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EFFECT OF STORAGE ON THE VIRULENCE NUCLEAR POLYHEDROSIS (NPV) EXTRACTED FROM *Spodoptera littoralis (Boisd.)*

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ABSTRACT: ***Nuclear polyhedrosis Inclusion bodies of Spodoptera littoralis (Boisd.) were stored as powders and as water suspensions under different conditions and different periods. Polyhedrosis Inclusion bodies were subject to room elevated temperatures, refrigeration and Freeze dried. Suspensions retained their potency for five years under Freeze dried, and for two years at room temperature. However, freeze-dried powder at 4˚C lasted for five years before losing its potency, slowly .freeze-dried powder and suspensions held at 35 ˚C for six month lost their potency.***

Key word : ***polyhedrosis virus,Storging time, Spodoptera littoralis.***

INTRODUCTION

Insect pathogenic viruses have long been recognized as potentially environmentally safe alternatives to chemical pesticides. The development of widespread resistance to chemicals have also encouraged the development of biopesticides based on insect viruses as a mean of overcoming this problem . Huber (2003) reported that the specificity of the baculoviruses to arthropods has led to them, being the preferred group of viruses for development as pest control agents . Within this group the nuclear polyhedron viruses (NPV) with their ability to infect many species of major lepidopterous pests such as *Heliothis spp*. and *Spodoptera spp.* combined with their faster mode of action, have been studied most (Vail, *et al.* 1999 ; Wood and Granados 1999) . Bergold 2001; Steenberg,T. *et al* 1998; Paschke, J. *et al* 2005; Jones, K. A. *et al* 1999 ; Hughes and Wood, 2002; Huber, J. 2003; Granados, *et al* 2001; Cherry, et al 1999; Breillatt, *et al* 2003 .

Several investigators have found that Nuclear polyhedrosis Inclusion bodies remain inefective for long periods of time. Lynn *et al*.2004 found that silkworm larvae hemolymph that contained polyhedraand had been stored mostly at 4 ˚C was still infective after 15 years . Butani *et al*.1997 ; Eilenberg, 2000 and Steenberg *et al*. 1998 found that polyhedrosis Inclusion bodies lost little or no activity after 9 years . Cherry, *et al.* 1999 found virus in dried *B. mori* retained its virulence for nearly 5 years at 8-14 ˚C and for least 2 years at room temperature .Virulence of *Trichoplusia ni* virus decreased after the 2nd years measured by the number of days required to kill Granados *et al.* 2001 .

The aim of this work is to determine the most practical storage conditions for retaining the potency of *Spodoptera littoralis (Boisd.)* Nuclear polyhedrosis Inclusion bodies (PIB) .

MATERIALS AND METHODS

Preparing of polyhedra Stock:

The concentrated of nuclear polyhedrosis virus (NPV) of the cotton leafworm, *Spodoptera littoralis (Boisd.)* was prepared according to Jons, *et al*.(1999). Newly hatched larvae were used for bioassay .Larvae were tested in groups of 10 / petri dish , there were 5 replicates for each . Each test used 5 groups of 20 untreated larvae as controls. At various times after dosing to determine the effect of dose and incubation time on NPV productivity. Bioassays were carried out using the droplet feeding method modified after Jons, *et al*.(1999). Larvae were allowed to drink from virus suspensions of known concentrations. The concentrated polyhedral suspensions was prepared by isopycnic centrifugation in a K-series centrifuge (Breillatt *et al*. 2003). Suspension were maintained in distilled water, and powders were made suspension that were dried in a freeze-dried vacuum. freeze-dried NPV was prepared by quick-freezing the suspension with dry ice-acetone and by lyophilizing.

Preparing Stock Suspension of Polyhedera :

10-mg sample of powder was placed in a sterile glass tissue homogenizer with 10 ml of steril Tris buffer (25ml of 0.2 M 2-2-amino-2-hydroxymethyl-1, 3-propanediol mixed with 47.0 ml of 0.1 NHCL and diluted to a final volume of 100 ml, pH 7.2). The polyhedral powder and Tris were blended for 3min; 1:100 dilution was prepared from the stock suspension polyhedral counts of the diluted samples were made with an improved Levy-Neubauer hemocytometer . Polyhedra in tested at ambient and elevated temperatures 35˚C had been held for one year at 4˚C before exposure for testing, as had the frozen powder and suspensions (-10 ˚C) .

Bioassay Technique :

PIB's were mixed into the diet (50 ˚C) by a variable speed stirrer (5000 RPM) for 30 sec. Test concentrations ranged from $1x10^7$ to $1x10^2$ PIB / ml of diet. Larvae were fed for 48 h on 1.25 cm^3 pieces of virus containing diet. Control larvae were fed nonvirus diet. Bioassays were conducted at 28˚ ± 2˚ C of light daily. The numbers of living and dead larvae were determined each day, dead larvae were examined microscopically to determine the cause of death andonly larvae containing large numbers of polyhedra were classified as virus killed.

Analysis of data :

Lethal concentrations were calculated by using Bergold (1953) logit x^2 method and a computer program developed by Paschke *et al.* (1968).

RESULTS AND DISCUSSION

Initial viral mortality occurred at 7 day post-inoculation . and all larvae died within 13 days. The average virus yield per larva increased until 11 day, and then remained relatively constant despite an increase in viral-induced mortality (Table 1). A similar trend was observed among living virus–killed cotton boldworm larvae *Heliothis zea* (Boddie). PIBs were extracted from larvae at 7.11days, and 15 when 0, 46, and 100% mortality occurred, respectively. Results indicated that viral potency increased significantly ($P < 0.05$) as the incidence of virus-induced mortality increased from 0 to 100% . Thus , virus from day 13 (100% mortality) was 4 x more active ($P < 0.05$) than virus from days 5, 6 and 7 (0% mortality) and 3x more active ($P< 0.05$) than virus from day 11) 50% mortality) . Similarly ,virus from virus-Killed *Heliothis zea* .. Occlusion bodies yields in *Spodoptera littoralis* reported here agree well with yield given in the literature. Smit (2002) reported maximum yields in *Spodoptera exigus* of $9.0 \pm 2.0 \times 10^8$ (± SA) when dosed in the late fourth instar .

All the bacterial isolated found were identified as belonging tothose species classified in the WHO lowest risk group (Collins and Lyue, 1984) . Most are commonly found free-living in the environment or are commensals in the gut of animals .Two of the isolates were identified as species belonging to a higher risk group (*Stophylococcus aureus* and *Actinomycetes israellii*) .

The only fungal isolate found was a yeast identified as *Torulopsis maris* , which was also found in the samples of healthy insect.

Our results demonstrated that virus potency, as measured by a standardized bioassay, increases in time with virus-induced. larval mortality . Whether this increase in potency is due solely to structural or bioconventional changes of the inclusion body or virions or extraneous factors, e.g. host-derived materials, bacteria. These results have practical implications. When virus is extracted and recovered at the proper time after inoculation, both maximal virus yield as well as maximal virus potency are obtained, resulting in a more efficient production.

Table (1): Yield and Biological Activity of Nuclear polyhedrosis Virus Collected From *Spodoptera littoralis (Boisd.)* Larvae at Different times After Infection .

Days post-infection[a]	Avg. % mortality ± SD mean	Avg. PIB per larvae ($x10^9$) ± SD mean	Avg. viral activity (LC_{50}) ± SD mean*
5	0.0 ±0. 0	0 0.2± 0.03	7.5± 1.9[a]
6	0.0 ± 0.0	1.10 ± 0.20	
7	0.0 ± 0.0	3.22 ± 0.95	
8	5± 1.8	12.7 ± 3.22	2.4± 1.1[b]
9	10 ± 3.7	13.0 ± 2.9	
10	39 ± 4.5	16.4 ± 3.0	
11	48 ± 6.6	22.2 ± 2.1	1.8 ± 0.9[c]
12	90± 7.1	19.9 ± 2.7	
13	100± 0.0	20.1 ± 1.9	

N.B.
a = Virus from day 5, 6 and 7 was used for bioassay . 4 replicates . 50 / larvae virus concentration .
b = Virus from day 8, 9 and 10 was used for bioassay . 4 replicates . 50 / larvae virus concentration .
c = Virus from day 11, 12 and 13 was used for bioassay . 4 replicates . 50 / larvae virus concentration .
*** Means followed by the same letter are not significantly different at the 5% level .**

Table (2) shows age, from and storaging conditions for the 1990 preparations of *S. littoralis* NPV and their changes in activity. However,Table (3) gives similar data for the 1995 preparation. From tables (2,3) there is no changes in activity of 1990 polyhedra in distilled water after 5 year of storage at 4˚C . The freeze-dried powder held at 4˚C seemed did not lose activity after 5 years measured as the LC_{50} dose . Steenberg (1998) reported no difference in activity of material stored for 2 and 5 years at both 4-5˚C and room temperature . However, this investigators demonstrated that virulence decreases as measured by the number of days required to kill after the 5 years storage, though percent mortality does not change appreciably. He also stated that decrease in virulence with age probably is common to most, if not all , polyhedral viruses. Our data differ from these of Jons *et al.* (1999)

Table (2): Form, age, storage, conditions and LC_{50} values of *S. littoralis* nuclear polyhedrosis polyhedra, 1990 preparation.

Storage form	Storage	Storage age	LC_{50} (PIB_s /ml diet) and fiducial limits

	condition	(months)	Lower limit	LC_{50}	Upper limit
Suspension	Frozen	0	$4.9x10^4$	$5.1x10^4$	$6.9x10^4$
		3	$6.3x10^4$	$7.5x10^4$	$8.8x10^4$
	4°C ±1°C	0	$4.9x10^4$	$6.7x10^4$	$7.5x10^4$
		3	$7.5x10^4$	$8.6x10^4$	$9.7x10^4$
		12	$8.4x10^4$	$1.1x10^5$	$5.8x10^5$
		24	$1.0x10^5$	$3.1x10^8$	$5.2x10^5$
		36	$1.9x10^5$	$3.9x10^5$	$6.1x10^5$
		48	$3.2x10^5$	$6.2x10^5$	$7.9x10^5$
		60	$4.2x10^5$	$7.2x10^5$	$9.7x10^5$
	Ambient	0	$4.9x10^4$	$6.1x10^4$	$8.5x10^4$
		3	$6.5x10^4$	$7.4x10^4$	$9.7x10^4$
		12	$7.4x10^4$	$8.5x10^4$	$2.4x10^5$
		24	$8.2x10^4$	$9.5x10^4$	$5.2x10^5$
	35°C ±1°C	0	$4.9x10^4$	$6.7x10^4$	$8.7x10^4$
		3	$2.9x10^6$	$7.2x10^6$	$5.2x10^7$
		6	potency lost	potency lost	potency lost
Powder (Freeze dried)	Frozen	0	$4.9x10^4$	$6.2x10^4$	$8.4x10^4$
		3	$6.7x10^4$	$9.5x10^4$	$2.3x10^5$
		6	$1.2x10^5$	$2.4x10^5$	$5.3x10^5$
	4°C ±1°C	0	$4.9x10^4$	$6.2x10^4$	$8.8x10^4$
		3	$5.3x10^4$	$7.5x10^4$	$9.4x10^4$
		12	$6.1x10^4$	$7.3x10^4$	$3.2x10^5$
		24	$6.5x10^4$	$7.1x10^4$	$2.2x10^5$
		36	$7.2x10^4$	$8.7x10^4$	$3.2x10^5$
		48	$8.4x10^4$	$9.4x10^4$	$5.2x10^5$
		60	$9.2x10^4$	$9.7x10^4$	$6.2x10^5$
	Ambient	0	$4.9x10^4$	$5.4x10^4$	$6.6x10^4$
		3	$5.8x10^4$	$6.3x10^4$	$7.8x10^4$
		12	$6.7x10^4$	$7.1x10^4$	$9.4x10^4$
		24	$7.3x10^4$	$8.2x10^4$	$9.9x10^4$
	35°C ±1°C	0	$4.9x10^4$	$6.8x10^4$	$7.8x10^4$
		3	$5.1x10^4$	$9.4x10^4$	$3.2x10^5$
		6	$9.8x10^5$	$3.2x10^6$	$4.8x10^7$

Table (3): Form, age, storage, conditions and LC_{50} values of *S. littoralis* nuclear polyhedrosis polyhedra, 1995 preparation.

Storage form	Storage condition	Storage age (months)	LC_{50} (PIB_s /ml diet) and fiducial limits		
			Lower limit	LC_{50}	Upper limit

Suspension	Frozen	0	$3.7x10^3$	$5.1x10^4$	$6.8x10^4$
		3	$5.3x10^3$	$7.5x10^4$	$8.6x10^4$
	4°C ±1°C	0	$3.7x10^3$	$6.7x10^4$	$7.9x10^4$
		3	$6.4x10^3$	$8.6x10^4$	$1.2x10^5$
		12	$7.3x10^3$	$1.1x10^5$	$6.8x10^5$
		24	$0.9x10^4$	$3.1x10^8$	$7.1x10^5$
		36	$1.5x10^4$	$3.9x10^5$	$8.6x10^5$
		48	$2.5x10^4$	$6.2x10^5$	$9.9x10^5$
		60	$3.4x10^4$	$7.2x10^5$	$1.2x10^6$
	Ambient	0	$3.7x10^3$	$6.1x10^4$	$7.5x10^4$
		3	$4.5x10^3$	$7.4x10^4$	$8.8x10^4$
		12	$6.4x10^3$	$8.5x10^4$	$1.3x10^5$
		24	$7.1x10^3$	$9.5x10^4$	$6.1x10^5$
	35°C ±1°C	0	$3.7x10^3$	$6.7x10^4$	$9.4x10^4$
		3	$9.1x10^3$	$7.2x10^6$	$4.3x10^8$
		6	potency lost	potency lost	potency lost
Powder (Freeze dried)	Frozen	0	$3.7x10^3$	$5.4x10^3$	$7.4x10^3$
		3	$5.4x10^3$	$8.4x10^3$	$5.2x10^4$
		6	$0.9x10^4$	$3.2x10^4$	$8.8x10^4$
	4°C ±1°C	0	$3.7x10^3$	$5.8x10^3$	$6.8x10^3$
		3	$4.2x10^3$	$6.9x10^3$	$8.4x10^3$
		12	$5.1x10^3$	$7.1x10^3$	$1.2x10^4$
		24	$5.5x10^3$	$6.9x10^3$	$3.2x10^4$
		36	$6.2x10^3$	$7.7x10^3$	$6.2x10^4$
		48	$7.2x10^3$	$8.5x10^3$	$8.7x10^4$
		60	$8.1x10^3$	$8.9x10^3$	$9.1x10^4$
	Ambient	0	$3.7x10^3$	$4.9x10^3$	$5.6x10^3$
		3	$4.4x10^3$	$5.8x10^3$	$6.2x10^3$
		12	$5.2x10^3$	$6.7x10^3$	$8.2x10^3$
		24	$6.2x10^3$	$7.5x10^3$	$9.7x10^3$
	35° ±1°C	0	$3.7x10^3$	$5.8x10^3$	$6.5x10^3$
		3	$4.9x10^3$	$8.2x10^3$	$1.5x10^4$
		6	$8.4x10^4$	$1.9x10^5$	$3.8x10^6$

in that we did not observe a decrease in virulence as measured by days to death. Comparisons were made (Table 3) with the original suspension, the suspension at room temperature for 36 month and the other at 4 ˚C for 5 years . Days to death and percent mortality did not indicate a loss of virulence even at week PIB concentrations. Neither freeze-dried polyhedral

powder held for 5 years nor polyhedral suspensions held for 60 month lost significant activity. Both the powder and suspension held at 35 ˚C for 6 months lost significant potency . The suspension lost all potency after 3 months at 35 ˚C, whereas the powder lost activity for 6 month at the same conditions. Polyhedral counts of the suspension held at 35 ˚C for 3 month did not change . The frozen freeze-dried powder did not lose activity at 3 month. The activity of *S. littoralis* NPV stored under refrigertion (4 ˚C) for 5 years as a suspension , or nearly 5 years as a freeze-dried powder , is comparable to that of fresh material. The 1990 preparation was established to further evaluating the effect of storage on polyhedral powders (Table 4) . As seen in Table 3, the freeze-dried powder held at elevated temperature conditions loses significant activity rapidly . The powder held at ambient temperature shows a quick loss of activity and then a fairly stable activity for nearly 2 years. freeze-dried powder, held at 4 ˚C maintains activity for 5 years and then exhibits deterioration and stabilization for the next year . Frozen freeze-dried powder maintain activity for 6 months .

The data indicate that *S. littoralis* NPV will maintain acceptable activity if stored at 4 ˚C as a suspension and as a frozen freeze-dried powder for five years. Suspension or freeze-dried powder held at ambient temperature will lose activity slowly and preparations held at elevated temperatures will degrade very rapid.

Table (4): Effect of storage on virulence and time of death of *S. littoralis* nuclear polyhedrosis virus

Storage condition	PIB / ML diet	Mortality %	Days of death

1 month	Control	0.00	-
	$1x10^2$	18.0	3
	$1x10^3$	23.1	3
	$1x10^4$	41.0	3
	$1x10^5$	80.2	3
	$1x10^6$	98.0	2
	$1x10^7$	100	2
36 month	Control	0.00	-
	$1x10^2$	17.8	3
	$1x10^3$	24.0	3
	$1x10^4$	42.1	3
	$1x10^5$	81.3	3
	$1x10^6$	97.9	2
	$1x10^7$	100	2
60 month	Control	0.00	-
	$1x10^2$	17.6	3
	$1x10^3$	22.9	3
	$1x10^4$	39.9	3
	$1x10^5$	81.0	3
	$1x10^6$	99.1	2
	$1x10^7$	100	2

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تاثير التخزين على حيوية فيرس (NPV) المستخلص

من يرقات دودة ورق القطن

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الملخص العربي

استهدفت الدراسة المقارنة بين طرق التخزين المختلفة لوحدات فيرس NPV الناتج من يرقات دودة ورق القطن الحية او الميتة و فاعليتها .

اوضحت النتائج ان التخزين وحدات الفبرس فى صورة مساحيق قابلة للبلل و لمدةو خمس سنوات تحت لادرجة حرارة الثلاجة و كذلك المجمدة لم تتاثر حيوتنها كما اوضحت النتائج تاثير الحرارة المرتفعة على وحدات فيرس NPV .