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SCREENING OF FOUR LIGNIN ADDITIVES AS UV PROTECTANTS TO BACULOVIRUS

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Abstract

Ultraviolet in sunlight is considered the main factor affecting persistence of the baculovirus under field conditions. Many efforts were devoted to improve the persistence of baculoviruses by using the lignin products as natural UV protectants. In present study, four lignin products designated as LP1, LP2, LP3 and LP4 were evaluated as UV protectant additives for the persistence of *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*MNPV). The irradiation tests were carried out in Egypt using three irradiation exposure systems; simulated UV lamps (OSRAM), natural sunlight on Petri-dishes and natural sunlight on cotton foliage. The addition of lignin increased the half-life period of the virus up to about 6.6 fold for simulated sunlight system in Petri-dishes. Also, the best UV protectant effect additive (LP4) proved to be more efficient than either magnesium lignosulfonate (Mg-lignosulfonate) or fluorescent brightener-28 (Tinopal LPW). Different concentrations of LP4 (1,5and 10%) resulted in a protection rate of 1.92, 3.65 and 151.89 folds, which are translated, to a calculated 5.31, 10.13 and 420.98 days exposure in the field.

The present study reveals the potential effect of lignin derivative as a UV protectant, especially under the sunny Egyptian field conditions.

Introduction

Lepidoptera insects such as the cotton leafworm, *Spodoptera littoralis* (Boisd.) are considered major agricultural pests, in Egypt and many other countries. Virus biocontrol agents, particularly baculoviruses (BV) have been shown to be highly effective against these insect pests, as well as being host specific and friendly environmentally. Entomopathogens such as viruses, bacteria and fungi are much affected by solar inactivation, which is the main problem of field application. NPV's lose most of their original activity, in a short time after spraying on plants especially under the sunny field conditions. Sunlight is the major factor determining the persistence of baculoviruses in the field and most of that effect is attributed to the Ultra Violet "UV" portion of sunlight below 320 nm (Jones *et al.*, 1993).

Additives such as feeding stimulants, UV protectants, and natural or chemical enhancers are necessary in order to increase the efficacy of such promising biocontrol agents in the Integrated Plant Health Management (IPHM).

Several UV-protectant additives provided a significant protection for nucleopolyhedroviruses such as carbon (Ignoffo *et al*., 1997). Congo red (Shapiro, 1989, Baskaran *et al*., 1998b and El-Salamouny *et al.*, 2000), pantothenic acid, pyridoxine, folic acid and riboflavin (Shapiro, 1985), Uric acid (Tuan *et al*., 1995 and Koa and Huang, 1992), activated carbon, and xantine (Koa and Huang, 1992). It is known that Fluorescent brighteners provided excellent protection and reduced the loss of virus activity. (Shapiro, 1992, Vail *et al.* (1993), Baskaran *et at.* (1998a), Farrar and Ridgway (2000), El-Salamouny *et al*., (2000), El-Salamouny *et al.* (2001) and Tamez-Guerra *et al*., (2000).

Also, different natural UV protectant additives were tested to prolong the activity of viruses such as Skim milk (Huber and Dickler, 1977; Jaques *et al.*, 1987 and Vail *et al*., 1991), larval extracts, whole milk, egg yolk, egg white, coconut water and crude sugar, also molasses (Im *et al*., 1990, El-Salamouny, 1998 & Jankevica and Zarins, 1997). It was noticed that addition of tissue extracts from larvae of *L. dispar* or uric acid to *Ld*NPV enhanced viral stability after exposure to UV radiation by 2- and 3.58 fold, respectively (Shapiro, 1984).

The lignin derivative as a natural UV protectant showed a high protecting effect when used as an additive (El-Salamouny *et al*., (2002), Tamez-Guerra *et al*., 2000 & Farrar and Ridgway, (2000)). Three different lignosulfonates

(Mg-lignosulfonates, Na-lignosulfonate and Ca-lignosulfonate) were evaluated as UV-protectants for *Hear*NPV and *Spli*NPV. Both the Mg- and Na-salt preserved virus activity better than Ca-lignosulfonate (El-Salamouny *et al.*, 2002). Preparations containing lignosulfonate showed the highest adhesion, dispersion and resistance to UV irradiation (Eglite and Zarin, 1987).

Under field conditions, the virus persistence was increased by Tinopal (Rabindra and Jayaraj, 1988). Tamez-Guerra *et al*. (2000) demonstrated that *Anfa*NPV in lignin-based spray dried formulations (SDF) has a shelf-life of up to 4 months at room temperature and at least 7 months in cold conditions, with longer residual activity in the field compared to NPV alone. Carbon was used in microencapsulation of *Heliothis* NPV to increase its sunlight stability (Ignoffo and Batzer, 1971). In addition, India ink, charcoal, yeast extract, brewer's yeast, peptonized milk, soy hydrolyzate, a commercially developed protectant, yellow stain, or the autoclaved supernatant from a crude suspension of virus-killed larvae were also evaluated. In field tests, a combination of egg albumen and India ink was the most effective protectant (Jaques, 1971).

In Egypt, very few studies are available concerning the possibilities of increasing the effectiveness of viruses by additives, whereas, comprehensive investigations have been made to prove the validity of baculoviruses in pest management. Nevertheless, all investigations indicated the necessity of protecting virus activity under field applications.

Therefore, the aim of the present study is to evaluate the impact of certain additives, with a special reference to natural products (Lignin derivatives), on increasing the activity of baculoviruses. In this respect, comparative tests were undertaken under both laboratory and field conditions to determine the role of the additives in protecting the virus. Extract of the results have been presented in the 53th German Plant Protection Congress (Elnagar *et al.*, 2002).

Materials and Methods

A laboratory colony of the cotton leafworm, *Spodoptera littoralis* (Boisduval), was established. A semi-synthetic diet was necessary for handling large numbers of the test insects, as well as standardizing experiments. The semi-synthetic diet described by Shorey and Hale (1965) was used, except for the exclusion of formaldehyde from diet ingredients.

A multiple embedded *S. littoralis* nucleopolyhedrovirus (*Spli*NPV) was used in the present investigation. Serial dilutions of the stock virus suspension were made in Tris buffer (pH 8), in addition to 2.5 % Teepol as a wetting agent. The tested additives were used in diluting virus suspensions.

Five additives were tested as UV protectants, F. brightener 28 (SIGMA) in addition to four lignin derivatives supplied by (LIGMEDA Consult, Leipzig, Germany) and Mg lignosulfonate (BORECHEL MG 845, (BORREGAARD-DEUTSCHLAND, Germany)), were tested at 1 % and 10 % concentrations. The lignin was obtained as a powder and diluted in distilled water and mixed with the virus suspension at a final concentration of 1 -10%. Teepol 2.5 % was added to the viral suspension and 50 µl were spread inside a Petri-dish (10 cm in diameter) using a fine pipette. After drying, the dishes with the virus films were exposed to the tested irradiation source.

Sunlight UV was simulated (SUV) using a series of four UV lamps (Ultra-Vitalux, OSRAM) that were built into a chamber (50cm width and 160 cm height). The biological effect is approximate 6-7 times greater than that of normal sunlight if the distance between sunlamp and dry deposit is 50 cm. The exposure time was fixed 0, 1, 4, 16, 32, 64 and 128 minutes. These lamps were used to simulate the sunlight UV spectrum that actually reaching the earth's surface.

In all experiments, samples were exposed to natural sunlight as a source of UV spectrum (radiation) in Giza region by using the same previous technique. The tested exposure time was carried out between 11.00 am to 03.00 pm. After the exposure of virus treatment to UV-irradiation (2000 fold LC_{90} PIB), the polyhedra deposits in a Petri- dish were resuspended in 10 ml Tris buffer, and filled into several test tubes, in order to be used in bioassay tests.

Diet Incorporation Bioassay

Activity of the virus after irradiation was determined by a bioassay test using 5 ml of the collected 10 ml of the PIB's suspension incorporated with 45 ml semi-synthetic diet at a diet temperature below 40°C. The contaminated diet was then distributed into special bioassay plates (LICEFA, Bad-Salzuflen, Germany). The plate, measuring 14x7x2 cm, contains 50 cells. One newly hatched larva was placed into each cell. The plates were covered with tissue paper and 14 x 7 cm glass plate fixed with rubber bands. All treatments were incubated at 26±2°C, for 16 days. Mortality due to virus infection was recorded every two days and up to 16 days. The larval mortality caused by untreated virus (control) was determined (El-Salamouny *et al.,* 2002).

Semi-field application tests

Seeds of the cotton variety (Giza 86) were cultivated in pots (21 cm in diameter x 24 cm in length), and ten pots were used for each replicate of each treatment. Three replicates were used in the test. The different virus suspension treatments were applied to cotton foliage at three different concentrations using a garden hand sprayer.

Leaf bioassay

The *Spli*NPV polyhedra suspension with or without additives was applied to the host plants using a 1.5 litre hand sprayer. The nozzle was directed towards the plants and spraying took place during sunset, 1-2 hr before darkness. After air drying, sprayed leaf from each treatment/replicate was collected (as a zero time) then a daily collection was made through 7 successive days. Test leaves were fed to neonate larvae for 48 hr. Mortality due to infection was recorded every two days and for 16 days (El-Sheikh, 1984).

Statistical analysis

Data of bioassay results were subjected to probit analyses using the method described by Finney, (1971). The relative potancies of the treatments were calculated according to the changes in LC50 value.

Results and Discussion

Rate of virus inactivation after UV exposure

Exposure of LC_{90} value of *S. littoralis* MNPV to simulated UV light (lamps) and natural UV light (sunlight) resulted in a gradual deterioration in virulence of the tested virus.

In the case of exposure to simulated UV light, the activity of virus deposits decreased sharply as a result of the exposure to the simulated UV light from 91.6 % to 10% after 188 minutes exposure to UV. Similarly, in the case of exposure to natural sunlight, the activity of virus deposits decreased sharply, from 92.51 % to 15.15 % after 188 minutes exposure to UV. *Spli*NPV sprayed on cotton foliage and leaf-bioassayed immediately after treatment, gave also a sharp decrease in virulence from 91.45 % to 7.07 % after 7 days exposure to solar light.

Values of estimated survival half-life values (SHL_{50}) for virus in Petri-dishes exposed to either simulated UV or natural sunlight were 58.73 or 69.82 minutes, respectively (Fig. 1). In case of virus on cotton foliage exposed to natural sunlight, the SHL_{50} was 56.97 hr. (2.37 days).

Previous reports (Tamez-Guerra *et al*., 2000 and El-Salamouny *et al*., 2002) demonstrated that, baculoviruses were rapidly inactivated after exposure to SUV or natural sunlight and under natural field conditions. Also, purified virus was more affected than the crude extract (Elnagar and Abul-Nasr, 1980). In Egypt, the effect of sunlight on *Spli*NPV was thoroughly investigated. The *Spli*NPV was inactivated (46 %) by the exposure to 300 nm (Elnagar, 1983), and lost most of its virulence on the next day of application onto cotton foliage (Elnagar and Abul-Nasr, 1980). The most destructive wavelength, under Egypt conditions, is lower than 308 nm (Jones *et al*., 1993). The computed survival half-life values for *Spli*NPV and *Se*NPV were 74.5 and 130.8 minutes, respectively (El-Salamouny *et al*., 2000). Other

studies demonstrated the same trend for the adverse effect of UV (Ignoffo and Garcia, 1992; Ignoffo and Garcia, 1996).

Lignin derivatives as UV protectants

Virus inactivation under simulated UV irradiation and the role of UV protectants

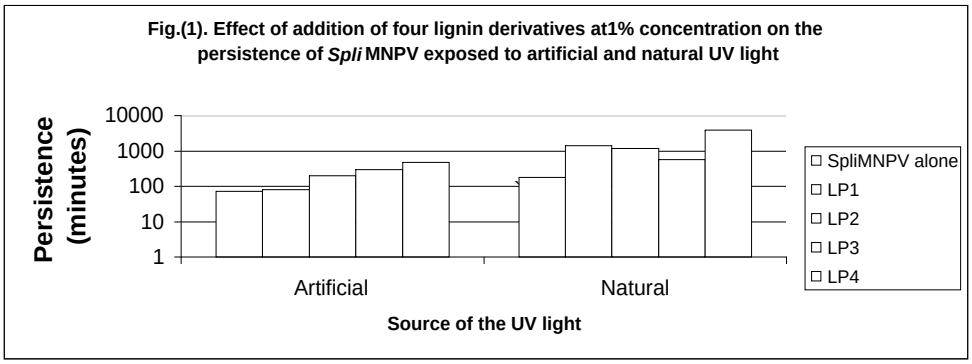


Fig.(1). Effect of addition of four lignin derivatives at1% concentration on the persistence of *Spli* MNPV exposed to artificial and natural UV light

In the case of *Spli*NPV virus alone treatment, the calculated inactivation time for 50 % reduction in virulence (survival half-life of the virus, SHL_{50}) was 71.5 minutes. Addition of lignin additives LP_1, LP_2, LP_3 and LP_4 prolonged the activity of the virus, where the calculated survival half-life of the virus for 50 % of original virulence was 80.68, 196.1, 297.06 and 478.16 minutes for the tested additive, respectively (Fig. 1).

The calculated values of potency (increase of virulence) were 1.13, 2.74, 4.16 or 6.69 fold for LP_1, LP_2, LP_3 or LP_4, respectively. In conclusion, LP_4 additive gave the best UV protection effect on virus treatment by 6.69 fold.

The concentration of 10 %. LP4 additive showed the highest rate of protection, Mg-lignosulfonate (10%) came second and F. brightener (1%) came third in that order, where the estimated SHL_{50} value were 1888.7, 301.49 and 248.51 minutes, after the tested progressive irradiation periods, respectively (Fig.2). The calculated potency values were 27.64, 4.41 and 3.63 fold for LP_4, Mg lignosulfonate and F. brightener, respectively.

Virus inactivation under natural sunlight radiation and the role of UV protectants

In this case, LP_4 additive also gave the highest protection value. The addition of lignin additives increased the calculated survival half-life of the virus (SHL_{50} value) (expressed as 50 % of original virulence) from 177.3 minutes in case of virus-alone treatment to 1386.69, 1193.5, 577.86 or 3874.25 minutes for LP_1, LP_2, LP_3 or LP_4 additive, respectively (Fig.1). The calculated potency values were 7.82, 6.73, 3.26 and 21.85 folds for LP_1, LP_2, LP_3 and LP_4, respectively.

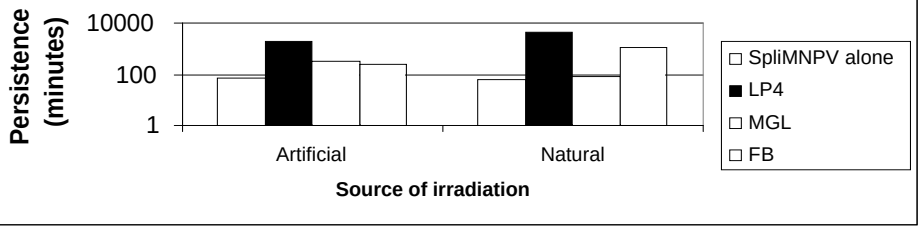
In the case of *Spli*NPV virus alone treatment, the calculated survival half-life of the virus for 50 % reduction in virulence (SHL_{50}) was 62.19 minutes. It is worthnoting that, the percentage of reduction in virulence was high (71.60

%) in the case of virus alone treatment which was reduced to the lowest level (20.89 %) with the addition of LP_4 additive (*i.e.*, increasing protection effect). However, it was reduced to 38.69 and 56 % in case of F-brightener and Mg lignosulfonate, respectively. The values of calculated survival half-life of the virus (SHL_{50}) were 4176.635, 79.89 or 1038.01 minutes for LP_4, Mg lignosulfonate and F. brightener additives, respectively. The calculated potency values were 67.16, 1.28 and 16.69 for LP_4, Mg lignosulfonate and F. brightener, respectively (Fig.2).

In conclusion, the LP_4 additive showed the best UV protection effect under either simulated UV or natural sunlight by 27.64 and 67.16 folds, respectively. LP_4 (10 %) showed the best UV protection effect under simulated UV compared to both Mg lignosulfonate and F. brightener additives.

The LP4 product showed the best UV protection (6.6 and 21.8 folds for both the simulated UV and natural sunlight, respectively). The LP4 was also superior to either Magnesium lignosulfonate (10 %) or Fluorescent brightener 28 (1 %) under the simulated UV as well as under the natural sunlight. Mg lignosulfonate (10 %), Fluorescent brightener and sodium lignosulfonate are reported active as UV protectant additives (El- Salamouny *et al*. 2002; Farrar and Ridgway, 2000).

Fig. (2). Effect of (10 %) LP4 product, Magnesium lignosulfonate (10 %) and Fluorescent brightener (1%) on the persistence of *Spli* MNPV exposed to artificial and natural UV light.



Effect of different concentrations of lignin derivative (LP_4), on virus protection

Three concentrations of the LP_4 additive were tested, and results revealed a direct relation between additive concentration and rate of mortality with virus exposed to radiation as follows:

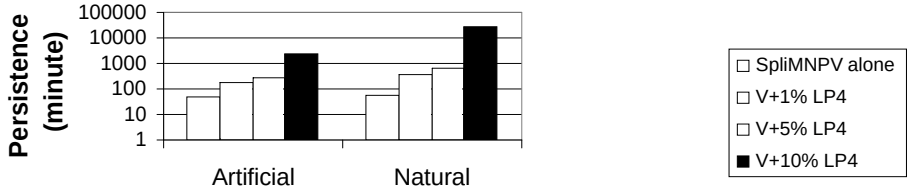
In the case of *Spli*NPV virus alone treatment, under simulated UV irradiation the survival half-life of the virus (SHL_{50}) was 51.55 minutes. With the addition of LP_4 concentration of 1 %, the survival half-life of the virus (SHL_{50} values) were 184.14, 262.21 or 2300.68 minutes for the three tested concentrations (1, 5 and 10 %), respectively (Fig. 3). The calculated potency values were 3.57, 5.09 and 44.63 folds for 1, 5 and 10 %, respectively. In conclusion, the concentration of 10 % additive gave a significantly high protection to the virus.

In case of *Spli*NPV virus alone treatment, under natural sunlight radiation the calculated survival half-life of the virus (SHL_{50}) value was 53.5 minutes, which was increased to 348.87 minutes in case of addition of LP_4 (1%). The calculated survival half-life values of the virus (SHL_{50}) were 348.87, 605.41 and 26684.18 minutes for the three tested concentrations 1, 5 and 10 %, respectively (Fig.3). The calculated potency values were 6.52, 11.31 and 498.76 fold for the LP_4 concentrations of 1, 5 and 10 %, respectively. In conclusion, the highest LP_4 concentration (10 %) showed the best UV protectant effect, 44.63 and 498.76 folds under simulated UV and natural sunlight, respectively.

In conclusion, the highest LP4 concentration (10%) showed the best UV protectant effect 44.63 and 498.76 folds under simulated UV and natural sunlight, respectively.

The calculated potency value was 44.6 and 498.7 folds under simulated UV and natural sunlight, respectively. In both

Fig. (3). Effect of different concentrations of LP 4 additive on the persistence of *Spli* MNPV exposed to natural and artificial UV light



trials, the calculated potency value as a survival half-life of the virus (SHL_{50}) was directly related to the concentration of protectant additive (LP4). Similarly, Tamez-Guerra *et al.*, (2000) reported that *Anfa*NPV formulated with potassium lignate and pre gelatinized conflour (PCF), retained almost full activity after 8 hr. of exposure to simulated sunlight (SUV).

Semi-field application test of a lignin additive (LP4) on cotton foliage

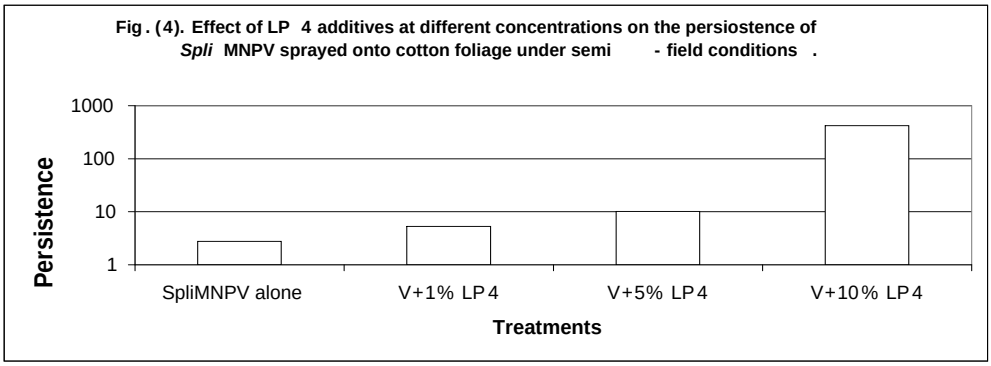
First trial: The LP_4 additive was used at relatively low concentration (0.1, 0.5 and 0.9 %). Obtained data showed that, the estimated SHL_{50} was 23.78 hrs after 7 days of exposure. The estimated SHL_{50} values were 34.14, 46.54 or 51.35 hrs for the LP_4 concentration of 0.1, 0.5 or 0.9 %, respectively. The calculated potency values were 1.43, 1.95 and 2.16 fold for LP_4 concentration at 0.1, 0.5 and 0.9, respectively.

Second trial: In this trial the LP4 additive was tested at a higher concentration of 1, 5 or 10 %. In the case of using *Spli*NPV virus alone (at LC_{90}), the estimated SHL_{50} values were 2.77, 5.31, 10.13 or 420.9 days for the virus alone treatment and for LP_4 treatment at the concentrations of 1, 5 and 10 %, respectively (Fig.4). The calculated potency values were 1.91, 3.65 and 151.89 fold for the LP_4 treatment at 1, 5 and 10 %, respectively.

In conclusion, the LP_4 concentration of (10 %) gave the best protection to the virus under field conditions.

Development of viruses as bioinsecticides has been limited because activity is rapidly lost during storage and after application in the field. Spray dried lignin based formulation (Tamez-Guerra *et al.*, 2000) successfully encapsulated the PIB's that retain insecticidal activity longer than unformulated virus after application. The virus in this formulation had a shelf-life of up to 4 months at room temperature and at least 7 months in cold temperature. The half-life values when lignin (1 %) was used with *Spli*NPV and *Se*NPV were 2306.0 min. and 1385.0 min., respectively (El-Salamouny *et al*., 2000), compared to 74.5 min. and 130.8 min., respectively for the untreated control. *Anfa*NPV and *Ac*MNPV formulations containing lignin retained > 50 % activity within 24 hr. after application in the field (McGuire *et al.*, 2001). It was concluded that, formulation additive that protects the virus from the effect of sunlight must absorb or reflect wavelengths between 300 and 320 nm (Jones *et al.*, 1993).

Lignin additive appears very promising as UV protectants to baculoviruses in the pest control strategies. Dellicolli, (1980) and Taiz and Zeiger, 2000 suggest that the aromatic chemistry of lignin makes it an excellent protective matrix for materials that are sensitive to UV radiation. The obtained results support the usage of lignin derivatives as protection material to the baculoviruses in order to be effective under the Egyptian sunny conditions.



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