In vivo Production of *Palpita vitrealis* Nucleopolyhedrovirus

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Abstract

Elkady, Y.T., E.A. Agamy and S. El Salamouny. 2025. *In vivo* Production of *Palpita vitrealis* Nucleopolyhedrovirus. Arab Journal of Plant Protection, 43(2): 178-184. https://doi.org/10.22268/AJPP-001310

A Preliminary bioassay was conducted to determine the suitable larval instar of the olive leaf moth, *Palpita vitrealis* (Rossi) and the needed concentration of its homologous baculovirus *Palpita viteralis* nucleopolyhedrovirus (PaviNPV) for in vivo production. The calculated lethal concentrations for killing 90% of the tested larvae (LC_{90}) were 8.3×10^4, 15×10^4, 9.1×10^4 and 1.7×10^4 PIBs/ml for the tested 2nd, 3rd, 4th and 5th instars, respectively. In production tests, leaf-bud dipping bioassays were used for larvae infection. Virus yield of PaviNPV progeny polyhedral inclusion bodies (PIBs) was calculated in relation to the inoculum used for larval infection. The mortality rates of the tested PaviNPV concentrations (10^4-10^7 PIB's/ml) against the tested fourth and fifth larval instars showed ascending mortality-concentration dependence. The productivity rate (folds) of PaviNPV in the case of the fourth instar larvae were 137000, 35540, 1471, and 134.8 folds for the tested inoculum concentrations of 10^4, 10^5, 10^6 and 10^7 PIBs/ml, respectively. The addition of fluorescent brightener 28 (Tinopal UNPA-GX) enhanced the mortality rate and the obtained PaviNPV progeny by 188800, 43600, 1333.2 and 177.66 folds for the same inoculum concentrations, respectively. The study suggests using the fourth instar *Palpita vitrealis* larvae with a PaviNPV concentration of 10^4 PIB's/ml for in vivo production.

Keywords: *Palpita*, olive leaf moth, production, alphabaculovirus.

Introduction

Olive leaf moth (OLM), sometimes called jasmine moth, *Palpita vitrealis* (Rossi) *(*Syn*: Palpita unionalis,* Hübner; *Margaronia unionalis*, Hübner); (Lepidoptera: Crambidae), is a serious insect pest of olive (Hegazi *et al.,* 2012). The larvae attack buds and young leaves; moreover, in heavy infestations, larvae attack olive fruits, especially table varieties (Badawi *et al*., 1976). This insect pest causes severe damage to olive orchards in different Mediterranean countries, i.e., Egypt (Hegazi *et al*., 2012), Syria (Lababidi & Haj Hammoud, 2008), Algeria (Chaouche *et al.,* 2019), Greece (Athanassiou *et al.,* 2005; Mazomenos *et al.,* 2002), Türkiye (Yilmaz & Genç, 2012), Italy and Spain (Balachowsky, 1972) and Portugal (Torres *et al.,* 2004).

Baculoviruses are safe, specific and harmless to human and non-target organisms such as bees, predators and parasitoids, and consequently they are environmentally friendly biocontrol agents. Due to these advantages, they are considered promising candidates for suppressing insect pest populations in organic production systems (Burges *et al*., 1980). Alphabaculoviruses (nucleopolyhedroviruses) are dsDNA viruses that infect lepidopterous insect larvae and have been used since the 1980s in crop protection as commercial biopesticides (Wilson *et al*., 2020).

Production of baculovirus in insect larvae is a simple process that can readily be undertaken without the need for complex or expensive production in vitro systems (Masetti *et al*., 2008). The first commercial development of biopesticides based on baculoviruses was Elcar during the 1960s in the USA, using in vivo production of the *Heliothis* NPV (Ignoffo, 1973). Since then, over fifty baculovirus products were developed and used against different insect pests worldwide **(**Moscardi *et al*., 2011; Szewczyk *et al*., 2006). The need to produce a reliable baculovirus product in adequate quantities of suitable quality and at an affordable cost is a fundamental requirement.

Because NPV production remains the major constraint to the use of baculoviruses in plant protection, in vivo production is still used (Shapiro *et al*., 1986). Several publications have reviewed the development and production of baculoviruses in general using artificial diet (Black *et al*., 1997; Huber & Miltenburger, 1986; Hunter-Fujita *et al*., 1998; Ignoffo, 1973; Shieh, 1989; Weiss *et al*., 1994). Large-scale production of baculoviruses can only be carried out through the propagation of the virus in the larvae of susceptible insect species (Eberle *et al*., 2012; Shapiro, 1986). The majority of commercial baculovirus production uses cultured larvae on artificial diets, rather than in wild-collected larvae or those reared on plant leaves (Moscardi, 2007). These viruses can be also produced when plant leaves are used for feeding the larvae (Valicente *et al*., 2013). In addition, field production in some specific cases may be less expensive **(**Grzywacz *et al*., 2014; Moscardi, 2007). Larvae of the last two instars of *Spodoptera frugiperda* are recommended for mass production of NPV under laboratory conditions (Rios-Velasco *et al*., 2012). It is well documented that fluorescent (optical) brightener 28 acts as a susceptibility enhancer by increasing the susceptibility of insect larvae to nucleopolyhedrovirus (Shapiro & Robertson 1992). Fluorescent brightener cannot change a non-susceptible host to be susceptible (El Salamouny *et al*., 1997). The enhancement mode was attributed to the degradation of the peritrophic membrane and the facilitation of the entrance of baculovirus particles (El Salamouny, 2007). The first record

https://doi.org/10.22268/AJPP-001310
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of baculovirus infection of the larvae of the olive leaf moth, *Palpita vitrealis* was recorded by Nasr *et al.* (2002) then the virus was isolated again by Awad (2008) and characterized and identified at the molecular level as PaviNPV by El-Salamouny *et al*. (2022).

In this study, we determined the virulence and productivity of PaviNPV and investigated whether the addition of fluorescent brightener enhanced viral activity and productivity.

Materials and Methods

Collection of the test insect

The olive leaf moth (jasmine moth), *Palpita virtuales* (Rossi) (Lepidoptera: Crambidae) was collected from Paradise Farm, Cairo Alexandria Desert Road, km 50, Giza, Egypt. A laboratory colony was established in order to provide enough numbers of *Palpita virtuales* larvae for bioassays and virus production tests. Larvae were maintained in the laboratory on olive leaf buds in petri dishes (12 cm in diameter) until pupation. Pupae were collected and placed in plastic jars lined on the inner sides by plastic sheets for egg laying. The moths were fed a 10% honey solution. The egg sheets were collected daily and kept at room temperature until hatching. Newly hatched larvae were fed on fresh olive leaf buds. The colony was maintained at room temperature (25ºC), 65% relative humidity, and light regime of 16 hours light: 8 hours dark according to the method described by Mansour *et al*. (2016) and El-Salamouny *et al*. (2022). Fourth and fifth instar larvae were tested for in vivo propagation of *P. vitrealis* alphabaculovirus (PaviNPV).

Viral isolate used

The origin of the viral isolate under investigation is *Palpita vitrealis* nucleopolyhedrovirus (alphabaculovirus) (PaviNPV), isolated by Awad (2008) and recently characterized as *P. vitrealis* NPV by El-Salamouny *et al*. (2022). To increase the susceptibility of *Palpita* larvae to its homologous baculovirus PaviNPV, fluorescent (optical) brightener 28 (Tinopal UNPA-GX, Sigma- Aldrich, Germany) was used at a concentration of 0.1% (w/v).

Virus purification

The viral isolate (PaviNPV) was prepared and partially purified as reported earlier (Awad, 2008). Briefly, the dead larvae were stored frozen and allowed to defrost, homogenized in sterile distilled water, and filtered through muslin cloth. In order to remove the larval debris, centrifugation of the suspension was done for 30 seconds at 4°C. The supernatant was then centrifuged at 10,000 for 15 min at 4°C, and the virus pellet was collected. The resulting pellets were resuspended in sterile distilled water. Further purification was conducted by using sucrose gradients (30-65%) using Hitashi ultracentrifuge Type 1610-8500Nm at 14,000 rpm (JKI, Darmstadt, Germany) for one hour at 4°C (El-Salamouny *et al*., 2022). The sucrose residue was removed by washing twice in distilled water and centrifugation at 14,000 rpm for 30 minutes at 4°C. The polyhedral inclusion bodies were counted using a double ruled hemocytometer (Neubauer, Germany) and diluted in distilled water in a serial concentration ranging from 10^4 to 10^7 PIBs/ml with a factor of fivefold.

Preliminary infection tests

Preliminary bioassays were conducted to estimate the susceptibility of *P. vitrealis* second, third, fourth, and fifth instars to different virus concentrations ranging from 10^3 to 10^6 polyhedral inclusion bodies (PIB's)/ml in leaf disc dipping bioassays according to the methods described by El-Salamouny *et al*. (2022).

Production tests

For mass production of polyhedral inclusion bodies (PIBs) of PaviNPV, virus suspensions at concentrations ranging from 10^4 to 10^7 PIBs were used for infection of fourth and fifth instar larvae by dipping the olive leaf buds in 5 ml of the baculovirus-purified suspension. Triton-X 100 was used at a concentration of 0.01% as a surfactant to decrease the surface tension of the virus suspension on the olive leaves. After dipping the buds in virus suspension, they were dried at room temperature. Third, fourth and fifth instar larvae were tested, with three replicates for each treatment. The contaminated leaf buds were exposed to the larvae for 24 hours then individually reared on untreated olive leaf buds. Control larvae were fed on leaf buds treated only with water. Larval mortality was recorded daily based on the viral symptoms of the disease caused by NPV until death or pupation. Dead larvae were kept frozen.

Virus harvesting

A modified method by Rios-Velasco *et al*. (2012) was used to facilitate the collection of the PIB's. The collected dead larvae (cadavers) were placed in plastic 12-ml Falcon tubes containing 5 ml of distilled water and kept frozen. Cadavers in each tube were homogenized using a glass stem and partially purified by centrifugation at 500 rpm for 1 minute to remove the sclerotized parts of larval body debris. Polyhedral counts in the supernatant were taken using the Neubauer Hemocytometer under a light microscope (Zeiss, Germany) at 400X using three samples of each larval group. The cadaver weight was determined using the digital balance (Electronic Scale, China).

Statistical analysis

The ANOVA, Probit analysis (Finney, 1971) and LSD tests were used for data analysis. Mortality rates were corrected according to the Abbott (1925) formula.

Results and Discussion

Susceptibility of different *Palpita vitrealis* instars to PaviNPV

Larval mortality is a key factor in the productivity of baculoviruses. In order to define the susceptibility of *P. vitrealis* instars its homologous nucleopolyhedrovirus (alphabaculovirus) (PaviNPV) under investigation, a leaf disc bioassay was used. The results showed a positive relationship between larval mortality of *P. vitrealis* and PaviNPV concentrations. In general, the tested 2nd, 3rd, and 4th instar larvae were more susceptible to *P*aviNPV than the fifth instar larvae. Results obtained showed a mortality–

concentration dependent positive response (Figure 1). The calculated lethal concentrations killing 90% of the tested *Palpita* larvae (LC_{90}) were 8.3×10^4, 15×10^4, 9.1×10^4, and 1.7×10^4 PIBs/ml for the tested 2nd, 3rd, 4th and 5th instars, respectively. The high susceptibility of *Palpita* larvae to the tested virus concentrations could be due to the absence of the peritrophic membrane, which is similar to the results obtained with the tortricid larvae by El Salamouny (2007).

Mortality response of the fourth and fifth instars in the dipping olive bud's production test

In order to optimize the production of the polyhedral inclusion bodies (PIBs) of PaviNPV, different viral concentrations ranging from 10^4 to 10^7 PIB's/ml were tested against fourth and fifth instar larvae. The mortality rates of fourth instar larvae fed on olive leaf buds treated with *Palpita* alphabaculovirus (PaviNPV) were 39.32, 82.55, 90.56, and 100% for the tested virus concentrations of 10^4, 10^5, 10^6 and 10^7 PIBs/ml, respectively. The addition of fluorescent brightener (FB) increased the mortality rate to 53.65, 84.14, 92.72 and 96.36% for the same tested concentrations, respectively. The calculated LC_{50} value for virus alone treatment was 3.09×10^4 PIBs/ml which decreased to 1.9×10^3 PIBs/ml by the addition of FB as a mortality enhancer. The result of the low enhancement rate of the susceptibility of *Palpita* larvae by the addition of fluorescent brighteners is similar to that obtained by El Salamouny *et al.* (1997) who confirmed that the high rate of enhancement by fluorescent brightener was more observed in susceptible hosts than in highly susceptible hosts.

The fifth instar larvae were found to be less susceptible to PaviNPV than the fourth instar larvae (Figure 2). Mortality rates for virus alone treatment did not exceed 27% at the highest virus concentration of 10^7 PIBs/ml. When FB was added as an enhancer, the mortality rate increased to 69.23% with 2.64-fold increase. The result obtained that the fifth instar larvae were less susceptible to infection than the fourth instar larvae with PaviNPV agrees with previous finding (Rios-Velasco *et al*., 2012). Thus, fourth instar larvae are recommended for PaviNPV virus production.

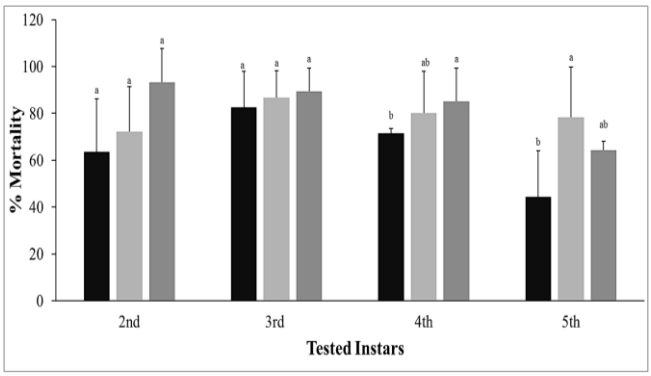

Figure 1. Mortality response of *Palpita vitrealis* alphabaculovirus against different *Palpita vitrealis* instar larvae. Bars marked with similar letters are not significantly different at P=0.05.

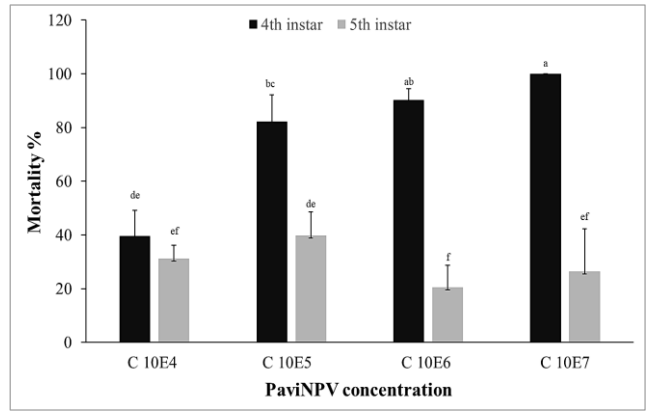

Figure 2. Mortality response of PaviNPV against fourth and fifth instar larvae of *Palpita vitrealis* fourth, and fifth instar larvae. Bars marked with similar letters are not significantly different at P=0.05.

Effect of PaviNPV concentrations on the amount of produced polyhedral inclusion bodies progeny

The effect of virus concentration and larval instar on the amount of polyhedral inclusion bodies (PIBs) progeny produced in infected *Palpita* larvae is shown in Table 1. There was a significant difference between the total number of progenies PIBs recovered from the fourth and fifth instars. The highest average virus yields of 177.7×10^8 PIB's/ml was obtained when the fourth instar larvae were exposed to the concentration of 10^5 PIB's/ml. However, in the case of using the fifth instar larvae, the average of virus yield obtained decreased to 67.3×10^8 PIB's/ml at the same inoculum concentration, which was less than that obtained from the fourth instar larvae. The increase in virus yield by the fourth instar larvae could be due to the high rate of mortality (82.55%) compared to the fifth instar larvae (37.16%). The lower productivity of the fifth instar larvae could be due to the early pupation of the larvae before the infection cycle was completed, thus reducing the virus yield.

Cadaver weight and PIBs yield per gram of cadaver

Data presented in Table 1 summarizes the relationship between cadaver weight and virus progeny. The cadaver weight was higher when FB was added to the virus. In addition, cadaver weight was higher in case of using fifth instar larvae at a virus concentration of 10^7 PIBs/ml with FB added. Results obtained showed that cadaver weight was found to be higher at 0.58 and 0.82 g when the fourth instar larvae were treated with concentrations of 10^5 and 10^6 PIB/ml, respectively. However, in case of the fifth instar larvae, the cadaver's weight was 0.45 and 0.62 grams when using concentrations of 10^4 and 10^5 PIBs/ml, respectively. Fluorescent brightener increased the cadaver weight when lower concentrations (10^4 and 10^5 PIBs/ml) were used. However, the cadaver weight decreased when concentrations of 10^6 and 10^7 PIBs/ml were used. The productivity of PIBs progeny per gram was 306.379×10^8 and 110.327×10^8 PIBs/g for the fourth and fifth larval instars, respectively.

Table 1. Productivity of PaviNPV in fourth and fifth instar larvae.

Inoculum Concentration*	Total yield (PIB's)**	Cadaver weight/g	Productivity PIB's/g	Dead/Tested	Mortality rate (%)
PaviNPV against fourth instar larvae					
10^4	68.50×10^8 ab	0.33	207.58×10^8	35/89	39.32
10^5	177.70×10^8 a	0.58	306.38×10^8	71/86	82.55
10^6	73.55×10^8 b	0.82	89.69×10^8	48/53	90.56
10^7	141.06×10^8 ab	0.38	37.12×10^8	57/57	100.00
PaviNPV + Fluorescent brightener (0.1%) against fourth instar larvae					
10^4	94.40×10^8	0.46	205.22×10^8	44/82	53.65
10^5	2180×10^8	0.74	294.60×10^8	69/82	84.14
10^6	66.66×10^8	0.43	155.02×10^8	51/55	92.72
10^7	88.83×10^8	0.37	240.08×10^8	53/55	96.36
PaviNPV against fifth instar larvae					
10^4	19.97×10^8 cd	0.45	44.38×10^8	46/147	31.29
10^5	67.30×10^8 bc	0.61	110.33×10^8	55/48	37.16
10^6	10.34×10^8 d	0.22	47.00×10^8	17/82	20.73
10^7	17.47×10^8 cd	0.29	60.24×10^8	22/84	26.19
PaviNPV+ Fluorescent brightener (0.1%) against fifth instar larvae					
10^6	10.34×10^8	0.17	60.82×10^8	17/91	18.68
10^7	54.52×10^8	0.76	71.73×10^8	63/91	69.23

*= 5 ml of each concentration was used. **= Polyhedral Inclusion Bodies. Values followed by the same small letters in the same column are not significantly different at P=0.05.

Effect of Fluorescent brightener on the amount of virus progeny produced in fourth instar larvae

The addition of FB at 0.1% (v/w) to the concentration of 10^4 PIBs/ml increased the virus progeny in one fourth instar larva from 5.9×10^8 (when using virus alone) to 6.2×10^8 PIBs/ml (when using virus +FB). Adding FB to the inoculum concentration of 10^5 PIB's/ml also increased the yield per larva from 7.3×10^8 to 9.5×10^8 PIBs/ml. However, no change in the amount of progeny per larvae was obtained at concentrations of 10^6 and 10^7 PIBs/ml (Figure 3). This could be due to the fact that FB speeds up the larval death before forming enough polyhedral inclusion bodies, therefore reducing the virus yield. Thus, the best concentration was 10^5 PIBs/ml in the presence of the brightener, which gave a higher virus yield (Figure 3).

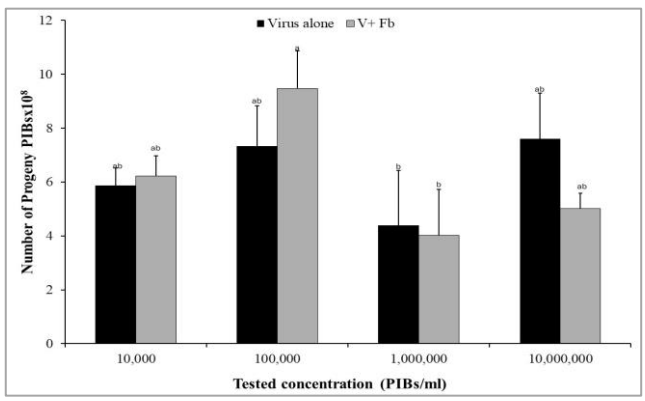

Figure 3. The average number of progenies PIBs per larva in the presence or absence of fluorescent brightener in the fourth instar. Bars marked with similar letters are not significantly different at P=0.05.

Potency of PaviNPV on production rate (folds) against the fourth instar

The production rates (fold) presented in Figure 4 were higher in cases of lower virus concentrations, resulting in a low rate of mortality. In the case of virus alone treatment, the obtained potency values were 137000, 35540, 1471, and 282.112 folds, for the tested inoculum of 10^4, 10^5, 10^6 and 10^7 PIB's/ml, respectively, increased to 188800, 43600, 1333.2 and 177.66 folds by the addition of FB at the same tested concentrations, respectively.

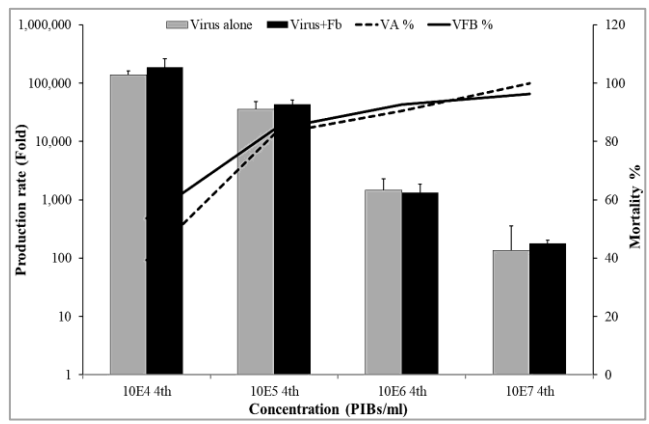

Figure 4. Relative potencies (folds) of four PaviNPV concentrations against *Palpita* fourth instar larvae in relation to mortality rates.

The same trend of increase in PIBs yield was shown in the case of *Helicoverpa armigera* by Narayanan & Jayaraj (2002). Lower concentrations kill the larvae slower than higher concentrations, thus allowing the virus to effectively infect the maximum amount of host tissue (Stinguel *et al.,* 2022).

It can be concluded from this study that a concentration of 1×10^4 PIBs/ml against the fourth instar larvae can be recommended for olive leaf moth alphabaculovirus production. Future studies will be conducted to optimize the virus yield by using an artificial diet.

Acknowledgements

The authors express sincere thanks to Mrs. Fatma Abdelmohsen for her valuable technical assistance in the mass rearing of the test insects, to Paradise Farm for providing the source of the test insect, and to Dr. Hashim Abdellatif for his valuable help in analyzing the data. The authors deeply thank Prof. Merle Shepard, Clemson University, USA, for reading the manuscript.

الملخص

القاضي، يحيى، عصام عجمي وسعيد السلاموني. 2025. إنتاج فيروس متعدد الوجوه (nucleopolyhedrovirus) متخصص بدودة براعم الزيتون (*Palpita vitrealis*) مختبرياً. مجلة وقاية النبات العربية، 43(2): 178−184. https://doi.org/10.22268/AJPP-001310

أجريت اختبارات حيوية لتحديد العمر اليرقي المناسب لدودة براعم الزيتون/فراشة الياسمين (*Palpita vitrealis*) والتركيز المناسب من الفيروس متعدد الوجوه المتخصص بها والتابع للجنس Baculovirus (PaviNPV). قدّرت التراكيز المميتة لـ 90% من اليرقات (LC_{90}) باستخدام التراكيز 8.3×10^4، 15×10^4، 9.1×10^4 و 1.7×10^4 أجسام محتواة/مل للأعمار المختبرة (الثاني، الثالث، الرابع والخامس، على التوالي). ولإعداء اليرقات بهدف إنتاج الفيروس، استخدمت طريقة غمر البراعم الورقية بمحلول الإعداء، وتمّ حساب المحصول الفيروسي الناتج بعدد الأجسام المحتواة متعددة الوجوه/مل وعلاقته بالتركيز الأصلي المستخدم في العدوى. تراوحت معدلات الموت ما بين 10^4 و 10^7 جسماً متعدد الوجوه/مل وفقاً للتراكيز المختبرة ضدّ كلٍّ من العمرين الرابع والخامس لليرقات، وكانت العلاقة طردية بين نسبة الموت والتراكيز المستخدمة. كان معدل تضاعف الفيروس بمفرده في حالة العمر اليرقي الرابع 137000، 35540، 1471 و 134.8 ضعفاً للتراكيز 10^4، 10^5، 10^6 و 10^7 جسماً متعدد الوجوه/مل، على التوالي. وعند إضافة مادة المشعع الفلوروسنتي (Tinopal UNPA GX) زاد معدل الموت وكذلك معدل التضاعف ليصبح 188800، 43600، 1333.2 و 177.66 ضعفاً للتراكيز المذكورة أعلاه، على التوالي. توصي نتائج هذه الدراسة باستعمال العمر الرابع لدودة براعم الزيتون مع التركيز الفيروسي 10^5 جسم متعدد الوجوه/مل للإكثار الكمّي للفيروس في العائل الحشري.

كلمات مفتاحية: فراشة ورق الزيتون، الإنتاج الكمي للفيروس، جنس ألفا باكيولو فيروس.

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Received: October 23, 2023; Accepted: March 13, 2024

تاريخ الاستلام: 2023/10/23؛ تاريخ الموافقة على النشر: 2024/3/13