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Histopathological studies of mulberry leaf webber (*Diaphania pulverulentalis*) (Hampson) larva infected with nuclear polyhedrosis virus

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Abstract
Leaf webber, *Diaphania pulverulentalis* (Hampson) (Lepidoptera : Pyralidae) is a key pest of mulberry. Use of chemicals to control the pests, developed resistance and destroyed beneficial insects. Nuclear Polyhedrosis Virus plays an vital role in management of leaf webber due to its restricted host specificity. In the present study, histopathology of NPV infected leaf webber larvae was carried out to know the mechanism underlying behind the extent of infection by Nuclear Polyhedrosis Virus. The feral dead larvae collected from the mulberry field, were examined under the microscope for the presence of virions and the polyhedral bodies were isolated, purified and maintained under lab conditions. The production of virus was performed in healthy third instar larvae of *D. Pulverulentalis* through leaf contamination method and histopathology was done on the NPV infected fifth instar larvae. The results showed that the virions were scattered throughout the granular virogenic stroma and accumulated peripherally with the advancement of infection. The midgut region of uninfected larvae of *D. pulverulentalis* did not show any evidence of damage or disruption of cells. Hence, it is proved that *Dp*NPV could be an excellent potential and safe biopesticide for the management of leaf webber in mulberry eco system.

Keywords: Leaf webber, *Diaphania pulverulentalis*, nuclear polyhedrosis virus, histopathology, biopesticide

1. Introduction
Leaf webber, *Diaphania pulverulentalis* (Hampson) (Lepidoptera : Pyralidae) is a devastating pest of mulberry plantations and it was first reported in Karnataka during the 1995 [1], and spread to Tamil Nadu and Andhra Pradesh on local, M5, MR2, S36 and V1 mulberry varieties [2]. The incidence of *D. pulverulentalis* was found to be 22 per cent during June and increased to 85 and 100 per cent during September and December respectively. It decreased slowly to 50, 25 and 14 per cent during the months of January, February and March, respectively [3, 4]. Rajadurai *et al.* [5] reported that the pest caused the leaf yield loss of 12.8 per cent with an average incidence of 21.77 per cent [3, 6, 7, 8].

Leaf webber, *D. pulverulentalis* was reported to serve as a secondary host, harboring pathogens which entered silkworm through contaminated mulberry leaf and causes diseases in silkworm affecting the cocoon crop production [9, 10]. The natural population of leaf-roller was found to be infected by fungus, *Beauveria bassiana* (Vuill.) (12%), microsporidians (50%) and Nuclear Polyhedrosis Virus (38%) [11]. The *Dp*NPV larvae inoculated with microsporidians and *B. bassiana* caused 100 per cent mortality and NPV isolated from leaf roller when inoculated to silkworm did not cause any mortality due to its high restricted host specificity [9, 11, 12, 13, 14].

The progress in the use of insect pathogens especially baculoviruses, is one of the components in control of crop pests. NPV caused natural epizootic disease in pest populations and have an excellent potential as biological pesticides (Dent and Jenkins, 2000; Moscardi, 1999) [1, 12]. There is a possibility for development of resistant insect populations with chemical pesticides but baculoviruses does not produce any residual problems. It will not infect non arthropod host and other beneficial insects. It replicates only in host insects leading to biological magnification and virus production can be done with low energy input technology.

To study the underlying mechanism behind the extent of infection of virus, it is necessary to study the histopathology of Nuclear Polyhedrosis Virus of leaf webber. Hence, the present study documented the histopathology of leaf webber, *D. Pulverulentalis* larvae infected with NPV.

2. Materials and Methods

2.1 Collection of feral colonies

Nursery and newly established gardens were examined sequentially for the presence of dead leaf webber. The presence of faecal pellets and webbing of apical portion of mulberry plants were the typical visual symptoms which made the collection easier. The collected dead larvae were transferred to sterilized vials, isolated and maintained under laboratory conditions.

2.2 Establishment and mass culturing of *Dp* larvae

A stable culture of *D. pulverulentalis* was established from the field collected larvae and utilized for the present study. The larvae were reared on mulberry leaves throughout the larval period. Healthy larvae were selected after careful screening of pathogens [17].

2.3 Multiplication of *Dp*NPV

*Dp*NPV was purified from feral colony and maintained in the Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore. The virus was propagated *in-vivo* by leaf surface contamination method. The production of virus was performed in healthy third instar (6^{th} day) larvae of *D. pulverulentalis.* Larvae were starved overnight before inoculation with virus. The whole mulberry leaf was sterilized with one per cent $NaCl_2$, washed with distilled water for three times and dried before being inoculated with occlusion bodies. Larvae that consumed the whole leaf contaminated with virus were transferred to individual container and supplied with additional mulberry leaves daily [18]. The freshness of the mulberry leaves was maintained by keeping the moistened, sterilized cotton cloth under the containers. NPV infected larvae were stored at -20^{0}C for histology study.

2.4 Histopathology study

Procedure included sequential steps *viz.*, fixation, dehydration, clearing, impregnation, embedding, staining and sectioning of the larvae [19, 20] are as follows:

2.4.1 Fixation

*Dp*NPV infected larvae were fixed in 10 per cent formal saline (40 % formalin 10 ml, sodium chloride 0.9 g and tap water 90 ml). A couple of hours after fixing in formal saline, the larvae were cut into three equal pieces in the region of thorax and abdomen to ensure proper fixing of the tissues in the fixative. The larvae were then left in the formal saline for 24 hours. After fixation, they were subjected to washing in running water for one hour. Healthy larvae of same age were collected simultaneously and treated as above.

2.4.2 Dehydration

The larvae from the running water were transferred to different concentrations of propanol *i.e.,* 30, 50, 70, 90 and then in 100 per cent. The larvae were kept for two hours in each of the concentrations except in 100 per cent propanol, where they were subjected for dehydration only for an hour. At 100 per cent propanol, two changes of an hour duration were given to ensure that larval tissues contained only the dehydrant.

2.4.3 Clearing

The larvae were then transferred to xylene, a clearing agent. Before transferring to pure xylene, they were transferred to xylene-propanol solution in the ratio of 1:1 for
15 min and then to pure xylene again for 15 min. The larvae became clear and transparent.

2.4.4 Impregnation and Embedding

Impregnation of the larval tissues was done by keeping the larvae in xylene-paraffin at 1:1 ratio for 15 min and then in melted paraffin in oven for about an hour. Embedding of the larval tissues were carried out with the melted paraffin (melting point 56-58^{0}C) in glass beaker kept in oven at 70^{0}C temperature. The paraffin blocks were made using aluminium L angles with the orientation of the larvae perpendicular to the plane.

2.4.5 Sectioning

Paraffin blocks were trimmed and fixed on to wooden blocks with the help of scalpel and spirit lamp. The blocks were placed in water for cooling. Thin sections of 5 µ thickness were made with the help of microtome. The sections were fixed on the micro slides using 0.4 per cent gelatin solution and spread gradually on hot plate at 45^{0}C. Excess gelatin solution was drained off and the slides were kept overnight for drying before proceeding for staining.

2.4.6 Staining

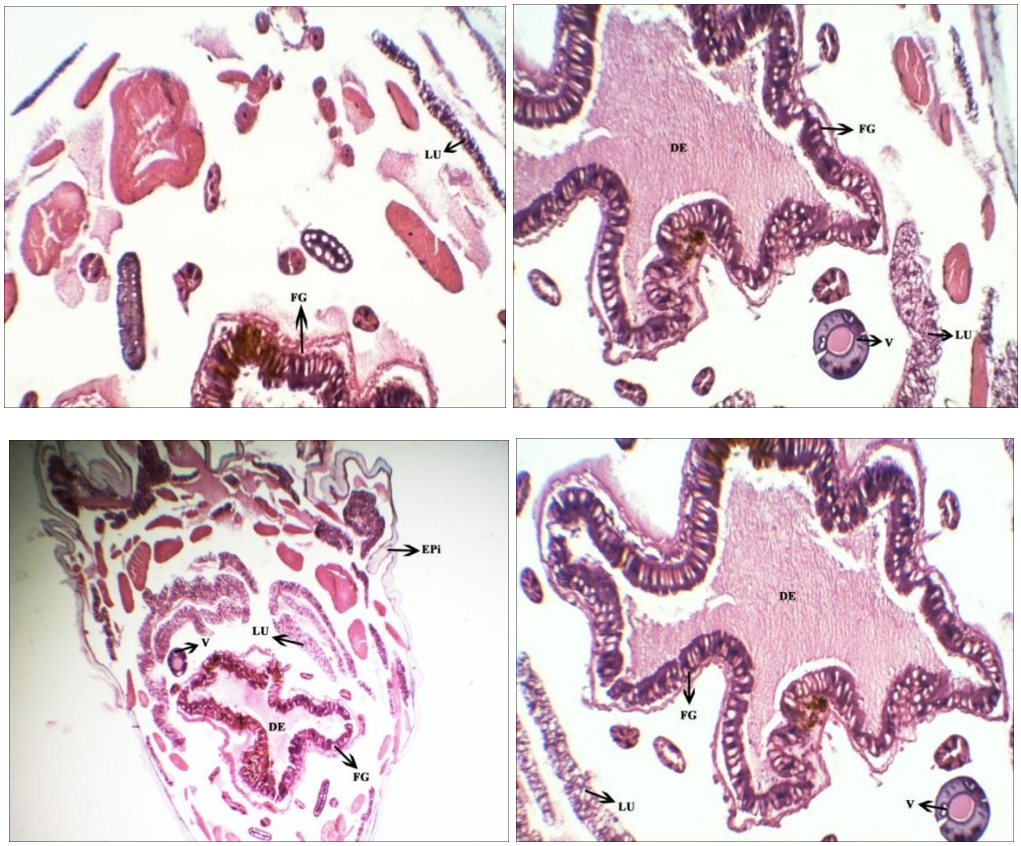
The slides along with sections were processed in a series of chemicals taken in coupling jars as follows: xylene- 1, xylene-2 (5 min. each), absolute alcohol-1, absolute alcohol-2, 90 per cent alcohol, 80 per cent alcohol, 70 per cent alcohol (2 min each).

The slides were then washed with running water for 5 min and then dipped in haematoxylin solution for 20 min. After washing the slides in running water for one min, they were just dipped in acid alcohol for 30 sec and the slides were washed in running water again for 5 min to remove the acid alcohol from the sections. The sections were counterstained in eosin for 30 sec and dipped in water for 10 sec to one min. The slides were then processed in a series of alcohol concentrations of 80 per cent, 90 per cent and absolute alcohol for 30 sec each. The slides were then dried in air. The dried sections were sealed after giving two changes in xylene, with DPX mounting media and observed under phase contrast microscope.

3. Results and Discussion

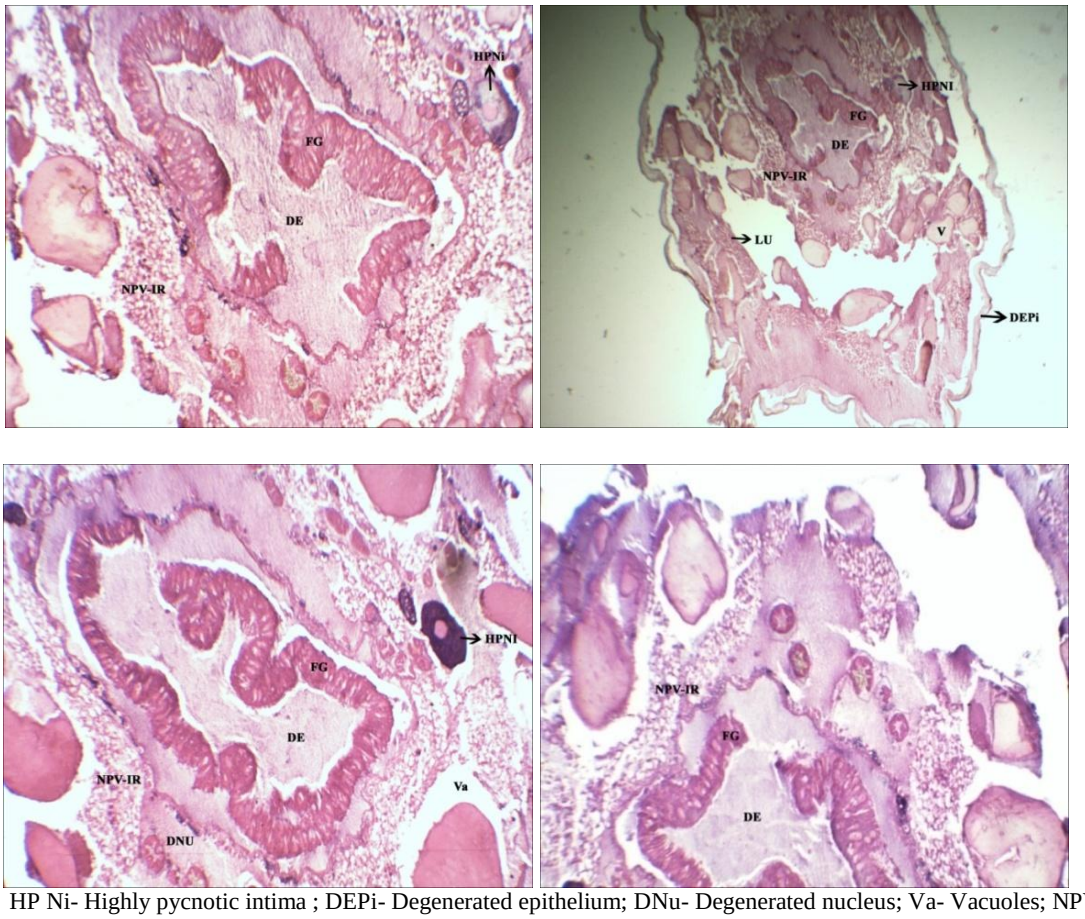
The mid gut cross sections of NPV infected and uninfected larvae of *Diaphania pulverulentalis* were diagnosed for the presence of polyhedral bodies in various parts of midgut regions.

In the early stage of infection, virions were scattered throughout the granular virogenic stroma and accumulated peripherally with the advancement of infection. Virogenic stroma were also present in body cells and get hypertrophied, filled with large numbers of polyhedral bodies. The midgut region parts *viz.,* epithelial layer, peritrophic membrane, fat bodies, gut lumen, food granules and vesicles of uninfected larvae of *D. pulverulentalis* did not show any evidence of damage or disruption of cells (Fig. 1). Whereas, cross section of mid gut tissues of the *Dp*NPV infected 5^{th} instar larvae of *D. pulverulentalis* showed irregular shaped epithelial layer, pertitrophic membrane, fat bodies, gut lumen, food granules and vesicles. Nuclei of infected larvae examined under electron microscopy showed advanced stage of infection and virogenic stroma was filled most of the nucleus (Fig. 2). Larvae showing external symptoms of NPV infection also showed inclusion bodies in the tissues such as hypodermis, fat body and tracheal matrix cells. It was possible to observe the outline of intra nuclear virus occlusion bodies throughout the cell membrane.



LU- Lumen; Epi- Epithelium; DE- Digestive enzyme; FG- Food granules; V- Vesicles

Fig 1: Cross section of midgut cells of *D. pulverulentalis* larva uninfected with *Dp*NPV LU- Lumen; Epi- Epithelium; DE- Digestive enzyme; FG- Food granules; V- Vesicles



HP Ni- Highly pycnotic intima ; DEPi- Degenerated epithelium; DNu- Degenerated nucleus; Va- Vacuoles; NPV-IR- NPV infected region; Lu- Lumen; DE- Digestive enzyme; FG- Food granules

Fig 2: Cross section of midgut cells of *D. pulverulentalis* infected with *Dp*NPV HP Ni- Highly pycnotic intima ; DEPi- Degenerated epithelium; DNu- Degenerated nucleus; Va- Vacuoles; NPV-IR- NPV infected region; Lu- Lumen; DE- Digestive enzyme; FG- Food granules

The present results are in agreement with Gothama *et al*. [21] and Laoh *et al*. [22] observed that the organs of young larvae were weak, especially the mid gut, the primary target of pathogen and NPV could be more easily penetrated and caused damage to mid gut cells. Advancement of infection at 72 and 96 hours post infection, peritrophic membranes became less and less intact. Thus, the longer the contact time between NPV virions with host cells, the level of damage was higher. The damage that occured in advanced stage caused the ability of epithelial in shaping peritrophic membrane was distorted [23].

In the present study, midgut epithelial tissues of *D. pulverulentalis* larvae were refractive to *Dp*NPV infection. Utari [24], reported that the integrity of the peritrophic membrane was decreased with increased doses of NPV infection in *Helicoverpa armigera* larvae. Similarly, Vinod and Singh [25] made histology on NPV infected *Spodoptera litura* and observed the integrity of peritrophic membrane decreased with increased dose of infection. It was noticed that there was no such disruption in uninfected larvae in the present study. Vail *et al.* [26] recorded similar infection of the midgut columnar epithelium by *Ac*NPV. The present observations are in conformity with the findings of Smith Johanssen *et al.* [27].

Kikhno [28] made histopathology studies in *Sl*NPV infected insects and observed typical hypertrophy in cell nuclei of the fat body, hypodermis, tracheal matrix and haemocytes. Priyadharshini *et al.* [29] revealed that the proteinic viral inclusions were observed in the nuclei of any other tissues, regardless of the severity of the infection in the midgut epithelium. The cells of the midgut epithelium of larvae infected with *Dp*NPV showed irregularly shaped hypertrophied nuclei filled with large numbers of polyhedral bodies compared to normal size. Yayan *et al*. [36] reported that the damage of histological structure caused by *Sl*NPV (0, 315, 390, 465, 540 and 615 PIBs/ml) after

0, 12, 24, 72 and 96 hours of post infection. Results showed that longer exposure of virion in the midgut lumen caused more damage on peritrophic membrane 96 hrs post infection and also damaged epithelial cells, brush border, nuclei and basement membrane [30].

Histopathology studies of *Dp*NPV determined the extent of infection of viruses in various tissues of the insect. The mechanisms and pathology of infection by *Dp*NPV was also well documented in this study. From the present study, it is clear that the virions of *Dp*NPV destroyed midgut tissues of the leaf webber thereby causing natural death of larvae which in turn keeping pest population low and reducing leaf yield loss.

4. Conclusion

Histopathological studies of *Dp*NPV infected larvae showed the greater infection of NPV in midgut, characterized by hypertrophy of nuclei filled with virions caused the death of leaf webber larvae. Due its restricted host specificity and non toxicity to beneficial insects, *Dp*NPV could be an excellent potential and safe biopesticide for the management of leaf webber in mulberry eco system.

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