BIOLOGICAL CONTROL

Stability and Persistence of Two Formulations Containing *Anticarsia gemmatalis* Nuclear Polyhedrovirus (AgMNPV)

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Estabilidade e Persistência de Formulações à Base de Nucleopoliedrovírus de *Anticarsia gemmatalis* (VPNMAg)

RESUMO - A estabilidade e persistência de duas formulações de VPNMAg desenvolvidas pelo Instituto Biológico e Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo foram estudadas. As formulações foram armazenadas em condições ambiente e expostas à radiação ultravioleta em condições de laboratório e à radiação solar em condições de campo. A formulação pó molhável (PM) foi preparada através da impregnação da suspensão de vírus e adjuvantes em inerte mineral (Caolin). A formulação óleo emulsionável (OE) foi preparada pela mistura da suspensão do patógeno com óleo + adjuvantes. Durante 20 meses, com intervalos de 120 dias, amostras das formulações e do vírus original eram adicionadas à dieta artificial de lagartas de quarto ínstar de *Anticarsia gemmatalis* (Hübner). Após 20 meses de armazenamento, a formulação OE perdeu apenas 18,3% de sua atividade original, enquanto a formulação PM teve reduzida sua eficiência para 11,7% após 12 meses. Nenhuma diferença significativa foi observada para as duas formulações quando expostas à luz ultravioleta. Ambas protegeram o vírus quando comparado ao tratamento controle. Em condições de campo a formulação OE teve melhor persistência com cerca de 60% da atividade original presente após 14 dias da aplicação. Grande parte da formulação PM foi removida das folhas pela chuva.

PALAVRAS-CHAVE: Insecta, nucleopoliedrovirus, armazenamento e radiação.

ABSTRACT - The stability and the persistence of two AgMNPV formulations developed by the Instituto Biológico and the Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Brazil were studied. Formulations were stored in environmental conditions and exposed to ultraviolet radiation in laboratory and to solar radiation in the field. The wettable powder formulation (WP) was prepared by impregnating viral suspensions + adjuvants on inert mineral, chiefly kaolin. The emulsifiable oil formulation (EO) was prepared by mixing the pathogen suspension with corn oil + adjuvants. At 120-day intervals for 20 months, samples of formulations and the standard treatment (crude virus) kept in a laboratory cabinet were added to artificial diet fed to 4th-instar *Anticarsia gemmatalis* (Hübner) larvae. After 20 months in storage, the formulation EO lost only 18.3% of its original activity, whereas the formulation WP had its efficiency reduced to 11.7% after 12 months. No significant differences between the two formulations were observed when exposed to the ultraviolet germicidal light, and both formulations protected the virus when compared to the standard treatment. Under field conditions, the EO formulation enhanced the viral persistence, with >60% of the original activity present 14 days after the application. Most of the WP formulation was removed from leaves by the rain.

KEY WORDS: Insecta, nuclearpolyhedrovirus, storage, ultraviolet radiation.

The expansion of programs for microbial control of pests depends on the large scale production and formulation of the entomopathogens. The formulation of microorganisms, through the addition of inerts and adjuvants, can result in products with better field performance, improved handling and application. Most importantly, formulations must guarantee better viability in storage under ambient conditions with minimum loss of desirable characteristics. Little information exists on the stability of formulated entomopathogens in storage at ambient conditions (Couch & Ignoffo 1981). These authors suggested that a microbial insecticide to be economically viable should remain active for at least 18 months when stored under environmental conditions.

Microbial formulations must allow uniform distribution of the pathogen and keep it viable in the environment for the time necessary for its action. Laboratory and field studies suggest that the solar radiation, especially the ultraviolet portion of the spectrum, is probably the most important factor affecting the persistence of microbial insecticides. This radiation directly affects the nucleic acids, modifying or denaturing them, preventing growth and reproduction of the microorganism (Ignoffo *et al*. 1977, Jacques 1985, Pawar *et al*. 1995). In general, the entomopathogenic viruses are very stable at low temperatures. Viruses can remain highly viable for several years, especially those with intact inclusion bodies stored in insect cadavers, dry powders or in suspensions sheltered from light and kept at 0-4°C (David & Gardiner 1967, Dulmage & Burgerjon 1977, Jacques 1985). On the other hand, the free particle viruses are less stable even at low temperatures.

The baculoviruses may be unstable at environment temperature, but they can remain active for long periods depending on the storage method. Studies on viral storage in Brazil have shown a great variability in results, due to differences in formulations and the duration of such studies (Batista Filho *et al*. 1991, 1994). The microencapsulation and use of ultraviolet protectants with the Heliothis NPV were studied by Ignoffo & Batzer (1971) and Bull (1978). Also, attempts to enhance the photostability of viral products have been carried out with the addition of compounds that absorb or reflect the ultraviolet light (Martignoni & Iwai 1985).

The objective of this research was to evaluate the stability of formulations developed by the Instituto Biológico and the ESALQ, Universidade de São Paulo, Brazil, containing the *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV). The formulations were tested after storage, exposure to ultraviolet radiation in laboratory, as well as sunlight and other climatic factors in a soybean field. Results may contribute to an eventual industrial production and use of this entomopathogen in the field.

Material and Methods

Production and Formulation of Crude AgMNPV. The crude AgMNPV was obtained by differential centrifugation from *Anticarsia gemmatalis* (Hübner) larvae, infected with virus obtained in 1984 from Embrapa Soja, Brazil. This virus was stored in the collection of entomopathogenic microorganisms of the Instituto Biológico, in Campinas, SP, Brazil, under the denomination CB-50. After centrifugation at 8000 rpm, the precipitate was suspended in distilled water. The crude AgMNPV suspension was then formulated as a wettable powder (WP) or a emulsifiable oil (EO) (Table 1). The WP formulation was prepared by direct impregnation of the viral suspensions into kaolin with the addition of adjuvants including wetting and suspending agents. After the impregnation of all components, the material was dried at room temperature and ground with an air mill. The EO formulation was prepared by mixing the viral suspension with refined corn oil, a emulsifier and a feeding stimulant. The mixture was homogenized in a blender.

Stability of the AgMNPV Formulations in Storage at Room Temperature. Three treatments were used: crude AgMNPV, WP formulation and EO formulation. The liquid formulations (EO and crude viral suspension) were stored in glass bottles and the WP formulation in plastic bags. Each sample contained 5 ml or 5 g of product. The material was stored at room temperature, sheltered from light, in a wooden laboratory cabinet. A thermohydrograph was placed in the cabinet to record the air temperature and relative humidity during the storage period.

Table 1. AgMNPV formulations composition.

Wettable Powder	
Suspension of polyhedral inclusion bodies	200 g
Kaolin	500 g
Silica	50 g
Talc	50 g
Sucrose	150 g
Surfactant and suspending agent (Ethoxylated Nonyl Phenol and Naphthalenesulfonic Acid)	50 g
Emulsifiable Oil	
Suspension of polyhedral inclusion bodies	200 g
Refined corn oil	500 g
Emulsifier (Polyoxyethylene Glycol Esters of Fatty Acids)	50 g
Glycerin	50 g
Distilled water	q.s. to 1000 ml

The viability tests were conducted with the fresh preparations and then at 120-day intervals for 20 months. On these occasions, three samples were obtained from each treatment. Each sample was used to inoculate one of the three replicates with 20 4th-instar *A. gemmatalis* (approximately 1.5 cm in length), for a total of 60 insects per treatment. Water suspensions of the formulations were added with an automatic pipette to the surface of artificial diet (Greene *et al*. 1976, minus the formaldehyde) in glass tubes (2.5 cm of diameter x 8.5 cm high). Each tube received 2 x 10^4 polyhedral inclusion bodies (PIBs) and one caterpillar. Insects were incubated at 27±1°C, 75±5% RH and 14h photophase. Insects were observed daily until they reached the pupal stage. A completely randomized design was used. Mortality data were corrected by Abbott's formula (Abbott 1925) and submitted to analysis of variance (ANOVA) after arcsin transformation. The treatments were compared using Fisher's protected least significant difference at 5% level of significance.

Effects of Ultraviolet Radiation on Formulations Under Laboratory Conditions. The level of protection conferred by the formulations to the pathogen exposed to UV light was evaluated in absence of other detrimental factors (rain, temperature, etc.) that occur concomitantly in the field. Soybean leaves (cultivar IAC-8, 12 cm^2 average foliar area) were collected from a field approximately 30 days after planting. The material was disinfested with a 0.2% solution of sodium hypochloride for five minutes, washed with distilled water and dried on paper towels. Batches of 200 leaflets were fixed with insect pins to a Styrofoam board (3000 cm^2) covered with a filter paper. The board was placed, in the vertical position, in a spraying chamber (90 x 50 x 60 cm, with spray nozzle mounted on top), where the treatment applications were conducted. The viral formulations were diluted in sterile distilled water to a concentration of 1.0 x 10^5 PIBs/ml and sprayed to the leaves under constant pressure (0.5 atm). Aliquots of 3 ml were applied to the leaflets to provide a concentration of 1.0 x 10^2 PIBs/cm^2 of foliar surface, and sterile distilled water was used as control.

Samples of 100 leaflets of each treatment were exposed to the germicidal light (253.7 nm UV radiation) for five min. at 25 cm from the radiation source. The other 100 leaflets treated with each formulation were not exposed to UV radiation. Twenty-five leaflets from each treatment and condition (irradiated or not) were used in each of four replicates. The leaflets were placed, individually, in glass tubes (8.5 cm of height x 2.5 cm of diameter) with one 4th-instar *A. gemmatalis.* Tubes with caterpillars were maintained in incubator for 17h until the insects consumed all the treated leaves. After this period, the caterpillars were transferred to glass tubes containing artificial diet and handled as described above.

Insects were observed daily for mortality due to virus and other causes, and for surviving pupae. Mortality caused by the pathogen was analyzed using factorial ANOVA after arcsin-transformation, and means were compared using Fisher's protected least significant difference at 5% level of significance. Effects of the UV radiation were evaluated within and between formulation treatments.

Persistence of the Formulations Under Field Conditions. The protection conferred by the formulations against degradation by solar radiation and washing-off caused by rains was evaluated under field conditions. The field assay was conducted in the Centro Experimental do Instituto Biológico (CEIB/IB), in Campinas, SP (22° 54' South, 47° 5' West, 694 m above sea level). The four treatments were carried out as described before, including a water control. A randomized block design with four replications (25 m^2 plots of soybean cultivar IAC-8) was used. Three lines of border plants were left between plots within block and 4 m were left uncultivated between blocks.

The treatments were sprayed starting at 8:00 AM with a backpack manual sprayer BRUDDEN P5. A concentration of 1.0x10^{11} PIBs/ml and a volume of 200 liters of water/hectare were used. Two hours after spraying, 25 leaflets from the upper third of the plants were collected from each plot and taken to the laboratory. These leaflets were handled as described above for UV experiment. These procedures were repeated one, three, seven and 14 days after application of the formulations to the soybean field. Climatological data including maximum, minimum and average temperatures, number of sun hours and average relative humidity were collected. Mortality caused by the pathogen was analyzed using ANOVA after arcsin-transformation, and means were compared using Fisher's protected least significant difference at 5% level of significance.

Results and Discussion

Stability of the AgMNPV Formulations in Storage at Room Temperature. Temperature and the relative humidity conditions during the storage experiment had little variation with averages of 24.5°C and 64.2% respectively for the 20 months. By 16 months of storage, the liquid preparations (crude virus and EO) had not suffered significant decrease in activity (Table 2). After 20 months in storage, the pathogen formulated in EO had lost only 18.3% of activity, less than half the reduction observed for the virus suspended in water (crude virus). This performance was better than that of similar formulations used by Batista Filho *et al*. (1991) which did not retain 40% of original activity, after 12 months at environmental conditions.

The WP formulation had different behavior, and starting on the eighth month, the activity of this formulation decreased significantly. After 20 months this formulation caused only 5% mortality compared with 80% mortality caused by the EO formulation. Probably, the kaolin used in this formulation was incompatible with the pathogen, deactivating it almost completely within the first year. This result differs from previous work (Batista Filho *et al*. 1991) in which 71.2% of activity was retained with virus impregnated into talc and stored for 12 months. Leucite, another clay tested previously (Batista Filho *et al*. 1994), was more efficient in maintaining the activity of the virus, and retained 73.3% of its original activity after 24 months in storage. One of the factors that could have contributed to the low stability of the WP formulation is the pH of the formulation (Gudauskas & Cannerday 1968). With the inert leucite, the pH value was close to 6.0 whereas for the formulation in kaolin the pH was 9.4.

Table 2. Percent mortality (means ± SEM) of *A. gemmatalis* larvae fed diet treated with AgMNPV formulations stored under environmental conditions for different periods of time.

	Months in storage					
Formulation	0	4	8	12	16	20
Control (water)	0.0 C a	0.0 B a	0.0 C a	0.0 B a	0.0 B a	0.0 C a
Crude virus	83.3 ± 3.33 B c	100 A a	98.3 ± 1.67 A ab	98.3 ± 1.67 A a	95.0 ± 0.00 B c	58.3 ± 4.41 B d
WP	98.3 ± 1.67 A a	100 A a	58.3 ± 6.67 B b	11.7 ± 4.41 B c	10.0 ± 5.00 B c	5.0 ± 2.89 C c
EO	98.3 ± 1.67 A ab	100 A a	98.3 ± 1.67 A ab	83.0 ± 3.33 A bc	93.3 ± 4.41 A abc	80.0 ± 2.89 A c

Data corrected by Abott's formula. Means followed by the same capital letter (within the columns) and small letter (within the rows) do not differ significantly (Fisher's protected least significant difference at 5%).

The stability of the crude virus was significantly affected only after 20 months, with 40% reduction in larval mortality. The remaining activity of the EO formulation, as demonstrated by the capacity to kill 80% of the insects after 20 months in storage, is within the requirements for economically viable formulations (Couch & Ignoffo 1981).

Effects of Ultraviolet Radiation on Formulations Under Laboratory Conditions. Different AgMNPV formulations exposed to UV radiation had their activities significantly reduced by the radiation (F=413.4; P=<0.0001), independent of its components. These results demonstrate the great sensitivity of the pathogen to this radiation (Table 3). Despite the large reduction in mortality of insects treated with irradiated formulations, both formulations were significantly better than the crude virus (WP: F=2.45, P=0.022; and EO: F=2.43, P=0.023). The addition of vegetable oil or clay to the polyhedron suspension allowed better protection of the microorganism and an extension of its activity. Using the same UV radiation (253.7 nm), Jacques (1971) demonstrated fast inactivation of the *Trichoplusia ni* NPV after 10-minute exposure to ultraviolet radiation. A protectant combination including egg albumen and India ink also allowed the virus to survive a 60-minute exposure to the ultraviolet radiation and maintain 74% of its original activity.

Table 3. Percent mortality (means ± SEM) of *A. gemmatalis* larvae fed foliage treated with AgMNPV formulations and exposed or not to the ultraviolet radiation (253.7 nm) for five min.

	Exposure to UV radiation	
Formulation	Not exposed	Exposed
Control (water)	0.0 C a	0.0 C a
Crude virus	92 ± 2.8 B a	19 ± 3.0 B b
WP	99 ± 1.0 A a	37 ± 6.4 A b
EO	98 ± 1.2 A a	37 ± 4.1 A b

Data corrected by Abott's formula. Means followed by the same capital letter (within the columns) and small letter (within the rows) do not differ significantly (Fisher's protected least significant difference at 5%).

The WP and the EO formulations had similar performances after exposure to UV radiation. Because our formulations were chemically and physically very different, the mechanisms that conferred protection may also be different. Jacques (1971) suggested that organic material protects the virus by absorbing the ultraviolet light and by blocking the radiation, due to the color or protein content of these materials. This explains why the impure or raw viral suspensions guarantee certain protection to the pathogen, maintaining it viable for considerable longer time on foliage than purified suspensions (David & Gardiner 1966). The extension of viral activity for two or more days, as by the incorporation of adjuvants to the polyhedron suspensions to provide UV protection, may increase the efficiency of the entomopathogen the field.

Persistence of the Formulations Under Field Conditions. The WP formulation was significantly affected by the precipitation that occurred 6h after application of the entomopathogen in the field. This is demonstrated by the sharp drop (ca. 50%) in mortality of *A. gemmatalis* fed on treated foliage collected just one day after spraying (Table 4). Other formulations were still as active on soybean foliage as on the day of application. The intensity of rain reached 49 mm/h, sufficient to remove considerable proportion of the WP formulation from the foliage but not the other formulations. This conclusion is corroborated by the lack of similar drop in *A. gemmatalis* mortality with the other treatments in the first 24h. Also, previous work with similar WP formulations in the absence of rain (Batista Filho *et al*. 1992a) showed only 9% reduction of the insecticidal activity in the first 24h after application. In contrast with our observations with the WP formulation, other studies with baculoviruses demonstrated that the solar radiation, and not rain, had the most harmful effect on the activity of viral preparations (David & Gardiner 1966, Bullock 1967).

The crude preparation lost 20% activity by the third day, when it was significantly less active than on the day of applica-

Table 4. Percent mortality (means ± SEM) of *A. gemmatalis* larvae fed soybean (cultivar IAC-8) foliage treated with AgMNPV formulations in the field and exposed to climatic factors for 0-14 days.

Formulation	Days after spraying very different				
	0	1	3	7	14
Control (water)	0.0 B a	0.0 C a	0.0 D a	0.0 C a	0.0 C a
Crude virus	98.0±2.00 A a	91.0±2.89 A ab	78.0±4.16 B b	45.0±3.66 B c	32.0±5.16 B c
WP	94.2±2.33 A a	42.2±5.41 B b	40.5±5.10 C b	40.2±5.66 B b	22.0±5.29 B c
EO	93.1±2.92 A a	93.3±4.71 A a	100.0 A a	61.8±5.02 A b	64.1±2.85 A b

Data corrected by Abott's formula. Means followed by the same capital letter (within the columns) and small letter (within the rows) do not differ significantly (Fisher's protected least significant difference at 5%).

tion. The EO formulation had a superior performance when compared with other preparations, and significant losses of the viral activity (31%) were only detected after seven days for this formulation in oil (Table 4). The protective effect conferred by the oil formulation was cleared by the 3rd day after application when it was significantly different from the other two formulations. The high persistence of the EO formulation in the first three days in the field can be due, among others factors, to the low number of sunshine hours during the period (Alves *et al*. 1992), since this formulation was not washed off the plant as the WP formulation and possibly the crude virus were. In previous work with oil formulations of AgMNPV (Batista Filho *et al*. 1992b), the virus activity decreased similarly but at a faster rate, perhaps due to an average 9.1 daily hours of sunshine.

For the WP formulation, there was no decrease in insecticidal activity during this period. This fact corroborates the conclusion that the initial reduction was due to the removal of virus by rain. Between the 7th and 14th days, 101 mm of rain were recorded, most concentrated on the 7th and 8th days. Again, the activity of the WP formulation was significantly affected during this period, in contrast with the activity of the EO and the crude preparation.

Our results demonstrate the protective effect of the crude material due to the presence of fats, proteins and other residues from the insect host. However, given the superior protective action of the EO formulation, its use would allow the application of AgMNPV products during the day, in contrast to the late afternoon applications recommended for the unformulated virus. Late applications, or the EO formulation, minimize the ultraviolet effects and guarantee a longer period of activity of the preparation.

In conclusion, the EO formulation provided good protection to AgMNPV in storage, and when exposed to ultraviolet radiation in the laboratory and climatic factors in the field. Although the WP formulation was adequate in protecting the virus from artificial UV radiation, its persistence in the field was compromised when heavy rains occurred. Also, the WP formulation performed poorly in storage. Given the results presented herein, the oil formulation of AgMNPV is recommended for application in the field. Similar formulation can be produced by commercial companies with reasonable expectation of good results.

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