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Nucleoplyhedrovirus (NPV) Infecting the Cabbage Small Butterfly, *Pieris rapae* L. (Lepidoptera: Pieridae)

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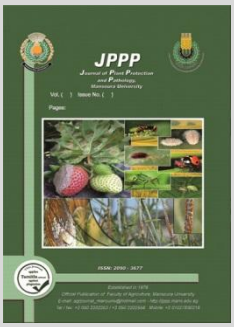

ABSTRACT

Dead larvae of *Pieris rapae* L., showing symptoms of nucleoplyhedrovirus (NPV), were collected from cabbage and cauliflower fields. Microscopical examination of corpus body fluid exhibited polyhedral occlusion bodies (POBs). Larvae of the 4th instar (L4) were infected with the virus suspension at the concentrations of $4x10^4$, $4x10^6$, $4x10^8$, and $4x10^{10}$ POBs/ml. Bioassay tests revealed that egg incubation period of P. rapae, reared on a semi-synthetic diet, was 4.29±0.24 days, total larval period was 16 ± 1.67 days and pupal period was 5 ± 0.6 days). Seven days after treatment of larvae with POBs, larval mortality of L2 increased by increase of POBs concentration to 30, 42, 58 and 62% and reached 60, 70, 82 and 100% on the 8th day for the successive respective POBs concentrations. Treating larvae of L4 with the same respective concentrations, showed death among treated larvae one day earlier than in case of L2. Mortality values of treated L4 recorded 60, 66, 72 and 84% on the 7th day after treatment. These values increased to 84 and 92% for the concentrations $4x10^4$, $4x10^6$ and 100% for the concentrations $4x10^8$, and $4x10^{10}$ POBs/ml, on the 8th day, respectively.

Keywords: *Pieris rapae*, NPV, bioassay, histopathology

INTRODUCTION

The cabbage small butterfly, *Pieris rapae* (L.) (Lepidoptera: Pieridae) is a worldwide pest causes extensive damage to all crops of the family Cruciferae (Allawy and Payne, 1984; Jögar *et al.*, 2003*;* Saleh *et al.*, 2023), especially to cabbage and cauliflower (Moiseeva, 1984; Stewart and Sears, 1988; Shah and Rafi, 2016). Due to its extremely high relative growth rate, it became a global pest with highly abundance and migratory nature (Wei *et al.*,1983; Konno, 2023, Kour *et al.*, 2023). It was accidentally introduced to China in 1989, North America in 1860, Australia in 1937 and New Zealand in 1930 (Yin *et al.*, 1989). Controlling larvae of this insect pest in the field is mostly done by applying chemical insecticides (Dempster,1967; Theunissen, 1984; Arya and Dey, 2007; Khan *et al.* 2017), which was not always successful and posed environmental and health hazard problems (Khan *et al.*, 2017). Thus, the need to an alternative and ecologically safe pest management tactics become urgent. The entomopathogenic viruses (EPVs) are considered to be an alternative agent group for controlling many lepidopteran insect pests (Lacey 2012). Microbial control of insect pests with nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) is a favorable alternative to chemical pesticides, because they are naturally occurring in the agroecosystems and sometimes outbreaking causing epidemics sharing in regulation of insect populations (Moore, 1972; Harper, 1987). They are also specific having a very narrow host range and, accordingly are safe to non-targeted organisms (Griiner, 1990). Granulosis virus (GV) was early reported by Kitazima (1938) in Japan and is common as natural infection in *P. rapae* larvae under field conditions (Akutsu K, 1967; Kour *et al.*, 2023) and used for managing populations of this pest (Akutsu 1967, Su 1986, 1989, 1991, Saito and Goto, 1992; Kour *et al.* 2023). Also, nucleoplyhedrovirus was reported to infect *P. rapae* and applied for its control (Battu, 1995; Shah and Rafi 2016). In the present study, some of the field collected larvae of *P. rapae* died showing symptoms of NPV infection. The virus was isolated, *in vivo* propagated and bioassyed versus 2nd and 4th instar larvae of the pest. Also, a histopathological study was carried out.

MATERIALS AND METHODS

Rearing *P. rapae*: Wild adult butterflies of *P. rapae* were collected from cabbage and cauliflower fields in Giza region by an insect net and transferred to a large sunny out-door wooden cage with wire mesh sites (2x2x2 m), provided with 10% honey solution in a soaked cotton wool piece placed in a small container hanging in middle of the cage. Sun is necessary for enhancing mating of the encaged butterflies (Watanabe and Ando, 1994). Potted radish plants served as an oviposition site for the butterfly females in the cage and were inspected daily for eggs laid. Eggs were collected using a wetted fine camel hair brush, placed onto a wetted filter paper furnished in a Petri-dish and transported to the laboratory at room temperature (25 °C ± 2) till hatching. The newly hatched larvae were transferred to small diet plates (10x15 cm) and reared gregariously till reaching the 4th instar, then they were transferred individually using highly soft forceps to square plastic units/cell (3x3 cm) inserted in large diet plates (30x35 cm), covered with a sheet of filter paper placed under perforated plastic cover fitted in place with rubber bands. Diet was previously poured in all the rearing plates in about 1.5 cm thick layer. Each large plate contains 100 square units. The semisynthetic diet for rearing *P. rapae* was prepared as described by Webb and Shelton

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(1988). The diet amount in each cell is enough for feeding the encaged larval stage till pupation occurs. The rearing system is somewhat similar to that of Troetschler *et al.* (1983) and Emilie *et al.* (2023) with some local modifications. The pupae were collected from the rearing trays and placed in hard paper box seated on holder surrounded by water to prevent ants from reaching the pupae in the out-door cage till adult emergence, that occurred after about 5-6 days. Eggs of F1 were similarly collected, and the newly hatched larvae were further reared on the same diet as previously mentioned. Larvae of the 2nd and 4th instars were used for bioassay of the NPV and the latter instar for the histopathological study.

NPV Propagation, purification and Bioassay: Field collected dead larvae of *P. rapae* showing typical symptoms of NPV infection were macerated in distilled water and filtered through a piece of two layers' muslin cloth to obtain a crude suspension of their body content free from large body fragments. Microscopical examination of a drop from the crude suspension smeared on glass slide, air dried and stained with 10% Giemsa's solution for 10 minutes, then washed with running water for 10 seconds (Wigley, 1976; Khattab, 2013) showed viral polyhedral occlusion bodies (POBs) typical for NPV. The crude was sprayed to surface of the diet in the rearing trays and 4th instar larvae were placed individually in the square cells for feeding on the contaminated diet. One week later, death occurred in larvae that reached the 5th instar.

Dead larvae were carefully collected before skin bursting and kept freeze till needed for purification. Using a sterilized glass mortar, the dead larvae were well macerated in distilled water and filtered as described above. The crude suspension was centrifugated at 4000 rpm for 20 minutes, and the pellet (POBs) was suspended in 5ml distilled water as stock suspension. A Neubauer hemocytometer was used for counting the POBs in the stock suspension that kept at 4°C till used in the bioassay test and histopathological examination. A solution with high POBs concentration was initially prepared, followed by serially diluted concentrations to obtain the required lower concentrations for the bioassay test. The number of POBs was calculated by using the formula:

Number of POBs per ml = D X X/N X K

Where,

D = dilution factor, X = total number of squares counted, N = Number of squares counted, K = Volume of above one small square in cm3

For the bioassay test, 4 concentrations of POBs were prepared from the stock suspension in distilled water, *i.e.,* $4x10^{4}$, $4x10^{6}$, $4x10^{8}$, and $4x10^{10}$ POBs/ml. A thin diet layer (1 mm thick) in four rearing trays was sprayed each with one of the tested POBs concentrations and left for one hour to enable the sprayed material to be absorbed in the diet. Another 4 similar diet plates were sprayed with distilled water and served as control. Thereafter, the motionless full-grown larvae of the 1st instar (L_1) with swollen neck area showing clearly visible coronal suture on the head, attached to the filter paper cover of the rearing units waiting for molting to 2nd instar (L_2), were transferred individually using fine wetted camel brush onto a small filter paper disc placed on diet in the cells in both plates of the treated diet and control. The filter paper disc helps the molting larva to fix her body with the prolegs hooks and molt successfully minimizing mortality. Newly molted larvae (L_2) were left feeding on the treated and control diet for 48 hours then supplied with non-treated diet. Mortality among larvae of the test was recorded from the 4th to the 8th day post treatment. The same procedure was carried out for bioassay with 4th instar larvae (L_4), which were collected as motionless full-grown 3rd instar larvae (L_3) showing the same pre-molting symptoms as in case of the 1st instar larvae. Thus, the newly molted individuals are obtained in the exactly required tested larval instars (L_2 and L_4) for the bioassay test. The test was carried out at room temperature of 25 ± 2°C.

The recorded mortality values were processed through the "LdpLine" program (Bakr, 2005) for calculating the 90% lethal (LD_{90}) and the median lethal concentration (LC_{50}) and the 90% lethal and median lethal time (LT_{50}) values which were used for evaluating the efficacy of the present studied NPV against larvae of *P. rapae.*

Histopathological study: Using the concentrated suspension extracted from dead treated larvae, the diet in a small tray was sprayed with, and 20 4th instar larvae of *P. rapae* were allowed to feed on. Following the procedure described by El Husseini (1976), six days post treatment, the larvae were pinched several times with fine pin on the abdominal region to facilitate fixation in alcohol Buin's solution for 48 hours. Then washed in 70% ethyl alcohol for 1 day, followed by dehydration through an alcohol series (90 and 100%) each twice for 12 hours, then placed in xylene for 24 hours to be ready for infiltration with paraffin wax through a serial of 1: 3, 1:2, 1:1 wax: xylene at 40°C, then transferred into pure paraffin wax 2 times each for 24 hours. The larvae were individually embedded in wax blocks for cutting with rotary microtome in serial sections of 6-7 microns. The sections were floated on water on glass slides previously smeared with very thin layer of egg albumin and glycerin (1: 1) as adhesive for the catted tissues, and placed in oven at 30°C for 24 hours. Thereafter, slides carrying the larval sections were processed for single staining with hematoxylin, and double staining with eosin-hematoxylin, mounted in Canada Balsam and dried in oven at 35°C, then examined with light microscope, and selected photos were shot by a Zeis camera mounted on the microscope.

RESULTS AND DISCUSSION

As in the present study, the naturally dead young larvae of *P. rapae* in the field due to infection with a nucleoplyhedrovirus was also mentioned by Dempster (1967). According to Harper (1987) and Fuxa and Tanada (1987), presence of such dead larvae seasonally on the long term, could be expected to launch an epizootic with NPV among populations of *P. rapae* under certain natural conditions.

Reared F1 of *P. rapae* on the semisynthetic diet showed that egg incubation period lasted 4-5 days, with a mean of 4.29±0.24. The total larval duration reached 16 ± 1.67 days. Pupal period recorded 5-6 ± 0.6 days. Adult emergence was high reaching 94%. These values are nearly in agreement with those of Emilie and Kobiela (2023). It is worth to mention, that our procedure for handling and selecting the exact desired larval instars minimized the larval mortality in both treatments and control to the zero level along the 8 days of the bioassay test. Accordingly, both Abbott's formula (1925) for correcting the mortality and

Finney's Probit Analysis (1964) were not needed for the statistical analysis of the bioassay data. Instead, data was processed using the "LdpLine" software (Bakr, 2005) for calculating the values of lethal concentrations (LCs), and lethal times (LTs).

Bioassay results of the isolated NPV from naturally dead larvae of *P. rapae* versus larvae of L_2 and presented on Table (1), showed no larval mortality during the first 6 days' post treatment as well as in the control. On the 7th day, mortality values of 30, 42, 58 and 62% were recorded for the treatment with the concentrations of $4x10^4$, $4x10^6$, $4x10^8$, and $4x10^{10}$ POBs/ml, respectively.

Table 1. Mortality % among larvae (L_2) of *Pieris rapae* fed on diet contaminated with different NPV concentrations

Treatment	Days after treatment				
	4th	5th	6th	7th	8th
$4x10^4$ POBs	0	0	0	30	60
$4x10^6$ POBs	0	0	0	42	70
$4x10^8$ POBs	0	0	0	58	82
$4x10^{10}$ POBs	0	0	0	62	100
Control	0	0	0	0	0

These mortality values were much less than those mentioned by Arya and Dey (2007) using chemical insecticides (100%); but nearly similar to those recorded by Akutsu (1967) for controlling larvae of *P. rapae* using nucleoplyhedrovirus. Furthermore, these values increased on the 8th day reaching 60, 70, 82 and 100% mortality for the same respective POBs concentrations. Meanwhile, mortality among the control larvae remained by zero%. Accordingly, mortality correction following Abbot's formula (1925) was not needed. The procedure previously described for handling and selecting the targeted instar larvae in the present and other studies proved highly effective in minimizing mortality of the tested larvae (El Husseini, 2020, El Husseini *et al.*, 2023).

Treating the 4th instar larval of *P. rapae* with different concentration of POBs resulted one day earlier larval mortality than in case of L2 starting on the 6th day post treatment (Table 2). It recorded 12, 22, 28 and 36% mortality for the same respective POBs concentrations. On the 7th day, these values increased to 60, 66, 72 and 84%, respectively. The 100% mortality was recoded on the 8th day post treatment for the highest two tested concentrations of $4x10^8$, and $4x10^{10}$ POBs/ml. Meanwhile, it was 60 and 70% for the lower tested concentrations of $4x10^4$, $4x10^6$ POBs/ml.=, respectively. Khan *et al.* (2017) recorded acceptable control level against the cabbage butterfly, *P. brassicae* using both NPV and chemical insecticides. High control results with *P. rapae* NPV were recorded by Akutsu (1967) as in the present study.

Table 2. Mortality % among larvae (L_4) of *Pieris rapae* fed on diet contaminated with different NPV concentrations

Treatment	Days after treatment				
	4th	5th	6th	7th	8th
$4x10^4$ POBs	0	0	12	60	84
$4x10^6$ POBs	0	0	22	66	92
$4x10^8$ POBs	0	0	28	72	100
$4x10^{10}$ POBs	0	0	36	84	100
Control	0	0	0	0	0

The calculated values of LC_{50} and LC_{90} for L_2 reached $9.3x10^6$ and $6.9x10^{10}$, respectively as seen in Table (3). Meanwhile, these values were $6.5x10^2$ and $6.6x10^{10}$ for L_4, respectively.

Table 3. Efficacy of *Pieris rapae* NPV against larvae of L_2 and L_4 calculated on 7th day post-treatment using the "LdpLine" software

Larval instar	POBs/ml	Log.	Treated	Observed response%	Linear response%	Linear probit	LC	
L_2	$4x10^4$	4.6021	100	30.000	31.3230	4.5132	LC_{50}	LC_{90}
	$4x10^6$	6.6021	100	42.000	42.1580	4.8012		
	$4x10^8$	8.6012	100	58.000	53.6274	5.0911		
	$4x10^{10}$	10.6021	100	62.000	64.8007	5.3800		
Upper limit	$9.7x10^7$							
Lower limit	$1.1x10^6$							
Slope	0.1445±0.0289						$9.3x10^6$	$6.9x10^{10}$
L_4	$4x10^4$			60.000	58.2316	5.2078		
	$4x10^6$			66.000	67.0284	5.4407		
	$4x10^8$			72.000	74.9695	5.6736		
	$4x10^{10}$			81.000	81.7605	5.9064		
Upper limit	$4x10^4$							
Lower limit	64x10							
Slope	0.1164 ±0.0303						$6.5x10^2$	$6.6x10^{10}$

Histopathological study

The tested *P. rapae* NPV (*Pr*MNPV) infected nuclei of all ectodermal tissue epithelial cells (body wall, trachea, salivary glands, for- and hindgut), and mesodermal epithelial cells (fat body, Malpighian tubules, nerve system) of the diseased larvae. The infection started with changes in mid-gut peritrophic membrane (PM) during invading the epithelial cells (EC). Derksen and Granados (1988) reported that such alteration of a lepidopteran peritrophic membrane by baculoviruses are caused by a factor present in the polyhedral occlusion bodies POBs) of baculoviruses inducing specific biochemical and structural changes in the PM. Moreover, this factor enhances viral infectivity. The budded virus replicated first in mid-gut epithelial cells (EC) and is liberated in the hemocytic flued filling the body cavity invading ECs of all other body tissues to replicate in their cell nuclei. Fig. (1 a-c) shows the POBs in the attacked hypodermal cells under the cuticle of the larval body. The virus replicated and formed its POBs also in the tracheal epithelial cells surrounding the trachea (Fig 2 a and b). After lysis of the tracheal ECs, the spiral cuticle of the trachea can be clearly seen floating in the body fluid filled with POBs (Fig. 2 c). nuclei of for- and hindgut were infected and lysed leaving only the chitinous intima as seen on Fig. (3). Nuclei of the fat bodies were also attacked by the virus leaving its POBs inside (Fig. 4 a and b). Cells of the cortex

layer in the ganglia of nerve system showed the POBs of the virus in their nuclei (Fig. 5a and b). Also, nuclei of the Malpighian tubules were found infected with the NPV leaving its POBs inside. As well, nuclei of ECs of the salivary gland showed POBs inside. Thus, the present *P. rapae* NPV infect all ecto- and mesodermal organ tissues like other NPVs, invading and replicating in their nuclei causing lysis of all cells and death of the host larvae showing the typical symptoms of NPV infection described by many authors on different lepidopteran species (Abo Ela *et al.*, 1995 on semi-loopers*;* Cunningham & Entwistle,1981 on the sawflies; Cunningham, 1982 on forest pests; Abou Bakr *et al.*, 1985 on *Heliothis armigera*; El Husseini *et al.*, 2012 on *Spodoptera littoralis* and 2020 on *Galleria mellonella*).

Fig. 1. Cross section in infected larvae showing POBs of *P. rapae* NPV in epidermal cells (ECs) of the body wall, (a) cells still intact with the cuticle; (b) cells detached from the cuticle; (c) total lysis of cells releasing POBs in the haemocoel.

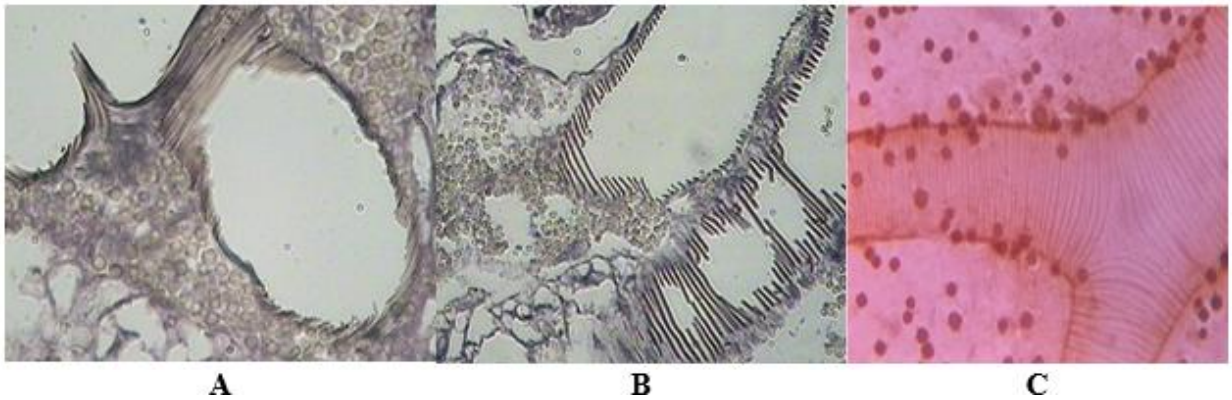


Fig. 2. Cross (a) and longitudinal (b) sections POBs of Pieris rapae NPV in EC of the trachea; (c) the spiral chitinous intima of the trachea after lysis of the ECs releasing POBs.

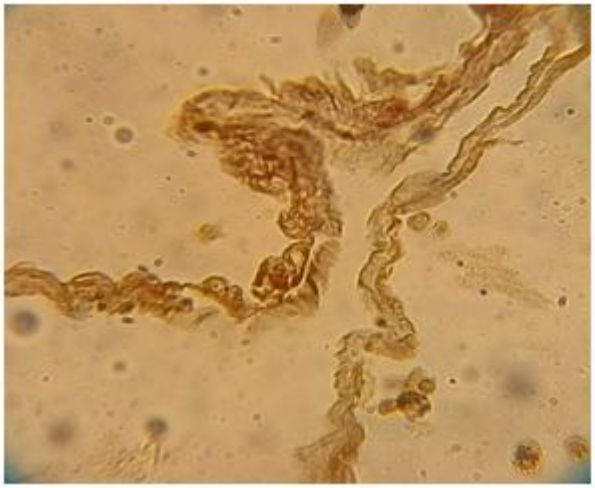
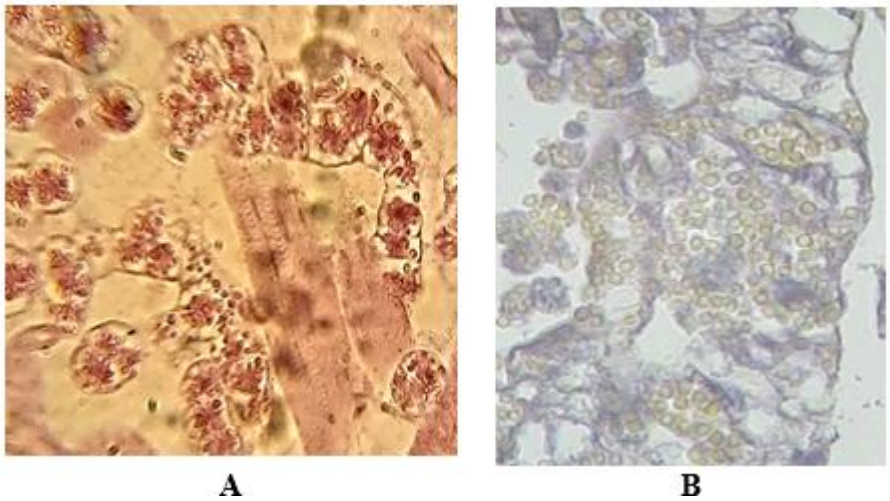
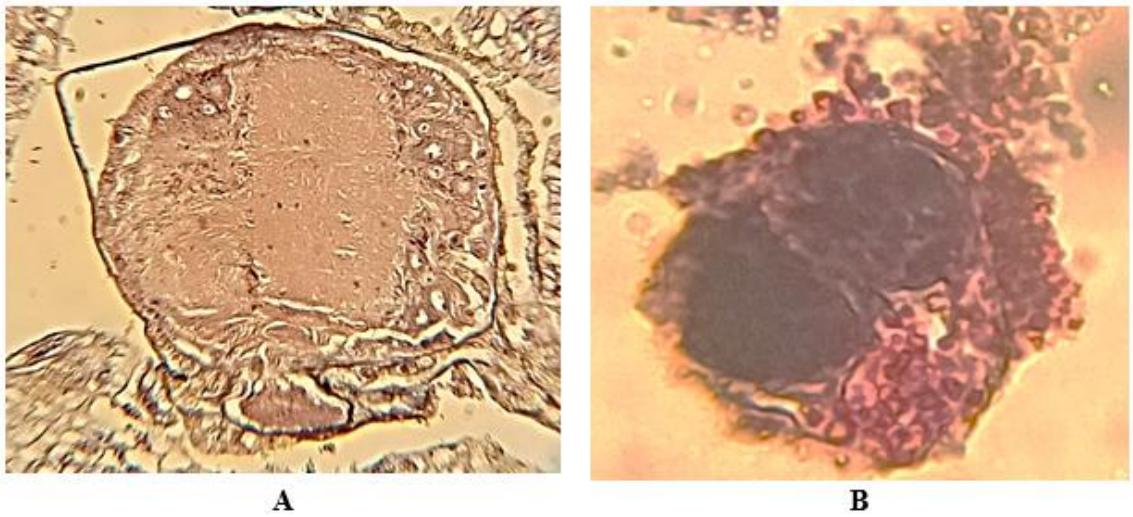


Fig. 3. Longitudinal section in hind gut of NPV infected Pieris rapae larvae showing complete lysis of the ECs leaving only the chitinous intima behind.



A B

Fig. 4. POBs of P. rapae NPV in nuclei of the fat body cells (a & b).



A B

Fig. 5. POBs of P. rapae NPV in nuclei of cortex cells in the ganglia of the nerve system (a & b).

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اصابة فراشة ابى دقيق الكرنب الصغيرة، Pieris rapae L. (Lepidoptera: Pieridae) فيروس (NPV)

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الملخص

جمعت يرقات دودة الكرنب L *Pieris rapae* التى ظهرت عليها أعراض فيروس نوكليوبليهدروفيروس NPV. أظهر الفحص المجهري لسائل جسم الدودة وجود أجسام الفيروس. تم عدوى يرقات العمر الرابع بتركيزات4x108, 4 x106, . أظهرت الاختبارات الحيوية أن فترة حضانة بيض الحشرة المُربّاة على نظام غذائي شبه صناعي كانت 4.29±0.24 يومًا، وفترة اليرقات الكلية 16±1.67 يومًا، وفترة العذراء 5±0.6 يومًا. بعد سبعة أيام من معالجة اليرقات، ارتفع معدل نفوق يرقات العمر الثانى بزيادة تركيز POBs/mlإلى 30 و42 و58 و62%، ووصل إلى 60 و70 و82 و100% في اليوم الثامن لتركيزات POBs/ml المتتالية. أما معاملة يرقات L4 بنفس التركيزات، فقد أظهرت نفوقها قبل يوم واحد من حالة .L2 وسجلت قيم نفوق اليرقات L4 المعاملة 60 و66 و72 و84% في اليوم السابع بعد المعاملة. وارتفعت هذه القيم إلى 84 و92% للتركيزات 4 ,x106 4x108, d 6و100% في اليوم الثامن، على التوالي.