma doses used. Larvae at 8- day after treatment were maintained in formalin 10% until histology.

E- Histology:

The specimens from *S. littoralis* larvae samples were collected and fixed in 10% buffered neutral formalin solution. Paraffin sections of 5 microns thickness were prepared and stained with haematoxylin and eosin (H & E) according to Bancroft, *et al.* (1990) and examined microscopically. The integument, muscle and fat bodies were investigate microscopically (X 200), While, mid gut (x 400).

All sections of *S. littoralis* larvae were done at Animal Health Research Institute, Agriculture Research Center.

RESULTS AND DISCUSSION

The cotton leaf worm, *S. littoralis* treated as 4th instars larvae by gamma irradiation doses of 15, 30 & 60 Gy alone or in combination with *Bacillus thuringiensis*, Kurs., *Metarhizium anisopliae*, Metsch. and chitosan. The LC₅₀'s of bio-agent compounds exposed to gamma doses were recorded after 3-days from treatment as in Table (1) to study the histopathological structure of the larval integument, muscles, fat bodies and mid-gut of *S. littoralis* larvae.

Table (1): Half lethal dose of bio-agent compounds exposed to gamma doses after 3-days against *S. littoralis* treated as 4th instars larvae

Treatments	LC ₅₀ (IU/L) 95%Confidence limits	Treatments	LC ₅₀ (CFUS/L) 95%Confidence limits
<i>B. thuringiensis</i>	1133 x10 ⁶ 965.1x10 ⁶ ± 1551x10 ⁶	<i>M. anisopliae</i>	62.23 x10 ⁸ 32.55x10 ⁸ ±90.38 x10 ⁸
<i>B. thuringiensis</i> +15 Gy	810.2 x10 ⁶ 581.8x10 ⁶ ±1257x10 ⁶	<i>M. anisopliae</i> +15 Gy	62.1 x10 ⁸ 32.56x10 ⁸ ±90.42 x10 ⁸
<i>B. thuringiensis</i> +30 Gy	337.9 x10 ⁶ 136.9x10 ⁶ ±643.8 x10 ⁶	<i>M. anisopliae</i> +30 Gy	61.41 x10 ⁸ 30.42x10 ⁸ ±89.58 x10 ⁸
<i>B. thuringiensis</i> +60 Gy	163.9 x10 ⁶ 29.7x10 ⁶ ±484.2 x10 ⁶	<i>M. anisopliae</i> +60 Gy	60.22 x10 ⁸ 30.12x10 ⁸ ±87.87 x10 ⁸
Treatments	LC ₅₀ (m/L) 95%Confidence limits	Treatments	LC ₅₀ (m/L) 95%Confidence limits
Chitosan	24.41 18.88 ±40.28	Chitosan +30 Gy	20.45 13.25±33.54
Chitosan +15 Gy	21.22 15.46±35.38	Chitosan +60 Gy	18.82 10.89±30.98

3. Histopathological studies.

A. Integuments:

Normal integument structure of *S. littoralis* larvae consists of the inner basement membrane, a single cell layer and outer cuticle which are differentiated into an outer epicuticle and endo cuticle (Fig. 1). Severe damage was occurred on the integument of *S. littoralis* larvae when they were treated by bio-agent compounds combined with gamma doses. Thickening of outer cuticle fibrous layer was shown in the most treatments. In addition, sever thickening of outer cuticle resulted from treatments of gamma dose 60Gy and its combination with *B. thuringiensis*, *M. anisopliae* and chitosan.

Hypodermis layer had swelling in gamma treatments (15, 30 and 60 Gy) and *M. anisopliae* +15 Gy. Meanwhile, *M. anisopliae* +30 Gy caused swelling in hypodermis layer in some parts and necrosis of other parts. Also, chitosan + 30 Gy caused swelling and vaculation in hypodermis layer; while, chitosan +15 Gy and chitosan + 60 Gy had slurping from hypodermis layer. They elicited a lack of differentiation between outer cuticle and endo cuticle, destruction of the basement membrane and appearance of vacuoles between cuticle and hypodermis in the most treatments.

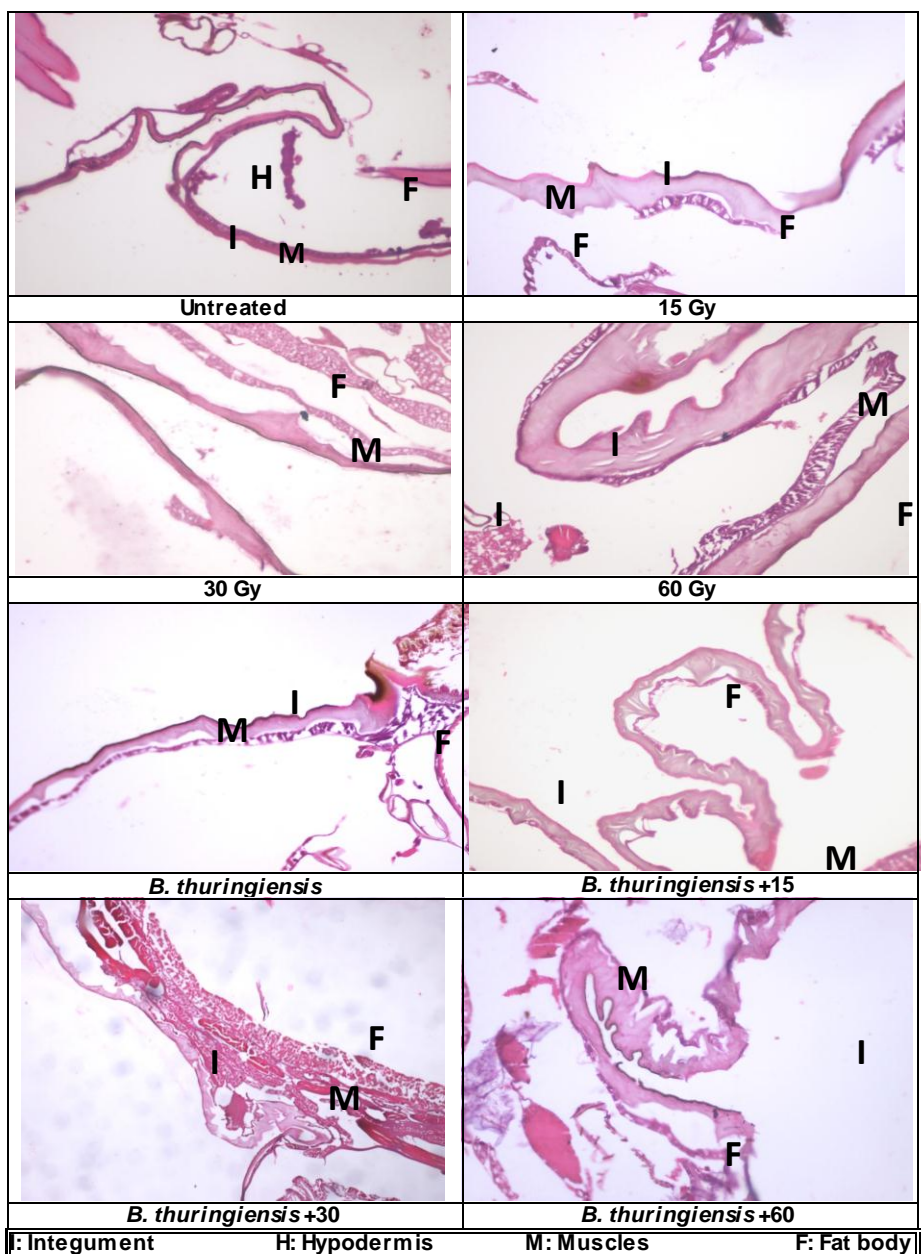


Figure (1): Longitudinal sections in the larval cuticle of *S. littoralis* treated as 4th instars larvae by bio-agents and gamma irradiation (X 200).

B. Muscles:

The muscles are composed of striated fibers. Each fiber consists of parallel fibrillate numbers or sacrostyles, occupying the whole cross section of the fiber and laid down in plasma or sacroplasm. The nuclei of the sacroplasm are disposed immediately beneath the sarcolemma. The appearance of fissure and breaking down of muscles into small parts are attributed to the destruction of the sarcolemma (Fig.1,2).

C. Fat bodies:

Histological structure of the normal fat bodies indicated that they are composed of two layers. An outer or partial layer which is formed of ribbons beneath the body wall and an inner or visceral layer surrounding the various organs. The ribbon consists of many irregular cells. Their cells surrounded by sheath (Fig.1). The histological changes were caused by the bio-agent treatments used in this study that showed a noticeable destruction on the fat body cells, as vacuolization of the fat cells and destruction of the membranous sheath (Fig. 1,2).

D. Mid guts:

1. Normal mid gut:

Mid gut of *S. littoralis* is the main site for digestion and absorption of the digestion products and is a very metabolically active tissue. The normal mid gut consists of single layer of epithelium placed on a basement membrane. The epithelium is made up of columnar cells, secretory cells. These epithelial cells are relatively high and form a regular and compact wall. An oval nucleus is located in the central part of each columnar cell. The apical surface of each columnar cell bordering with the gut lumen is covered with microvillae which create the tight structure called the brush border. The regenerative cells are another type of cells within the epithelium. These tiny cells form the regenerative nidi that are regularly located at the base of the columnar cells (Fig. 3). Goblet cells are interspersed among the columnar cells. The cytoplasm of these pear-shaped cells is reduced, and the apical border of the cell surface invaginates to form a deep cavity. In this cavity there are numerous cytoplasmic extensions. Flat nucleus of the goblet cell is located basally, below its cavity. The epithelium rests on well-developed basement membrane that is surrounded by a layer of circular muscles and an outer longitudinal muscle coat.

2. Changes in the mid gut induced by irradiation:

The following cross changes in the mid gut of *S. littoralis* larvae were found in Figure (3). On the 8th day after *S. littoralis* 4th instars larvae exposed to gamma irradiation with a doses of 15, 30 & 60 Gy, the epithelial cells of larvae were elongated and vacuolated. Their nuclei were distinctly enlarged. Brush border was seen on a large surface of epithelial cells, but it disappeared on the most affected cells. Cytoplasmic extensions of the goblet cells were degenerated, and their fragments were often noted in the cavity of goblet cells. All regenerative cells were lost, and the basement membrane formed many folds as a result of the muscle contraction (Fig. 3). At the same time, the mid gut of *S. littoralis* larvae treated with a dose of 60 Gy was much more affected. The regular structure of the epithelium was disturbed. Most of columnar cells elongated into the gut lumen, and their

apical part swelled distinctly. Cell nuclei were enlarged, and they moved into the direction of the gut lumen. The basement membrane formed large folds as a result of the distinct muscle contraction.

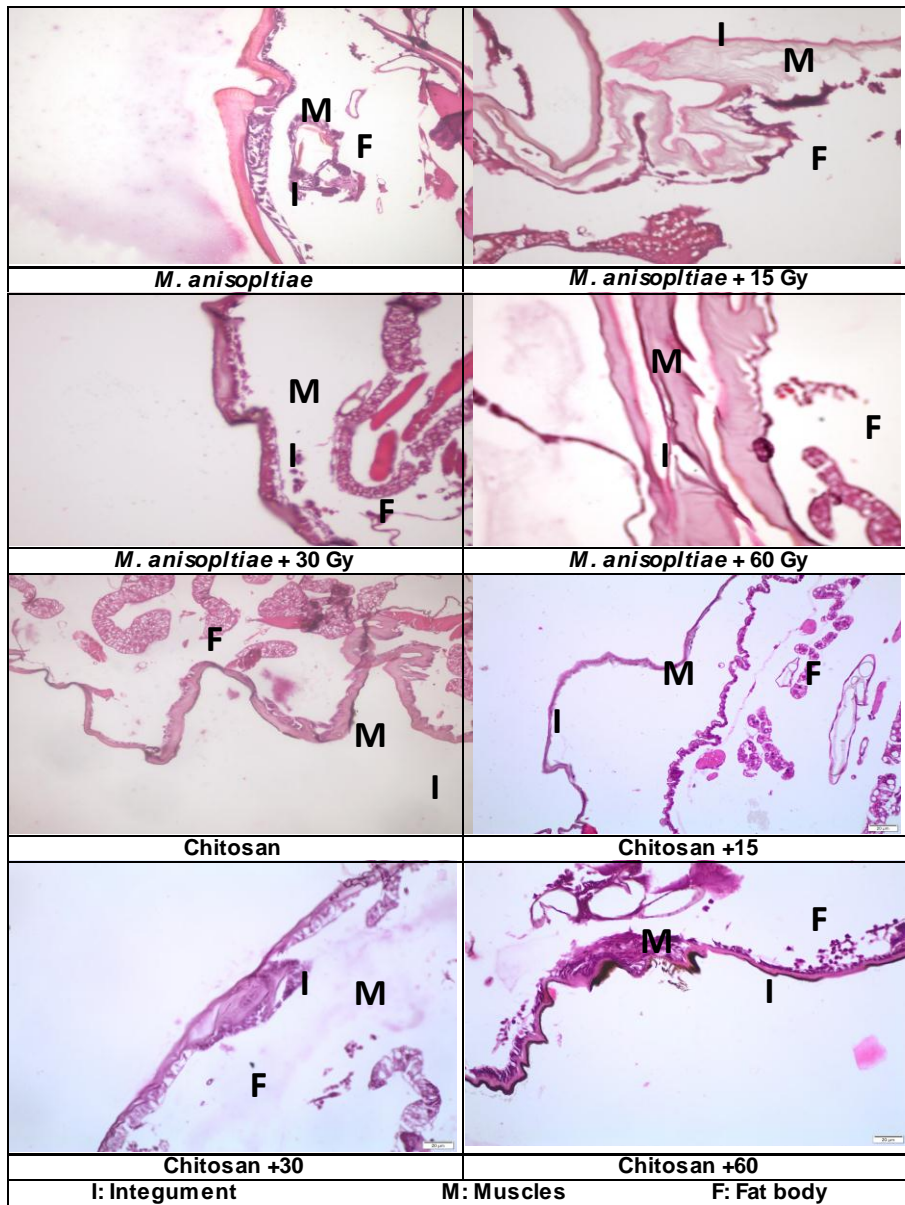


Figure (2): Longitudinal sections in the larval cuticle of *S. littoralis* treated as 4th instars larvae by bio-agents and gamma irradiation (X 200).

3. Changes in the mid gut induced by bio-agents exposed to gamma irradiation:

Bio agent compounds exposed to gamma irradiation doses of 15, 30 & 60 Gy caused many deleterious in the mid gut of *S. littoralis* as distraction of columnar cells lining mid gut as well as basement membrane. Also, bio-agents exposed to gamma dose of 15 Gy caused hyperplasia of cells lining mid gut, losses of its brush border with increase of goblet cells. In addition, appear ghost of cells lining mid gut and sever prolipherantion of columnar as well as goblet cells lining mid gut especially with bio-agent exposed to 60 Gy (Fig. 3,4).

Undifferentiated cells of the mid gut forming the regenerative cells of the *S. littoralis* larvae that were found to be the most sensitive to bio-agents used combined with gamma irradiation. Damage to them resulted in the total disruption of the epithelium by preventing the replacement of the secretory cells exhausted by secretory activity. Degree of damage to the regenerative cells seems to be dependent on the dose of radiation and on the pest susceptibility to bio-agent compound exposed to gamma irradiation (Fig. 3,4).

Similar results were gained by El-Sinary and Rizk (2007) mentioned that gamma irradiation (50, 100 and 150 Gy) combinations with the fungal concentrations of *B. bassiana* increased the damage in the larval mid gut treated as fourth larval instar of *G. melonella*. The present study also agree with Haiba *et al.* (2008) who exposed potato tuber moth, *Phthorimaea operculella* to gamma radiation at doses 50 and 150 Gy and showed various forms of changes, there was direct relationship between the dose levels and the observed effects. At dose level 150 Gy, the effects were more advanced than those at 50 Gy dose level. Current results are in agreement with findings of Amer (2010) who treated *P. gossypiella* as newly hatched larvae by LC₅₀ of Dipel-2x. Also, it combined with gamma irradiation doses of 5, 10, 20, 40 & 80 Gy. Dipel-2x had destructive acts in disintegration of testicular epithelial septa, degeneration the germ cells and the sperm bundles; also, the peritoneal membrane leaving vacuolated areas and shrinking in the male testis. In addition, it reduced the size of ovaries, absence the nurse cells, dissolved the most follicular epithelial cells, vacuolated and shrinking of the oocyte or ovaries as compared with the control. Gamma irradiation doses of 5, 10 & 20 Gy had nearly the same effects caused by Dipel-2x. While, in case of irradiation by 40 & 80 Gy gamma doses had drastically effect. In addition, Lauzon and Potter (2012) reported that mid gut from adult sterile male *Ceratitis capitata* (Wiedemann) and *Anastrepha ludens* (Loew), the Mediterranean fruit fly and Mexican fruit fly, respectively, were examined microscopically to determine if radiation used in sterile insect technique (SIT) affected this non target tissue and/or the microorganisms associated with the mid gut. Their comparisons revealed that newly emerged and two day-old irradiated flies exhibited signs of damage to mid gut tissue, cellular organelles, and gut micro biota not observed in non-irradiated flies of the same ages. Cellular damage of mid gut tissue from irradiated flies included distorted, small nuclei that lacked nuclear material, and mitochondria that

were dilated and/or vacuolated. No visual evidence of cellular damage was observed in non irradiated flies.

Figure (3): Longitudinal sections in the larval mid gut of *S. littoralis* treated as 4th instars larvae by bio-agents and gamma irradiation (X 400).

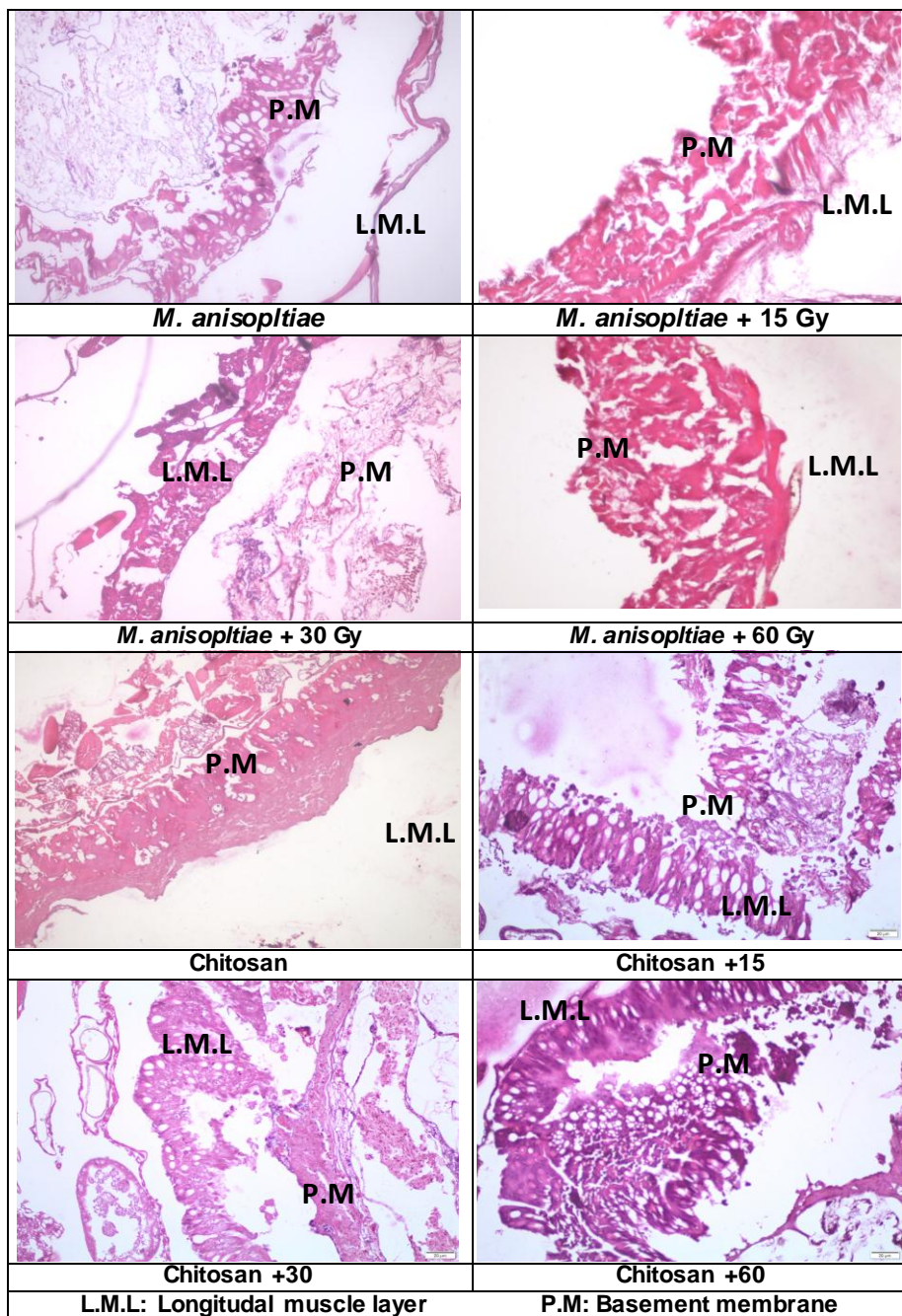


Figure (4): Longitudinal sections in the larval mid gut of *S. littoralis* treated as 4th instars larvae by bio-agents and gamma irradiation (X 400).

Abd-El Wahed, *et al.* (2011) stated that mid gut histological sections of 6th instar larvae of *S. littoralis* treated as 4th instars larvae with LC50 of protecto, *B. thuringiensis* was effective product in causing aberrations in the mid gut layers. Also, Abd El-Mohsen, *et al.* (2013) showed that, the effect of *B. thuringiensis* on 2nd and 4th instar larvae of PBW. These effects are complete destruction of mid gut vigorous degeneration of fat bodies (IFB and OFB), sometimes degeneration of some epidermal cells and mid gut in the 2nd and 4th instars larval compared with untreated larvae.

Generally, Histopathological study cleared that bio-agent compounds (*B. thuringiensis*, *M. anisopltiae* and chitosan) exposed to gamma doses of 15, 30 and 60 Gy had destructive effects on larvae of *S. littoralis* in integument, muscle, fat body and mid gut treated as 4th instars larvae compared to bio-agent or gamma doses when used singly. Also, dose of 60 Gy is considered the best dose enhance from bio-agents efficiency, followed by doses of 30 and 15 Gy.

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التأثيرات الهستوباثولوجية لبعض المركبات الحيوية المعرضة لأشعة جاما على دودة ورق القطن

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تم تعريض المركبات الحيوية (*B. thuringiensis*, *M. anisopltiae* and chitosan) لجرعات أشعة جاما (١٥ - ٣٠ - ٦٠ جراى) وذلك بهدف زيادة كفاءتها ضد دودة ورق القطن التى عوملت فى العمر اليرقى الرابع بالتركيز النصفى المميت للمركبات الحيوية منفردة أو المعرضة لجرعات أشعة جاما وذلك بهدف دراسة التأثيرات التشريحية على طبقة الكيوتيكول والعضلات والاجسام الدهنية والمعى الأوسط . وأظهرت النتائج أن معظم المعاملات سببت زيادة سمك طبقة الكيوتيكول الخارجية ليرقات دودة ورق القطن. حدوث تورم وتآكل فى طبقة الكيوتيكول الداخلية فى معاملات أشعة جاما بالإضافة إلى معاملات *M. anisopltiae* and chitosan المعرضين لجرعة ٣٠ جراى من أشعة جاما. كما حدث تشققات وتكسير فى طبقة العضلات إلى أجزاء صغيرة . كما سببت كل معاملات المركبات الحيوية تحطم ملحوظ وحدث فجوات فى خلايا الاجسام الدهنية وسببت تحطم فى التجويف العشائى للاجسام الدهنية . وقد تم إكتشاف حدوث تغيرات فى المعى الأوسط مثل تحطم وتضخم فى الخلايا العمادية المحاذية للمعى الأوسط كما زادت الخلايا الكأسية مع فقدان فى أطراف الخلايا الهدبية . وكان لإستخدام أشعة جاما ٦٠ جراى هى الأفضل بالمقارنة بالجرعات الإشعاعية الأخرى المختبرة ، حيث أدت إلى زيادة فعاليات المركبات الحيوية على يرقات دودة ورق القطن .