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OPTIMIZATION OF THE NUCLEOPOLYHEDROVIRUS SpltMNPV-P7 PRODUCTION IN ITS HOMOLOGOUS HOST, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)[1]

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ABSTRACT

An efficient mass production protocol was developed for the Philippine strain of *Spodoptera litura* nucleopolyhedrovirus, SpltMNPV-P7. A three-step study was designed to determine the larval age, amount of inoculum and post-infection harvest time that would efficiently yield the highest number of occlusion bodies (OBs) in *S. litura* larvae. The highest number of OB produced was observed in 9 days old larvae ($2.667 \times 10^7 \pm 0.602 \times 10^7$), indicating that 9 days is the most ideal age of the host larvae for SpltMNPV-P7 mass production. Among the four inoculum concentrations used to infect 9 days old larvae, 10,000 OBs/larva produced the highest yield of OBs ($4.167 \times 10^8 \pm 0.361 \times 10^8$), thus, the combination of 9 days old larvae and 10,000 OBs/larva was used to determine the optimum post infection period for harvesting SpltMNPV-P7, which was found to be 6 days post-infection (DPI) that gave the highest OB yield ($7.004 \times 10^9 \pm 1.513 \times 10^9$). Thus, the best larval age, inoculum concentration and post-infection harvest time for optimum OB yield of SpltMNPV-P7 in *S. litura* are 9 days old, 10,000 OBs/larva and 6 DPI, respectively.

Key words: *Spodoptera litura* nucleopolyhedrovirus, SpltMNPV-P7, common cutworm, efficient protocol, *in vivo* mass-production of occlusion bodies

INTRODUCTION

Spodoptera litura (Fabr.), the common cutworm, is a polyphagous species that infests a number of economically important crops, except those with hard, woody stems. Among the important crop species attacked by *S. litura* in the tropics are alfalfa, taro, cotton, flax, groundnut, jute, maize, rice, soybean, tea, tobacco (Pathak and Khan, 1994) and vegetables, such as aubergines, *Brassica*, *Capsicum*, cucurbits, *Phaseolus*, white potatoes, sweet potatoes and *Vigna*. Other host plants include ornamentals, wild plants, weeds and shade trees like *Leucaena leucocephala*, the shade tree of cocoa

plantations in Indonesia (Mochida, 1973). In the Philippines, 28 plant species, mostly agricultural crops, were reported as hosts (Gabriel, 2000). *S. litura* is a cosmopolitan species found in the United Kingdom, tropical and subtropical Asia, Hawaii and Australia, which includes Northern Territory, New South Wales, Queensland, Solomon Islands, Western Samoa and most other Pacific Islands (Mochida, 1973, Aitkenhead et al., 1974).

On most crops, *S. litura* damage results from extensive feeding by the larvae on the leaves and soft stems, leading to complete stripping of the plants. On cotton, leaves are heavily attacked and bolls have large holes in them from which yellowish-green to dark-green larval excrement protrudes. On tobacco, leaves develop irregular, brownish-red patches and the stem base may be gnawed off. On maize, the stems are often mined and young grains on the ear may be injured (Mochida, 1973).

S. litura is susceptible to nucleopolyhedroviruses (NPVs), which are highly specific to insects. NPVs belong to the family Baculoviridae, consisting of insect viruses with covalently closed double-stranded DNA (dsDNA) genome of 80 to180 kilo base pairs (kbp) (Burgess, 1977). Baculoviridae consists of two genera, namely, the Granulovirus (GV) and NPV. GVs and NPVs contain virions occluded within inclusion bodies (IBs) of crystalline protein called granulin and polyhedron, respectively. The polyhedron, also called occlusion body (OB), occurs in a form similar to those in other virus families, but apparently exclusive to invertebrate viruses (Tweeten et al., 1981). The GVs differ from the NPVs in that each granulin contains only one virion, while the polyhedron contains several virions. Symptoms noted in infected larvae include whitening or yellowing of the gut. Rapid melanization leading to blackening of the body occurs after death. The outer skin easily ruptures, thus releasing the liquefied body contents. Research shows that NPVs have no harmful effect on vertebrates or living organisms other than the insect host (Ignoffo, 1973). The host specificity of viruses allows their use without killing beneficial insects, such as parasitoids and predators, in an agroecosystem.

NPV is one of the biological control agents that can be used in an integrated pest management program (Rao et al., 2007). An NPV-based biopesticide Elcar, with *Helicoverpa zea* NPV as its active ingredient, was first commercialized in 1975 (Ignoffo and Couch, 1981). To date, NPV formulations are being marketed in Australia, Austria, Brazil, China, India, Italy, Finland, Norway, United Kingdom, the USA and USSR (McCutchen and Flexner, 1998). However, the annual market share of this group of biopesticides was only a few million US dollars by the end of the last century (Szewczyk et al, 2006).

The limited market expansion for NPVs can be attributed to the technical and economic difficulties in *in vivo* commercial production. Larval age is crucial in mass

production of NPV so as to maximize virus yield. A study by Legacion and Gabriel (1975) noted that *S. litura* larvae become less susceptible to NPV as they mature. Ignoffo (1966) observed the same phenomenon wherein larval age is inversely related to NPV susceptibility, that is, susceptibility decreases with larval maturity. The characteristic liquefaction of NPV-infected cadaver also poses a problem in *in vivo* OB mass-production. Proper timing in the collection of diseased larvae close to death is equally important to reduce the OB loss due to liquefaction. It is of utmost importance to study at what larval age SpltMNPV-P7 is best inoculated to maximize the OB production in infected insects as well as the post infection harvest time. These information are essential to enable harvesting of the optimum amount of viral particles from cutworm larvae for use in formulating NPVs into biological pesticide. Thus, information on efficient mass-production protocol with specific NPV dose, larval age for infection and timing of NPV collection in the infected larvae that will result in optimum OB production are needed. This study aimed to develop a protocol that optimizes the determine the: age of *S. litura* larvae, optimum amount of inoculum per larva, and post-infection harvest time that will yield the optimum number of SpltMNPV occlusion bodies.

MATERIALS AND METHODS

Collection and Mass Rearing of Test Insects

Egg masses and larvae of *Spodoptera litura* were collected from the Central Experiment Station, U.P. Los Baños, Laguna and brought to the laboratory for mass rearing. All rearing containers and castor leaves were properly disinfected with 0.5% sodium hypochlorite solution. The larvae were placed in clean rearing trays and fed with fresh castor leaves. The pupae were collected and transferred to an emergence cage to allow mating of the emerged adults. Fresh sweet potato leaves with stems dipped in water in a bottle were placed in the emergence cage for oviposition. The egg masses were collected and placed in acrylic pans for hatching to serve as stock culture for the experiment.

Preparation of Nucleopolyhedrovirus Inoculum

The viral inoculum of *Spodoptera litura* nucleopolyhedrovirus, SpltMNPV-P7 (Laviña et al., 2001), used in this study was obtained from the Insect Pathology Laboratory, Crop Protection Cluster, College of Agriculture, U.P. Los Baños. All materials used in this study were properly sterilized. A stock NPV solution was prepared from 100 *S. litura* larvae infected with OBs purified from an insect cell line. Morbid larvae were placed in an Erlenmeyer flask, triturated using a glass tissue homogenizer, the homogenate filtered through two layers of muslin cloth, and the

filtrate centrifuged at 11,600 x g, at 4°C for a minute to partially purify the occlusion bodies (OBs). OBs were washed twice and then resuspended in sterile distilled water and the OB concentrations estimated using New Improved Neubauer Hemacytometer. This viral suspension was used as stock solution and suspensions with different OB concentrations were prepared by serial dilution.

Determination of Larval Age Suitable for NPV Production

Different larval age groups, namely: 6 days old, 9 days old and 12 days old larvae, were used in this study. Each larva was fed with 100 OBs by overlaying 10 µl of 10,000 OBs/ml onto an artificial diet cube (approximately 2.5 cm x 2.5 cm x 2.5 cm). The control group was fed with 10 µl sterile distilled water overlaid onto a similar diet cube. Three trials were conducted with five replications per trial and ten larvae per replicate. The parameters measured were larval weight and OB production. *S. litura* larvae in each treatment, including the control, were pre-weighed individually prior to infection, reweighed at 2, 4 and 6 days post infection (DPI) to determine larval weight gains. The data gathered were statistically analyzed. OB production was estimated from fifteen *S. litura* larvae from each replicate per treatment, pooled in a sterile amber bottle at death or at 6 DPI, whichever came first. OBs were harvested as described in the inoculum preparation, quantified using an Improved Neubauer Hemacytometer. OB counts were obtained from three trials with three replicates per trial. Data gathered were statistically analyzed.

Determination of the appropriate OB Inoculum Concentration for Optimum NPV Production

Using the larval age that gave the significantly highest OB production, the following OB concentrations were compared, namely, 10, 100, 1,000 and 10,000 OBs/larva plus the control. Using 10 larvae per treatment replicated three times, three trials were done. At 6 DPI, 15 NPV-infected larvae randomly selected from each replicate per trial were transferred to sterile amber bottles. Sterile distilled water was added to each bottle and mixed thoroughly before pouring into a sterile homogenizer. The homogenized putrefied solution of diseased larvae was filtered once through a double-layered sterile muslin cloth, then thrice through single-layered cheesecloth, each time collected into a new sterile amber bottle for harvesting of OBs by centrifugation as described by Rao and Meher (2004) and Trang and Chaudhuri (2002). The OB solutions were transferred to sterile amber bottles and stored at 16°C until quantified. The volumes of the OBs suspensions were standardized in sterile 100 ml volumetric flasks. A hundred fold dilution was prepared for each sample and 250 µl was loaded into improved Neubauer Hemacytometer. Nine counts for each replicate were made and the data gathered were statistically analyzed.

Determination of Optimum Post-infection Harvest Time

The average occlusion body production was computed as described by Cherry et al. (1997) with some modifications. It was suggested that OB yields in larvae harvested alive could be increased further after harvest by a second incubation period without the diet. To investigate this possibility, a total of sixty 9 days old larvae, divided into three replicates, were infected with OB at concentration that produced the highest number of OBs for each treatment. Fifteen larvae were ramdomly harvested from each group, at 5, 6 and 7 days post-infection (DPI). The larvae were pooled into a sterile amber bottle for each replicate, their OB yields determined as done earlier, and the data gathered analyzed statistically.

RESULTS AND DISCUSSION

Larval Age Best Suited for *in vivo* Mass Production of *Spodoptera litura* NPV, SpltMNPV-P7

The average weight gains of larvae after SpltMNPV infection at different ages as determined at varying post infection periods are summarized in Figure 1. At 2 DPI, the highest average larval weight gain was observed in 12 days old larvae (0.15330 g), which was significantly higher than those in 6 days (0.01228 g) and 9 days old (0.07735 g) groups. At 4 DPI, 9 days old larvae exhibited the highest average larval weight gain of 0.2479 g compared with the 0.0345 g and 0.1728 g of 6 and 12 days old larvae, respectively. However, the difference between the 9 and 12 days old groups was not significant. At 6 DPI, only the average larval weight gains in 6 and 9 days old groups, which were 0.0862 g and 0.3753 g, respectively, were statistically compared since 32.22% of the 12 days old larvae tested already pupated. The average larval weight gain in 9 days old larvae was significantly higher than that in the 6 days old larvae at 6 DPI.

A positive correlation between larval weight gain and larval age was observed in 6 and 9 days old larvae infected with SpltMNPV-P7. The same was noted by Sporleder et al., (2007) in granulovirus-infected *Phthorimaea operculella,* where they observed a logistic function between the larval weight gain and age. Virus infections result in physiological changes such as cell hypertrophy as viral replication progressed from the midgut to other tissues. Trang and Chaudhuri (2002) concluded that an increase in weight gain in NPV-infected larvae indicates more tissues as sites of viral replication. This may explain the trend of larval weight gain in 12 days old larva, wherein a slight increase was observed from 2 to 4 DPI and then decreased at 6 DPI, since most of the larvae infected at 12 days old were about to pupate at 6 DPI. At this time, limited larval tissues were available for NPV replication. The results in 12 days old larvae conform to the observation of Hernandez-Crespo et al. (2001) that there was a decrease in virus yield per unit of weight over time in *Mamestra brassica.* Biji et al. (2006) on the other

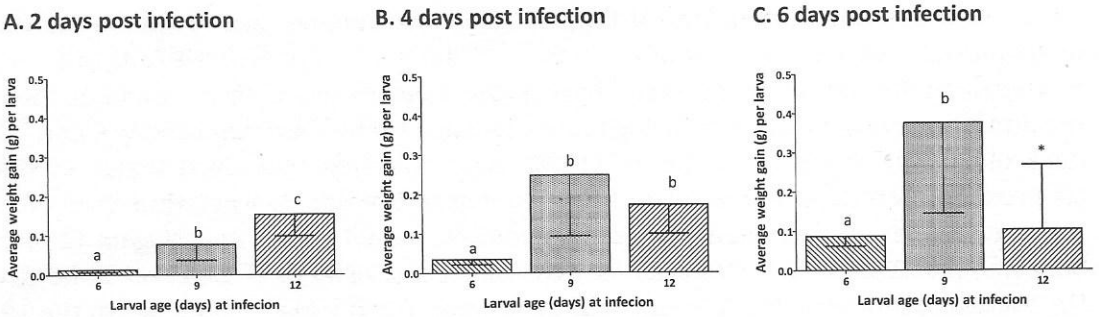

Figure 1. Average weight gain of *Spodoptera litura* infected with nucleopolyhedrovirus SpltMNPV-P7 at 100 occlusion bodies (OBs)/larva, as observed at (A) 2, (B) 4 and (C) 6 days post-infection. Bars with the same letter are not statistically different at $\alpha = 0.05$. (*) indicates pupation, thus was not compared statistically. Vertical bars indicate standard deviation.

hand, observed in *Hyblaea puera* that virus yield per milligram of body weight was not proportional to the increase in body weight.

To further verify the suitability of 9 days old larvae for mass production of OBs, the average numbers of OBs produced in the different larval age groups infected with 100 OBs per larva were determined at 6 DPI (Table 1). The highest number of OBs produced was in S. *litura* larvae infected at 9 days old ($2.667 \times 10^7 \pm 0.601 \times 10^7$), while the lowest ($0.622 \times 10^7 \pm 0.193 \times 10^7$), was in 6 days old larvae which manifested small larval weight gain at OB harvest. In addition, since early instar and younger larvae of S. *litura* are more susceptible to NPV infection (Legacion and Gabriel, 1975), 6 days old larvae succumbed faster to NPV-infection, thus, fewer OBs were produced. Although 12 days old S. *litura* larvae possess more tissues than 9 days old larvae for NPV infection, they were nearing pupation or have already pupated at harvest time at 6 DPI. At this time, NPV production no longer increased since the 12 days old larvae at infection had already infected all the available body tissues. The same was observed by Pourmiza (2000) in HearNPV- infected fifth instar *H. armigera* larvae.

Table 1. Average number of occlusion bodies (OBs) of SpltMNPV-P7 nucleopolyhedrovirus harvested from fifteen *Spodoptera litura* larvae after 6 days post infection with with 100 OBs/larva

Larval Age (days) at Inoculation	Average Number of OBs Produced[1]	Standard Deviation
6	$0.622 \times 10^{7\,a}$	$\pm 0.193 \times 10^7$
9	$2.667 \times 10^{7\,b}$	$\pm 0.601 \times 10^7$
12	$2.230 \times 10^{7\,b}$	$\pm 0.424 \times 10^7$

[1] Values followed with different letters are significantly different at $\alpha = 0.05$ based on Kruskal-Wallis test followed by Dunn's Multiple comparison test.

Our results showed that 9 days is the most suitable age of the larvae for infection with 100 OBs each for *in vivo* mass production of SpltMNPV-P7. At this age, *S. litura* larvae, although infected, have more time to grow, thus reaching their maximum weight gain and producing more occlusion bodies, as compared to 6 and 12 days old larvae at infection. Ebora (1987) also observed that third instar larvae (approximately 9 days old) of the common cutworm, lived the longest, thus giving the highest number of occlusion bodies. In addition, Monobrullah and Nagata (2000) observed that 9 days old *S. litura* larvae gave the highest virus yield. However, although the average OB production in 9 days old larvae was slightly lower than that in the 12 days old larvae, the difference was not significant and in economic terms, it is not practical to delay the NPV mass production process for 3 days. It can be concluded, then that among the three, the 9 days old group is the most ideal for optimized NPV production based on larval weight gain and actual OB count.

Least Amount of SpltMNPV-P7 Inolucum Needed for Optimum *in vivo* Mass Production of the nucleopolyhedrovirus

The average number of OBs produced in 9 days old *S. litura* larvae infected with 10, 100, 1,000 and 10,000 OBs/larva are summarized in Table 2. The highest average OB production was observed in larvae infected with 10,000 OBs/larva ($4.167 \times 10^8 \pm 0.361 \times 10^8$) while the least was in those infected with 10 OBs/larva ($2.689 \times 10^8 \pm 0.678 \times 10^8$). Statistical analyses showed significant difference in average OB production in larvae between the groups infected with 10,000 OBs/larva and all the other inoculum doses used in the study. However, no significant difference in OB yield was observed between inoculum doses 10 and 100 OBs/larva. The results in this study are in contrast with those observed by Kumar et al. (2005). They noted that in *S. litura* the virus concentration of the inoculum and the resulting virus yield had a negative relationship because high NPV concentration resulted in death of the test insects before attaining their maximum weights, thus, resulting in low OB production. Narayanan and Jayaraj (2002) and Kalia et al. (2001) also noted this trend in *Helicoverpa armigera*. Similarly, Jankevica and Zarins (1999) observed an inverse relationship between inoculum concentration and virus yield in *Malacosoma neustria*. Nonetheless, our results indicate that 9 days old *S. litura* larvae infected with SpltMPNY at a dose of 10,000 OBs/larva produced a significantly higher amount of OBs at 6 DPI.

Post Harvest Time for Optimum *in vivo* Mass Production of SpltMNPV-P7

It is of utmost importance to study the ideal time to harvest OBs from infected *S. litura* so as to maximize its virus yield and to minimize yield losses due to the characteristic liquefaction of the infected larva at the terminal stage of infection, which results in the release of OBs in the environment. In this study, the average OB yields in

Table 2. Average number of occlusion bodies (OBs) of nucleopolyhedrovirus SpltMNPV-P7 harvested at 6 days post infection from fifteen *Spodoptera litura* larvae infected at 9 days old with various inoculum concentrations

Inoculum Dose (OBs/larva)	Average OBs Produced[1]	Standard Deviation
10	2.689×10^8 a	$\pm 0.677 \times 10^8$
100	2.767×10^8 a,b	$\pm 0.432 \times 10^8$
1,000	3.326×10^8 c	$\pm 0.522 \times 10^8$
10,000	4.167×10^8 d	$\pm 0.361 \times 10^8$

[1] Values followed with different letters are significantly different at $\alpha = 0.05$ based on Kruskal-Wallis test followed by Dunn's Multiple comparison test.

Table 3. Average number of occlusion bodies (OBs) of nucleopolyhedrovirus SpltMNPV-P7 harvested at different periods post infection from fifteen *Spodoptera litura* larvae infected at 9 days old with 10,000 OBs/larva

Harvest Time (Days post infection)	Average OB Production[1]	Standard Deviation
5	3.956×10^9 a	$\pm 0.655 \times 10^9$
6	7.004×10^9 b	$\pm 1.513 \times 10^9$
7	6.822×10^9 b	$\pm 1.756 \times 10^9$

[1] Values followed by different letters are significantly different at $\alpha = 0.05$ based on Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. OBs were harvested from fifteen randomly selected larvae in the three replicates. Three trials were done with three replicates per treatment per trial. Average OB production was estimated from nine counts per replicate per treatment.

9 days old *S. litura* larvae infected with 10,000 OBs/larva and harvested at 5, 6 and 7 DPI are shown in Table 3. As shown, average OB harvest at 6 DPI was highest ($7.004 \times 10^9 \pm 1.513 \times 10^9$), the least harvest at 5 DPI ($3.956 \times 10^9 \pm 0.655 \times 10^9$), and the second highest average OB yield was at 7 DPI ($6.822 \times 10^9 \pm 1.756 \times 10^9$). However, average OB yield difference was only significant between 5 DPI and 6 DPI or 7 DPI but not between 6 DPI and 7 DPI.

The primary cycle of nucleopolyhedrovirus replication occurs in the midgut cells at the first three days post infection. This can be divided into three phases, namely: early, late and very late. OBs are produced during the late phase of the primary cycle of replication but production is still low (Miller, 1997). The secondary infection starts immediately in other tissues, such as hemolymph, muscle cells and malphigian tubules, once the budded viruses produced during the late phase of primary infection are released to infect adjacent cells. By this time, virus replication doubly increases, thus, producing more OBs. At the final stage of NPV infection, the cells lyse due to production of cathepsin and chitinases by the NPV, releasing the OBs to the environment (Hawtin et al., 1997). At 7 DPI, average larval mortality was 28.89% and

liquefaction of infected cadaver already occurred, thus, leading to lower OB yield due to release to the environment. However, at 6 DPI the average larval mortality was only 11.67% and the infected cadaver are still intact, thus, preventing OB loss due to liquefaction. This results in more OBs harvested at 6 days post infection from 9 days old larvae infected with 10,000 OBs per larva.

SUMMARY AND CONCLUSION

To date, *in vivo* infection remains the most practical method of NPV mass production. Results of the studies conducted showed that adoption of the most appropriate larval age, inoculum concentration and post harvest time combination could further improve *in vivo* OB yield. Among the three *S. litura* larval groups (6, 9, 12 days old) tested, 9 days-old larvae gave the highest NPV yield. Among the tested inoculum concentrations (10; 100; 1,000; 10;000 OBs/larva), 10,000 OBs/larva gave the highest NPV yield in *S. litura* larvae infected at 9 days old. Higher NPV harvest was obstained at 6 days post infection compared to those at 5 or 7 days, thus it is the most ideal time to harvest the infected insects for NPV production optimization. Based on results of the study conducted, it can be concluded that the most appropriate larval age, inoculum concentration and post-infection harvest time for optimum OB yield of SpltMNPV-P7 in *S. litura* are 9 days, 10,000 OBs/larva and 6 DPI, respectively.

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