PROTECTION MECHANISM BY LIGNIN ADDITIVES FOR BACULOVIRUSES AGAINST THE NEGATIVE EFFECT OF UV RADIATION

SALAH ELNAGAR[1], MOHAMED A.K. EL-SHEIKH[1], AHMED A. AMIN[2], GILLES FÉDIÈRE[3], ATWA A. ATWA[2] AND MAGDA M. KHATTAB[2]

[1]Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Giza, Egypt.
[2]Plant Protection Research Institute, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza.
[3]Center of Virology, Institut de Recherche pour Le Dèveloppement (IRD) - Faculty of Agriculture, Cairo University, Egypt.

ABSTRACT

The virus suspension containing 1% of any of the tested lignin additives had almost the same pH value of around 7.55. Therefore, it was difficult to state that the pH value of lignin additive played a key role in protecting the virus.

The spectrophotometer tests, showed also that, all the lignin additives were similar in their high rate of UV-absorption (from 210-900 nm), especially in the most effective UV inactivation region (300-320 nm). Scaning Electronmicrograph (SEM) of polyhedra combined with the lignin additive indicated the possibility of physical protection of the polyhedra by the tested additive. The latter appeared to surround the polyhedra with a continuous layer which was visible in the dark field of transmission electromicroscopic examination.

INTRODUCTION

Baculoviruses are most promising biological control agents of lepidopterous insect pests. The deleterious effect of sunlight (UV radiation) on virus deposits in field application has led to investigations on protectant additives to viral formulations. The role of lignin natural product (lignin derivatives LP1, LP2, LP3, LP4 and Mg Lignosulfonate at 1% concentration) was investigates as a protective additive for Baculoviruses (Elnagae *et al.*, 2002). However, the mechanism of protection is yet requires clarification. The spectrum from 290-400 nm is the main factor affecting the persistence of baculoviruses (Ignoffo *et al.*, 1997). The success

of these additives was as well as absorption in (UV-A region 320-400) (Shapiro, 1985). Absorption spectra of products Mg lignosulfonate (10 %) and Tinopal LPW (1 %) showed a high extinction rate in the UV-range (< 440 nm) to which the protection effect is referred (El-Salamouny *et al.*, 2001-2002). Shapiro (1992) concluded no inactivation of NPV occurred if the pH of the NPV + brighteners additives is between 3.5 and 8.7. Also, Argauer and Shapiro (1997) found no correlation between pH and activity enhancement when eight brighteners were compared as enhancers for *Ld*MNPV (pH's of the brighteners ranged from 7 to 10).

Baculovirus preparations containing lignosulfonate showed the highest protection to baculoviruses against UV irradiation (Eglite and Zarin, 1987). The computed half-life values for *Spli*NPV were 74.5 and 2306 minutes with untreated virus, and that treated with the lignin (1%), respectively. Using the *Spex*NPV, the half-life values were 130.8 and 1385.0 minutes for the same treatments, respectively (El-Salamouny *et al.*, 2000). Also, sodium lignin sulfonate (Lignosite$^{(R)}$) was active as a UV protectant when tested with *Anfa*NPV against *S. exigua* in the laboratory (Farrar and Ridgway, 2000) as well as *Hear*NPV and *Spli*NPV (El-Salamouny *et al.*, 2001). Scanning electron micrographs of *Anfa*NPV formulations revealed discrete particles and transmission electron micrographs showed virus embedded within microgranules (Tamez-Guerra *et al.*, 2000).

The purpose of present investigation is to study the mechanism by which lignin additives, provide protection to the virus against UV effect.

MATERIAL AND METHODS

1. Tested additives

1.1. Lignin additive

Four samples of lignin derivatives (LP1, LP2, LP3 and LP4) supplied by Ligmeda, Leipzig, Germany, (Elnagar *et al.*, 2002) were tested in virus suspensions.

1.2. Fluorescent brightener- 28

The fluorescent brightener- 28 (Sigma®) was obtained as a powder. The brightener used was Calcoflour white M2R; Tinopal LPW.

2. Virus purification

Dead larvae of *Spodoptera littoralis* in distilled water at 4°C were blended and the resulting suspension was filtered through several layers of muslin cloth. The

suspension was centrifuged at 1000 rpm for 5 min. and the pellet was discarded. The obtained supernatant was centrifuged twice at 4000 rpm for 20 min.The pellet of NPV was resuspended in Tris-sodium dodecyl sulfate (SDS) 0.1% by centrifugation at 7000 rpm for 10 min. then, resuspended in NaCl 0.9 % at 7000 rpm for 10 min. using Beckman J2-21 MIE centrifuge rotor 20 JA. The collected pellets containing relatively pure polyhedral inclusion bodies (PIB, s) were resuspended in Tris buffer, pH 8 and stored at 4°C, for further purifications. The PIB,s were counted using a haemocytometer slide (B.S. 748 Weber England, Neubauer Depth 0.1 mm, 1/400 mm^2). The pellet with or without lignin was resuspended in Tris buffer (50 mM, pH 7.8) ,deposited on 30-70 % (w/w) continuous sucrose gradient and centrifuged at 15000 rpm for 30 min. using Beckman L7-65 ultracentrifuge, rotor SW 28. The band containing virus was drawn-off with a pasteur pipette, resuspended in Tris buffer (50 mM, pH 7.8), and centrifuged at 10000 rpm for 30 min. using Beckman J2-21 MIE centrifuge, rotor 20 JA (Khamiss,1997). The pelleted highly purified polyhedra were then resuspended in Tris buffer. The viral suspension was stocked in Tris buffer at-20°C for Transmission electromicroscope examination. Polyhedra suspensions plus lignin additives were also subject to sucrose gradient preparation.

3. pH tests

The hydrogen ion concentration (pH) of additive formulations (either F-brightener 1 % or lignin derivative 10 %) was measured using a Beckman Zeromatic SS-3 pH-meter (Beckman Instruments, Fullerton, CA).

4. Spectrophotometer

Spectrophotometer (Lambda EZ 201) was used to determine the absorption of two different concentrations of UV-protectant additives.

5. Transmission electromicrograph

The highly purified (sucrose gradient) suspension of *Spli*NPV stained in 2 % phosphotungistic acid (PTA) pH 7.8 and layered onto special grids (carbon coated) was examined by Phillips (FP 6010/20; EM 208S) transmission electron microscope (TEM) (Smith *et al.*, 1990).The purified stock of *Spli*NPV suspension was layered on sucrose gradient The collected bands were further pelleted and divided into two groups in two separate Ependorf tubes. The first tube was considered as a control (lignin free). 10 % concentration of lignin was added to the second pellet-group of tested *Spli*NPV, which was, layered onto the sucrose gradient. The resulted pellet with or without lignin additive was examined with (TEM).

6. Scanning electron microscope

Scanning Electron Microscope (SEM) was used in examining the suspension of diluted polyhedra with or without additives as well as the additive alone. The tested suspension was mounted on top of stubs and left at room temperature till air dried.The mounted suspention on SEM stubs,was coated with gold (SPi – module™ spatter coater, SPi supplies P.O. Box 656 West Chester, PA 19381 - 0656 USA) and examined with a JEOL, JSM-5200 Scanning Electron Microscope operating at 5KV.

RESULTS AND DISCUSSION

1. Effect of pH of the additives

Table (1) shows pH values of the tested protectant additives. F. brightener - 28(Tinopal LPW) at a concentration 1 % had pH 8, while pH values for lignin additives LP_1, LP_2, LP_3, LP_4 and Mg lignosulfonate at the same concentration (1 %) were 7.55, 7.65, 7.80, 7.50 and 7.60, respectively. However, at the concentration 10 %, the pH values for lignin additives were 4.95, 5.25, 6.95, 5.10 and 5.05, respectively.

TABLE (I)

pH values of certain additives; four lignin derivatives, Mg lignosulfonate and F.brightener-28 tested at two different concentrations (1 % and 10 %).

Tested additive	Additive concentration	
	1 %	10 %
Lignin (LP_1)	7.55	4.95
Lignin (LP_2)	7.65	5.25
Lignin (LP_3)	7.80	6.95
Lignin (LP_4)	7.50	5.10
Mg lignosulfonate	7.60	5.05
F. brightener	8.00	--

All lignin additives tested at1%concentration. had almost the same pH value (about pH 7). Whereas, F. brightener (Tinopal LPW) at1 % concentration was pH 8. In the UV-protectant bioassay, the most effective lignin additive, LP4 (Khattab *et al.*, 2004) had a pH of 7.50 which is nearly the same pH value of the less effective lignin additives; *i.e.* LP1 (pH 7.55), LP2 (pH 7.65) and LP3 (pH 7.80) at a concentration of 1%. The LP4 at concentration 10 % that provides highest degree of

protection was acidic (pH 5.10) as well as all other lignin additives (*i.e.*, pH 4.95), except LP3 that had pH 6.95. Therefore it is difficult to state if pH played a key role in the mechanism of virus protection.

2. UV- absorption by tested additives

Lignin additives (LP_1, LP_2, LP_3, LP_4) and Mg lignosulfonate were diluted (1 % and 10 %) in Tris buffer (pH 8). At 1% concentration, the maximum absorbance occurred from 230-480 nm for all lignin additives. At the concentration of 10 %, all the lignin additives absorbed UV over the complete UV- spectrum (210-900 nm).

In the present study, all lignin additives showed a high rate of absorption (from 210-900 nm) especially in the very effective UV region of the inactivation 300-320 nm at a concentration 1 % or 10 %. F. brightener at concentration 1 % can absorb UV 450 nm. Shapiro (1989) demonstrated that dyes that absorb both UV-B and UV-A radiation were more effective as radiation protectants than those dyes absorb UV-B alone. The brighteners absorb ultraviolet energy and convert it to visible light (Villaume, 1958). In conclusion, the lignin additive that protects the virus from the adverse effects of sunlight (UV light of the wavelengths between 300-320 nm) seems a very promising component in virus formulation for UV-absorbance.

3. Electromicrograph of the status of lignin additive in the polyhedra suspension

During the investigation, many attempts were made; using the transmission electron microscopy (TEM) to reveal the status of *Spli*NPV combined with lignin additive, hence, the mechanism of UV-protection by that additive. In one successful examination, the lignin formulation, added to the suspension, appeared surrounding the polyhedra and forming a continuous thin layer around each inclusion body (PIB) of the *Spli*NPV (Fig.1d). This formation was particularly clear in the dark field examination by transmission electron microscopy. On the other hand, the examination of virus-alone suspension did not reveal such formation (Fig.1B). Fig (2) shows the scanning electronmicroscopic examination for the polyhedra either with or without the lignin additive. It is possible that the polyhedra with lignin appeared more rounded (Fig.2-T) unlike the usual appearance of polyhedra alone (Fig.2-C).

In the present study, the examination through TEM of the mixure of *Spli*NPV + lignin additive gives a direct indication that lignin may form a layer surrounding the PIB, which provides the physical protection. This physical formation appeared stable after runs of centrifugation at 15,000 rpm for 30 min. The formation was particulary clear in the dark field examination by TEM of either *Spli*NPV + lignin additive or

lignin alone. On the other hand, the examination of virus- alone suspension did not reveal such formation. It is worth mentioning here, the finding of Tamez-Guerra *et al.* (2000) who reported that transmission electron micrograph of a spray-dried lignin formulation showed a single inclusion body of *Anfa*MNPV surrounded by the formulation ingredients and enclosed within the microgranule. In this respect, lignosulfonate showed the highest adhesion, dispersion and resistance to UV irradiation when added to *Malacosoma neustria* NPV (Eglite and Zarin, 1987).

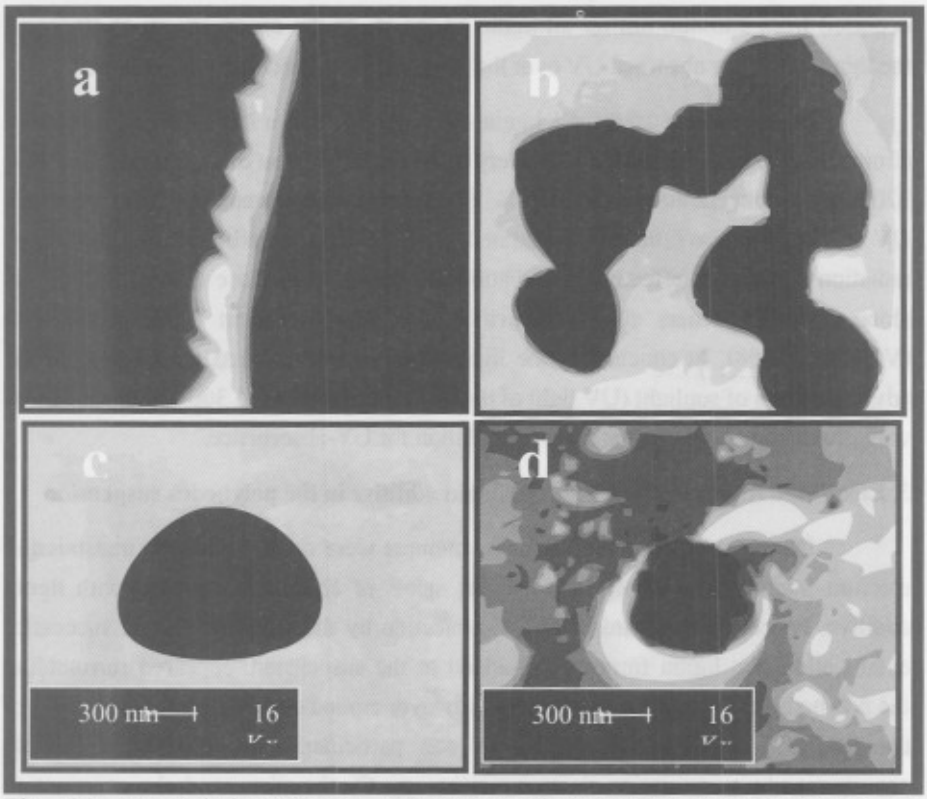

Fig. (1): Transmission electron microscopy of a purified viral suspension of *Spli*NPV mixed with lignin additive (Bar represents 300nm.). a: Lignin alone. b:Virus alone c: Virus + lignin additive examined through bright field. d:Virus + lignin additive examined through dark field.

Comparing the polyhedra with and without additives Fig.2 (a & b) (control and treatment), respectively, the polyhedron with additive appears more rounded

which is probably an indirect indication of lignin adhesion to the surface of the polyhedra. Similar observation was emphasized by Tamez Guerra *et al.* (2000) on the NPV of *Anagrapha falcifera*.

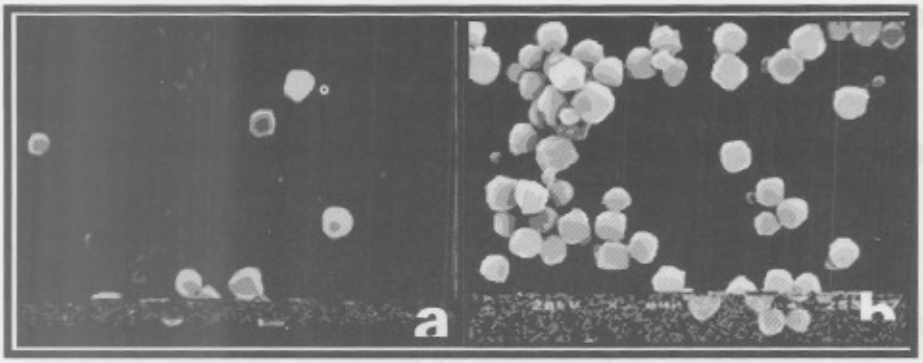

Fig. (2): Scanning electron microscopy of a purified viral suspension of *Spli*NPV mixed with lignin additive. a: Virus + lignin. b: virus alone (untreated control).

The present study indicates evidence on the mechanism of protection provided by lignin additive in virus suspension. Nevertheless, further confirmation is required using sectioning and transmission electron microscope in order to support the present evidence.

REFERENCES

ARGAUER, R. and M. SHAPIRO (1997): Fluorescence and relative activities of stilbene optical brighteners as enhancers for the gypsy moth (Lepidoptera: Lymantriidae) baculovirus. (*J. Econ. Entomol., 90 (2): 416-420).*

EGLITE, G. and I. ZARIN-H (1987): Effectiveness and properties of the virus preparation Virin-KSh formulated with different carrier materials. (*Trudy Latviiskoi Sel'skokhozyaistvennoi Akademii, 236: 48- 53).*

ELNAGAR, S., M.A.K. EL-SHEIKH, S. EL-SALAMOUNY, A. AMIN and M. KHATTAB (2002): Screening of four lignin additives as UV protectants to Baculovirusses. (*Mitt. Biol. Bundesanst.Land - Forstwirtsch.390. 53. Deutsche Pflanzenenschutztagung , Bonn, 16 -19 Sept).*

EL-SALAMOUNY, S.; M.A.K. EL-SHEIKH; S. ELNAGAR and J. HUBER (2000): Prolongation of the UV-persistence of nucleopolyhedroviruses by the lignin derivative product. *(33rd Annual Meeting Society for Invertebr. Pathol., Guanajuats Mexico University of Guanajuato, 13-18 Aug., pp. 39).*

EL-SALAMOUNY, S.; A. HERZ and J. HUBER (2001): Stability of three lignin products as a UV protectants for two nucleopolyhedro-viruses. *(Annual Meeting of Society of Invertebrate Pathology, Netherlands, 25-30 August).*

EL-SALAMOUNY, S.; A. HERZ and J. HUBER (2001-2002): Suitability of three lignin products as UV-protectants to baculovirus. *(Bull. ent. Soc. Egypt. Econ. Ser., 28: 103-111).*

FARRAR, R.R., JR. and R.L. RIDGWAY (2000): Laboratory evaluation of selected spray adjuvants as ultraviolet light protectants for the nuclear polyhedrosis virus of the celery looper (Lepidoptera: Noctuidae). *(J. Ent. Sci., 35 (3): 239-250).*

IGNOFFO, C.M.; C.GARCIA and S.G. SAATHOFF (1997): Sunlight stability and rain fastness of formulations of baculovirus *(Heliothis. J. Environ. Entomol., 26 (6) : 1470-1474).*

KHAMISS, O.A.A. (1997): Studies on the natural genomic interference of *Spodoptera littoralis* bacloviruses and their stability in the presence of insecticides. *(Unpublished Ph.D. Thesis, Fac .Agric., Cairo Univer).*

KHATTAB, M. M., EL- S.SALAMOUNY, M.A.K. EL- SHEIKH, A. AMIN, and S. ELNAGAR (2004): Positive effect of Fluorescent brightener 28 and Neemazal T/S on the activity of *Agrotis sgetum* granulovirus tested against the black cutworm, *Agrotis ipsilon* larvae (Lepidoptera: Noctuidae).*(1st Arab Conference of applied Biological Pest Control, Cairo, Egypt, 5-7 April 2004 pp57-58).*

SHAPIRO, M. (1985): Effectiveness of B vitamins as UV screens for the gypsy moth (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus. *(Environ. Entomol., 14 (6) : 705-708).*

SHAPIRO, M. (1989): Congo red as an ultraviolet protectant for the gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *(J. Econ. Entomol., 82 (2) : 548-550).*

SHAPIRO, M. (1992): Use of optical brighteners as radiation protectants for gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *(J. Econ. Entomol., 85 (5) : 1682-1686).*

SMITH, D.H.; V.J. DAGRACA and V.H. WHITLOK (1990): Granulosis virus from *Cacoecia occidentalis* : isolation and morphological description of a granulosis virus of the citrus leafroller, *Cacoecia occidentalis.(J. Invertebr. Pathol., 55 : 319-324).*

TAMEZ-GUERRA, P.; M.R. MCGUIRE; R.W. BEHLE; J. J. HAMM ; H.R. SUMNER and B. S. SHASHA (2000): Sunlight persistence and rainfastness of spray- dried formulations of baculovirus isolated from *Anagrapha falcifera* (Lepidoptera: Noctuidae). *(J. Econ. Entomol.* 93(2): 210-215).

VILLAUME, F.G. (1958): Optical bleaches in soap and detergents. *(J. Am. Oil Chem. Soc., 35: 558-566).*