IMPROVING BACULOVIRAL PREPARATION FOR THE CONTROL OF THE FALL WEBWORM MOTH (*Hyphantria cunea* DRURY)

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Abstract. Potential agricultural production loss caused by the activity of pest organisms in the world was always a concern at international level. In many countries there were established viral preparations applied in practice in order to reduce the number of pests. In such context, the problem is connected to large application of baculoviral preparations that have become a reality only by elaboration and organization of production of such biological means, work registered after execution of deep biotechnological researches. They are ubiquitous in the environment and are known to be an important contributor to insect population regulation. These characteristics make them good candidates for the management of crop and forest insect pests with minimal or no off-target impacts. Commercial production of baculoviruses for the use as biological control agents of insect pests is carried out worldwide at different scales depending on the market. In order to obtain viral insecticides, it is necessary a mass multiplication of phytophagous insect larvae to obtain a significant biomass baculovirus. For a normal result of the infection process it is necessary to ensure optimal conditions of temperature, humidity and aeration. This has been developed by elaborating special technological equipment for the production of viral insecticides in combating *H. cunea*

Keywords: *Hyphantria cunea* DRURY, improving, baculoviral preparation, VG, VPN.

Rezumat. Ameliorarea preparatului baculoviral pentru combaterea omizii păroase a dudului (*Hyphantria cunea* DRURY). Pierderile potenţiale de producţie agricolă cauzate de către activitatea organismelor dăunătoare în lume a fost întotdeauna o preocupare la nivel mondial. În multe ţări au fost puse la punct preparate virale care se aplică în practică pentru reducerea numărului insectelor nocive. În acest context, problema este legată de aplicarea largă a preparatelor baculovirotice care a devenit o realitate doar prin elaborarea şi organizarea producerii a asemenea mijloace biologice, lucru înregestrat după efectuarea cercetărilor biotehnologice profunde. Ele sunt prezente în mediu și sunt cunoscute ca un factor important care contribuie la reglementarea populației de insecte. Aceste caracteristici sunt bune exemple pentru gestionarea insectelor dăunătoare la plantele agricole şi forestiere cu impact minimal. Producția comercială a baculovirusurilor pentru utilizarea ca agenți de control biologic al dăunătorilor se efectuează la diferite niveluri, în funcție de piață. La obținerea insecticidelor virale este necesară îmulţirea în masă a larvelor de fitofagi pentru a obține o biomasă semnificativă de baculovirusuri. Pentru obţinerea rezultatului normal a procesului de infecție este necesar de-a asigura condiții optime de temperatură, umiditate și aerare. Acest lucru a fost dezvoltat cu elaboratrea echipamentelor speciale tehnologice pentru productia insecticidelor virale în combaterea *H. cunea.*

Cuvinte cheie: *H. cunea,* ameliorarea, preparat baculoviral, VG, VPN.

INTRODUCTION

Over the last years, the global environmental and health issue has a central role in the social life of the modern civilization. For that reason, ecological agriculture spreads largely, covering more states and continents. Over the last decades, there have been consolidated the researches in the application of ecological agriculture that, among many activities, pursue the transition from chemical protection to the application of alternative methods of control of pest organisms.

The necessity of control of pest organisms is determined by the fact that agricultural crops are attacked by a wide range of pests (insects, mites, pathogens of diseases). Cultivation loss is 25-30%, and sometimes it exceeds 40-50 %, or in some cases, agricultural crops are completely compromised. At the international level, cultivation loss is $50 trillion, and in the Republic of Moldova it exceeds 2 billion lei (VOLOŞCIUC, 2007; 2009).

In order to reduce the impact of pest invasions there are elaborated technologies of control and minimization of the output of crops that need presentation of a wide range of biological means alternative to chemicals, ensuring increased biological parameters. That requires the elaboration of methods of selection and improving of biological efficiency, modifying the parameters of individual development both of housing plants and of the capacities of agents used for controlling pests (BEKAGE & DREZEN, 2012).

Microbiological products have an important role in the biological control. Application of entomopathogenic baculoviruses aims at a big number of pathogens that produce in ~~the~~ nature epizootics on large surfaces. Large scale application of viruses that have proven their economic advantages in comparison with other microbiological methods of plant protection may put into practice the production of viral insecticides (BERCA, 2004; LENTEREN, 2003).

Baculoviruses only infect insects, are ubiquitous in the environment and are known to be important in the regulation of many insect populations. Baculoviruses are host specific, infecting only one or a few closely-related species, helping them to make good candidates for the management of crop and forest insect pests with minimal off-target impacts (HEWSON et al., 2011; RAYMOND et al., 2005). In fact, baculoviruses have been recognized as being amongst the safest of pesticides and have been included on lists of "low risk" biocontrol agents by the European Union (LEUSCHNER, 2010). As of 2010, over 24 baculovirus species have been reported to be registered for use in insect pest management throughout the world (KABALUK et al., 2010; QUINLAN & GILL, 2006). Market share of baculoviruses is

6% of all microbial pesticides (MARRONE, 2008; QUINLAN & GILL, 2006) and millions of hectares have been treated with registered baculovirus products over the years (KABALUK et al., 2010; MOSCARDI, 2011; SZEWCZYK et al., 2009).

The efficiency of baculoviral insecticides is ensured by an active ingredient and by a number of advantages in comparison with chemical methods, among which the most important is their specificity. Wide application of baculoviral preparations has become a reality only after the organization of their production thanks to deep biotechnological researches. Thus, it has become possible the production of efficient and cheap viral preparations necessary for the control of crop pests (CIUHRII & ARMENESCU-CIUHRII 2008; MATTIAS et al., 2008; VOLOŞCIUC, 2010). Technologies of baculoviral preparation production may be realized on the grounds of mass growth of plant feeder insects (CIUHRII et al., 1990; VOLOŞCIUC, 2000).

In this review of baculoviruses, we discuss how baculovirus evolution, host range determination and pathogenesis have contributed to their inherent safety for non-target organisms including humans. The article represents a generalization aimed to the obtaining of viable preparations and improvement of active strains.

MATERIAL AND METHODS

The researches have been realised on the caterpillars of 2-3 ages of the *H. cunea.* In the study, we used the Nuclear Polyhedrosis Virus, selected and identified in the laboratory of the insect viruses.

For the contamination of the laboratory insects, we used the dosed feeding, which contains 10 polyhedrons for each caterpillar. The monitoring of the insects lot and the estimation of the dead caterpillars has been carried out daily, beginning with the 3rd day of the contamination. The caterpillars *H. cunea* were kept under laboratory conditions at 27˚C.

For the infection of larvae there was necessary a preliminary preparation of viral suspensions, using for that purpose pure or initial suspensions and applying dosed infection of insects according to the Vago C. procedure (1972) and its different modifications (CHUKHRII et al., 1990; 1991).

During the process of identification and determination of biological activity of baculoviruses there was necessary its purification. At initial phases purification of VPN and VG does not differ substantially. Dead larvae were soaked with the help of a mixer, and the biological mass was mixed with sterile filtered bidistilate through an apron screen.

For the purification of VPN there were used several methods, for which we have used the modifications of our institute, consisting of the following phases. Filtered viral suspension is centrifuged within 30 min at 1000 rpm in ŢLN-2 centrifuge. The obtained deposition is washed three times with water. The obtained suspension is centrifuged in the gradient of sucrose concentration (70-20%) and is centrifuged at 3000 rpm within 10 min. Zones with concentration of 40-50% were put together and layered in the gradient of 50-60% and after 15 min of centrifugation there was obtained the fraction of SPVC.

For determination of concentration of baculoviral suspensions there were used different methods, especially electronic microscope (CHUKHRII et al., 1991). Titration of baculoviruses with the help of quantum microscopy depends on the kind of virus. Thus, if VPN may be examined with all kinds of optical microscopes, because they have relatively big size (0.5-10 mcm), then VG having much smaller size (0.01-0.5 mcm), is at the edge of optical microscope resolution, that is why they were mostly treated with the help of electronic microscopes.

For the determination of baculoviral concentration there are used different methods, especially of electronic and optical microscopy (CHUKHRII et al., 1991). Titration is carried out with the help of Goreaiev chamber or in the fixed and coloured preparations. There were elaborated different methods of determination of the biological activity of baculoviruses. At the initial phase, viral suspension is titrated, determining its concentration. Then, there is prepared a series of successive dilutions with the help of which the larvae of the second age are infected (it is rational to use 40 larvae of the same physiological state). After the third day there is determined the morality of larvae by options, and is prepared the diagram of "dose-effect" relation. For that reason there is applied the method of sample analysis. Then, there are made some additional calculations which allow the transformation of axis for obtaining the "dose-effect" relation in the form of straight line, and not in the form of asymmetrical curve. The construction of diagram allows us to determine the logarithm of the viral suspension dose, which ensures the death of 50% of the experimental larvae. Knowing the virus concentration and volume of viral suspension it is easy to determine lethal concentration (CL_{50}).

The mathematical treatment was registered on the 15th day after contamination; the statistical treatment was made according to DOSPEHOV, 1985; GAR, 1963; SĂVESCU & RAFAILA 1978; CIUHRII et al., 1990.

RESULTS AND DISCUSSIONS

Preferential nature denotes the forms of biological control and biological particularities of the baculoviral preparations for the purpose of local epizootic release that would regulate the density of population of *H. cunea* insects.

The reproduction of baculoviruses on the basis of plant feeder insects remains the main way of insect production. That was confirmed by the researches carried out in different scientific and production centres (BEKAGE & DREZEN, 2012; CIUHRII & VOLOŞCIUC, 1988).

The results obtained within some multiple experiments carried out with larvae of the second and the third age of *H. cunea*. lead to the conclusion that surviving of insects and baculoviruses has become possible only at obtaining a

moderate biological activity. Thus, there were registered substantial results at the examination of the biological activity of viral biological mass obtained from larvae which died on different days after infection with the viral suspension (Table 1).

The results rendered in the above table show the difference between the parameters of biological activity of biological mass obtained on different days from the infection with baculoviruses. There are not noticed any substantial differences of biological activity in the case of viral suspension with the same concentration (10^7 pol./ml). Good results were registered at the analysis of lethal time necessary for obtaining a death rate of 50% of larvae (TL_{50}). That parameter has minimal value 0 the first 5 days after infection. In the terms of that aspect, biological mass obtained from dead larvae after these days is characterized by parameters specific to wild strains obtained from natural conditions, that aspect induces the difference of biological activity of biological mass obtained from dead larvae on different days of infection and denotes the possibility of application of that measure in the process of improving baculoviral strains applied for the elaboration of viral insecticides (CIUHRII & VOLOŞCIUC, 1988; VOLOŞCIUC, 2007, 2009). Other authors have also confirmed the results of the investigations in that field, (ADAMS et al., 1991; DENOTH et al, 2002; ILIENIH, 2007).

Table 1. Biological activity of viral suspension obtained from dead larvae on different days after the infection with baculoviruses.

Options	Concentration, Pol./ml	Number of larvae	Biological activity, %	TL_{50}, days
Day 3	10^7	100	91.4	5.3 ± 0.57
Day 4	10^7	100	9.8	5.0 ± 0.41
Day 5	10^7	100	96.4	7.8 ± 0.75
Day 6	10^7	100	92.4	5.6 ± 0.63
Day 7	10^7	100	100.0	6.7 ± 0.72
Day 8	10^7	100	93.5	6.6 ± 0.65
Day 9	10^7	100	97.2	5.5 ± 0.52
Day 10	10^7	100	98.3	7.9 ± 0.73

The results of experiments are expressed in Table 2. The production of SVC was not modified in case ~~when~~ larvae are kept at the temperature of 23°C, 26°C and 29°C, between 1 x $I0^9$ SVC/larva and 1.04 x $I0^9$ SVC/larva. TL_{50} decreases together with the temperature increase: at the temperature of 23°C it was 8 days, at 26°C – 6.5 days, and at 29°C – 6 days. In the option with 32°C in the growth chamber, TL_{50} was of 12 days, and the percent of obtained infected larvae (62%) and amount of SVC has considerably decreased in comparison with other options. To minimise the loss of larva tissue rupture or of establishment a superior infection, we have taken into account also TL_{30}, as a transition moment of the infected larvae in plastic bags in order to be stored in freezer till processing. Thanks to the fact that about 20-25% of SVC obtained from larva tissue remain in the medium used for filtration, that biological material needs to be recovered by repeated suspension in distilled water, increase and filtration.

Table 2. Biological activity of the granulosis virus depending on the temperature in the chamber of larvae growth.

Temperature	Concentration, Pol./ml	Number of larvae	Biological activity, %	TL_{30}, days	TL_{50}, days
23˚C.	10^7	20	78	7.2	8.2
26˚C.	10^7	20	80	6	6.5
29˚C.	10^7	20	82	4.7	6
32˚C.	10^7	20	62	8.5	12

For the purpose of explanation of essential differences of the biological activity of polyhedral, there were carried out ultrastructure researches upon them. Ultrastructure analysis of VG H.cunea shows that the size of polyhedral varies a lot. The research data have shown that SVC have an average length of 398.2 - 414.5 nm and an average width between 293.4 and 339.5 nm (Table 3). In the analysed preparations, there were noticed, besides normally formed SVC, other ones different by form and size, the presence of much bigger virion capsids and other ones 2-5 times bigger than the usual ones, of irregular form.

Table 3. Dimensions of isolated superior virion capsids from larvae of *H. cunea* infected with granulosis virus.

Year	Length (nm)			Width(nm)		
	minimum	maximum	average	minimum	maximum	average
2009	232	629	403.1	206	418	314.2
2010	212	593	398.2	198	306	293.4
2011	243	608	414.5	217	483	339.4

Larvae, presenting the symptoms of infection with baculoviruses, could be stored in plastic bags of 0.5 kg, in fridge at temperature of -15 -20°C, before the end of the collection process which was carried out in phases. Vacuum compaction in bags was made for longer time storage: from one week in fridge up to at least two years in freezer (Table 4).

Table 4. Storage time of infected larvae of *H. cunea* depending on the storage method.

Option	Storage method	Temperature	Storage time
Option 1	Plastic bags, in fridge	+ 4°C	Maximum 24 hours
	Plastic bags, in freezer	- 10°C	A few days
	Plastic bags, in freezer	-15°C	Two weeks
	Plastic bags, in freezer	- 20°C	One year
Option 2	Vacuum bags, in fridge	+ 4°C	One week
	Vacuum bags, in freezer	- 10°C	One year
	Vacuum bags, in freezer	-15°C	Two years
	Vacuum bags, in freezer	-20°C	Minimum two years

In this respect, putting into practice of an integrated control system would allow the inclusion of a production process that could be used simultaneously or succeeding, on the background and for the purpose of maximization of the efficiency of restricting action of enthomophagy, pathogens for insurance of big and high quality production, diminishing the registered loss (JOOP VAN LENTEREN, 2011).

Given that all published reviews unequivocally state that baculoviruses are safe and support their use as low-risk biological control agents for the control of insect pests, we propose that human and environmental toxicity tests and studies related to the residual fate of baculoviruses not to be required for the registration of baculoviruses.

CONCLUSIONS

Modern microbiological means, including baculoviral preparations, made of natural biological agents, is characterized by smaller quality parameters, which need its constant improving. In order to obtain efficient baculoviral mass there were proposed efficient techniques of increase of activity parameters, which represent an efficient lever on the way of elaboration of viral insecticides.

The application of viral biological preparations in the field of control of pests induces the initial form of influence and impact in the biological control of insect pests, aspect which in the applicable approach substantially reduces the dependence on wide application, sometimes abusive, of pesticides.

The storage of biological activity of baculoviruses and insurance of ecological and economic efficiency of preparations made on their basis, needs application of deep knowledge regarding the creation of optimal technological conditions for their use in the control of insect pests, synergic action between the baculoviral preparation and natural virus strains, as well as the application of efficient forms of the elaborated preparations.

Following this article we conclude that baculoviruses are safe for animal and human consumption and are, therefore, acceptable for use in the control of insects that cause damage to plants.

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