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The Efficacy and Persistence of *Spodoptera littoralis* Nucleopolyhedrovirus (*Spli*MNPV) Applied in UV Protectants against the Beet Armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) under Saudi Field Conditions

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SES, KGR and ASA conceived the project. All the authors designed the experiments, analyzed the data and wrote the manuscript. SS and MT finalized the analysis and manuscript with constructive discussions.

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Biological control, Baculovirus, *Spodoptera exigua*, Persistence, Ultraviolet.

ABSTRACT

Baculoviruses are known biocontrol agents of several crop pests, however, their activity is deteriorated rapidly when exposed to the sunlight. Many UV protectants were used to improve the efficacy of these viruses under field conditions. The objective of the current study was to evaluate the effectiveness of plant extracts to improve the persistence of *Spodoptera littoralis* multiple nucleopolyhedrovirus (*Spli*MNPV) against beet army worm, *Spodoptera exigua* (Hübner) under harsh sunny field conditions in Saudi Arabia. A preliminary test of *Spli*MNPV was performed to determine the lethal concentrations of this virus against the first and second larval instar of *S. exigua*. The potency of ten plant extracts as UV absorbers were done by measuring the absorption spectra of the 0.5% extracts using UV spectrophotometer. Based on preliminary data, clove, green tea and henna extracts at 10% (v/v) concentration were added to the virus and tested under semi-field conditions on the cabbage plantations for 7 days. The preliminary experiment showed that the LC_{50} and LC_{95} for the first instar larvae were 1.59 x 10^3 PIB and 4.91 x 10^7 PIB, respectively. Whereas, these values for the second instar larvae were 4.99 x 10^6 PIB and 1.06 x 10^8 PIB, correspondingly. The field experiment data indicated that the mortality of the first instar larvae was highest at day 0 of the sunlight exposure. In virus alone treatments, after 7 days of sunlight exposure, the efficacy recorded was 88%. However, in clove-virus treatment, the mortality was higher (96%) than other treatments. These data also revealed that the second instar larvae were more resistance than the neonates. The efficacy of *Spli*MNPV at 0 and 7-day against the second larval instar was 88% and 5%, respectively. In clove-treated applications, the mortality was was 96% and 32%, at 0 and 7 d post treatment, respectively. Findings of this study demonstrated that the addition clove extract enhanced the effectiveness and persistence of *Spli*MNPV under arid conditions against the beet armyworm.

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is one of the most serious pests of agricultural crops including vegetables and flowers worldwide (Pogue, 2002; Saeed *et al*., 2010). This pest is also present in Al Qassim region of Saudi Arabia (Al-Kherb, 2014). The beet armyworm is considered as one of the most important pests of cauliflower (*Brassica oleracea*), pea (*Pisum sativum*) and wheat (*Triticum aestivum*) (Saeed *et al*., 2010). The larvae consume the foliage, flowers, buds and fruits of the crops, which affect the plant growth and ultimately the quality and quantity of the harvest. The control of *S. exigua* on vegetable crops includes the cultural, physical, biological and chemical methods. Chemical pesticides, such as spinosad, fenvalerate, phoxim, methomyl, abamectin and cyfluthrin,

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have been used to control *S. exigua*. Some synthetic insecticides have been studied in the laboratory conditions for the IPM of *S. exigua* in Pakistan (Saeed *et al*., 2012). However, recent reports have shown that use of these chemical pesticides lead to the resistance of the insect pests after five generations. Wang *et al*. (2006) have reported a 345.4 fold increase in resistance of *S. exigua* to spinosad.

The increasing awareness of the environmental pollution and the growing demand for safe food products lead to find the biopesticides as an alternative to chemicals. Baculoviruses are promising candidates due to their safety and target-specificity (Burges *et al*., 1980).

Baculoviruses (family baculoviridae) are rod-shaped viruses that can infect many insects. Family Baculoviridae has four genera that include Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses (NPVs), Betabaculovirus (lepidopteran-specific Granuloviruses (GVs), Gammabaculovirus (hymenopteran-specific NPVs) and Deltabaculovirus (dipteran specific NPVs) (Jehle *et al*., 2006). Generally, baculoviruses cause infection at the larval stages of the insects and the success of multiplication depends on dose, temperature, nutrition, physical character and the age of the larvae (Federici, 1997). Pathogenicity of baculoviruses depends on the age of larvae, for example, in *S. littoralis,* third instar larvae died in 8-8.5 days, whereas the neonates died in 3-3.5 days after *Spli*NPV application (Toprak *et al*., 2005).

A NPV was isolated from the larvae of cotton leafworm, *S. littoralis* in Egypt (Abul-Nasr, 1956) and its characterization has been done (Seufi, 2008). The virus was tested under the field conditions and it is available in the market under the commercial name of Spodopterin ® and Littovir®. Under the sunny conditions, baculoviruses are affected by ultraviolet (UV). It has been proved that UV in sunlight has negative impact on persistence of microbial control agents (Jaques, 1977; Behle *et al*., 1997; Jones *et al*., 1993; Ignoffo *et al*., 1989). There is a considerable evidence that the UV-B portion of sunlight between 280-320 nm is responsible for baculoviruses inactivation (Ignoffo and Batzer, 1971). In order to overcome the inactivation problem and improve the persistence of baculoviruses under the field conditions, several additives like fluorescent brightener (Dougherty *et al*., 1996), Congo red, indigo carmine (Shapiro and Robertson, 1990) and carbon (Ignoffo *et al*., 1991) have been used.

Recently, the use of natural UV protectants in virus formulations has received attention of the researchers involved in biocontrol studies. Natural UV protectants like lignin, a side product of paper industry, has shown a potential as a UV protectant of *S. littoralis* MNPV (Elnagar *et al*., 2003; El Salamouny and Huber, 2004). Magnesium lignosulfonate also has been reported to improve the persistence of *Helicoverpa armigera* MNPV (El Salamouny *et al*., 2003). Potassium lignite in the presence of pre-gelatinized corn flour has the ability to improve the activity of *Anagrafa falcifera* MNPV. Moreover, beverages such as black tea, green tea, cocoa and coffee are shown to act as UV protectants for *S. exigua* MNPV (Shapiro *et al*., 2008; El Salamouny *et al*., 2009a, b). Extracts of spices, medicinal herbs and weeds showed a promise as UV protectants (Shapiro *et al*., 2009b; Shepard *et al*., 2010). Plant extracts also improve the persistence of biopesticides under the field conditions (Shapiro *et al*., 2008).

Saudi Arabia has arid weather in which the habitat is dominated with less humidity and rich sunny conditions. The activity of baculovirus, as a biocontrol agent, is rapidly declined when exposed to these harsh conditions. Finding an effective natural UV protectant will lead to resolve the issue of rapid inactivation of baculoviruses under the harsh sunny field conditions of Saudi Arabia.

MATERIALS AND METHODS

The insect mass rearing

The beet armyworm, *Spodoptera exigua* (Hübner) adults were collected by light trap from Al-Amariyah, Riyadh, Saudi Arabia. The captured moths were kept in plastic jars (20 cm x 35 cm) provided with sugar soaked-cotton as a food source and opaque paper as an egg laying substrate. The eggs were surface sterilized by dipping the egg mass in 1% chlorox solution (v/v) for 10 seconds and then washed in the running tap water. After hatching, the neonate larvae were transferred into white bean artificial diets (Shorey and Hale, 1965) placed in plastic cups (50 mm x 80 mm). The 4th instar larvae were then transferred and maintained individually on the artificial diet until the pupal stage. The pupae were surface sterilized in chlorox solution (1%) and then washed in the running tap water. The pupae, 20-25 individuals were then placed in the plastic jars until adults emerge. The colony was maintained under controlled conditions at 25°C and 60-70% RH.

Virus source and natural additives preparations

The virus used in the present study was *Spodoptera littoralis* multiplenucleopolyhedrovirus (*Spli*MNPV) available as commercial product Littovir® (Biocontrol, Switzerland). The concentration of the product *Spli*MNPV was 2x10^{12} PIB/L. Dilutions of virus suspensions were prepared as required using autoclaved distilled water. The serial solutions were kept in glass tubes (Lab. Glass, India) at 4°C until further use.

Three plant extracts used as natural UV protectants were: henna, *Lawsonia inermis* L. (Myrtales: Lythraceae), Green tea leave, *Camellia sinensis* (L.) Kuntze (Ericales: Theaceae) and clove flower, *Syzygium aromaticum* (L.)

Merrill and Perry (Myrtales: Myrtaceae). The plant materials were air dried for at least 72 h, then ground to make powder. 100 gram of each powder was soaked in a 1000 mL (10% w/v) distilled water for 24 h at the room temperature. The mixture then blended and filtered using two layers of muslin cloth. The filtrates were kept at 4°C until use.

The prepared filtrates were added to virus solution to get the final concentration of *Spli*MNPV as $2.23x10^7$ PIBs/mL according to the method described by Shapiro *et al*. (2008). This preparation was done prior to the field experiment.

Preliminary bioassay

Preliminary laboratory tests were performed to determine the lethal concentration of *Spli*MNPV against the beet army worm by using diet surface contamination bioassays. For this bioassay, six concentrations of $2x10^1$, $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$ and $2x10^8$ PIB/mL of the virus were tested against first and second instar larvae of the beet army worm. While distilled water was used as the control treatment. For bioassay, one mL of each concentration was evenly poured onto artificial diet that was prepared in bioassay plates (5 x 10x 1.5 cm) (LICEFA, Bad Salzuflen (DE), Germany). Then a 50 bioassay cells plate (1cm x 1cm) was fixed into the bioassay plate. The diet was then air died at room temperature for 2 h and 1 larva was introduced in each cell. Each bioassay plate was then covered with two layers of tissue paper and a glass plate that was fixed with rubber band to prevent escaping of the larvae. The preliminary experiments were done against 1st and 2nd instar larvae. Each treatment was replicated 3 times using 50 larvae in each. Larval mortality was observed daily for 10 days post-application.

The UV absorption spectra of the plant extracts

To study the UV absorption capacity of the plant additives, the absorption spectra of the green tea, olive, henna, beetroot, lemon, grapes, dates, kiwi, clove and pomegranate were measured by spectrophotometer (JENWAY, 6705 UV/Vis. England) at the range of 190-500 nm. One mL of each sample at 0.5% concentration was used for the UV absorption measurement.

Field trials of natural UV

Field trials were conducted to evaluate the UV protectants for their effectiveness to enhance the persistence of the virus by using 6 week old cabbage plants cultivated at the Educational Farm, King Saud University, Dierab, Saudi Arabia. The cabbage plants were planted at 25 cm by 50 cm distance between plants.

The virus formulations with the best three natural additives (clove, henna and green tea) were prepared six hours before the application. The virus concentration of 100 X LC_{95} was diluted with 10% plant extracts and 1% surfactant (final concentration) (Triton X-100, Bio Basic Inc., CA) to obtain a final volume of 500 mL with 10 folds of LC_{95} concentration. The formulated virus solution was then placed into a 1000 mL hand sprayer for application. The treatment was done by evenly spraying the upper surface of eight cabbage leaves per plant in the field. Each treatment was using three replicates (as mentioned above) while six different exposure timings of 0, 1, 3, 5 and 7 days post-application were utilized to test efficacy under field-sunlight conditions. After desired exposure timing, one treated-leave was taken from the field, kept in zip plastic bag and stored at -20°C until used for the bioassay. The field trials were conducted during beginning of the summer in 2015 when the average temperature recorded was 36°C.

For bioassays, a frozen leaf sample (kept at -20°C) for each treatment was taken (30 mm diameter) and placed onto the surface of 10 mm thick bacteriological agar provided in the plastic cup (50 mm diameter 80 mm height) almost six hours before the analysis. The leave discs were then thawed at room temperature for about 1 h. After thawing, 20 1-day-old larvae were released on each leaf disc. The cabbage leaves treated with H_2O and exposed for 7 days under the sunlight were used as the control treatment. The larvae were allowed to feed on the leaf disc for 24 h and then were transferred to an artificial diet in 50 cells bioassay plate. The effectiveness of the treatment in terms of larval mortality was recorded daily for 10-d post-application. A further bioassay following the same protocol was done by using only clove, the best natural additive against the 2nd instar larvae of *S. exigua*.

Statistical analysis

Probit analysis was used to calculate LC_{50} and LC_{95} values (Finney, 1977). A complete randomized design and ultimately with two factorial analyses of variance (ANOVA) was used to determine the effectiveness of treatments against the *S. exigua*. The means were separated using LSD test. All analyses were done using SPSS 13.0 (SPSS, 2005).

RESULTS AND DISCUSSION

Preliminary bioassay of Spli*MNPV efficacy against 1st and 2nd instar larvae of* S. exigua

The preliminary bioassay of *Spli*MNPV effectiveness was conducted against the first and second instar larvae of *S. exigua* to determine LC_{50} and LC_{95} values in the laboratory. The effectivity of virus against *S. exigua* larvae

was presented in Table I. *Spli*MNPV showed 76%, 70.67%, 77.33%, 96.67 and 100% mortality at the concentrations of $2x10^4$, $2x10^5$, $2x10^6$, $2x10^7$ and $2x10^8$ PIB/mL, respectively against the 1st larval instar. Whereas, mortality in 2nd instar larvae was 64%, 64.67%, 64%, 84.67% and 98.67%, at the above concentrations. The LC_{50} and LC_{95} values obtained against the 1st instar larvae were 1.59×10^3 and 4.91×10^7 PIB/mL, respectively. Whereas in second instar larvae the LC_{50} and LC_{95} values were 4.99×10^6, 1.06×10^8 PIB/mL, respectively.

These data suggest that the baculovirus *Spli*MNPV is pathogenic to both the larval instars of the beet armyworm although this insect is a non-homologous host of the virus *Spli*MNPV, a virus strain isolated from the other lepidoperan pest *Spodoptera littoralis*, as it has been reported to be effective for *S. exigua* (Pudjianto *et al*., 2016). It was reported that NPV application might also have some impacts on non-target Lepidoptera (Takatsuka *et al*., 2007). *Spli*MNPV was reported to be highly virulent to the 2nd larval instar of *Spodoptera littoralis* with the lethal time 50% of 7.32 days (Masetti *et al*., 2008). *Spli*MNPV is also reported to be pathogenic to *S. frugiperda,* whereas the baculoviruses isolated from *S. exigua* (*Se*NPV) could not cause infection to *S. frugiperda* or *S. littoralis* (Murillo *et al*., 2003) and termite (*Reticulitermes speratus*) (Takatsuka *et al*., 2007). Similar to that of *Spli*MNPV, a non-homologous pathogenicity has also been observed in cabbage moth (*Mamestra brassicae*) NPV when applied to non-lepidopteran species (Doyle *et al*., 1990). The most recent study of the local *Se*NPV has been reported from Egypt which causes 91.02% and 6.66% mortality against 5th instar larvae and pupal stage of the beet army worm, respectively (Khattab, 2013).

Table I.- The mortality percentage (means± SE) of the 1st and 2nd instar larvae of *S. exigua* at 10 days pot treatment of *Spli*MNPV under laboratory conditions and its lethal concentration values.

Concentration (PIB/mL)	Mortality (%)	
	1st instar	2nd instar
Control	5.33±0.67a	3.33±0.67a
$2x10^4$	76.00±10.26b	64.00±1.15b
$2x10^5$	70.67±1.76b	64.67±1.76b
$2x10^6$	77.33±5.21b	64.00±2.00b
$2x10^7$	96.67±1.33c	84.67±3.33c
$2x10^8$	100±0.00c	98.67±0.60c
Significance	F=51.19; df= 5, 12; *P*<0.0001	F=311.45; df= 5, 12; *P*<0.0001
LC_{50} (PIB/mL)	1.59×10^3	4.99×10^6
LC_{95} (PIB/mL)	4.91×10^7	1.06×10^8

Means in the same column followed by the same letterwere not significantly different at α: 0.05. PIB indicates concentration of polyhedral inclusion bodies of the virus.

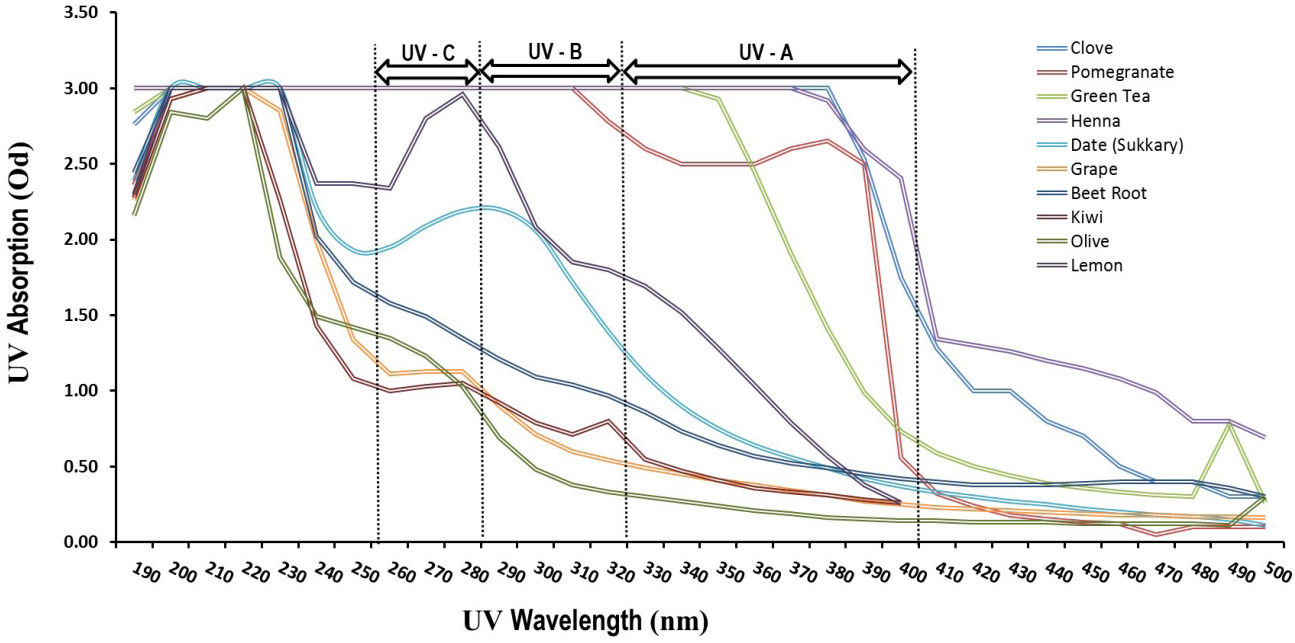

Fig. 1. Absorption spectra of green tea, olive, henna, beetroot, lemon, grape, dates, kiwi, clove and pomegranate measured by the spectrophotometer at the wavelength range of 190-500 nm.

Table II.- The mortality percentage (means± SE) of the 1st instar larvae of *S. exigua* in response to *Spli*MNPV applications formulated with henna, green tea and clove as natural UV protectants on the cabbage plants under Saudi Field conditions.

Exposure timings (days)	Mortality (%)				Significance
	Virus alone	V+HN	V+GT	V+CV	
0	98.33±1.67cA	100±0.0bA	91.32±2.94bcA	100±0.0cA	F= 1.59; *P*= 0.27
1	87.97±6.27bcAB	88.33±4.41bAB	66.10±12.85cA	95.76±2.26cB	F= 2.87; *P*= 0.10
3	84.83±4.92bcA	81.57±8.43bA	76.80±9.48cA	75.97±7.22bcA	F= 0.29; *P*= 0.830
5	80.47±5.37bA	78.53±8.23bA	83.33±16.67cA	91.10±8.90cA	F= 0.27; *P*= 0.846
7	88.00±3.81bcA	77.97±4.88bA	75.00±14.43cA	96.47±1.77cA	F= 1.54; *P*= 0.278
Control H_2O	8.33±1.67a	8.33±1.67a	8.33±1.67a	8.33±1.67a	
Additive only		3.42±1.71a	3.42±1.71a	3.42±1.71a	
Significance	F= 81.36; *P*< 0.001	F= 5.92; *P*= 0.003	F= 4.01; *P*= 0.015	F= 53.02; *P*< 0.001	

Means in the same column followed by the same letter (small letter) are not significantly different at α: 0.05. Means in the same row followed by the same capital letters are not significantly different at α: 0.05. V, HN, GT and CV are the virus, henna, green tea and clove.

The UV absorption spectra of the plant extracts

The absorption spectra of the tested natural additives are presented in Figure 1. Among the 10 natural additives, clove, henna and green tea have shown the highest rate of UV absorption. The UV absorption ability of these additives reveals that these natural additives might have the potential to be used as UV protectants for virus when applied under field sunlight conditions. Based on our absorption spectra experiments, clove, henna and green tea extracts showed a good potential as UV protectants even against a wide range of UV light starting from UV-B (280-320 nm) to UV-C (<280 nm). Moreover, our previous findings indicated that black tea is also a good UV absorber of UV-B (El Salamouny *et al*., 2009b) and provides the UV protection to the *S. exiqua* MNPV (*Se*MNPV) when used against beet armyworm.

Evaluation of Spli*MNPV persistence with natural UV protectants against the 1st and 2nd larval instars of* S. exigua *under field conditions*

The persistence of *Spli*MNPV after blended with natural plant extracts (clove, henna and green tea) against *S. exigua* was tested under field-sunlight conditions in Riyadh, Saudi Arabia. The data presented in Table II revealed the effectiveness of virus formulations on the mortality (%) of *S. exiqua*. The highest mortality was recorded on day 0 of sunlight exposure and was declined in parallel to increase of the sunlight exposure timings. It is worth mentioning that in the virus alone treatment, the mortality was decreased up to 10% on day 7 of sunlight exposure as compared to those of un-exposed treatment. Meanwhile in henna, green tea and clove treated virus applications, the decrease in mortality was up to 23, 16 and 4%, respectively. The results indicated that the virus (*Spli*MNPV) treated with henna and green tea has presented less protection as compared to clove treated-virus where the persistence remains high on day 7 of sunlight exposure.

Table III.- The mortality percentage (means± SE) of the 2nd instar larvae of *S. exigua* in response to *Spli*MNPV application formulated with clove as the best natural UV protectant on the cabbage plants under Saudi field conditions.

Exposure timings (days)	Mortality (%)		Significance
	Virus alone	Virus+Clove	
0	88.33± 3.33cA	78.33± 13.02dA	t= 0.74; *P*= 0.498
1	37.28± 7.22abA	77.72± 5.97dB	t= -4.32; *P*= 0.012
3	43.27± 13.18bA	67.63± 11.22cdA	t= -1.41; *P*= 0.232
5	8.70± 1.87aA	46.67± 10.93bcB	t= -3.42; *P*= 0.027
7	5.37± 3.21aA	31.67± 11.67bB	t= -2.17; *P*= 0.059
Control H_2O	0±0.0a	0±0.0a	
Control additive	0±0.0a	0±0.0a	
Significance	F= 29.71; *P*< 0.001	F= 13.73; *P*< 0.001	

Means in the same column followed by the same letters (small letters) are not significantly different (in the exposure timings effect on the insect mortality) at α: 0.05. Means in the same row followed by the same capital letters are not significantly different (in the formulation effect on the insect mortality) at α: 0.05.

A further experiment on effectiveness of virus formulated with clove, the best UV protectant in this report, against second larval instar of *S. exigua* was also performed under field conditions (Table III). The results

indicated that the mortality (%) of *S. exiqua* in virus alone treatment was decreased from 88% at day 0 to 5.37% (F= 29.71; $P< 0.001$) at day 7 of sunlight exposure. On the other hand, the mortality in clove-treated virus applications was 78% on day 0 and 32% on day 7 of sunlight exposure. The low mortality (%) in 2nd larval instar as compared to that of the 1st larval instar suggests that the 2nd larval instar is probably less susceptible than that first larval instar. The addition of clove provides at least some preliminary UV protection, because the mortality of tested larvae was higher than virus alone treatments.

Studies have documented that persistence of baculoviruses is affected by the ultraviolet sunlight. Ultraviolet portion in sunlight is deleterious to the virus activity as it causes DNA and protein matrix degradations (Bandyopadhyay *et al*., 1999) by producing two types of pyrimidine dimers; cyclobutane pyrimidine dimers and pyrimidine–pyrimidine 6-4 photoproducts (Friedberg *et al*., 1995). Based on our finding, the virus alone (without additives) lost its 83% pathogenicity after 7 days of sunlight exposure when applied against the 2nd larval instar of beet army worm. These results are in parallel to those reported by Shapiro *et al*. (2008) where 98.7% decrease in pathogenicity was observed.

The finding of clove as a good UV protectant agrees with the results reported by Shapiro *et al*. (2008) since it contains high concentration of antioxidant thus able to absorb the ultraviolet (Shapiro *et al*., 2009b; El Salamouny *et al*., 2009a, b). It is known that clove extract contains tannins and flavonoid that play an important role in absorbing the UV light (Shapiro *et al*., 2009a). Clove gave more improvement in the persistence of *Spli*MNPV in the field as compared henna and green tea.

The addition of clove extract improved the persistence of *Spli*MNPV under field conditions. The present results are in agreement with the previous findings on green tea, black tea and kudzu as *Se*MNPV additives against larvae of the *S. exigua* (Shapiro *et al*., 2009a; Shepard *et al*., 2010). The most recent research on additives to increase the virus persistence under ultraviolet A (UV-A) has been done by using microencapsulation polymer methods of starch (3%), gelatine (3-5%), sodium alginate (3%) against another lepidopteran pest, the cotton bollworm (*Helicoverpa armigera*). It was reported that gelatine (5%) provides high protection up to 93.3% after being exposed under UV-A for 72 h, while starch (3%) has shown only a protection of 3.7% (Gifani *et al*., 2015).

CONCLUSIONS

The present findings conclusively demonstrate that *Spli*MNPV that was originally isolated from *Spodoptera littoralis* has high potential to be used as a biopesticide to control another lepidopteran pest, the beet army worm (*Spodoptera exigua*). Although, the effectiveness of commercial formulation remains high until 7 days of field application, however, the addition of clove extract as a natural UV protectant provides more persistence than that of virus alone applications for the later instar infestations. This extract will inhibit the fast inactivation of baculoviruses as a potent UV protectant under the harsh Saudi sunny field conditions. Further screening of natural UV protectants, especially from the local resources to enhance the effectiveness/persistence of the virus is recommended.

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Statement of conflict of interest

There is no conflict of interest or otherwise.

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