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Latent and Histopathological Impacts of Three Bioinsecticides on The Female Reproductive System of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

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ABSTRACT

Recently, scientists searched for safe alternatives to synthetic insecticides to manage the cotton leafworm *Spodoptera littoralis*. Of these alternatives are microbial-based insecticides. These compounds are known for their unique larvicidal activity, especially against Lepidopterous insects. In this regard, the present investigation was carried out to evaluate the latent and histopathological influence of *Bacillus thuringiensis* var. *kurstaki* (*Btk*) and *Spodoptera littoralis* nuclear polyhedrosis virus (*Spli*NPV) either individually or in a mixture against the adult female of *S. littoralis*. The LC_{50} values of the tested entomopathogens were determined. The impact of median lethal concentrations of tested entomopathogens on adult longevity, fecundity, and fertility was assayed after treating 2nd instar larvae. In addition, the histological changes in female ovaries due to treatment were investigated. Results showed that the low LC_{50} value showed that *Btk*+*Spli*NPV was the most hazardous against the 2nd instar larvae, followed by *Spli*NPV and *Btk*. All tested entomopathogens did not cause high immediate larval mortality; however, larval mortality increased throughout the larval stage until pupation. There was also a significant reduction in fecundity and fertility of emerged females resulting from 2nd instar larvae treatment. In addition, the treatment caused severe alterations in the histological structure of ovarioles. These findings support entomopathogens as a non-toxic alternative to conventional pesticides for controlling the cotton leafworm. Because of this, the population density of succeeding generations may decline since these organisms kill the treated larvae and have delayed effects that appear to reduce the eggs deposited and hatching. The results explain the reduced reproductive potential since these entomopathogens influence the female reproductive system when applied alone or as a mixture.

INTRODUCTION

Insect pests pose a significant risk to many types of crops across the globe. Lepidoptera is the second-largest order in the class Insecta and is home to several common agricultural pests. *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), sometimes known as the Egyptian cotton leafworm, is a damaging polyphagous insect pest. About 90 host plant species from 40 families are affected by this pest, some of which are commercially important crops (Shaurub *et al.*, 2023). Since this pest causes significant economic losses,

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many control methods have been developed to combat it, including biopesticides (Barrania, 2019), nano-based pesticides (Thabet *et al.*, 2021), and genetically modified crops (Salman *et al.*, 2021). Rotating between insecticides such as organophosphates, carbamates, insect growth regulators, and pyrethroids annually has been fundamental to Egypt's cotton leafworm control approach (Shaurub *et al.*, 2023). However, widespread and long-term use of chemical pesticides has led to unintended consequences such as pesticide pollution of soil and water, pesticide residues on harvested food, and the evolution of insecticide resistance and insecticide biomagnifications. Therefore, regulatory measures are being implemented internationally to reduce the use of chemical pesticides. As a result, biopesticides are in high demand worldwide (Abd El-Kareem *et al.*, 2010; Bravo *et al.*, 2011; Yasin *et al.*, 2020). Biopesticides are sustainable alternatives to synthetic insecticides since they break down in the environment and are effective against pests (Morán-Diez & Glare, 2016; Rajamani & Negi, 2021). Biopesticides are made from non-toxic compounds, such as those found in fungi, bacteria, algae, viruses, nematodes, and protozoa, or their products (microbial, phytochemicals), or their byproducts (semiochemicals) (Rajamani & Negi, 2021). Bacteria of the genus *Bacillus*, specifically *Bacillus cereus*, *Bacillus sphaericus*, *Bacillus popilliae*, *Bacillus subtilis*, and *Bacillus thuringiensis*, which possess entomopathogenic properties, have been employed for controlling diverse insect pests (Stahly *et al.*, 2006). *Bacillus thuringiensis* (*Bt*) (Berliner) is widely recognized as one of the most extensively utilized bacteria for insect control in commercial applications. During bacterial sporulation, a crystal protein (δ-endotoxin) is generated, which can cause the lysis of gut cells in susceptible insects upon consumption (Jisha *et al.*, 2013; Olson, 2015). Compared to synthetic pesticides, *Bt* spores and parasporal crystals are considered more specific and safer. The sub-species of *B. thuringiensis*, namely *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*, exhibit a high level of toxicity towards lepidopteran larval species as per reference (Pinos *et al.*, 2021). Insect-specific baculoviruses have a rod-shaped envelope and measure between 40 and 50 nm in diameter and 200 and 400 nm in length. The DNA genomes of baculoviruses are circular and double-stranded; their sizes range from 80 to 200 Kbp. They have been extensively exploited to create systems for expressing genes, which have found applications in gene therapy and the synthesis of recombinant proteins (Ahmed *et al.*, 2019). Moreover, Nucleopolyhedrosis viruses (NPVs) are an effective alternative to synthetic insecticides for controlling *S. littoralis*. Phylogenetic analysis has classified the Baculoviridae family into four distinct genera: Alphabaculovirus (lepidopteran-specific NPVs), Betabaculovirus (lepidopteran-specific granuloviruses (GVs)), Gammabaculovirus (hymenopteran-specific NPVs), and Deltabaculovirus (dipteran-specific NPVs) (Jehle *et al.*, 2006). The baculovirus known as *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*NPV) has been studied, registered, and put into practice for the management of *S. littoralis*, *S. frugiperda*, and *S. litura* (tobacco cutworm) in Africa, the Americas, and Japan. According to research by Harrison *et al.* (2018), *Spli*NPV may be traced back to the genus Alphabaculovirus and the species *Spodoptera littoralis* nucleopolyhedrovirus. The main benefits of NPVs are their host specificity and lack of negative effects on beneficial insect and pollinator populations (Simón *et al.*, 2020). However, the NPVs' limited use in pest control might be attributed to the lengthy incubation time required before they become lethal. *Bt* and NPVs may increase the virus's pathogenicity (Magholifard *et al.*, 2020; Akhanaev *et al.*, 2022). Making a mixture of two microorganisms can be acceptable. There are three possible outcomes of interactions between microorganisms: cooperation, synergy, and antagonism. Synergism increases virulence as a consequence of contact, whereas antagonism decreases it. In theory, interactions of both synergy and antagonism might occur between distinct microbial agents and even between different strains of the same pathogen (Osman & Mahmoud, 2009; Abd El-Aziz *et al.*, 2019). Moreover, previous research confirmed the potential role of both *B. thuringiensis* and NPVs for controlling many agricultural pests, not only for direct killing effect but also for their latent impacts on insect's biological, physiological, and histological aspects (El-Banna *et al.*, 2012; Abd El-Aziz *et al.*, 2019; Shazdehahmadi *et al.*, 2019; Magholifard et al., 2020; Akhanaev *et al.*, 2022). Based on the information above, the purpose of this study is to compare the effects of solitary and mixture applications of *B. thuringiensis kurstaki* and *S. littoralis* NPV (*Spli*NPV) on the pathogenicity, fecundity, and fertility of adults and histological characteristics of *S. littoralis* adults.

MATERIALS AND METHODS

Tested Insect:

The cotton leafworm egg masses used to create the *S. littoaralis* strain used in the laboratory were obtained from the Cotton leafworm research department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. Under regulated circumstances at 25±2° C, relative humidity of 65±10%, and an 8:16 L:D (Light: Dark) photoperiod, castor leaf beetle larvae were successfully grown on clean castor leaves (*Ricinus communis* L.) (El-Sawaf, 1971). Ten generations of the acquired strain were raised in a chemical-free environment.

Tested Compounds and Toxicological Assay:

Under controlled conditions, two entomopathogenic microorganisms were evaluated for their pathogenicity against the 2nd instar larvae of *S. littoralis*. They were *Bacillus thuringiensis* var. *kurstaki* (*Btk*) (Bacillales: Bacillaceae) and *S. littoralis* nuclear polyhedrosis virus (*Spli*NPV) (WP 9.4% and 4%, respectively). Larvae were treated with either a single entomopathogen or a mixture (*Btk*+*Spli*NPV WP 5%+2%). All entomopathogens were supplied by the Plant protection research institute, Agricultural research center, Dokki, Giza, Egypt.

Seven concentrations were prepared by dissolving 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 gm in 100 ml of distilled water. Fresh castor oil leaves were dipped into each solution for 10 seconds and then left to dry at room temperature before being offered to larvae (Tabashnik et al., 1991). For each treatment, 20 newly molted 2nd instar larvae were offered treated leaves for 48h. they were replicated thrice. The same number of larvae were used as controls, whereas the larvae were given castor oil leaves dipped in distilled water. Larval mortality was recorded daily 48h post-treatment till pupation and was corrected according to Abbott's formula (Abbott, 1925). The LC_{50} values were estimated for further assayed using "LdPLine® "software [http://embakr.tripod.com/ldpline/ldpline.htm] following Finney (1971).

Effect on Fecundity and Fertility of Adults:

The 2nd instar larvae were treated with the estimated LC_{50} of each tested compound for 48h, as mentioned in the previous section, and the surviving larvae were kept until pupation. The resultant pupae were distinguished as males and females and placed in solitary pairs in a glass globe in one of the following pairings: i) a treated male & a treated female, ii) a treated male & an untreated female, and iii) a treated female & an untreated male. For the control pair, an untreated female & an untreated male was confined. All pairs were replicated fifth. All glass globes were supplemented with a cotton pad soaked with 20% sugar solution as a food source and stripes of paper as an ovipositional substrate. Daily collections of egg masses from each mating were made. Each adult moth's fecundity and fertility were documented throughout its whole life cycle (pre-oviposition, oviposition, and post-oviposition periods, measured in days).

Histopathological Studies:

The latent influence of the LC_{50} of each compound on the histological structure of the ovaries of the females that survived treatment as 2nd instar larvae were investigated. Adult females that had just emerged from each treatment and their respective controls were studied. After removal, their reproductive organs were placed in Bouin's fixative Eppendorf's for the night. These samples underwent histological preparation utilizing a stepwise dilution in ethanol. The material was sectioned longitudinally at a thickness of 4µ. Staining was done using Ehrlich's hematoxylin and eosin. The slides were analyzed using a light microscope and photographed by a high-definition digital camera. Several photographs were obtained at various magnification levels.

RESULTS

Pathogenicity of Tested Entomopathogens:

Results presented in Table (1) showed the LC_{50} values of tested entomopathogens against the 2nd instar larvae. The low LC_{50} value showed that *Btk*+*Spli*NPV (0.054 gm/ml) was the most hazardous against the 2nd instar larvae. Then came *Spli*NPV and *Btk* (0.061 and 0.121 gm/ml, respectively). All tested entomopathogens did not cause high immediate larval mortality; however, larval mortality increased throughout the larval stage until pupation. Results also showed that mixing both microorganisms raised their pathogenicity. This enhancement may be due to the synergistic action of mixing both *Btk* at high concentrations and *Spli*NPV at low concentrations. There have been reports of the synergistic effects of various biocontrol agents, such as *Bt* and NPV on *H. armigera*, *SI*NPV-*Btk* on *Culex pipiens* (L.) (Mahmoud *et al.,* 2012), *Btk*+*Spli*NPV against *S. littoralis* (Abd El-Kareem, 2016; Magholifard et al., 2020), and *Bt*-*Ha*NPV against *P. xylostella* (Magholli *et al.,* 2013; Kalantari *et al.,* 2014; Shazdehahmadi *et al.,* 2019). On the other hand, the lowest concentration of *Bt* could cause a delay in the development of the larvae and injure the cells of the targeted insect's intestine, resulting in an increase in the efficacy of *Spli*NPV (Salama *et al.,* 1993) and a general decline in fitness (Nouri-Ganbalani *et al.,* 2016). In the presence of *Bt*, the proportion of insects resistant to NPV infection is diminished (Hesketh & Hails, 2015). Following the findings of Cook *et al.* (1996), the combination of *BtSpli*MNPV at a modest concentration significantly increased larval mortality and decreased insect killing time. Typically, at low concentrations of *Bt*, toxins bind to specific receptors of the midgut epithelium of the targeted insect, causing the cells to lyse and facilitating virus entrance. At high concentrations of Bt, the mode of action of Cry toxins is to cause injury to the gut cells of the targeted insect, which may prevent *Spli*MNPV from entering the midgut cell. These observations were similar to formerly published studies on *S. littolais* and other insects (Abd El-Aziz *et al.,* 2019; Shazdehahmadi *et al.,* 2019; Magholifard *et al.,* 2020; Akhanaev *et al.,* 2022).

Table 1: The median lethal concentrations (LC_{50}) of tested entomopathogens against the 2nd instar larvae of *Spodoptra littoralis* under laboratory conditions

Tested entomopathogens	Median lethal concentration (LC_{50}) (gm/ml)	Slope
Bacillus thuringiensis kurstaki (*Btk*)	0.121^{**3}	1.61 ± 0.996
*Spli*NPV	0.061^{*2}	1.59 ± 0.879
Btk+*Spli*NPV	0.054^{*1}	1.6 ± 0.86

Numbers 1, 2, and 3 refer to sorting highly toxic compounds according to the LC_{50} value

*Corresponding to high toxicity according to LC_{50} value

** Corresponding to moderate toxicity according to LC_{50} value

Effect of Median Concentrations of Tested Entomopathogens on Adult Longevity, Fecundity, and Fertility:

Adult moths resulting from 2nd instar larvae that survived treatment with the LC_{50} of tested entomopathogens were assayed for their longevity, fecundity, and fertility (Tables 2 and 3). The oviposition duration of moths of both sexes that emerged after 2nd instar larvae were treated with LC_{50} of *Btk* was lengthened to 5.67 days from 4.33 days in the control group (Tables 2 and 3). During this time, females deposited an average of 543.99 eggs and 2.66 egg masses, decreases of 38.56 and 74.52 %, respectively, compared to the control. The percentage of these hatched oviposited eggs is shown in Tables (2) and (3). The oviposition duration and the mean number of deposited egg masses were identical between normal female moths and males emerging from treated 2nd instar larvae with LC_{50} of *Btk* (Tables 2 and 3). While only 48.72% of the eggs produced by each female hatched, the overall number of eggs laid was substantially greater, averaging 640.98 eggs. When female moths emerged from treated 2nd instar larvae and mated with untreated males, female fertility had little influence. These moths were 57.27% less fertile than the control population, laying an average of 921.33 eggs per female with a total of 2.31 egg masses per female during an oviposition period of 7.76 days. The hatch rate (as shown in Tables 2 and 3) for these laid eggs was 57.16%.

The lowest observed number of oviposited egg masses per female was produced by mated moths that emerged from 2nd instar *S. littoralis* larvae treated with LC_{50} of *Spli*NPV, with a total of 846.99 eggs laid throughout a 3.76-day oviposition period (Tables 2 and 3). In contrast, mating untreated females with treated male moths yielded 1283.66 eggs/female from an average of 3.99 egg masses laid. The number of egg masses laid and the total number of eggs laid were found to be reduced by 7.81 and 39.88%, respectively, compared to the control (Tables 2 and 3). When normal male moths were coupled with females emerging from treated second-instar larvae, the treatment's effects were attenuated. Table (2) showed that even though these pairings had lower fecundity than the control group by 46.42% and 70.09%, respectively, in terms of the number of egg masses deposited (2.32 egg mass/female). The number of eggs per mass (638.65 eggs/female) and egg hatchability were still excellent at 80.48%.

The longest oviposition duration (Table 3) was in the 2nd instar *S. littoralis* larvae treated with LC_{50} of *Btk*+*Spli*NPV, lasting 6.67 and 7.33 days. When moths of both sexes emerging from the second instar were coupled, they laid 573.65 eggs (Table 2), with an average egg mass of 3.65. These two numbers were significantly lower than the control by 15.70% and 73.13%, respectively. These eggs had a hatching success rate of 34.8 percent. The fecundity and egg mass produced by pairs of males and females that developed from treated 2nd instar larvae resulted in a reduced total of 586.98 and 255.66 eggs per female, respectively. The hatch rate of deposited eggs was 42.70% for the first coupling and 24.24% for the second. The severity and form of the observed abnormalities were consistent with those described for other insects infected with Btk, baculovirus, or a combination of the two (Abd El-Kareem *et al.,* 2010; Nouri-Ganbalani *et al.,* 2016). The great influence of tested entomopathogens against the reproductive potential of *S. littoralis* adults can be due to the used compounds interfering with egg formation or development and, consequently, reducing the number of laid eggs (Abd El-Kareem *et al.,* 2010). Aldebis *et al.* (1993) and Santiago-Alvarez and Osuna (1988) provide a possible framework for understanding the results. Researchers discovered that the number of eggs laid was unaffected when NPV-infected male *S. littoralis* were permitted to mate with untreated females (Patil *et al.,* 1989; Rothman & Myers, 1994; Cabodevilla *et al.,* 2011; Führ *et al.,* 2021). Still, the hatchability of those eggs was drastically reduced. It's also possible that treatment-related damage of eggs and/or sperm is to blame for the observed drop in hatch rate. Aldebis *et al.* (1993) hypothesized that the failure to deliver sperm to females during copulation might be a contributing factor. Researchers have shown that bioagent-treated cotton leafworm moths cannot reproduce (Cabodevilla *et al.,* 2011; El-Sheikh, 2012; Santiago-Alvarez & Osuna, 1988; Vargas-Osuna & Santiago-Alvarez, 1988).

Table 2: The effect of median lethal concentrations of *Btk*, *Spli*NPV, and *Btk*+*Spli*NPV on the fecundity and fertility of adult *Spodoptera littoralis* resulted from 2nd instar larvae treatment.

Compound	Mating combination	Mean no. of egg masses/♀ ± SE	Reduction %	Mean no. of eggs / ♀± SE	Reduction %	Mean no. of hatched eggs ± SE	Reduction %	Eggs fertility %
Btk	**T♀ x T♂**	2.66±0.03^{e}	38.56	543.99±57.2^{def}	74.52	259.65±34.4^{fg}	87.65	47.71^{h}
	N♀ x T♂	2.98±0.01^{d}	31.17	640.98±23.7^{d}	69.98	313.32±7.7 ^{ef}	85.09	48.72^{g}
	T♀ x N♂	2.31±0.04^{f}	46.65	921.33±36.0^{c}	57.27	526.66±15.4^{c}	74.95	57.16^{f}
***Spli*NPV**	**T♀ x T♂**	1.98±0.02^{g}	54.27	846.99±27.1^{c}	60.33	527.99±33.5^{c}	74.88	30.45^{k}
	N♀ x T♂	3.99±0.03^{b}	7.85	1283.66±48.3^{b}	39.88	1141.32±23.9^{b}	45.72	88.91^{c}
	T♀ x N♂	2.32±0.01^{f}	46.42	638.65±22.3^{d}	70.09	513.99±36.9^{c}	75.55	80.48^{d}
***Btk+Spli*NPV**	**T♀ x T♂**	3.65±0.01^{c}	15.70	573.65±42.5^{de}	73.13	199.66±22.9^{g}	90.50	34.80^{i}
	N♀ x T♂	2.31±0.01^{f}	46.65	255.66±32.1^{g}	88.02	61.99±6.9^{h}	97.05	24.24^{l}
	T♀ x N♂	2.98±0.02^{d}	31.17	586.98±50.2^{de}	72.5	250.66±29.2^{fg}	88.11	42.70^{i}
Control	**N♀ x N♂**	4.33±.01^{a}	-	2135.33±34.8^{a}	-	2102.66±30.4^{a}	-	98.47^{a}
F values	-	454.4***	-	139.97***	-	456.51***	-	5222.72***
L.S.D.		0.11		118.88		73.25		0.991

Means with the same letter are not significantly different ($p<0.05$).

Table 3: The effect of median lethal concentrations of *Btk*, *Spli*NPV, and *Btk*+*Spli*NPV on the fecundity and fertility of adult *Spodoptera littoralis* resulted from 2nd instar larvae treatment**.**

Compound	Mating combination	Total oviposition periods (days)			The mean of adult life span (days ± S.E.)	
		Pre	Ovi	Post	♀	♂
Btk	**T♀ x T♂**	4.67	5.67	2.33	12.67±0.17 ^{e}	13.33±0.19 ^{e}
	N♀ x T♂	4.33	5.67	2.33	12.33±0.25 ^{e}	12.67±0.23 ^{g}
	T♀ x N♂	3.00	7.67	3.33	14.00±0.28 ^{bc}	13.67±0.18 ^{f}
***Spli*NPV**	**T♀ x T♂**	4.67	3.67	4.67	13.67±0.12 ^{cd}	15.67±0.12 ^{bc}
	N♀ x T♂	4.00	4.00	4.33	12.33±0.1 ^{e}	14.67±0.27 ^{g}
	T♀ x N♂	3.33	6.33	3.67	13.33±0.19 ^{d}	14.67±0.07 ^{g}
***Btk+Spli*NPV**	**T♀ x T♂**	5.00	7.33	3.67	16.00±0.29 ^{a}	17.33±0.04 ^{e}
	N♀ x T♂	5.33	6.67	2.33	14.33±0.3 ^{b}	15.33±0.29 ^{e}
	T♀ x N♂	5.67	7.33	3.00	15.67±0.4 ^{a}	16.33±0.28 ^{c}
Control	**N♀ x N♂**	4.67	4.33	3.33	12.33±0.1 ^{e}	15.33±0.20 ^{d}
F values	-	-	-	-	31.196 ***	61.704***
L.S.D.					0.706	0.616

Means with the same letter are not significantly different ($p<0.05$).

Histopathological Impacts on Female Ovaries:

Spodoptera littoralis, like the vast majority of other lepidopterous insects, have identical ovaries. Each ovary consists of four polytrophic ovarioles. There is a lateral oviduct on each ovary. The connection between the two oviducts forms the common oviducts (Chapman, 2013). Each ovariole of the polytrophic type is enveloped by a thin epithelial membrane (the outer membrane sheath). Its tip is known as the terminal filament. The terminal filaments of each ovary's four ovarioles are united into a single filament. Together, the two primary filaments of both ovaries constitute a suspensory filament. The upper portion of each ovariole is known as the germarium zone, which contains the primordial germ cells (oogonia, trophocytes, and perifollicular cells), which become differentiated and mature into eggs in the vitelarium zone. Each oocyte is enveloped by follicular epithelium and has nurse cells arranged atop its follicle. Depending on the stage of development, the oocytes and the nurse cells vary in size; when the oocytes mature into an egg, the nurse cells degenerate (Fig. 1a).

Emerging females treated as 2nd instar larvae with LC_{50} concentrations of tested entomopathogens exhibited varying degrees of ovariole membrane or oocyte formation injury. The membrane effects were the separation of the follicular epithelial cells, rupture of the follicular cells, and separation between the developing oocytes and the follicular epithelium (Fig. 1b). Alteration in developing oocytes can be summarized as follows: a reduction in the oocyte's contents, resulting in the loss of its oval shape and the formation of clear space around it; a partial absence of the nurse cells; and a partial decline in the oocyte's contents. In addition, the ovarioles appear degenerated with irregularly separated oocytes and destruction of some follicular epithelial cell lining (Fig. 1c). Furthermore, microscopic examination revealed rupture of some ovarioles outer sheath and degeneration of oocytes with a wide space between them (Fig. 1b, 1c, and 1d), as well as degeneration of oocytes with separation of its epithelial cell lining (Fig. 1d).

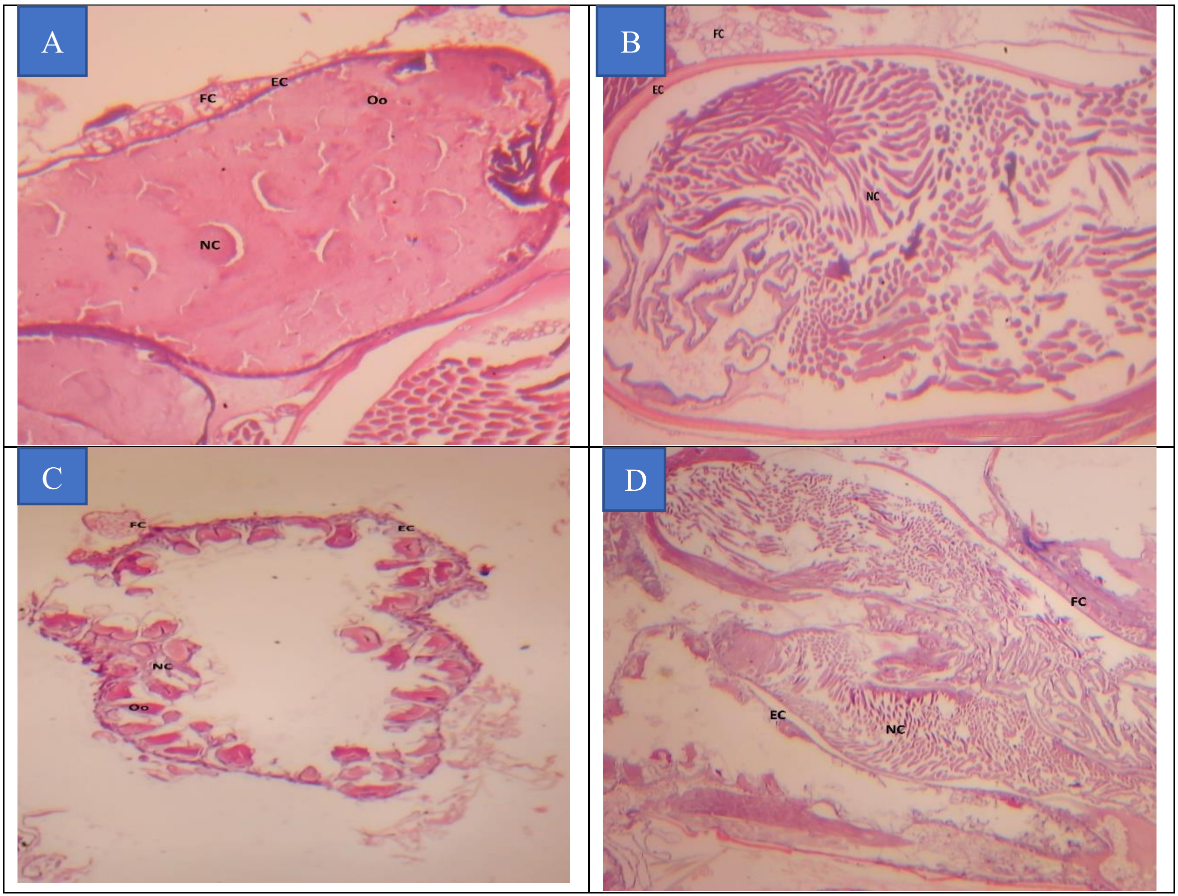

Fig. 1: Longitudinal sections of ovarioles of newly emerged females resulting from 2nd instar larvae treatment with LC_{50} of tested entomopathogens. a) Normal; b) *Btk*; c) *Spli*NPV; and d) *Btk*+*Spli*NPV. FC: follicular cells, EC: epithelial cells, Oo: oocytes, NC: nurse cells.

Conclusion:

The present study's findings provide compelling evidence regarding the effectiveness of insect pathogens in managing the cotton leafworm, serving as a secure substitute for traditional chemical pesticides. The findings indicate that these entomopathogens exhibit not only larvicidal properties but also residual effects that can inhibit oviposition and reduce hatching rates, potentially resulting in reduced population densities in subsequent generations. Furthermore, the impact of these microorganisms on the female reproductive system elucidates the reduction in reproductive potential as a delayed consequence when applied individually or as a mixture.

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