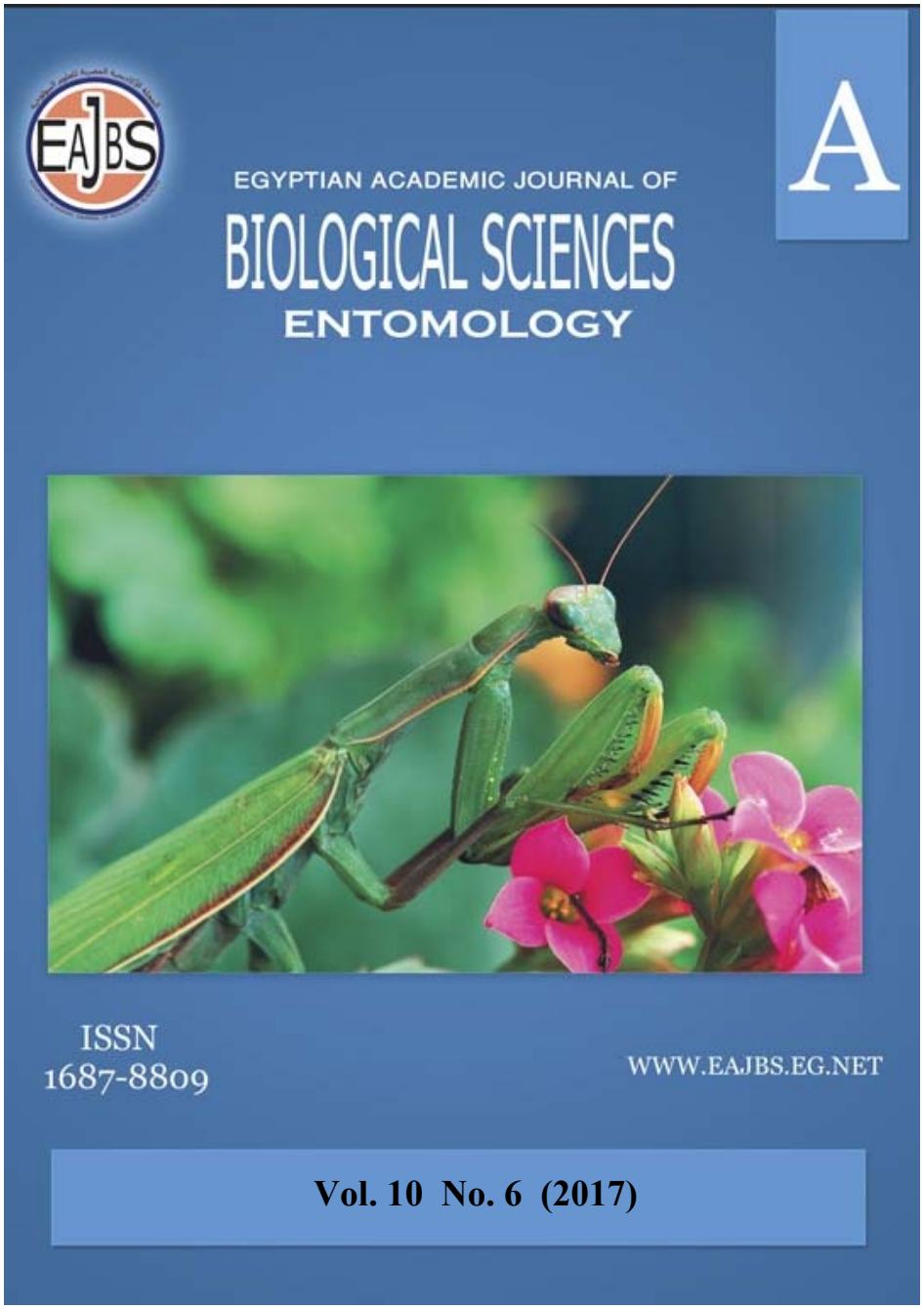

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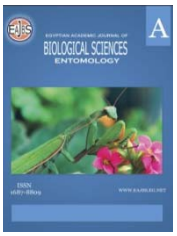

Examination of Temperature Fluctuations on The Viability of Egyptian Isolate of *Helicoverpa armigera* Nuclear Polyhedrosis Virus

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ABSTRACT

Present work aimed to Studies the effect of temperatures on the viability of entomopathogenic *H.armigera NPV* virus under vitro condition. In this study the nucleopolyhedral virus *(NPV*), belongs to baculoviridae was isolated from naturally infected larvae of *H.armigera.* The effect of different temperatures on the pathogenicity of the entomopathogenic virus *HaNPV* to the American bollworm *H. armigera* was examined. The viral pathogenicity in terms of larval mortality increased with the increase of temperature; with optimal temperature of 35^0C for the 2^{nd} and 4^{th} instars larvae but the incubation period was shortened with higher incubation temperatures. However, the pathogenicity significantly decreased with the increase of the exposure period. The virus lost its infectivity after exposer to 50^0 C for ten min. and therefore 50^0C considered as the thermal inactivation point of *HaNPV* virus.

INTRODUCTION

The American bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) is an important destructive pest in Egypt, It attacks most of cultivated crops. The development of pest control measures using microorganisms especially entomopathogens has received increasing attention in recent years (Brar *et al.,* 2004). Since viral pathogens are obligate in nature, they must be necessarily multiplied on their natural live hosts from which they have been collected (Narayana, 2003). Because of serious resistance to chemical insecticides among *H. armigera* larvae, Nucleopolyhedroviruses find extensive use in assisted control programs for early management of pests (Carner and Yearian, 1989; Rabindra *et al.*, 2003; Dolinski and Lacey, 2007). The Entomopathogenic nucleopolyhedral viruses *(NPVs*), belongs to baculoviridae, have been isolated from many insect orders, primarily from the lepidopterans and play a major role in and recognized as one of the most promising control agents for such pests. In this study, *NPV* was isolated from naturally infected larvae of *H.armigera*, the infected larvae are extremely fragile to the touch, rupturing to release fluid filled with infective virus particles. These viruses are insect specific used successfully as ecologically pesticides (biological control) in agriculture. *NPV* infection started when *H.armigera* fed on the infected diet, the OBs dissolve in the alkaline medium of the viral midgut and liberate virons which enter the epithelial cells, replicate in the nuclei thereby damaging the entire cellular of the midgut. A principle disadvantage of using baculovirus in field is their short residual activity due

to their inactivation by the high daily temperatures. Therefore, the present studies were undertaken to determine the effect of temperature fluctuations on viability of *HaNPV* on the 2nd and 4th instars larvae of *H. armigera*.

MATERIALS AND METHODS

Insect rearing:

The American bollworm, *Helicoverpa armigera* used in this study was reared for three generation under highly controlled conditions to avoid any insecticides contamination. The larvae were individually fed artificial diet described by Shorey and Hale, (1965) at 26 ± 1 ◦C and 70% RH, with a 14:10 h photoperiod; Smits, 1987, Smits *et al*., 1986, at plant protection research institute, Dokki, Giza, Egypt.

Isolatation and propagation of virus:

1.Detection of *HaNPV*:

The original *HaNPV* isolate was detected from diseased *H. armigera* larvae which collected from cotton and tomatoes fields of Agriculture Research Center, Qaha, Al-Fayom and El-Salhya during summer season. The larvae showed baculovirus infection symptoms were brought to laboratory and examined to confirm the presence of *HaNPV* by light microscope with Giemsa staining according to Mustafa, *et al*. (2001), in which a thin smear of infected worm tissue was prepared on glass slide and dried in air. The smear was immersed for 1-2 min in Giemsa, rinsed under running tap water for 5-10 sec then the smear was stained for two hours in 10% Giemsa stain (10g of Giemsa dissolved in 100ml distilled water). The dye was rinsed off in running tap water for 5-10 sec then the smear was stained for two hours in 10% Giemsa stain (10g of Giemsa dissolved in 100ml distilled water), the dye was rinsed off in running tap water for 5-10 sec and allowed to dry in air then examined under light microscope to detect the Occlusion Bodies (OBs)**.** After the examination the diseased larvae kept at -20^{o} C until the isolation and purification of OBs (polyhedra).

2.Isolation of *HaNPV*:

The healthy 3rd instars larvae were reared under controlled condition in laboratory were fed on diet contaminated with infected larvae tissue tested. These larvae were maintained under laboratory controlled condition for 7 days and investigated daily for external symptoms discovered. After seven days the larvae were tested with Giemsa stain for confirm their infection.

3.Virus propagation:

The propagation of the virus isolate was performed by inoculation of the 3rd instars larvae of *H.armigera* with *HaNPV* isolate which collected from the field and tested by light microscopy by surface contamination of the artificial diet. The inoculated larvae were observed daily to identify the *NPV* infected ones based on the sign and symptoms of disease. The tissues of dead larvae were examined as soon as possible with the naked eye and tissue smears under light microscopy as mentioned above.

4.Viral occlusion bodies (VOBs) purification:

The method of OBs purification was done as Sudhakar *et al*, 1997. The individually dead larvae showing symptoms of *NPV* were transferred to a micro centrifuge tube and homogenized in 300 µl of distilled water. The homogenates were filtered through a cheese cloth. The filtrate were subjected to sucrose layer (60% wt/vol) and centrifuged for 30min at 10.000 rpm. The band was formed on top of the layer containing OBs was collected and again subjected to sucrose layer (40%

wt/vol) and centrifuged at 10.000 rpm for 30 min. The band at the bottom of the gradient containing the OBs was collected and washed with distilled water. All the above steps were carried out at 4°C. Pure OBs were suspended in distilled water and stored at –20°C. For evaluation of OBs purification method, slide of OBs was stained with Giemsa stain as mentioned above and examined under light microscopy.

Effect of temperature on virus infectivity:

1.Effect of incubation temperature on mortality of virus infected larvae:

Ten second and fourth instars larvae of *H. amigera* were placed in diet surface infected by (50µl) of *HaNPV* (containing $6x10^9$ PIBs) separately and the cup covered and replicated three times. When the diet consumed completely the larvae transferred into another diet cups and incubated at different temperature ranged from 20^0C to 40^0C intervals 5^0C, and also the control treatment. The mortality for each treatment was recorded daily to obtain the highest mortality yield.

2.Virus stability to temperature (Thermal inactivation point):

The virus isolates ($6x10^9$ PIB) were suspended in distilled water (500 µl final volumes) in microtube, and heated for 10 min in water path at temperatures ranged from 20^0C to 60^0C intervals 5^0C and chilled into ice. The heated treated viruses were fed to 10 larvae (2^{nd} and 4^{th} instars) and replicate three times. Addition to the control treatment to obtained data then recorded.

RESULTS AND DISCUSSION

1. **Sample collection and light microscopy examination**

Viral symptoms of infected *H.armigera* which collected from the field can be summarized in: slow motion larvae, cuticle showing grey color, hanging larvae and liquefied larval body. Confirmation of infection of *H.armigera* by *HaNPV* after sample collection, virus propagation and OB purification by staining thin smear of infected larvae and drop of OBs with Giemsa stain was done by Light microscope and proved the presence of viral occlusion bodies which appear polyhedral (Fig.1) .

Fig (1) *HaNPV* polyhedra under light microscopy

2. **Effect of incubation temperature on infection development:**

Besides virulence and host specificity, temperature is an important factor in selecting an isolate for development as a mycoinsecticide. At high temperature the virus multiplied faster destroying the fate body before it could grow to provide greater substrate.

Studying of incubation temperatures effect on *HaNPV* against the 2^{nd} and 4^{th} instars larvae recorded that the mortality are increase with increasing the incubation

temperatures These results agree with the finding of Abul-nasr (1956) who stated that the incubation period of polyhedrosis virus shortened under warm and humid condition, this examination observed that In the case of 2nd instars larvae The highest mortality rate 96.6% was recorded with 35º C after six days of incubation, then the mortality reduced to 93.3% at 30° C and the lowest mortality percentage 26.6% was observed at 20º C after 12 day of incubation as shown in table (1)and illustrated in Figure 2.While The highest mortality rate for the 4th instar larvae was 83.3% at 35º C after 8 days of incubation, then the mortality reduced to 76.6% at 30º C. The lowest mortality percentage for the 4th instar larvae was 20% at 20º C after 12 day of incubation (Table 2 and Figure 3). This result was supported by Kershaw *et al.* (1999) where they stated that most isolates grow well between 5º C and 30º C; although some develop at temperatures as low as 5-10º C and others grow even at 35 - 40º C. On the other hand, obtained results disagreed with those obtained by Boueias *et al*. (1980) when incubated the *Anticarsia gernrnatalis* virus at 35º C effect was inferior to 30º C. Our results are further supported by the fact that protection of *HaNPV* from sunlight exposure by various parts of plants was more virulent to insects (Young & Yearian, 1974). It is thus suggested that the season with weak sunlight is optimal for applying the viral preparations in the field.

Table (1): Effect of incubation temperature on mortality of virus- infected 2nd instars larvae

Temperature degrees(º C)	No. of dead larvae/no. of total larvae	% Mortality
28*	27/30	90%
20	8/30	26.6%
25	20/30	66.6%
30	28/30	93.3%
35	29/30	96.6%
40	14/30	46.6%

*** Control treatment**

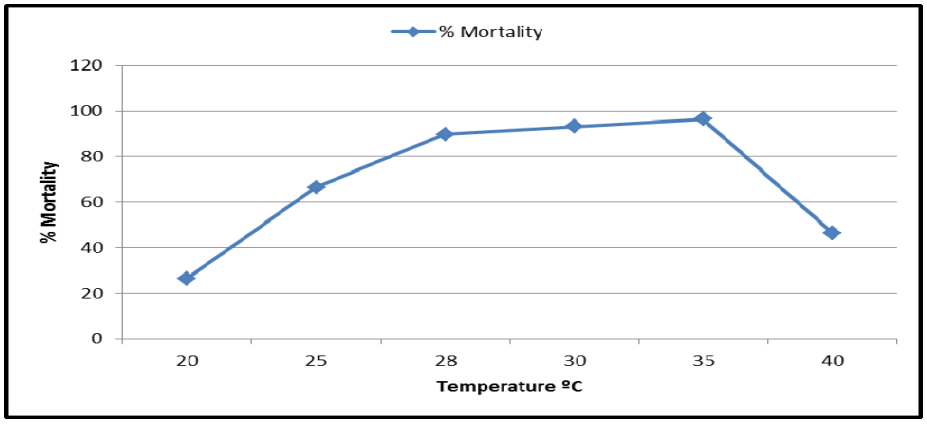

Fig. (2): Effect of incubation temperature on mortality of virus- infected 2nd instars larvae

Table (2): Effect of incubation temperature on mortality of virus -infected 4th instars larvae

Temperature degrees(º C)	No. of dead larvae/no. of total larvae	% Mortality
28*	24/30	80%
20	6/30	20%
25	17/30	56.6%
30	23/30	76.6%
35	25/30	83.3%
40	11/30	36.6%

*** Control treatment**

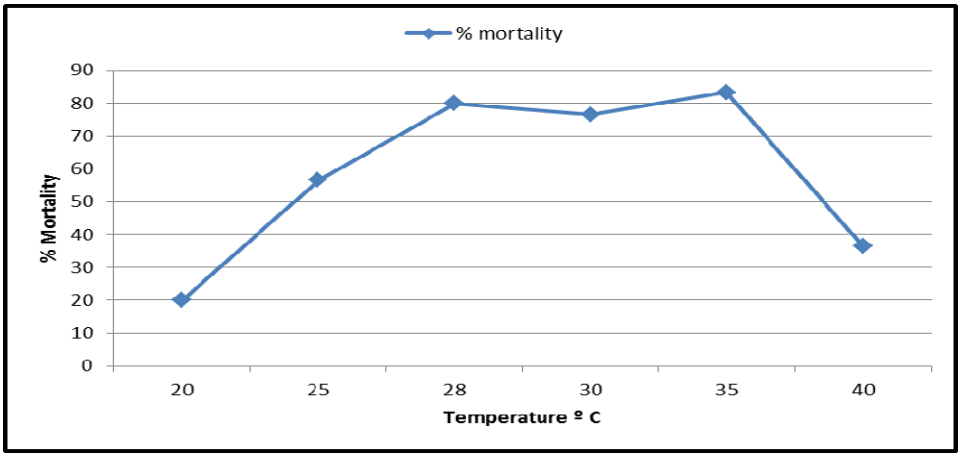

Fig. (3): Effect of incubation temperature on mortality of virus -infected 4th instars larvae

Effect of temperature on *HaNpv* infectivity:

On studying the virus stability to different temperature the researcher reported that temperature is one of the most important factors determining inactivation of virus Jeanne Dijkstra and Cees de Jager, 2014; therefore, there is a great variation in TIPs reported in the literature. these finding is insure our results after determination of Thermal inactivation point (TIP) of *HaNPV* as shown in tables (3 & 4) and illustrated in figures (4&5), which reported that in the case of the heat treated virus the virus activity against the 2nd and 4th instars when *NPV* suspension subjected at 20–60°C for 10 min indicated that the infectivity decreased as the temperature increased, and its complete absence at 50^0C for ten min. and therefore 50^0C considered as the thermal inactivation point of *HaNPV* virus. This result is agreement with many laboratory studies which reported that nucleopolyhedrosis virus is inactivated by exposed to high temperature, and with Stairs (1978) who observed that high temperature cause direct inactivation of virus, and affect the viral replication. While Manjunath (1978) concluded that The *NPV* suspension infectivity subjected at 60–90°C for 10 min decreased as the temperature increased, and significantly so at 70° C, followed by its complete absence at 90° C.

The reason for differences in mortality as affected by temperature was ascribed to variations in larval ingestion of virus-contaminated food; i.e., the larvae take up more food under higher temperatures (Ignoffo, 1966). But this did not occur in the present study because the larvae ingested the same dose of *HaNPV* when

inoculated at different temperatures. Obviously, effect of temperature on the viral infection is due to pathogenic process and not the rate of ingestion.

Table (3): Effect of virus stability to temperature on 2nd instars larvae

Temperature degrees(º C)	No. of dead larvae/no. of total larvae	% Mortality
28*	28/30	93.3
20	24/30	80
25	28/30	93.3
30	29/30	96.6
35	20/30	66.6
40	14/30	46.6
45	11/30	36.6
50	0/30	0
55	0/30	0
60	0/30	0

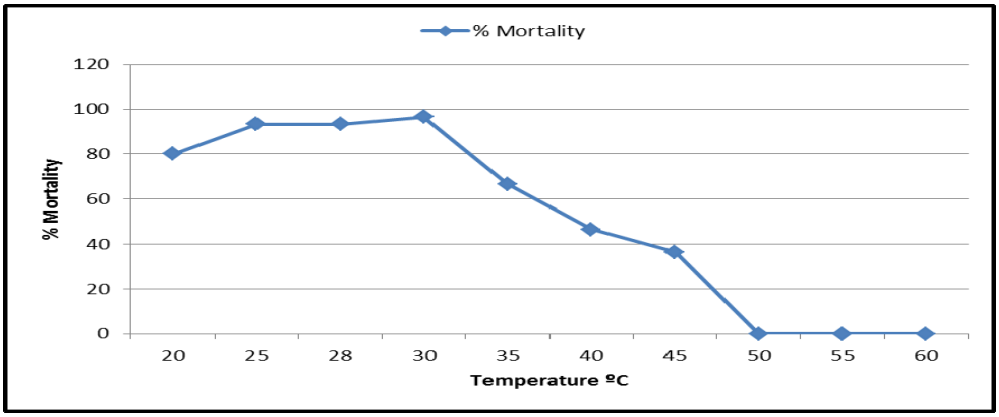

Fig. (4): Effect of virus stability to temperature on 2nd instars larvae

Table (4): Effect of virus stability to temperature on 4th instars larvae

Temperature degrees(º C)	No. of dead larvae/no. of total larvae	% Mortality
28*	25/30	83.3
20	19/30	63.3
25	22/30	73.3
30	26/30	86.6
35	16/30	53.3
40	11/30	36.6
45	10/30	33.3
50	0/30	0
55	0/30	0
60	0/30	0

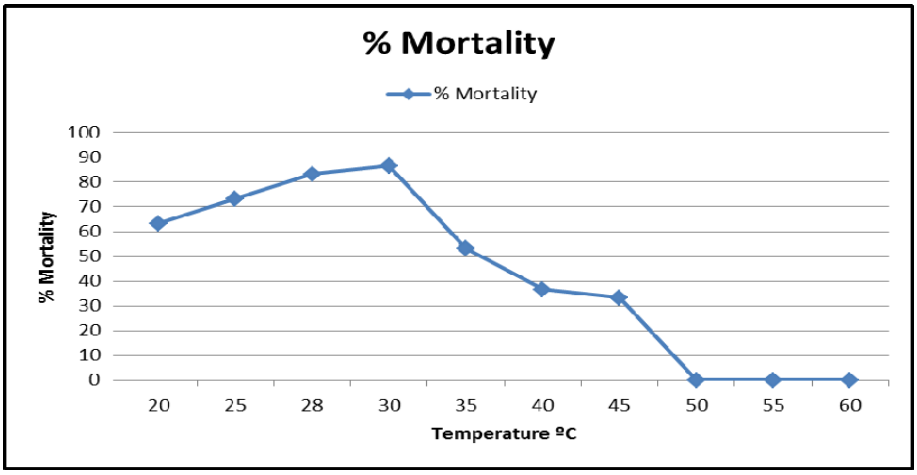

Fig. (5): Effect of virus stability to temperature on 4th instars larvae

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ARABIC SUMMARY

دراسة تأثيرات تقلبات درجات الحرارة على فاعلية العزلة المصرية للفيروس النووى لدودة اللوز الأمريكية

سوزان عبد الله إبراهيم

معهد بحوث وقاية النباتات، مركز البحوث الزراعية

الهدف من هذا العمل دراسه تأثيردرجات الحراره المختلفه علي كفأءه فيرس دوده اللوز الامريكيه علي حشره دوده اللوز الامريكيه واستخدامها كبديل امن للمكافحه الكيميائيه في الظروف المعمليه وقد تم إختبار القدرة المرضية للعزلة المصرية للفيروس النووى لدودة اللوز الأمريكيه على العمر الثانى والرابع حيث تم جمع وعزل الفيرس من الحشرات فور ظهور الأعراض المرضية عليها مثل التغير الى اللون الأحمر و الرمادى و قلة حركتها و كذلك السيولة المميزة لليرقة حيث تم التأكد من وجود الفيرس بإ ستخدام الميكروسكوب الضوئي. وقد بينت النتائج ان الحراره تعتبر مؤثر بيئى هام علي فاعليه فيرس دوده اللوز الأمريكيه حيث تؤثر علي معدلات موت اليرقات في العمرين الثاني و الرابع بشكل ملحوظ فقد لوحظ ان نسب موت اليرقات بالفيرس تزيد بزياده درجات حراره التحضين واعلي نسبه موت لوحظت عند ٣٥درجه تحضين يليها التحضين عند ٣٠ درجه مؤيه وأدني نسبه موت لوحظت عند ٢٠ درجه تحضين . كما تم إختبار أدنى درجة حرارة مطلوبه لإبطال كفاءه الفيروس تماما وهي ما يعرف بنقطة التعطيل الحراري وبإ ختبار درجات حراره تتراوح ما بين ٢٠ الي ٦٠ درجه مؤيه وجد ان الفيرس يفقد فاعليته تماما عند ٥٠ درجه مؤيه.