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MASS PRODUCTION OF NUCLEOPOLYHEDROVIRUS OF THE SATIN MOTH *LEUCOMA SALICIS* (LESANPV)

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Abstract: Nucleopolyhedrovirus (NPV) of the satin moth *Leucoma* (=*Stilpnotia*) *salicis* L. was produced by infecting the larvae with the LesaNPV strain obtained from epizootic center in Katowice. The infected larvae were reared under laboratory, greenhouse and insectarium conditions. Because *L. salicis* can not be reared on a semi-synthetic food, the insects were maintained on natural products. Efficiency of the mass virus production depended on an insect growth stage, virus concentration and number of infected larvae in a rearing container. The fourth-instar larvae were the best for LesaNPV replication. Inoculation of younger larval stages (third instar stadium) provided less number of inclusion bodies (insects were dying sooner and did not meet their maximum body weight). On the contrary inoculation of older stages (fifth and sixth instars) resulted in slower virus replication and low larva mortality. The virus concentration of 3×10^9 of inclusion bodies per container was the optimum inoculum for the mass virus production (double infection with the virus concentration of 1.5×10^9 inclusion bodies/1000 larvae). The larvae reared at high-density became more infected and it caused their earlier death and in consequences low virus efficiency. Rearing the insect at low density (less than 10 larvae per 1.0 L container) was conducive for both an increase of insect body mass and virus replication as well. The highest number of inclusion bodies per one larva (5.3×10^9–7.7×10^9) and the highest total number of inclusion bodies (152×10^{11}–188×10^{11}) were achieved under these rearing conditions in a greenhouse and insectarium.

Key words: *Leucoma salicis* L., nucleopolyhedrovirus (LesaNPV), efficacy of production, study of insect growth, density of larvae

INTRODUCTION

Mass virus replication might be carried out either *in vitro* or *in vivo*. While virus replication *in vitro* in cell cultures is expensive and rarely performed, replication *in*

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vivo is a common practice, also because it does not require highly specialized equipment. The insects are maintained on a semi-synthetic medium or natural diet and then fed with the food infected with virus. Virus replication in the insects reared on a semi-synthetic medium is convenient due to off-season availability of the insects. However, it does not concern all the insect species. The satin moth produces a single generation per year and can be reared only on natural food sources. Numerous efforts undertaken in the highly regarded research centers to develop a semi-synthetic medium for the satin moth rearing found this task very challenging and failed so far.

Establishing the most favoring conditions for the insect rearing and virus replication is very important in the mass production of a pathogen. Food, virus strain and a larval stadium of infected insects as well as density of insect population in a container are the most significant factors.

Rearing of *L. salicis* is very difficult due to specific food preferences of this species. The insects will eat only poplar leaves (particularly *Populus nigra* L.). Lack of the semi-synthetic medium makes the rearing even more complicated because *L. salicis* needs to be reared continuously on a natural diet (Ziemnicka 1981; Lameris *et al.* 1985; Grijpma 1989). Feeding of the satin moth on some poplar species such as *Populus alba* L. and *Populus tremula* L. and willows such as *Salix elegantissima* Koch and *Salix purpurea* L. results in the death of the insects caused by bacterial infection (Wagner and Leonard 1979; Ziemnicka 1981; Avtzis 1990). Hormone instability was also observed among the satin moth maintained on *Populus trichocarpa* L. (Grijpma 1989).

The main objective of the study was to establish the best conditions for the mass virus production (LesaNPV) with particular attention to the selection of larval stages and rearing conditions.

MATERIALS AND METHODS

Insect collection sites

The satin moth (*L. salicis*) larvae were collected from an area with high insect infection occurring on black poplar tress in Poznań and Kórnik region.

Virus isolation

LesaNPV virus was extracted from the bodies of satin moth larvae that died of NPV infection. They were collected from an epizootic center appearing in Katowice. The dead insects were homogenized in large amount of distillated water and then filtered three times through cheese clothes. The filtrate was centrifuged at 500 rpm (30 x g) for 15 minutes. The pellet was resuspended in distillated water with Triton X-100 (final concentration 0.001%) and again centrifuged at 1500 rpm (270 x g) for 15 minutes. This step was repeated several times until a clear supernatant was obtained. Each time after centrifuging the pellet was evaluated for its clarity under a light microscope (400x). The cleared pellet of inclusion bodies was placed in small amount of sterile distillated water and stored at +4°C.

Inoculation, rearing and collection of infected insects

Poplar leaves (*P. nigra*) sprayed with the virus suspension were placed in Erlenmeyer flasks filled with water. When water evaporated the leaves were moved into the large glass vessels (each 1–10l). The infected larvae were reared in laboratory con-

ditions at 20–24°C and 35–45% relative humidity and 18:6 photoperiod, in a greenhouse conditions at 20–28°C, 40–70% relative humidity and the same photoperiod and in an insectarium at conditions corresponding to the weather conditions in May and June in Poland.

In the greenhouse rearing conditions, the leaves sprayed with the virus suspension were placed in the glass vessels and in the glass cage with a double net (60 x 40 x 70 cm).

The larvae were placed on the dry leaves and the containers were either closed with the lids or covered with cheese clothes. The infected food was provided again after the larvae ate the first supply. The third and fourth leaf samples were not treated with the virus. Dead and dying insects were collected and stored at +4°C without water.

Collection and standardization of virus powder

The purified LesaNPV virus obtained from the replication in laboratory, greenhouse and insectarium conditions was dried in a thermostat at 25–30°C for 7–10 days until water entirely evaporated. The sediment was ground in porcelain mortal into fine powder and then standardized i.e. an enumeration of inclusion bodies (PIBs) per unit weigh in mg was performed with a hemocytometer (Bürker, Marienfeld, Germany) following the method described by Lipa and Śliżyński (1973). The viruses were stored in plastic containers at temperature +4°C.

Biological evaluation of virus powder activity, statistical analysis

Biological activity of the virus was evaluated based on a probit analysis that computes mean of mortality concentration LC_{50} according to Finney method (Lipa and Śliżyński 1973) and mean mortality time LT_{50} according to Litchfiled and Wilcoxon method (Śliżyński and Lipa 1973). The results were compared by statistical analyses with the t-Student test and analysis of variance ANOVA.

RESULTS

Effect of insect growth stage on the efficacy of virus LesaNPV replication

Rearing conditions, an insect growth stage and a virus dose are the crucial factors for the most efficient mass virus replication. The satin moth life cycle consists of several larval stages and the third, fourth and fifth instars were the most suitable for the study (Table 1). The larvae were infected with the virus at five different concentrations; $1x10^6$–1×10^{10} PIBs/90 larvae. The third larval stadium was the most susceptible to an infection and the fifth stage was the least. The corresponding values of LC_{50} are; the lowest 1.8×10^6 PIBs/90 larvae and the highest 1.8×10^{10} PIBs/90 larvae. The analysis of a mean mortality time showed that the larvae at the stage L_3 were dying the fastest (7.7 days) and at the stage L_5 the slowest (19.1 days) (Table 2).

The detailed data on efficacy of virus replication in the satin moth larvae at various larval stages are presented in the Table 3. The results indicate that the highest number of inclusion bodies per one larva and per weight mass unit was obtained from the larvae infected at the fourth instar larval stage. The number of inclusion bodies per mg of body mass of *L. salicis* infected at L_4 is 7-fold higher than for the insects infected at L_3 stage. Such a high number of inclusion bodies per body mass unit allowed to collect the highest amount of virus powder (1.25 g/300 larvae). Efficacy in-

dices calculated for the examined stages L_3, L_4 and L_5 were 16, 176 and 4 respectively. Based on these results, the larval stage L_4 and the virus concentration 10^9 were chosen as the most suitable for further study on the mass virus production.

Table 1. Mean mortality concentration (LC_{50}) of the satin moth (*L. salicis*) infected with LesaNPV at different larval stages

larval stage	No. of examined larvae	Virus conc. PIBs/90 larvae	LC_{50} PIBs/90 larvae	Confidence interval [95%]	Regression equation y = bx + a
L_3	450	1×10^6 1×10^7 1×10^8 1×10^9 1×10^{10}	1.8×10^6	$6.2 \times 10^5 – 5.0 \times 10^6$	y = 4.21x + 2.37
L_4	450	1×10^6 1×10^7 1×10^8 1×10^9 1×10^{10}	3.4×10^8	$6.4 \times 10^7 – 1.8 \times 10^9$	y = 1.74x + 3.52
L_5	450	1×10^6 1×10^7 1×10^8 1×10^9 1×10^{10}	1.8×10^{10}	$3.1 \times 10^9 – 1.0 \times 10^{11}$	y = 2.99x + 1.94

Rearing of the satin moth in laboratory conditions in the glass containers (30 larvae/1 l)
Three replications with 30 larvae/concentration
LC_{50} calculated 12 days after infection

Table 2. Mean mortality time (LT_{50}) of the satin moth *(L. salicis)* infected with LesaNPV at different larval stages

larval stage	No. of examined larvae	LT_{50} value [days]	Confidence interval [95%]	Regression equation y = bx + a
L_3	90	7.7	7.4–8.0	y = 5.43x + 0.18
L_4	90	11.4	10.8–12.0	y = 3.77x + 1.01
L_5	90	19.1	14.1–25.7	y = 1.75x + 2.76

Rearing of the satin moth in laboratory conditions in the glass containers (30 larvae/1 l)
Three replications with 30 larvae/larval stage
Virus concentration 1×10^9 PIBs/90 larvae

Table 3. Efficacy of LesaNPV replication at different larval stages of the satin moth (*L. salicis*) larvae

Larval stage	No. of examined larvae [Ni]	No. of provided inclusion bodies (PIBs)	No. of dead larvae (in %)					Weight of virus powder [in mg]	Total No. of PIBs [PIBc]	PIBs/mg powder	PIBs/dead larva	PIBs/mg insect body weight	Efficacy index [WW]
			total [Nz]	larval stage									
				L_3	L_4	L_5	L_6						
L_3	300	3×10^9	297 (99.0)	147 (49.0)	144 (48.0)	6 (2.0)	0 (0.0)	119 a	4.7×10^{10} a	4.0×10^8 a	0.2×10^9 a	0.5×10^7 a	16
L_4	300	3×10^9	251 (83.7)	–	140 (46.7)	89 (29.6)	22 (7.3)	1255 b	63.0×10^{10} b	5.0×10^8 a	2.5×10^9 b	3.9×10^7 b	176
L_5	300	3×10^9	42 (14.0)	–	–	15 (5.0)	27 (9.0)	167 a	8.0×10^{10} a	4.8×10^8 b	1.9×10^9 b	1.2×10^7 c	4

Rearing of the satin moth in laboratory conditions in the glass containers (100 larvae/6 l)
Three replications with 100 larvae
Numbers followed by the different letter differ statistically
[WW] = [Nz]/[Ni] x [PIBc]/[PIBi] where [PIBi] = 3×10^9

Table 4. Amount of obtained virus material in correlation to population density of the satin moth (*L. salicis*) in greenhouse conditions

No. of larvae/ 1L	No. of examined larvae [Ni]	No. of dead larvae (in %)				Total body mass of dead larvae [in g]	Weight of virus powder [in mg]	Total No. of PIBs [PIBc]	PIBs/mg powder	PIBs/dead larva	PIBs/mg insect body weight	Efficacy index [WW]
		total [Nz]	larval stage									
			L_4	L_5	L_6							
6.0	1000 x 3 **	2450 (81.6)	567 (18.9)	1153 (38.4)	730 (24.3)	557.4 c	36.6 b	188.0×10^{11} c	5.1×10^{8} a	7.7×10^{9} c	3.4×10^{7} b	1705
9.5	1600 x 3 **	4387 (91.4)	2164 (45.1)	1488 (31.0)	735 (15.3)	424.1 b	28.7 b	139.1×10^{11} b	4.8×10^{8} a	3.2×10^{9} b	3.3×10^{7} ab	1413
18.6	186 x 3 *	429 (76.9)	273 (48.9)	129 (23.1)	27 (4.8)	37.2 a	2.4 a	11.6×10^{11} a	4.8×10^{8} a	2.7×10^{9} a	3.1×10^{7} a	99
28	280 x 3 *	680 (80.9)	306 (36.4)	199 (23.7)	175 (20.8)	53.3 a	3.3 a	16.3×10^{11} a	4.9×10^{8} a	2.4×10^{9} a	3.1×10^{7} a	147

Insect rearing conditions: * glass vessels (10 l); ** cages (168 l). Examined stage L_4
Numbers followed by the different letter differ statistically
Total number of provided inclusion bodies per 3 cages/glass vessels was 9×10^{9}
[WW] = [Nz]/[Ni] x [PIBc]/[PIBi] where [PIBi] = 9×10^{9}

Effect of density of larvae on efficacy of virus LesaNPV replication

Due to a demand of collecting high amount of virus material the rearing conditions of the satin moth were changed from laboratory to greenhouse and insectarium. The density of insect population in the rearing containers is one of the most significant factors affecting efficacy of this process.

The insects were maintained in the greenhouse in the cages of 168l capacity and the glass vessels each of 10l capacity at different densities (from 6 to 28 larvae/l). The results presented in the Table 4 show some regularity. The larvae infected with the virus and reared at a low density of 6 larvae/l lived longer and died at the stage L_5–L_6. The larvae kept at higher density died earlier at the stage L_4–L_5. The LT_{50} value presented in the Table 5 confirmed these data. The mean mortality time at the density of 6 larvae/l was 12.6 days and it was twice longer than the mean mortality time for the density of 28 larvae/l. Besides, the larvae reared at the low density not only lived longer until the fifth instar larval stage but also had greater increase of mean body mass than these living at higher density (557.4/2450=0.228 g; 53.3/680=0.078 g; respectively), (Table 4). The larvae that were reared at the low density gave the highest total number of inclusion bodies (188.0 x 10^{11}) and number of inclusion bodies per one larva (7.7 x 10^9). The data from the study carried out in the insectarium conditions showed similar relations (Tables 6, 7). However, the indices were higher for the satin moth reared in the greenhouse than in the insectarium conditions.

Table 5. Mean mortality time (LT_{50}) of the satin moth (*L. salicis*) larvae infected with LesaNPV and reared at different population density

No. of larvae/ 1l	No. of examined larvae	No. of provided inclusion bodies [PIBs]	LT_{50} value [days]	Confidence inetrval [95%]	Regression equation y = bx + a
6.0	1000 x 3 **	9 x 10^9	12.6	12.3–12.9	y = 5.01x - 0.50
9.5	1600 x 3 **	9 x 10^9	11.4	11.0–11.8	y = 3.83x + 0.96
18.6	186 x 3 *	9 x 10^9	9.7	9.3–10.2	y = 5.47x - 0.41
28	280 x 3 *	9 x 10^9	6.9	6.5–7.3	y = 4.24x + 1.44

Insect rearing conditions: * glass vessels (10 l); ** cages (168 l). Examined stage L_4

Table 7. Mean mortality time (LT_{50}) of the satin moth (*L. salicis*) larvae infected with LesaNPV and reared at different population density in insectarium

No. of larvae/ 1l	No. of examined larvae	No. of provided inclusion bodies [PIBs]	LT_{50} value [days]	Confidence inetrval [95%]	Regression equation y = bx + a
6.5	1100 x 3 **	9 x 10^9	12.2	11.8–12.7	y =3.16x + 1.57
9.6	1616 x 3 **	9 x 10^9	12.0	11.6–12.5	y = 3.75x + 0.95
11	110 x 3 *	9 x 10^9	8.6	8.1–9.1	y = 3.00x + 2.19
28	280 x 3 *	9 x 10^9	6.2	5.7–6.7	y =3.32x + 2.37

Insect rearing conditions: * glass vessels (10 l); ** cages (168 l). Examined stage L_4

Tabela 6. Amount of obtained virus material in correlation to population of the satin moth (*L. salicis*) in insectarium conditions

No. of larvae/ 1L	No. of examined larvae [Ni]	No. of dead larvae [in %]				Total body mass of dead larvae [in g]	Weight of virus powder [in mg]	Total No. of PIBs [PIBc]	PIBs/mg powder	PIBs/dead larva	PIBs/mg insect body weight	Efficacy index [WW]
		total [Nz]	larval stage									
			L_4	L_5	L_6							
6.5	1100 x 3 **	2892 (87.6)	703 (21.3)	1350 (40.9)	839 (25.4)	616.2 c	30.9 b	152.4 x 10^{11} c	4.9 x 10^8 a	5.3 x 10^9 c	2.5 x 10^7 c	1483
9.6	1616 x 3 **	4650 (95.9)	2302 (47.4)	1346 (27.8)	1002 (20.7)	515.3 b	25.1 b	125.6 x 10^{11} b	5.0 x 10^8 a	2.7 x 10^9 b	2.4 x 10^7 bc	1338
11	110 x 3 *	282 (85.5)	177 (53.7)	87 (26.4)	18 (5.4)	32.7 a	1.5 a	7.3 x 10^{11} a	4.9 x 10^8 a	2.6 x 10^9 b	2.2 x 10^7 b	69
28	280 x 3 *	672 (80.0)	531 (63.2)	84 (10.0)	57 (6.8)	76.5 a	2.9 a	14.8 x 10^{11} a	5.1 x 10^8 a	2.2 x 10^9 a	1.9 x 10^7 a	132

Insect rearing conditions: * glass vessels (10 l); ** cages (168 l). Examined stage L_4
Numbers followed by the different letter differ statistically
Total number of provided inclusion bodies per 3 cages/glass vessels was 9 x 10^9
[WW] = [Nz]/[Ni] x [PIBc]/[PIBi] where [PIBi] = 9 x 10^9

DISCUSSION

The highest efficacy of the virus mass production can be achieved while using the strain of high virulence. The Polish strain of LesaNPV applied in the presented paper originated from the insects collected in Katowice and was highly virulent, and easily replicating in host tissues. The studies of Lameris and others (1985) revealed that the Polish strain was seven times stronger than the Yugoslavian. Its inclusion bodies contain more virons than the inclusion bodies of other *Lymantriidae*. Ciuhrii (1996) using an electron microscopy showed that there were 2096 virions in each single Polish inclusion body. It is 3.4 and 1.7 times more than in Canadian and Moldavian strain *Lymantria dispar* L. of NPV virus, and 3.2 more than in Moldavian strain of *Euproctis chrysorrhoea* L. of this virus and 1.5 times more than in American strain of *Orgyia leucostigma* (J.E. Smith) of NPV. Only the inclusion bodies of the Moldavian strain of *L. salicis* had more virons by 1.2 times than the Polish strain LesaNPV.

Another important factor in the virus mass production is the stage of the insect life cycle. The amount of biologically active virus that replicates in the host tissues is depended upon the insect growth stage. The larvae infected too early die before they reach maximum body mass. On the contrary, at late infection fewer tissues get infected because there is not enough time for a virus replication and the insects turn into a pupa stadium. Thus, the stages third, fourth and fifth instars are the most common for infections. The infection of *L. dispar* at these stages with the virus concentration of 5×10^6 PIBs/ml gave the following amount of inclusion bodies; 5.63×10^8, 1.40×10^9 and 2.86×10^9 respectively (Shapiro 1981). Smith *et al.* (1976) infected the larvae of *L. dispar* at late L_3 larval stage and collected 9×10^8 of inclusion bodies per larva. Głowacka (1989) showed that infection of *Lymantria monacha* L. at stages L_3 and L_4 resulted on average in 3×10^9 PIB/larva.

These data indicate that late infection (the last growth stage shouldn't be used) is better for higher replication of the inclusion bodies. Similar relation was found in the satin moth infected with LesaNPV. It was proved that the best time for infection and maximum virus efficacy was the stage L_4. The larvae at this stage increased the most body weight within about 10 days (Ziemnicka 1981). The duration of this period is similar to the mean mortality time LT_{50} (11.4 days) evaluated in the other assays. The insects infected at the stage L_3 die sooner and thus do not meet critical mass and in consequences there are less inclusion bodies present in their body. The larval stage L_5 is not the best for the infection as at this stadium the larvae are less sensitive and the mean mortality time LT_{50} is high (19 days). The insects have less tissue infected despite a large body mass that they can reach.

Successful infection also depends on an insect population density in a rearing container. Generally it is known that an increase of population density affects the insects' health conditions. Their disease resistance is lower and infections occur more frequently. Such situation has some benefits during an infection outbreak but not for the mass virus production. The insects reared at lower density are better source for virus replication. The data from the greenhouse and insectarium study show that relation. The differences in weather conditions such as wind and daily temperature fluctuation had no effect on the replication process. Lower density was not only more favored for total number of inclusion bodies but number of PIBs per one larva as well. These results were the same as the observations from the study of Shapiro *et al.* (1981) on *L. dispar* larvae and LesaNPV

The method described in this paper of replication of LesaNPV in the greenhouse and insectarium conditions became the basis for the patents for production of virus preparation Virin-LS recommended for *L. salicis* control (Ziemnicka *et al.* 1995a, 1995b).

CONCLUSIONS

1. The greenhouse and insectarium conditions favor the mass production of virus LesaNPV in *S. salicis* larvae.
2. The highest efficacy of production of LesaNPV inclusion bodies was achieved at the low population density (less than 10 larvae per 1l container) and the infection of the fourth instar larval stadium of *L. salicis* (double infection of 1.5 x 10^9 inclusion bodies/leaves/1000 larvae).

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POLISH SUMMARY

MASOWA PRODUKCJA NUKLEOPOLIEDROWIRUSA BIAŁKI WIERZBÓWKI *LEUCOMA SALICIS* (LESANPV)

Nukleopoliedrowirus (NPV) białki wierzbówki *Leucoma* (=*Stilpnotia*) *salicis* L. był produkowany metodą inokulacji gąsienic szczepem wirusa LesaNPV, wyizolowanym z ogniska epizootycznego w Katowicach. Inokulowane owady hodowano w warunkach laboratoryjnych, insektaryjnych i szklarniowych. Ze względu na brak możliwości hodowli owada na półsyntetycznym pokarmie hodowlę prowadzono na pokarmie naturalnym. O wysokiej wydajności w pozyskiwaniu materiału wirusowego decydował dobór odpowiedniego stadium owada, koncentracja wirusa oraz zagęszczenie inokulowanych gąsienic w naczyniu hodowlanym. Stadium L_4 białki było optymalnym do replikacji wirusa LesaNPV. Inokulacja młodszych stadiów larwalnych (L_3) dostarczała mniejszej liczby wtrętów wirusowych (owad zamierał szybciej nie osiągając maksymalnej masy ciała). Z kolei inokulacja stadiów starszych (L_5 do L_6) powodowała zbyt wolną replikację wirusa i niską zamieralność gąsienic. Koncentracja wirusa 3×10^9 wtrętów na naczynie była optymalnym inokulum zastosowanym w masowej produkcji wirusa (dwukrotna inokulacja wirusem o koncentracji około $1,5 \times 10^9$ wtrętów/bukiet liści/1000 gąsienic). Hodowla owadów w dużym zagęszczeniu przyspieszała rozwój infekcji powodując wcześniejsze zamieranie gąsienic, co w efekcie dawało niską wydajność. Hodowla owada w niskim zagęszczeniu (poniżej 10 gąsienic na litr pojemności naczynia hodowlanego) była korzystna zarówno dla zwiększenia masy ciała owada jak i replikacji wirusa. W takich warunkach hodowli (insektarium i szklarnia) uzyskiwano najwyższą liczbę wtrętów wirusowych przypadających na jedną gąsienicę ($5,3 \times 10^9$–$7,7 \times 10^9$) oraz najwyższą łączną liczbę wtrętów (152×10^{11}–188×10^{11}).