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Histological and nucleic acids alterations in *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) induced by *Streptomyces lavendulae* (Streptomycetaceae) culture filtrate

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Abstract

Histological and nucleic-acid alterations induced by *Streptomyces lavendulae* (Streptomycetaceae) culture filtrate on the 2nd instar larvae of *Spodoptera littoralis* (Boisd.) were studied. Using thin-film techniques, larvae were exposed to a concentration of 226 CFU cm^{-2} for 48 h, which caused 84% larval mortality. The filtrate induced deleterious histological changes, with clear signs of apoptosis in the mid-gut and brain tissues. Using gel electrophoresis, the intensity of RNA decreased while fragmented DNA increased relative to controls. These DNA fragments are from apoptosis, as supported by histopathological observations. Histochemically, total protein content decreased in mid-gut cells, consistent with the electrophoretic pattern of the RNA. These effects clearly indicated that *S.lavendulae* releases a toxin containing bioactive compound(s) whose mode of action could be on the nervous system and /or insect immune system.

Keywords: toxicity, *Streptomyces lavendulae*, histopathology, *Spodoptera littoralis*, RNA, DNA, protein.

Introduction

Like many higher plants, entomopathogenic microorganisms including bacteria, fungi and viruses can release toxic materials (secondary metabolites) usable as pesticides. Lacey *et al*. (2001) reported that microbial agents are safe for humans and other non-target species, free from residual toxicity, preserve natural enemies and increase the biodiversity of the ecosystem. These make microbial agents an attractive alternative to broad-spectrum insecticides.

Many authors have investigated high-molecular-weight insecticidal compounds of natural origin and their mode of action. Quesada-Morga *et al.* (2006) purified a soluble protein from the crude filtrate of the fungus *Metarhizium anisopliae*. They found that this protein caused dramatic effects on the mid-gut epithelium of *Spodoptera littoralis* larvae and caused 83% larval mortality.

A number of insecticidal proteins expressed and secreted during the vegetative growth phase (Vips) of *Bacillus thuringiensis* have been identified, which contrast with the widely investigated *B.thuringiensis* δ-endotoxins, or insecticidal crystal proteins, which form parasporal inclusions primarily during the sporulation phase. The released δ-endotoxin, when fed to susceptible insects, binds to a receptor present on the microvilli of the mid-gut epithelial cells (Gill 1992) and generates pores on the cell membrane (Lane et *al.* 1989). The Golgi-associated membrane appears to be susceptible to the effect of this toxin, suggesting that this intracellular compartment could be the secondary target site (Lane et *al.* 1989).

A high-molecular-weight protein-toxin complex (Tca) purified from the entomopathogenic bacterium *Photorhabdus luminescens* was found to be toxic against *Leptinotarsa decemlineata* (neonate larvae) and *Bemisia tabaci* (first nymphal instar). Ingestion of Tca led to extensive apical swelling and blebbing of the epithelium, and the gut lumen became packed with cytoplasmic vesicles (Blackburn *et al.* 2005). The relationship between *Photorhabdus* and the host (*Manduca sexta*) was studied by Eleftherianos *et al*. (2006). They reported that the immune system of *M.sexta* can recognize the pathogen by three different inducible microbial-recognition proteins: hemolin, immulectin-2 and peptidoglycan recognition protein. The toxin released by *Photorhabdus* contains specific double-strand

ribonucleic-acid interference (RNAi). The RNAi knock-down the genes of the recognition proteins, causing great reduction in insect resistance to the infection and subsequent death.

Streptomyces lavendulae is known to release many secondary metabolic compounds considered as important sources of antibiotics and pesticides. The antitumor antibiotic mitomycin C (MC) produced by *S.lavendulae* is a bioreductively activated alkylating agent that crosslinks DNA at 5'CpG sequences: it has been widely used clinically for antitumor therapy (Johnson *et al.* 1997). A high-molecular-weight transglutaminase inhibitor has been purified from the culture filtrate of *S.lavendulae* Y-200 (Ikura *et al.*, 2000). *S.lavendulae* SNAK 64297 releases a novel compound, 1100-50 (1), which has been isolated and purified by Takatsu *et al.* (2003). They reported that this compound has a nematocidal activity against *Meloidogyne hepla* larvae, and that activity may be derived from inhibition of RNA synthesis. The insecticidal activity of different concentrations of *S.lavendulae* culture filtrate on 3rd-instar larvae of *Sp.littoralis* was studied by Sakr (2007a). She found that a concentration of 226 CFU cm^{-2} of *S.lavendulae* culture filtrate exhibited 83% cumulative larval mortality.

In the course of searching for natural-origin insecticides and trying to find out their mode of action, the present study aims to evaluate the effect of a sub-lethal concentration of *S.lavendulae* culture filtrate on 2nd-instar larvae of *Sp.littoralis*, and to find out its mode of action in terms of the histology of two important organs (the mid-gut and brain), a histochemical determination of total protein content in mid-gut cells, and the electrophoretic pattern of RNA and genomic DNA.

Materials & Methods

The actinomycete *S.lavendulae* culture filtrate (at a concentration of 1 x 10^4 CFU ml^{-1}) was kindly provided by Dr SM El-Sabagh (Botany Dept, Faculty of Science, Menuofia University). The 100-base-pair ladder was obtained from Pharmacia Biotech. The cotton leafworm *Sp.littoralis* was obtained from a stock colony maintained (28 ± 2 ˚C and 65 ± 5 % RH) on castor bean leaves (*Ricinus communis*): 2nd-instar larvae were used in the bioassay.

To study sensitivity to the crude filtrate of *S.lavendulae*, a concentration of 226 CFU cm^{-2} was used (Sakr 2007a). Bioassays were performed by applying the filtrate as a thin-film on a glass Petri dish (15-cm diam); control dishes were treated with water. After water evaporation at room temperature, 20 healthy 2nd-instar larvae were placed in the middle of each Petri dish, in triplicate. After 6h of treatment, each dish was provided with a small piece of fresh castor-bean leaf, changed daily. 2 days post-treatment larvae were transferred to glass jars provided with fresh castor-bean leaves daily until pupation. Larval mortality was corrected using Abbot's (1925) formula. Mortality data were subjected to Finney's (1971) method to estimate the LT50 values and their 95% confidence limits.

The effect of *S.lavendulae* culture filtrate at the concentration of 226 CFU cm^{-2} (thin-film method) on the histological structure of the mid-gut and brain of 2nd-instar *Sp.littoralis* was determined. Mid-gut and brain tissues were examined at intervals of 24 h, 48 h and 5 d post-treatment by removing parts of the mid-gut and head capsules and fixing them overnight in either Bouin's solution or 10 % neutral formalin. Specimens were then dehydrated in a series of ethanol, cleared in xylene and embedded in parablast. Embedded tissues were sectioned on a rotary microtome at 5 µm. For the histopathological study, these sections were stained with hematoxyline and eosin; the mercury bromophenol blue stain (Bonhag 1955) was used for protein determination. Stained sections were finally mounted on glass slides with DePeX-mounted medium. Microscopic examination and photography were done using a Leitz microscope.

To ascertain the effect on nucleic acids under the same conditions, the electrophoretic pattern of RNA and DNA was determined. The intensity of RNA of treated and control larvae

was determined according to Hassab El-Nabi *et al.* (2001). Briefly, each larva was squeezed thoroughly in an eppendorf. Then 400 µl of lysing buffer (50 Mm NaCl; 10 Mm Na2 EDTA; 0.5% SDS, pH 8.3) was added to 20 mg of the squeezed tissue. After 30 min, 20 µl of the lysated tissue was loaded into the well of the gel and then 4 µl 6x loading buffer was added on tissue lysate. The RNA was analyzed on 1.8 % agarose gel and visualized by ethidium bromide staining. The RNA bands were visualized using a UV Transilluminator at 312 nm, photographed using a digital camera, and measured using Gel-Pro software.

Detection of DNA fragmentation was carried out with the method of Aljanabi & Martinez (1997) as modified by Hassab El-Nabi (2004). The genomic DNA was extracted as follows. 600 µl of lysing buffer (50 Mm NaCl; 10 Mm Na2 EDTA; 0.5% SDS, pH 8.3) was added to 20mg of squeezed larval tissue in an eppendorf, shaken gently and incubated overnight at 37˚C. 200 µl of saturated NaCl was then added to each tube (to precipitate the protein content), shaken gently and centrifuged at 12000 rpm for 10 min. In a new eppendorf tube, the supernatant was transferred and the nucleic acids precipitated by 700 µl of cold *iso*propanol. These tubes were inverted, shaken and finally centrifuged at 12000 rpm for 10 min. The supernatant was removed, while the pellet was washed by 500 µl of 70% ethanol and finally centrifuged at 12000 rpm for 10 min. Then the supernatant was removed and the tubes were turned over onto filter paper (to remove the ethanol completely). The resultant pellet (the extracted DNA and RNA) was re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDETA, pH 8) overnight at 37˚C. After that, a loading mixture of 5 µl of RNase and 10 µl of loading buffer was added and the tubes incubated overnight at 37 ˚C. Finally, the DNA with 6x loading buffer (25 µl of each sample) was loaded directly into the well of the gel. DNA was analyzed on 1.8 % agarose gel and visualized by ethidium bromide staining. The DNA bands were visualized using a UV Transilluminator at 312 nm and photographed using a digital camera. The fragmented DNA bands appeared to be located at 200 bp and multiples, and were measured using Gel-Pro software.

Results

The culture filtrate of *S.lavendulae* was found to be very toxic to the 2nd-instar larvae of *S. littoralis*. The cumulative mortality was 40% and 84% after 12 h and 48 h, respectively (Fig. A). After 10 d post-treatment, all the tested larvae were dead. The LT50 was found to be 18.5 h (95% confidence limits = 12.06 h - 25.5 h); slope = 1.646 ± 0.057.

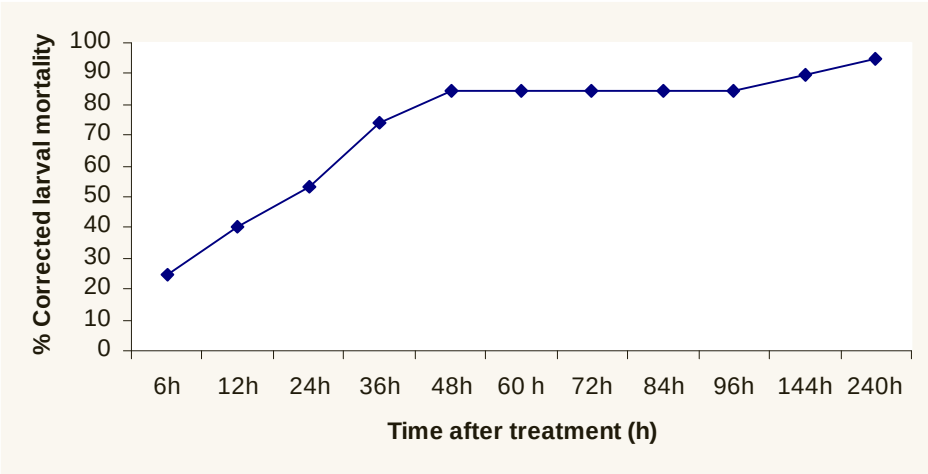


Fig. A: Cumulative larval mortality of the 2nd-instar larvae of *Spodoptera littoralis* exposed to *Streptomyces lavendulae* culture filtrate at a concentration of 266 CFU cm^{-2} for 48 h.

The histological structure of the mid-gut of control larvae (Fig. 1) shows many histological changes from that of treated larvae (Figs. 2 & 3). After 24 h, many vacuoles were observed in the gut epithelium and the apical parts of the columnar cells began to bleb into the gut lumen (Fig. 2a). These blebs contained condensed nuclei (Fig. 2b). Elsewhere, the epithelial cells were completely dissolved after 48h of treatment, and separated from the basement membrane (Fig. 3a). Destruction of the mid-gut epithelium with cells lacking nuclei was also observed (Fig. 3b).

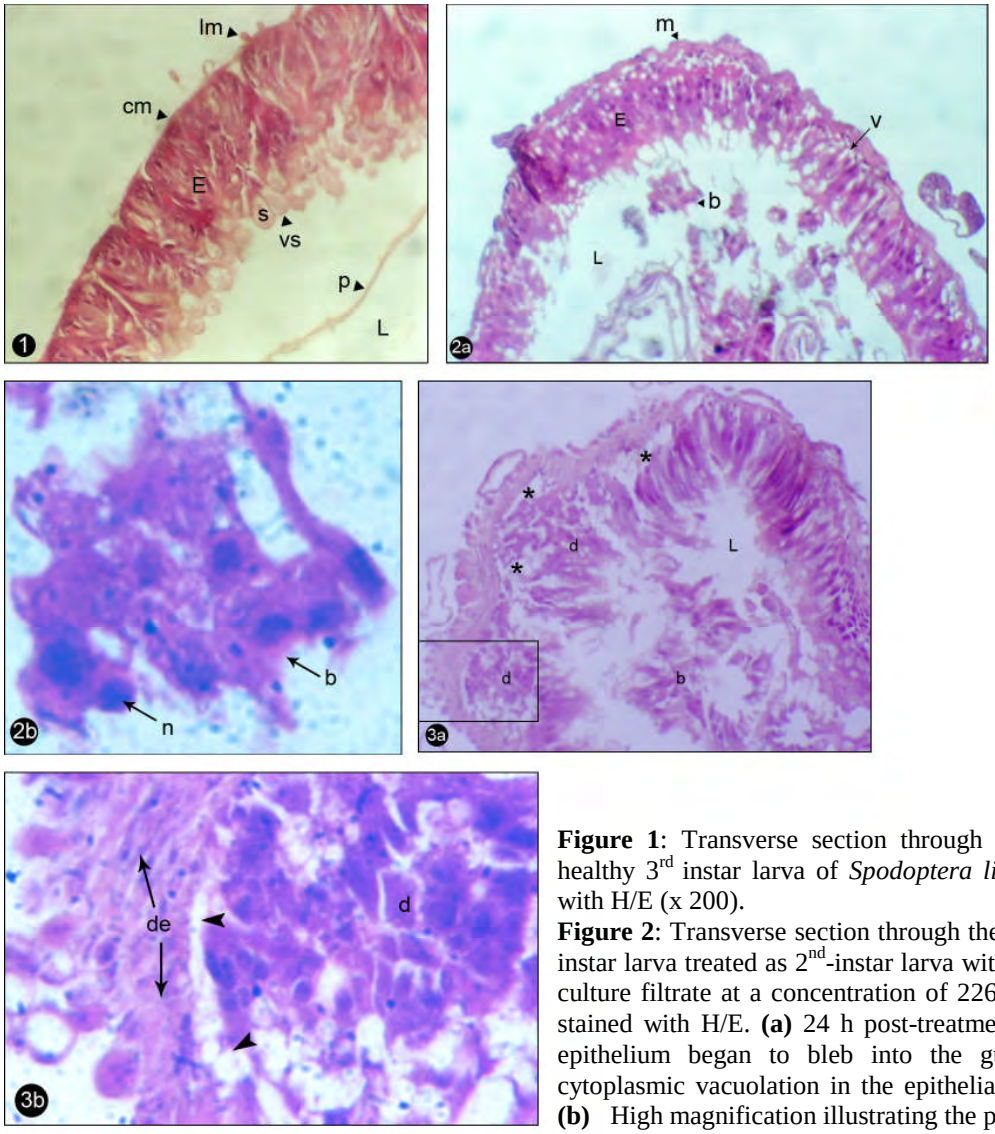


Figure 1: Transverse section through the mid-gut of healthy 3rd instar larva of *Spodoptera littoralis* stained with H/E (x 200).

Figure 2: Transverse section through the mid-gut of 2nd instar larva treated as 2nd-instar larva with *S. lavendulae* culture filtrate at a concentration of 226 CFU cm^{-2} and stained with H/E. **(a)** 24 h post-treatment, the mid-gut epithelium began to bleb into the gut lumen with cytoplasmic vacuolation in the epithelial cells (x 200). **(b)** High magnification illustrating the pyknotic nuclei within the cellular blebs, suggesting that they are apoptotic (x 1000).

Figure 3. As in Fig. 2 but 48 h post-treatment. **(a)** x 200; destruction of the gut epithelium more obvious, leaving a space in between (asterisk). **(b)** High magnification (x 1000), showing the destroyed epithelium with cells lacking nuclei, and the degenerate muscle layers. cm = circular muscles; d = destroyed epithelium; de = degenerate muscle layers; E = epithelial layer; L = lumen; lm = longitudinal muscle; p = peritrophic membrane; vs = vesicles.

Histologically, the larval brain consists of an outer sheath (neural lamella) which envelops the less densely packed layer of neurons and glial cells. The central part of the brain is occupied by a dense mass of fibrous tissue, the neuropile (Fig. 4a,b). Treated larvae exhibited many histological changes in the brain (Figs. 5-7). Degeneration marked by vacuoles occurred in the synaptic neuropils (Fig. 5a,b) (5 d post-treatment) and in the area where the glial cells were situated (Figs. 6, 7a). After 48h of treatment, some glial cells appeared with pyknotic nuclei (Fig. 7a) while the neural lamella was separated and became irregular in shape (Fig. 7b,c).

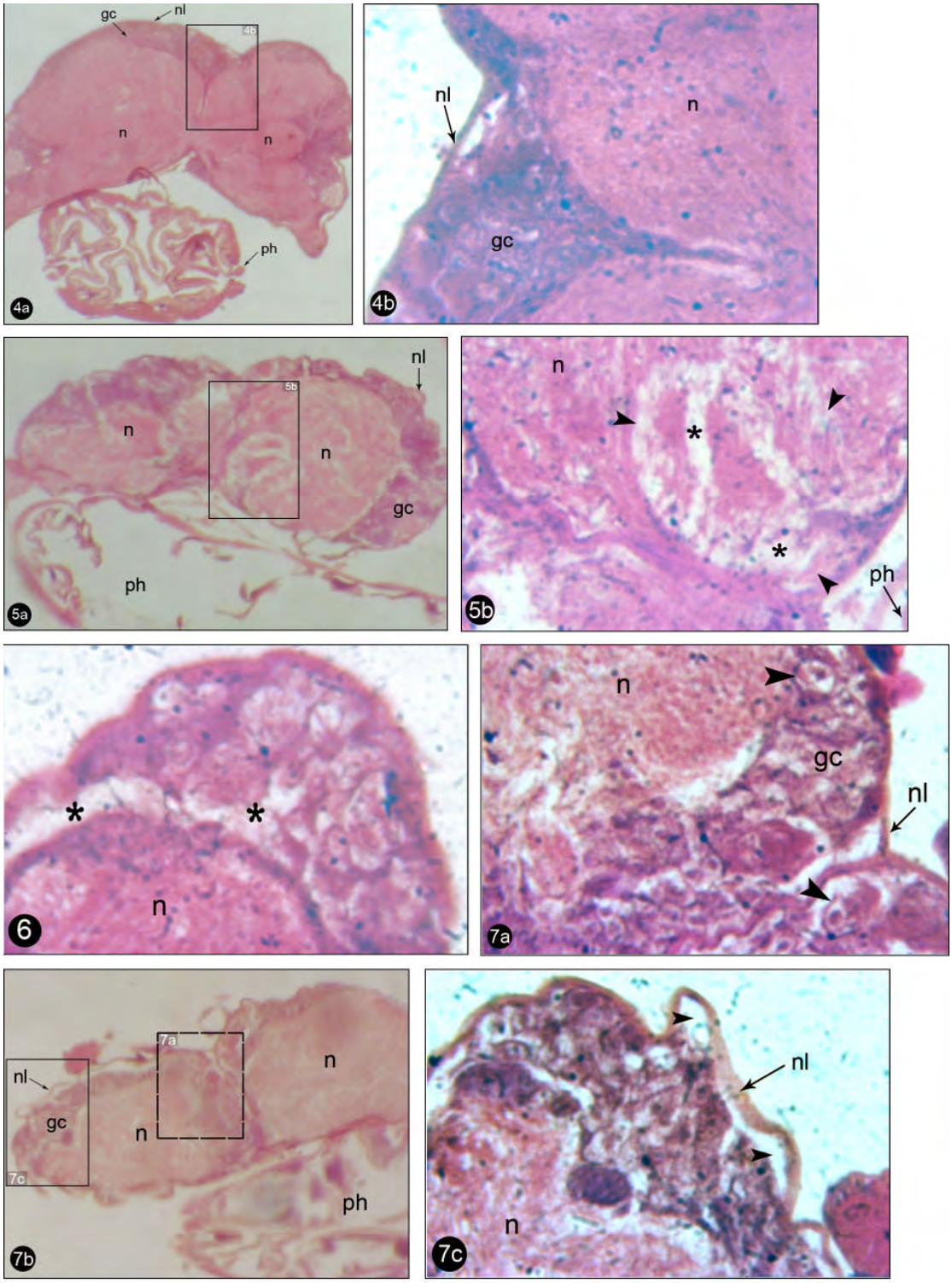
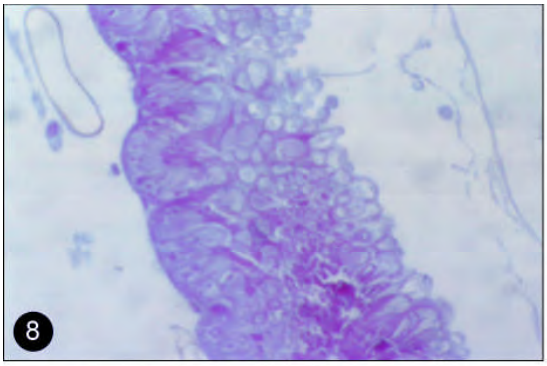
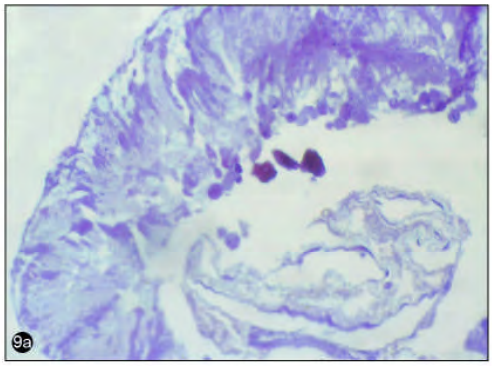


Fig. 4: Frontal section of the brain of the healthy 3rd instar larva of *Spodoptera littoralis* stained with H/E. **(a)** the normal structure of the brain (x 200). **(b)** high magnification (x 1000). Figs. 5-7: Frontal section of the brain of 2nd instar larva of *Sp. littoralis* treated with *Streptomyces* and stained with H/E. **Fig. 5**: five days post-treatment, degeneration in the synaptic neuropile (asterisk) leaving a vacuolation areas (arrow head); **(a)** (x 200); **(b)** (x 1000). **Fig. 6**: 24 h post-treatment, vacuolation in the glial cells area (x 1000). **Fig. 7**: 48 h post-treatment: **(a)** glial cells with condensed chromatin (arrow head) (x 1000); **(b)** general structure of the brain (x 200); **(c)** the separation of the neural lamella leaving a space (arrow head) and some glial cells with condensed chromatin (x 1000). gc = glial cells; n = neuropile; nl = neural lamella ph = pharynax.





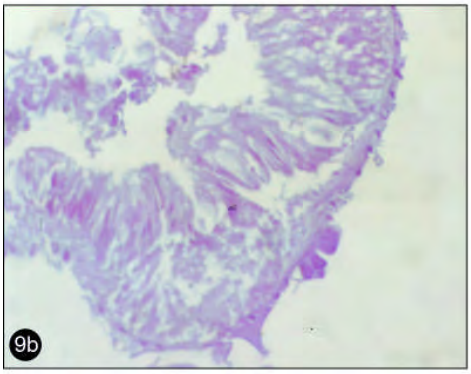
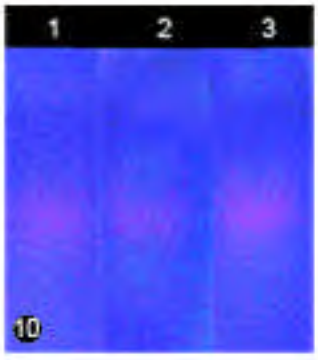
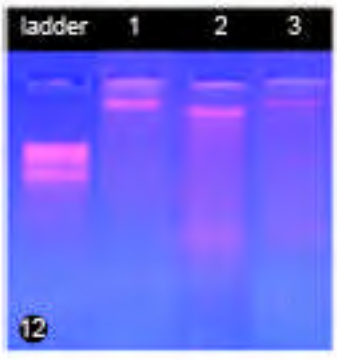


Fig. 8: Transverse section through the mid-gut of healthy 3rd instar larva of *Spodoptera littoralis* and stained with mercury bromophenol blue, notice all cell structures exhibit positive stain ability (bluish colouration) (x 200).
Fig. 9: Transverse section through the mid-gut of 2nd instar larva of *Sp. littoralis* exposed to *Streptomyces* as 2nd instar larvae and stained with mercury bromophenol blue.
(a) showing the slight decrease in the total protein content after 24 h of treatment (x 200); **(b)** 48 h post-treatment, showing an obvious decrease in the total protein content in the epithelial cells, especially in the cytoplasm (x 200).

In the mid-gut epithelial cells of the control *Sp. littoralis* larvae, the total protein content appeared as an intensely bluish colouration as demonstrated by the mercury bromophenol blue method (Fig. 8). Treated 2nd-instar larvae exhibited a marked decrease in the total proteins of the mid-gut cells (especially in the cytoplasm): 24 h post-treatment there was a slight decrease (Fig. 9a), but it became obvious after 48 h of treatment (Fig. 9b).





Photographs showing the RNA and DNA electrophoretic pattern of *Spodoptera littoralis* larvae exposed to *Streptomyces lavendulae* culture filtrate
Fig 10: the electrophoretic pattern of RNA, lane 1= 24 h post-treatment, 2 = 48 h; 3 = control;
Fig 12: the apoptotic fragmented DNA in the body tissues of *Spodoptera littoralis* larvae; lane 1 = control, 2 = 24 h post-treatment, 3 = 48 h. Ladder :100 bp.

The intensity of RNA (expressed as optical density) was determined in the 2nd instar larvae. 24 h and 48 h post-treatment, the optical density of RNA was lower, with values of 250 and 212, respectively, compared to the 296 of the control (Figs. 10, 11). The intensity of fragmented DNA in the tissues was also measured as optical density using gel electrophoresis. The intensity increased in treated larvae relative to the control, with higher values at 24 h than 48 h post-treatment. (Figs. 12, 13).

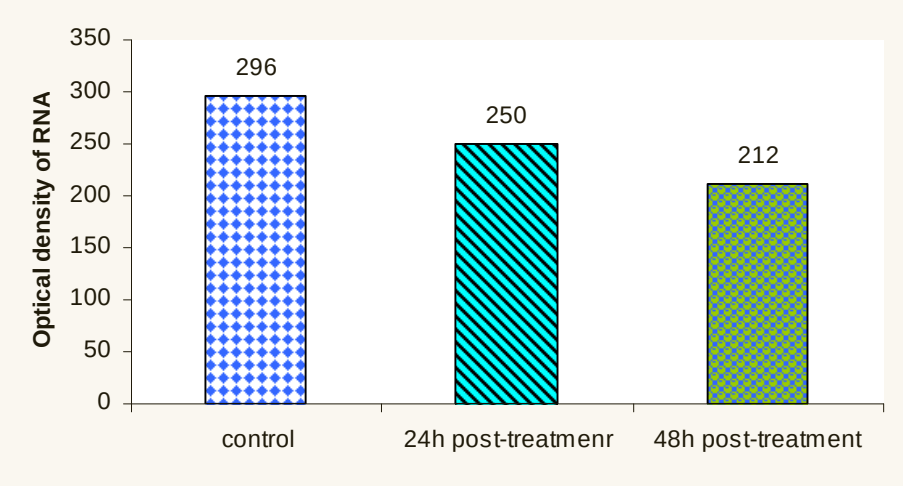


Fig.11: Optical density of RNA of 2nd-instar larvae of *Spodoptera littoralis* treated with *Streptomyces lavendulae* culture filtrate.

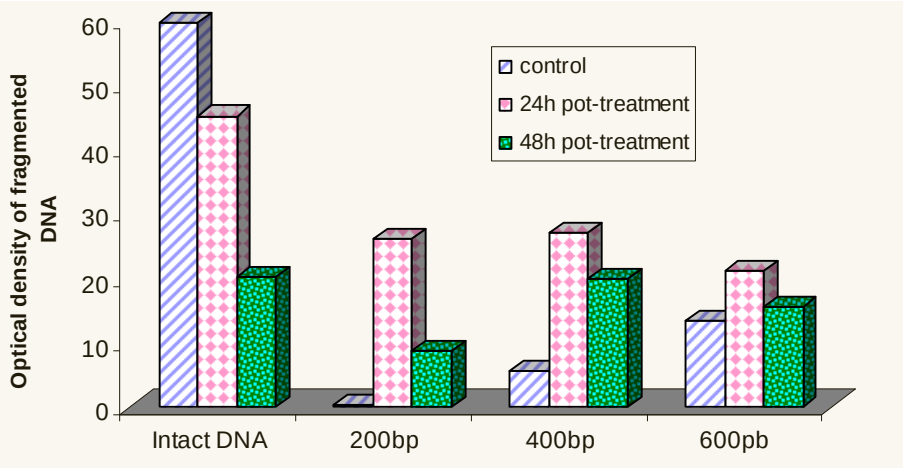


Fig. 13: Optical density of intact and apoptotic fragmented DNA of *Spodoptera littoralis* larvae treated with *Streptomyces lavendulae* culture filtrate.

Discussion

In the present study, 2nd-instar larvae of *Spodoptera littoralis* were very sensitive to the *Streptomyces lavendulae* culture filtrate, as shown by the LT50 value. This filtrate contains secondary metabolite compound(s) released by the microorganism. As a result, larval mortality was caused mainly by moult disruption induced by *S. lavendulae* toxin. This is consistent with results reported by other researchers. 4th-instar larvae of *Spodoptera litura* fed on castor leaves treated with 0.75ml of Spodlure (*Spodoptera* polyhedrosis virus) caused 79% larval mortality 96 h post-treatment (Prasad & Wadhwani 2006). The toxic soluble protein (extracted from *Metarhizium anisopliae*) fed to 2nd-instar larvae of *Sp. littoralis* at concentration of 6 mg protein ml^{-1} (for 5 consecutive days) caused 83% larval mortality (Quesada-Morga *et al.* 2006). Blackburn *et al*. (2005) found that Tca mixed with an artificial medium and offered to 2nd-instar larvae of *Leptinotarsa decemlineata* for 48 h was very toxic with LC50 = 2.7 ppm. Sakr (2007a) studied the effect of *S.lavendulae* culture filtrate at different concentrations on the 3rd-instar larvae of *Sp. littoralis*. She reported that the LC50 was found to be 151.4 CFU cm^{-2} after 7 d post-treatment, while a concentration of 226 CFU cm^{-2} produced 83% cumulative larval mortality.

The deleterious histopathological observations in the mid-gut and brain of *Sp.littoralis* showed that this instar is very sensitive to *S.lavendulae* culture filtrate. The cells of these organs showed histolysis with clear signs of apoptosis. We considered that the fragmented DNA bands detected on the gel electrophoresis are apoptotic fragments of the genomic DNA, consistent with the histopathological findings. The same finding was reported by Sakr (2007b). She found that the compound 5-aminopyrazolo-pyrazolone induced DNA damage in 3rd-instar larvae of *Sp.littoralis* (3 d post-treatment) in a dose-dependant manner. The antitumor antibiotic mitomycin C produced by *S.lavendulae* is a bioreductively activated alkylating agent that crosslinks DNA at 5'CpG sequences (Johnson *et al.* 1997).

The histopathology we saw is similar to that produced by other gut-active toxins. Dabron *et al*. (2002) reported that *Manduca sexta* (5th-instar larvae) injected with Mcf (*make caterpillars floppy*, a single *Photorhabdus* 8.8-kb gene secreted by *P.luminescens* bacteria) encoding *Escherichia coli* show massive destruction of the mid-gut epithelium; where the mid-gut epithelial cells began to bleb into the gut lumen, they often contain pyknotic nuclei. Cavados *et al*. (2004) observed serious damage in the mid-gut epithelium of *Simulium pertinax* larvae exposed to δ-endotoxin of *B. t.* Serovar *israelensis* at a dose of 4 mg ml^{-1} for 1 h. Columnar cells were vacuolized, microvilli damaged, epithelial cell contents passed into the gut lumen and finally the cells died. Blackburn *et al.* (2005) reported that the high-molecular-weight protein-toxin complex was toxic when ingested by neonate larvae of *L. decemlineata*, leading to an extensive apical swelling and blebbing of the epithelium, and the gut lumen becoming packed with cytoplasmic vesicles. Prasad & Wadhwani (2006) fed 4th-instar larvae of *Sp.litura* on castor leaves treated with 0.75 ml *Spodoptera* polyhedrosis virus at 2 x 10^9 polyhedra ml^{-1}); complete destruction of the mid-gut epithelium with large vacuoles was observed 48 h post-treatment. Quesada-Morga *et al.* (2006) found that crude soluble protein extracted from *Metarhizum anisopliae* elicited many histological changes to the mid-gut of 2nd-instar *Sp.littoralis* larvae: the mid-gut epithelium showed deterioration, there was destruction of the microvilli of the columnar cells, and formation of vacuoles .

In the present study, the intensity of the total protein decreased in the larval mid-gut cells after treatment with the *S.lavendulae* culture filtrate, and the amount of RNA decreased compared to the control. The filtrate of *S.lavendulae* contains mitomycin C that crosslinks DNA at 5'CpG sequences (Johnson *et al.* 1997), and also a novel compound, 1100-50 (1), which inhibits the synthesis of RNA (Takatsu *et al.* 2003).

Photorhabdus-released toxin contains a specific double-strand ribonucleic acid which knocks-down the genes of the recognition proteins, and caused great reduction in both RNA and insect resistance to the infection. This compound could be a specific RNAi which causes a reduction of RNA (Eleftherianos *et al.* 2006). The reduction of protein in the present study could be also due to a bioactive RNAi in the filtrate of *S.lavendulae* causing a reduction of RNA. The present histochemical findings are also consistent with the results of Abdel-Aal (2006), Abuo El-Mahasen (2007) and Sakr (2007a).

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الملخص العربي

التغيرات المحدثة للأنسجة و الأحماض النووية لدودة ورق القطن الكبرى Spodoptera littoralis (Boisd) (حرشفية الأجنحة: نوكتويدى) باستخدام المستخلص الخام لبكتيريا Streptomyces lavendulae (Streptomycetaceae)

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تم دراسة التغيرات المحدثة للأنسجة والأحماض النووية نتيجة استخدام المستخلص الخام لبكتيريا Streptomyces lavendulae (Streptomycetaceae) على العمر اليرقى الثانى لدودة ورق القطن الكبرى Spodoptera littoralis (Boisd) ، وذلك لمحاولة التعرف على طريقة عمل هذا المستخلص . و باستخدام طريقة الملامسة بتركيز 226 /سم2 و تحت الظروف المعملية أظهرت النتائج ما يلى: عند معاملة العمر اليرقى بهذا المستخلص لمدة 48 ساعة ، أحدث موت للأفراد المعاملة بنسبة 84,2 %. كما وجد أن لهذا المستخلص القدرة على إحداث العديد من التغيرات الهستولوجية للمعى الوسطى والمخ للأفراد المعاملة ، مع ظهور لعلامات الموت المبرمج للخلايا فى هذين النسيجين. وعلى المستوى الجزيئى وباستخدام طريقة الفصل الكهربى للأحماض النووية ، وجد إنخفاض في كثافة الحامض النووى الريبوزى (الرنا) مع زيادة كثافة الأجزاء المتفتة للحامض النووى الديوكسى ريبوز (الدنا) ، مقارنة بالمجموعة الضابطة. وفى ذات الوقت ، أدت معاملة اليرقات بالمستخلص البكتيرى إلى نقص ملحوظ فى المحتوى البروتينى لخلايا المعى الوسطى مقارنة بالمجموعة الضابطة وتتفق هذه النتيجة مع المشاهدات الخاصة بصورة الرنا.
أوضحت هذه النتائج أن المستخلص البكتيري يحتوي علي مجموعة من المركبات ذات النشاط البيولوجي. و يمكننا القول أن طبيعة عمل هذه المركبات قد يرجع إلي تأثيرها علي الجهاز العصبي و كذلك الجهاز المناعي للحشرة.