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Histopathological and ultrastructural impacts of nuclear polyhedrosis virus on infected tissues of the leopard moth *Zeuzera pyrina* (Lepidoptera: Cossidae) using transmission electron microscopy

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Abstract:

Leopard moth, *Zeuzera pyrina* (L.) (Lepidoptera: Cossidae) is considered one of the most dangerous and destructive wood boring pests that attack several varieties of the horticultural fruit trees mainly the apple trees, *Malus domestica* B. (Rosales: Rosacease). In Egypt, this borer causes severe crop loss resulting in serious detrimental and economic damage thus continuous control trials are taken place aiming to avoid these key problems. Through the ongoing study surveying visits were done to collect and sample the natural infected stage of this cossid borer. Then a series of experiments was performed to isolate and trace of the viral pathogen which is identified as the *Zeuzera pyrina* nuclear polyhedrosis virus **(**ZPNPV). Then testing the susceptibility of *Z. pyrina* larvae to this virus was done at three different controlled temperatures (25±5°C) in the laboratory. Screening tests revealed high mortality rates for the larval stage at 20°C recording 85.7±0.38 %. Ultimate sequence of experiments was applied to conduct histopathological and ultrastructural studies using transmission electron microscopy. Gained results showed severe malformation and distortion of the mid-gut cells confirming the susceptibility of *Z. pyrina* to the nuclear polyhedrosis viral infection and possibility of ZPNPV to be applied within the control programs of this cossid borer. It is concluded that *Z. pyrina* revealed a definite susceptibility to the infection with ZPNPV and it is highly provoked to be involved in the integrated pest management programs against this dangerous moth.

Introduction

Leopard moth, *Zeuzera pyrina* (L.) (Lepidoptera: Cossidae) is a highly destructive wood boring pest attacking several horticultural fruit trees mainly the apple trees, *Malus domestica* B. (Rosales: Rosacease) in several global regions especially the Mediterranean area including Egypt causing serious damage and considerable crop loss (Navon, 1977; Katlabi, 1989; Ismail *et*

al., 1988 and 1992; Guario *et al.*, 2000; Hegazi and Khafagi, 2005; Kutinkova *et al.*, 2006; Merghem, 2012; Mani *et al.*, 2014 and Manja and Aoun, 2019). Thus it was of great importance to combat this devastating boring pest, control measures for this borer are mainly focused chemical applications.

In Egypt, the control and management programs against this borer are depending mainly on pesticides applications for many years and still up till now. Excessive chemical sprays lead to high hazard risk, resistance problems and dangerous health effects for human, livestock and beneficial insects (Tadros *et al.*, 1993; Haniotakis *et al.*, 1999; Sarto, 2001; Osuna and Patanita, 2006; Patanita *et al.*, 2009 and Almanoufi *et al.*, 2012).

Consequently, searching for safe and non-chemical or alternative agents is encouraged aiming at maximizing the efficiency of that insect boring pest's control program and preventing the detrimental, residual and adverse effects resulting from chemical pesticide applications such as biocontrol agents. Unfortunately, biological control elements were restrictedly applied against *Z. pyrina* such as entomogenous nematodes and ectoparasitoids in addition to some scanty trials using microbial bioagents such as bacteria and fungi (Abdel-Kawy *et al.*, 1992; Nashnosh *et al.*, 1993; Tawfik and Ramadan, 2006; Lawrence *et al.*, 2007; Japoshvili and Hansen, 2013; Merghem and Hassan, 2014 and Labaude and Griffin, 2018).

Viral disease infections, especially the nuclear polyhedrosis viruses (NPV), as microbial entomopathogenic agents are still used on a limited scale for the control of the wood boring pests especially this cossid borer thus there was a need to estimate the efficiency of such biocontrol agents against this boring pest.

Present study was undertaken to explore the susceptibility of the wood boring pest *Z. pyrina* to the viral disease as a biocontrol agent for it. Moreover, focusing on the isolation of viral diseases that naturally infect the stages of this cossid borer was undertaken. Ultimately concern with the viral impacts on the larval tissues of *Z. pyrina*, through histopathology and electron microscopy investigations was studied.

Materials and methods

1. Field surveyed visits:

During the current study, different localities of the host horticultural trees of *Z. pyrina*, mainly the apple trees, had been visited throughout 2016 to 2017 searching for the naturally diseased individuals in Qalubeia and Beheira Governorates.

2. Sampling, preservation and identification:

Collected intact, health and moribund specimens of this target boring pest were collected and preserved separately in glass vials containing 0.55% sterile saline solution. Then samples were subjected to a preliminary identification for the collected stages of the boring insect done at the Department of Wood Borers and Termite in the Plant Protection Research Institute (PPRI) followed by a confirmation of this borer identity undertaken at the Entomological Collection of the Classification and Taxonomy Research Department of the same research institute (*i.e.* PPRI) and finally it was revealed that it is the leopard moth, *Z. pyrina*.

Diseased specimens were collected from the surveyed localities of the fore-mentioned Governorates. Then the viral-shown specimens were separated in the saline solution for the further pathological experiments and the moribund symptoms were recognized according to the identification keys after Vlak and Grőner (1980) and Evans and Shapiro (1997). Dissection of infected larvae was done and the digestive guts

were obtained for the following microscopic examination elucidating the internal symptoms as the presence of the virions and inclusion bodies virus (IBV) in the infected tissues with considerable numbers, dispersion of air sacs and vacuolation within cells and destruction of the nuclear membrane of target cells. Both histological and electron microscopy studies confirmed the identity of a NPV infection to this cossid borer called *Zeuzera pyrina* nuclear polyhedrosis virus (ZPNPV).

3. Laboratory screening tests:

On identifying the viral pathogen type, a second series of experiments dealing with the susceptibility of this leopard moth borer to the infection of ZPNPV, was undergone. Firstly, a stock viral suspension that would be used further for the screening tests was prepared through using the diseased larvae. Moribund larval guts were grinded with a sterile mortar till complete dissolve in 2ml sterile distilled water (SDW) tube with a ratio of 1:1 to tissues used. Then, the obtained suspension after a skimming process was stored as a stock for the further viral infectious treatments against healthy larvae of *Z. pyrina* and was keep in 4°C. Then, a suspension solution of $3x10^{10}$ IBV/ 100ml SDW concentration (*i.e.* $3x10^{8}$ IBV/ml) was prepared and was applied for each individual larva of this moth borer and the exposure treatment was done within a suitable Petri dishes. The screening tests were done at three controlled temperatures (25±5°C) in the laboratory with inspection process for a week and a fortnight periods, and seven replicates were used for each temperature degree both to control and treated checks. Mortality rate percentages of the inoculated *Z. pyrina* larvae were then recorded and corrected with Abbott's formula according to Abbott (1925).

4. Histopathology and electron microscopy studies:

4.1. Histopathological study:

To achieve the purpose of the histopathological study and the further investigation with electron microscope, a sequence of laboratory steps was followed beginning with the dissection of the larval body, removal of visceral and fat tissues and getting the gut (*i.e.* the mid-gut). Then, a procedure of micro-technique preparation was provided following by the dehydration, clearing, embedding, sectioning, staining, and fixation processes till the full examination, this procedure was according to Hamm (1999).

4.2. Electron microscopy study:

IBVs were randomly selected then suspended in a 50:50 glycerin/water solution and measured at x1,250 (phase contrast) with a micrometer hence were pelleted from an aqueous suspension at 15,000 rpm for 10min in an Eppendorf centrifuge. The bodies were fixed in 1.5% glutaraldehyde (pH 7.2) at 4°C for 2 hr; washed in 5% sucrose-sodium cacodylate buffer (pH 7.2) for 4 hr; and then fixed in 2% osmium tetroxide for 1 hr), washed in sucrose-sodium cacodylate buffer (10 min), and dehydrated in a series of 20, 40, 60, 80,90, and 100% ethanol. After remaining in 100% ethanol for 30 min, they were pelleted at 15,000 rpm for 10 min. The pellets were infiltrated and embedded in Spurs medium, sections ranged from 0.7 to 1.0 mm were cut on an Ultratome and stained with 1% ethanolic uranyl acetate for 10min, followed by a deionized water rinse and lead citrate stain for 2 min. They were then rinsed in sterile SDW, dried, and observed with the electron microscope at 80 kV as transmission electron microscopy (TEM) studies. This offered electron microscopy procedure was presented by Bud and Kelly (1977), Mclntosh and Ignoffo (1986) and Tanada and Kaya (1993) and it was taken place at the Electron Microscopy Unit, National Research Center.

5. Statistical analysis:

Obtained data were statistically analyzed according to Finney (1971).

Results and discussion

1. The viral isolation and identification:

Figure (1) showed that an electron micrograph of a viral suspension smear, at different magnifying powers (x), prepared from the infected larval stages of *Z. pyrina*. It reveals the dimensional hexagonal shape of the inclusion bodies (IBVs) for ZPNPV; the nuclear polyhedrosis virus isolated from the infected larval stages of *Z. pyrina*.

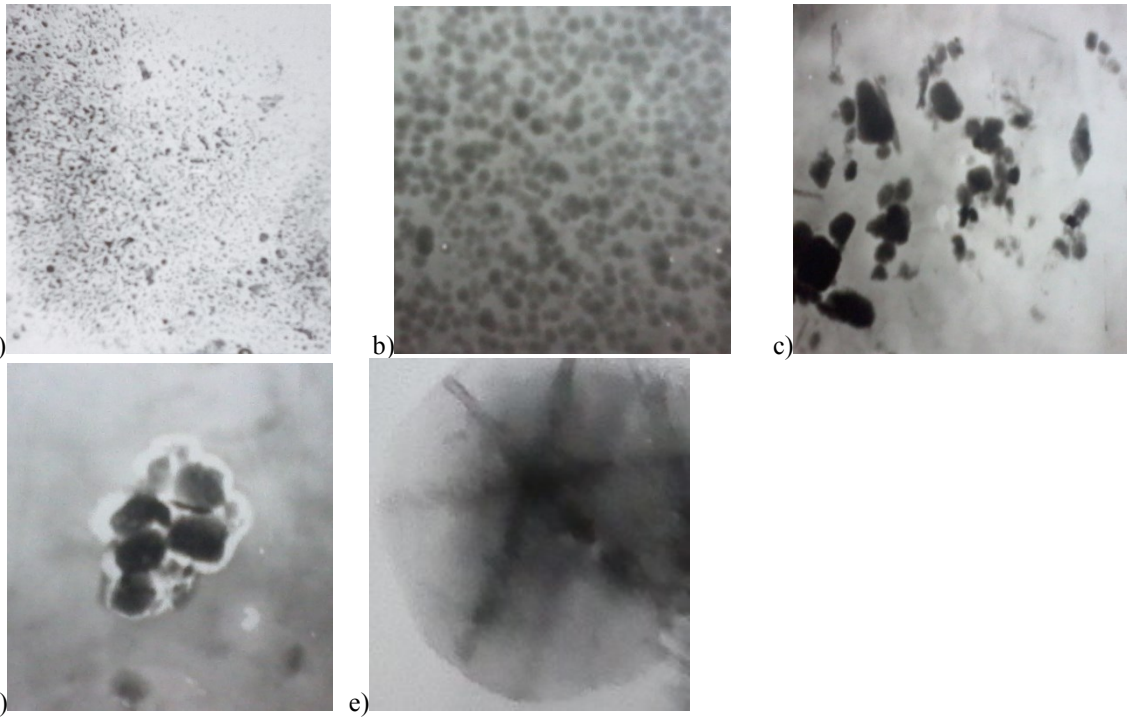

Figure (1): Electron micrograph of a smear of the viral suspension showing hexagonal inclusion bodies virus (IBVs) of the *Zeuzera pyrina* nuclear polyhedrosis virus (ZPNPV) at magnifying power ($x10^3$): a)12.5, b)16, c)20, d)40, e)60.

The finding that leopard moth borer, which belongs to the lepidopteran members, is susceptible to the NPV infection is matching with the results of Bud and Kelly (1977), Vlak and Grőner (1980) and Mclntosh and Ignoffo (1986) with noctuids and Shapiro (1992) with lymantriids which confirm the infectivity of the nuclear polyhedrosis viruses to lepidopterous families.

2. Laboratory screening tests:

Figure (2) elucidates the efficacy of ZPNPV; the nuclear polyhedrosis virus of *Z. pyrina* when inoculated to the larvae of this cossid borer at the controlled temperatures 25±5°C.

This efficacy is represented by the average mortality rates resulted from the inoculation treatments with the concentration of $3x10^8$ IBV/ml. Mortality rates were found to vary significantly at ($P<0.05$) as each temperature degree resulted in an average mortality rate, indicating the efficacy of the viral suspension, significantly different from the two other average rates of the two rest temperatures. It is also concluded that the increase of the temperature degree is diversely proportional with the viral efficacy as it is obvious that the highest controlled temperature (30°C) revealed the least mortality 42.9±0.54 % meanwhile the figure is reflected with the lowest degree (20°C) which recorded a rate of 85.7±0.38 %.

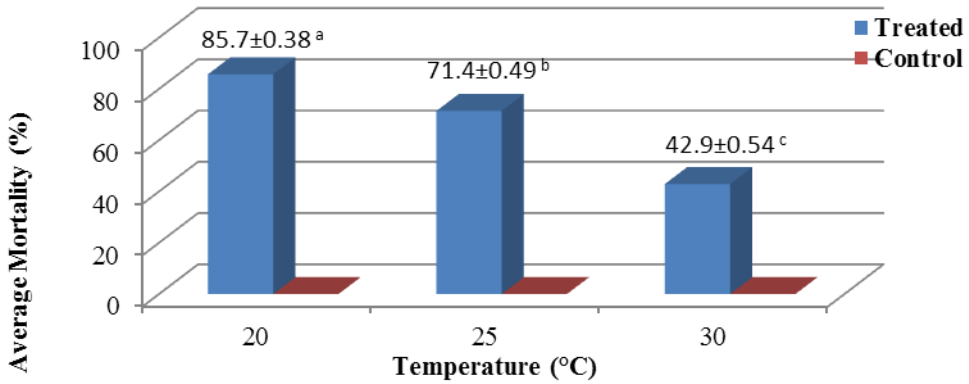

Figure (2): Efficacy of 3x10^8 inclusion bodies virus(IBV) /ml *Zeuzera pyrina* nuclear polyhedrosis virus (ZPNPV) concentration when inoculated to *Zeuzera pyrina* larvae at controlled temperatures (25±5°C).

3. Histopathological study:

Figure (3a) indicates the invasion of the nuclear polyhedrosis virus of the *Z. pyrina*; ZPNPV at the concentration of 3x10^8 IBV/ml. showing the viral replication and IBVs distribution through the mid-gut tissue cells of this cossid borer. The basement membrane of the epithelial tissue section reflects the impact of the viral replication and the invasions of the IBVs appearing the distortion of the epithelial cells near the membrane layer. The cytoplasm is filled with a lot of air sacs and vacuoles due to the viral infections.

Figure (3b) reveals an apparent contrast with the former Figure (3a) as it show an intact tissue lining with a firm in row of the adjacent cells. It is free from the IBVs presence in addition to the lack of either the air sacs or any vacuolation.

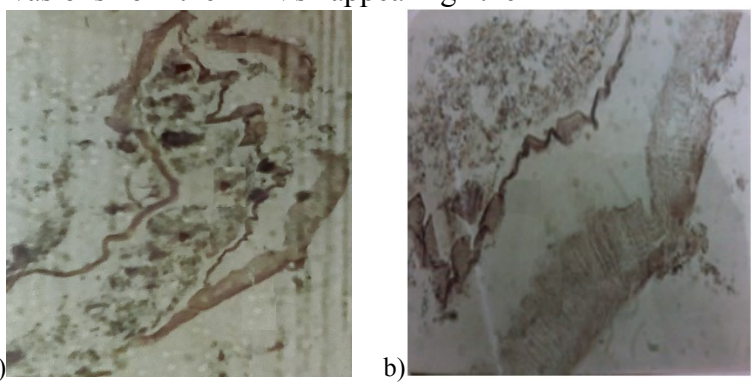

Figure (3): A photomicrograph of a longitudinal section of the mid-gut region of *Zeuzera pyrina* showing: a) the invasion of the inclusion bodies virus (IBVs) , distortion of the basement membrane and air sacs; and b) the normal intact membrane layer and lacking of vacuolation.

The photomicrograph in Figure (4) demonstrates the severe and detrimental effects of the advancing infection resulted due to the viral dispersion through the mid-gut epithelial cells of *Z. pyrina*. Figure (4a) represents the complete invasion of the IBVs to the epithelial cells of the mid-gut and Figure (4b) verified the destruction of the basement membrane layer with the bursting of the included epithelial cells with its cellular contents.

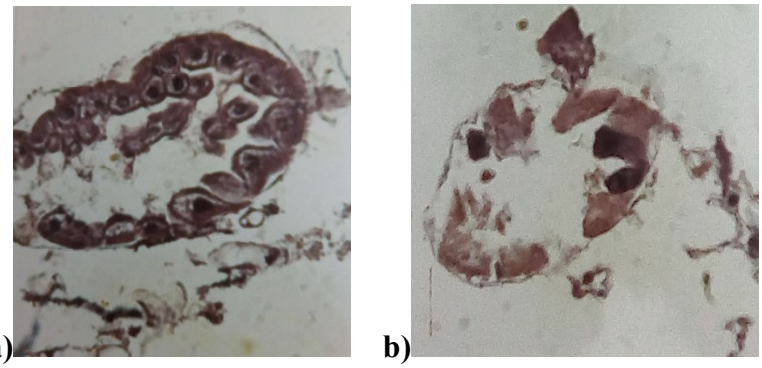

Figure (4): A photomicrograph of a longitudinal section of the mid-gut region of *Zeuzera pyrina* showing: a) the advanced and complete invasions to the epithelial cells; and b) the nearby detrimental symptoms of the inclusion bodies virus (IBVs) invasions leading to the distortion of the basement membrane and the epithelial cells.

The forementioned findings confirmed the susceptibility of this cossid borer to the infection by the viral attacks through its specific NPV *i.e.* ZPNPV. Similar findings were found by Hostetter *et al.* (1990) about the infectivity of a nuclear polyhedrosis virus of the yellow striped army worm (Lepidoptera: Noctuidae) encouraging the wider usage of the viral diseases infections to control such lepidopterous borers.

4. Electron microscopy study:

Figure (5) demonstrates the infection with the IBVs of ZPNPV to the mid-gut cells of *Z. pyrina* on the scale of ultrastructural technique using the transmission electron microscopy (TEM).

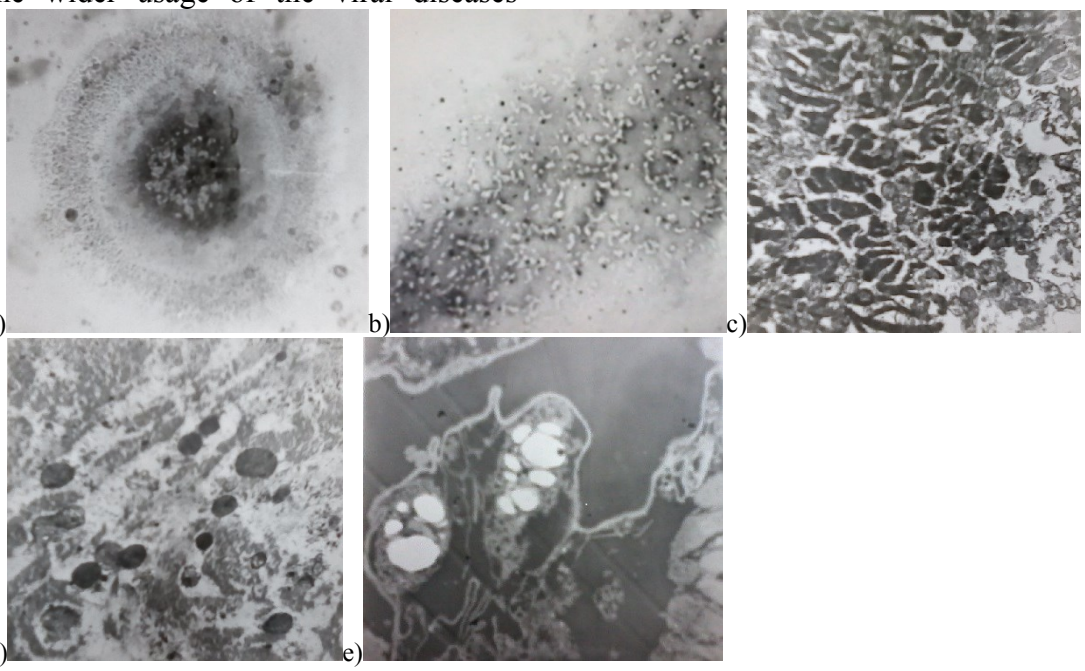

Figure (5): Electron micrograph of a transverse section of the infected mid-gut of *Zeuzera pyrina* with inclusion bodies virus (IBVs) of the *Zeuzera pyrina* nuclear polyhedrosis virus (ZPNPV) showing: a) infection of the mid-gut section with IBVs; b) magnified part of the basement membrane indicating the infection; c) scattering of the inclusion bodies virus (IBVs) invasions through the mid-gut tissues; d) multiplied IBVs within the nuclear matrix; and e) appearing of multiple vacuoles and air sacs; this was at magnifying power (x10^3): a)6.3, b)12, c)10, d)16, e)25.

This electron micrograph of the TEM in Figure (5) points out the infected mid-gut of *Z. pyrina* with IBVs of the ZPNPV showing different symptoms resulting from these viral infections to the mid-gut region and facilitates understanding the infection process of the NPV through the tissues of this lepidopterous borer. The TEM examinations revealed the presence of a

ZPNPV virus which is specific for *Z. pyrina* invading the nuclei of the epithelial cells of the mid-gut region. These observations are concordant with those of Mclntosh and Ignoffo (1986) who studied the impacts of the viruses on the cell structure. Thus, it is observed that both histopathological and electron microscopy studies confirmed the infection of a NPV to this leopard moth borer named ZPNPV.

Subsequently, it is concluded that the leopard moth borer revealed a definite susceptibility to the infection with ZPNPV and it is highly provoked to be involved in the integrated pest management programs against this dangerous boring moth, *Z. pyrina*.

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