Protective effect of different additives and natural polymers on the stability of *Helicoverpa armigera NPV* (*HaNPV*) against ultraviolet radiation

Aida Gifani[1], Rasoul Marzban[2], Sodeh Khorramvatan[3], Mehdi Ardjmand[4], Ahmad Dezianian[5]

1. Research Center for Modeling and Optimization in Science and Engineering, South Tehran Branch, Islamic Azad University, Tehran, Iran
2. Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran
3. Department of Chemical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
4. Department of Chemical Engineering, South Tehran Branch, Islamic Azad University, Tehran, Iran
5. Agricultural and Natural Resources Research and Education Center, Shahrood, Iran

*Corresponding author. Email: aida_gifani@yahoo.com

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Abstract

Microbial biopesticides because of their less environmental impact ensure the management of agro–ecosystems. Among the microbial biopesticides, baculoviruses have been interested. Because of the low stability of baculoviruses against sunlight, preparation of UV protectant formulations is being focused in this study. Accordingly, the ultraviolet protective effect of some natural and chemical additives (green tea, black tea, coffee, cocoa, Tween 80, sodium lignin sulfonate and titanium dioxide) at two concentration levels (0.5% and 3% w/v) for the *Helicoverpa armigera* nucleopolyhedrovirus was evaluated. The investigation was expanded to include three natural polymers (sodium alginate, gelatin, starch) which have recently been used for microencapsulation of biopesticides. Results are based on larval insecticide bioassays to test original activity remaining (OAR). The results showed that titanium dioxide (3% w/v) had no protective effect after 8 hours exposure to UVA (26.66± 3.33). After 48 hours exposure, although titanium dioxide (3% w/v) provided a very little protection against UVA radiation (30.00± 6.35), it still remained the less effective additive. Unlike some reports, titanium dioxide did not provide adequate protection against UVA radiation in our study. In addition, it reduced the activity of occlusion bodies (OBs) in the absence of UV rays. After eight hours exposure to UVA radiation, sodium alginate (3% w/v) was an excellent UVA protectant (93.33± 3.33), whereas the mentioned polymer provided inadequate protection, 48 hours post UVA radiation (50.00± 3.46). The other applied polymers provided medium protection against UVA radiation. After 48 hours, the concentrations of 3% w/v of green tea and coffee provided significant UV protection on stability of *Helicoverpa armigera* nucleopolyhedrovirus activity at 80.00± 1.54 and 83.33± 3.33, respectively. Lignin at both concentration levels was a proper UV protectant after 48 hours (83.33± 6.66).

Keywords: microbial biopesticides, baculoviruses, additive, stability, ultraviolet radiation

1. Introduction

Within the last decades, microbial biopesticides have significantly expanded their market. Several microorganisms like bacteria, fungi, baculoviruses, are included in microbial biopesticides (Kumar *et al*., 2021). Microbial biopesticides are the group of pesticides, which act against invertebrate pests in agro–ecosystems (Ruiu, 2018).

Nucleopolyhedroviruses (NPVs), which belong to baculoviruses, have been taken into consideration in the integrated pest management programs. Because of their selectivity property, they are safe to non–target organisms and also compatible with other biological control options (Erayya *et al*., 2013). Previous studies however, have shown that natural sunlight is the most significant environmental factor that affects the stability of baculoviruses, destroying viral DNA and occurring viral inactivation, so that their half–life reduces from two to several hours (Villamizar *et al*., 2010; El–Helaly *et al*., 2013; El–Helaly, 2019; Sayed *et al*., 2020). Formulation is a technique to overcome this limitation. The cost effectiveness, application, storage, persistence, and handling can all be improved by formulation (Satinder *et al*. 2006; Grzywacz *et al*., 2017). Formulation of baculoviruses with various solar protectants preserves occlusion bodies (OBs) by different methods such as reflection of harmful UV–rays, selective absorption, or by conversion of shorter wavelengths to longer harmless wavelengths (Tamez–Guerra *et al*., 2000; Arthurs *et al*., 2008). Within the last decades, plant–derived substances have been known as UV protectants and sunscreens (Arthurs *et al*., 2006; Shapiro *et al*., 2007a, b; Shapiro *et al*., 2008; Shapiro, Jackson *et al*., 2009; Shapiro *et al*., 2009; El Salamouny, Ling *et al*., 2009). Sutanto *et al.* (2017) showed that 1% w/v green tea extracts caused considerable virus protection after UVB exposure. This result is consistent with Shapiro's (2008) publication in which green tea extracts were used as UV

protectant for Spodopteraexigua (Hubner) (SeMNPV). Subsequently, the black tea was introduced as an excellent UV protectant for SeMNPV (El Salamouny *et al*., 2009b). Later, cocoa and coffee were tested as UV protectants for the SeMNPV (El Salamouny *et al*., 2009a). Lignin, a plant–derived material with its aromatic chemistry is a proper material, due to its ability to adsorb UV wavelengths of light (Arthurs *et al*., 2008; El Salamouny *et al*., 2009a). Lignin and lignin derivatives have been used in *Bacillus thuringiensis* formulations to protect its crystal protein endotoxin from sunlight degradation (Shasha *et al*., 1998). A significant short–term improvement in residual virus activity has been achieved with raising lignin in CpGV/kraft lignin formulation (Arthurs *et al*., 2008). According to the laboratory and field research, the lignin encapsulated formulation provided higher OAR (88.3%) than the unformulated virus treatments (70.0%), as well as enhancement of the half–life (Behle *et al*., 2012). TiO2 was selected as a suitable UV absorbent in wax microencapsulated formulations of baculovirus. Their results showed that TiO_2 protects the NPV from degradation against simulated sunlight and so that the foresaid formulation greatly improved the stability of NPV activity when exposed to simulated sunlight. They reported that Titanium dioxide had no effect on larval mortality (Wilson *et al*., 2020).

In the present work, the UV protection of some natural and chemical additives (green tea, black tea, coffee, cocoa, Tween 80, sodium lignin sulfonate and titanium dioxide), including some natural polymers (Sodium alginate, gelatin, starch), were compared in formulation of *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV). Our purpose in this study is to provide the most effective additive for protecting HaNPV from degradation by sunlight.

2. Materials and Methods

2.1. Insect colony and virus inoculum

The colonized strain of the *Helicoverpa armigera* used in this work was established and maintained at Iranian Research Institute of Plant Protection (IRIPP). The HaNPV was obtained from Henan JiyuanBaiyun Industry Co., Ltd (China).

Viral OBs of isolated HaNPV were produced by infecting third–instar larvae in 28^{o}C and 55–60% humidity. After infecting larvae with HaNPV, the OBs were solubilized in the mid–gut of larvae, and the released virions initiated infection of the mid–gut cells. The OBs were later spread by the excrement of the infected insects (Catena *et al*., 2014). Dead and diseased larvae were collected five days after infection. Then, they were homogenized in water and purified by filtration through a muslin cloth sieve followed by centrifugation for 10 minutes at 8,000 rpm. It caused the extraction of OBs. The centrifuge–cleaning process was repeated twice. The final pellet was suspended in deionized water, and the number of OBs per ml was determined using a Neubauer hemocytometer. Purified polyhedra was lyophilized over 48h at –50^{o}C until the moisture content was less than 5%, and dried OBs were stored at –8 ^{o}C (Tamez–Guerra *et al*., 2000; Tufts *et al*., 2011).

2.2. Additive materials

Sodium alginate (Sigma Aldrich,USA), gelatin (Difco Co.), starch (Merck, Germany), green tea (Changsha NatureWay Co., Ltd.), black tea (Ahmad tea Co, Iran), coffee (Piggly Wiggly Corporation), cocoa (Cocoa Marketing Co.,gh, Ltd), Tween 80 (Sigma Aldrich,USA), sodium lignin sulfonate and titanium dioxide were taken from Kimia Sabzavaran Co. Iran.

2.3. Preparation of formulations

All additive materials were used as powder. For preparation of the additive and polymer suspensions in two different concentration levels (0.5% and 3% w/v), 0.5 and 3 grams of each of the above additive powders was blended in 100 ml of distilled water and filtered through coarse cheese cloth. In initial bioassays prior to UV irradiation, virus concentration that caused 90–95% mortality was determined. As a result of these assays, HaNPV was diluted in distilled water (non–formulated virus suspension) or in an aqueous filtrate of the above formulations (formulated virus suspension) to obtain a final virus concentration of 1×10^{6} OB ml^{-1}. Tween 80 (0.4%) was added. The formulations were then mixed with incubator shaker to obtain homogenize virus suspension. The filtrate was kept at 4°C until used (El Salamouny *et al*., 2009a).

2.4. UV radiation

Radiation was provided by UVA tube, 385 nm (Entela lamp model UVGL–25 of 4 W). It was mounted 150 mm above the test dishes (Shapiro *et al*., 2009). Ten ml of each virus formulation was poured into glass Petri dishes (three repeats for each treatment). Ten ml of the non–formulated virus solution was poured into glass Petri dishes individually (in three replications). The plates were then placed expose to UVA irradiation for 8 and 48 hours. The water evaporation was retrieved by addition of distilled water to Petri dishes. So the volume of suspension was kept constant in each dish. The dishes were then stored at 4°C with the lids on.

2.5. Bioassay test

The protection property of the materials tested against UV radiation is evaluated by bioassay test. To do this, 10µl of the irradiated and non–irradiated virus suspension (formulated and non–formulated), was applied to each 30–ml cup containing artificial diet (Abbasi *et al*., 2007) (10 cups per treatment per

replicate). Each treatment was carried out three times and 10 third instar larvae were applied each time. Plastic cups were used to place larvae with infected diet in each. The larvae were reared under a day–long photoperiod of 16 hours light and 8 hours dark at 25°C, 50% RH until death or pupation. Three untreated cups with ten larvae in each were used as control. Mortality was assessed initially at day 4 and every 2 days thereafter until day 14, when the test was terminated. In the entire experiment, the average number of calculated dead larvae in each treatment (over the three replications), is known as the mortality. The percentage of original activity remaining (%OAR) post UV–irradiation was used as a basis of UV protection and obtained by dividing the mortality on a given formulated treatment by the mean mortality of non–irradiated HaNPV/H_2O suspension in each respective experiment (El Salamouny *et al*., 2009a).

2.6. Data analysis and statistical methods.

Analysis of variance (ANOVA) in Statistical Package for the Social Sciences (SPSS 1998) was applied. Duncan test was used to discover the significant differences between treatment means.

3. Results

The Mortality percentage of *Helicoverpa armigera* larvae fed with the virus suspension (formulated and non–formulated), at both pre– and post– UVA irradiation (8 and 48 hours exposure) is presented in Table 1.

Table 1. Mortality percentage of *Helicoverpa armigera* larvae fed on the virus suspension (formulated and non–formulated) pre– and post– UVA irradiation (8 and 48 hours exposure)

Treatment	No–exposure[a]	8h[b]	48h[c]
Non–formulated (HaNPV/ water)	96.66±3.33[a]	26.66± 1.85[f]	16.66± 4.05[i]
Starch 0.5%	93.33± 3.33[ab]	60.00± 2.88[de]	53.33± 7.26[cde]
Starch 3%	96.66± 3.33[a]	53.33± 3.33[e]	46.66± 3.33[defg]
Sodium alginate0.5%	93.33± 3.33[ab]	60.00± 5.77[de]	53.33± 2.02[cde]
Sodium alginate3%	93.33± 3.33[ab]	93.33± 3.33[a]	50.00± 3.46[def]
Gelatin 0.5%	96.66± 3.33[a]	46.66± 3.33[e]	40.00± 1.73[efgh]
Gelatin 3%	96.66± 3.33[a]	46.66± 6.66[e]	36.66± 3.38[fgh]
Green Tea 0.5%	86.66± 8.81[ab]	83.33± 6.33[abc]	76.66± 5.69[ab]
Green Tea 3%	90.00± 5.77[ab]	86.66± 6.00[ab]	80.00± 1.45[ab]
Black Tea 0.5%	96.66± 3.33[a]	83.33± 3.33[abc]	76.66± 2.33[ab]
Black Tea 3%	86.66± 4.37[ab]	73.33± 8.81[bcd]	66.66± 6.17[bc]
Cocoa 0.5%	80.00± 1.15[b]	73.33± 3.33[bcd]	66.66± 4.48[bc]
Cocoa 3%	80.00± 2.30[b]	70.00± 1.52[cd]	60.00± 4.04[cd]
Coffee 0.5%	96.66± 3.33[a]	73.33± 8.21[bcd]	66.66± 3.33[bc]
Coffee 3%	90.00± 5.77[ab]	90.00± 5.77[a]	83.33± 3.33[a]
Sodium lignin sulfonate 0.5%	96.66± 3.33[a]	90.00± 5.77[a]	83.33± 6.66[a]
Sodium lignin sulfonate 3%	96.66± 3.33[a]	86.66± 3.33[ab]	83.33± 6.66[a]
Titanium dioxide 0.5%	46.66± 3.33[c]	30.00± 4.04[f]	33.33± 3.33[gh]
Titanium dioxide 3%	43.33± 5.45[c]	26.66± 3.33[f]	30.00± 6.35[h]

The mortality resulted from HaNPV/H_2O virus suspension declined from 96.66 to 26.66 after 8 hours of UVA exposure and reached to 16.66 after 48 hours.

According to the ANOVA, the mortality yielded from cocoa and titanium dioxide formulations were significantly less than the non–formulated virus suspension (P<0.05), prior to irradiation. This indicates that none of the additives used in this work (except for the cocoa and titanium dioxide) affects the viral activity. From statistical point of view there is no significant difference between the mortality results of the titanium dioxide formulations after 8 hours UVA exposure and the non–formulated virus suspension (Duncan Test, P< 0.05). Clearly, the titanium dioxide produced no protective effect against UVA radiation after 8 hours. After 48 hours exposure, it provided a very little protection against UVA radiation, though it was not enough and it remained the less effective additive. However, coffee (3% w/v) and lignin (0.5% & 3% w/v) showed high protection (Table 1).

Figure 1 and 2 shows the OAR percentage of HaNPV/H_2O suspension before UV exposure and HaNPV/additive combinations after 8 and 48 hours UV exposure, respectively. It is apparent that the OAR percentage of each HaNPV/additive

combination (except for titanium dioxide) after 48 hours of irradiation is lower than the mentioned value after 8 hours of irradiation. From Figure 1 and 2, it is assumed that after 8 hours of UVA exposure, sodium alginate (3% w/v) is an excellent UV protectant whereas the mentioned polymer provided inadequate protection, after 48 hours of exposure. Except for sodium alginate, other used polymers created mean protection against UVA radiation.

Green tea and coffee at the concentration level of 3% w/v provided significant protection for HaNPV against UVA irradiation. The mentioned additives at lower concentration level (0.5% w/v), nonetheless, provided UV protection properly. Lignin at both concentrations was an effective UV protectant (Figure 1 and 2).

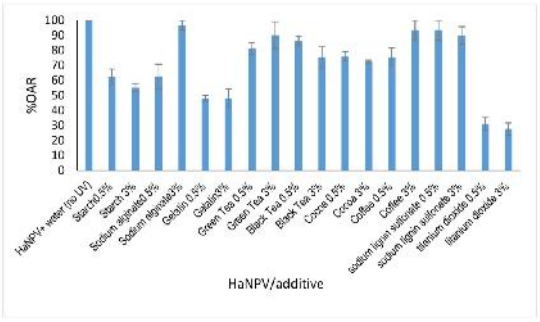

Fig. 1. The OAR percentage of non–irradiated HaNPV/H_2O and irradiated HaNPV/additives for 8 hours

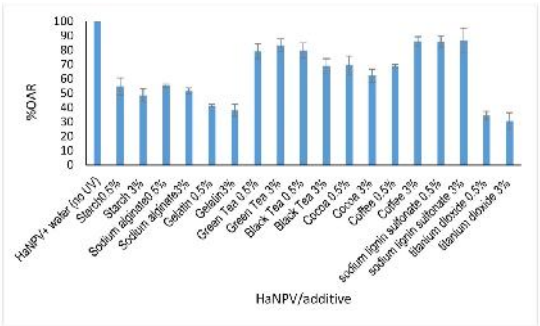

Fig. 2. The OAR percentage of non–irradiated HaNPV/H_2O and irradiated HaNPV/additives for 48 hours

4. Discussion

The specificity of baculoviruses with the host, ensure the management of agro–ecosystems and leads to environment and human health. Because of the low stability of baculoviruses in the environment and against sun light, preparation of UV protectant formulations are essential. There are different kinds of UV protectants such as absorbers, reflectors, and antioxidants. Antioxidants protect the microbial agent by counteracting the oxidative chemicals created by the UV radiation. Absorbers and reflectors do not let UV radiation reach the microbial agent (Farrar *et al*., 2003). Tea, coffee and cocoa beverages have been tested in baculoviruses formulations in order to inhibit the virus from inactivation after UV radiation (El Salamouny *et al*., 2009a). Lignin seemed to be a good absorber for both UVB and UVA radiation in the formulation of baculovirus biopesticides (Arthurs *et al*., 2006; Arthurs *et al*., 2008; El Salamouny *et al*., 2009b). However, black tea was primarily an absorber of UVB.

In the current study, we tested some natural and chemical UV protectants and expanded our investigation to include three natural polymers (sodium alginate, gelatin, and starch) applied for microencapsulation of biopesticides by Khorramvatan *et al.* (2013). For our study, we used these criteria to assess UV protection: (1) the virus concentration (OBs/ml) should cause approximately 90–95% larval mortality prior to irradiation; (2) UV protection for each of the formulation should be compared with initial pre–irradiation virus–caused mortality in virus/water suspension; and (3) our criterion for excellent UV protection should be above 90% OAR (Shapiro *et al*., 2009).

In our work, we presumed green tea as a good candidate for UV protection. The good UV protection of green tea is due to its phenolic compounds like Epigallocatechin Gallatewith antioxidant activity (Shapiro *et al*., 2008; Sutanto *et al*., 2017). Antioxidants because of OH group in their molecules, can remove the free radicals which are produced by UV radiation. Therefore, they protect cell against radiation–induced damage (Shin *et al*., 2014; Vishnoi *et al*., 2018).

Our statement about coffee as a good protectant against UV radiation is already favored by the results of El Salamouny *et al.* (2009a). It is due to the many of phenolics in coffee as chlorogenic acid, and some breakdown products such as melanoidins, caffeic acid, caffeine, and epicatechin, which are good UV absorbers and antioxidants (Pellegrini *et al*., 2003; George *et al*., 2008).

In order to provide a proper UV protection for viruses there is no need for the additives to have high UVA or UVB absorption. It seems that the main UV protection mechanism for viruses is counteracting the free radicals which cause DNA damage. But, it is not the same for some entomopathogenic fungus like *Beauveria bassiana*. The UV protectant substances for *B. bassiana* conidia must have high UV absorption capacity. So that it can provide a shield on the outside of spores to block the UV radiation (Kaiser *et al*., 2019).

El–Helaly *et al.* (2013) reults were in agreement with El Salamouny *et al.* (2009a) which recommended cacao as a good UVA protector. But our results in the present work showed that cocoa in

both concentrations of 0.5% and 3% w/v provided fair protection after exposure to UVA radiation.

Consequently, the titanium dioxide is not desirable as a UV protectant for the OBs of the NPV as supported by Farrar *et al*., (2003). They believe that although titanium dioxide is a reflector of ultraviolet light and can protect the OBs of NPVs from degradation by sunlight, it catalyzes the formation of hydrogen peroxide in the presence of sunlight and water. It is in compliance with Kim *et al*. (2020) experiment which used TiO_2–supported iron catalyst for activation of peroxide. They revealed that the interaction between H_2O_2 and TiO_2 was more effective at activating H_2O_2 than pure iron oxide (Kim *et al*., 2020).

There is no literature on using natural polymers as additives in the formulation of baculoviruses. They are commonly used for micro–encapsulation process. They have recently been used for micro–encapsulation of Bt by Khorramvatan *et al.* (2013). We used several natural polymers in the current research as additives for protection of HaNPV against UVA, out of which, only sodium alginate provided enough protection for HaNPV after 8 hours exposure to UVA, at concentration level of 3% w/v. Remarkably, the UVA absorbance ability of sodium alginate is decreased as time continues. Although our results are novel and unexpected, it can be explained with the following interpretation. Sodium alginate is a natural, water–soluble polysaccharide derived from brown algae. Phenolic compounds (PC) in brown algae named phlorotannins, are secondary metabolites that participate in many biological processes, such as ultraviolet radiation protection (Leonardo *et al*., 2007). The obtained results reveal the strong linkage between PC and alginates and that these linkages preserve the UV absorption capability of PC along with time and also for a longer period of time compare with the purified PC. Although the UV absorption properties of PC linked to alginates was strongly modified compare with the purified PC, the ability to absorb UV radiation is decreased as time goes on. For instance 18% of its absorbance at 210 nm is decreased after 72 hours (Leonardo *et al*., 2007).

5. Conclusion

Baculovirues is a group of microbial biopesticides which are not stable against sun light. To inhibit the virus from inactivation, the ultraviolet protective effect of some natural and chemical additives and three natural polymers at two concentration levels (0.5% and 3% w/v) for the *Helicoverpa armigera* nucleopolyhedrovirus was evaluated. In summary, coffee, green tea and lignin were effective in protecting HaNPV from ultraviolet radiation. Titanium dioxide does not provide adequate protection against UVA radiation. In addition it reduces the activity of OBs in the absence of UV rays (Table1).

Farrar *et al*. (2003) declared that in the absence of UV, the activity of the OBs was reduced by non–photostabilized titanium dioxide; though, it was unaffected by photostabilized titanium dioxide. Our results show that, after 8 hours, sodium alginate (3% w/v) was the most effective additive. Whereas, it was less effective when it was used for microencapsulation of virus in our previouse work (Gifani *et al*., 2015). Accordingly, due to the microencapsulation costs, the effectiveness of microencapsulation for virus protection and its necessity should be investigated. For this purpose, new borders of research on microencapsulation of HaNPV are needed. The difference of microencapsulated and non–microencapsulated formulations should be compared. Further studies in this point, will focus on different microencapsulation techniques to discover proper polymers as radiation protectants in the control of agriculturally–important insect pest populations.

Conflict of interest

No potential conflict of interest was reported by the authors.

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تأثیر حفاظتی افزودنی‌های مختلف و پلیمرهای طبیعی بر پایداری (*Helicoverpa armigera NPV* (*HaNPV*) در برابر اشعه ماوراء بنفش

آیدا گیفانی[۱]، رسول مرزبان[۲]، سوده خرم وطن[۳]، مهدی ارجمند[۴]، احمد دزیانیان[۵]

۱- مرکز تحقیقات مدلسازی و بهینه سازی در علوم مهندسی، دانشگاه آزاد اسلامی واحد تهران جنوب، ایران

۲- موسسه تحقیقات گیاه‌پزشکی کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران

۳- گروه مهندسی شیمی، دانشگاه آزاد اسلامی واحد علوم و تحقیقات، تهران، ایران

۴- گروه مهندسی شیمی، دانشگاه آزاد اسلامی واحد تهران جنوب، ایران

۵- مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی استان سمنان، سازمان تحقیقات، آموزش و ترویج کشاورزی، شاهرود، ایران

مسئول مکاتبات: آیدا گیفانی، ایمیل: aida_gifani@yahoo.com

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چکیده

آفت‌کش‌های میکروبی، به‌دلیل تاثیرات زیست‌محیطی اندک، پشتیبان بخش مدیریت در حوزه کشاورزی هستند. در میان آفت‌کش‌های میکروبی، باکولوویروس‌ها بسیار مورد توجه قرار گرفته‌اند. به‌دلیل پایداری کم باکولوویروس‌ها در برابر نور خورشید، در این پژوهش بر آماده‌سازی فرمولاسیون‌های محافظ در برابر UV، متمرکز شدیم. بر این اساس، اثر پوششی برخی از افزودنی‌های طبیعی و شیمیایی (چای سبز، چای سیاه، قهوه، کاکائو، توئین ۸۰، سدیم لیگنین سولفونات و دی اکسید تیتانیوم) در برابر اشعه ماوراء بنفش، در دو سطح غلظت وزنی (۰/۵٪ و ۳٪) برای ویروس *HaNPV* مورد ارزیابی قرار گرفت. علاوه بر آن، تحقیقات برای استفاده از سه پلیمر طبیعی (آلژینات سدیم، ژلاتین، نشاسته) که اخیراً برای میکروکپسوله کردن آفت‌کش‌ها بکار رفته‌اند، گسترش داده شد. برای تعیین میزان OAR، نتایج بر اساس زیست‌سنجی لاروها ثبت شد. نتایج نشان داد که دی اکسید تیتانیوم (۳٪)، پس از ۸ ساعت در معرض اشعه UVA قرار گرفتن، تاثیر محافظتی نداشته است. پس از ۴۸ ساعت، با وجود اینکه دی اکسید تیتانیوم (۳٪) محافظت بسیار کمی در برابر اشعه UVA ایجاد کرد، لیکن در بین مواد افزودنی مورد استفاده در تحقیق، کم تاثیرترین ماده افزودنی به‌شمار آمد. در پژوهش حاضر، دی اکسید تیتانیوم نه تنها بر خلاف بسیاری از گزارش‌ها، پوشش‌دهی کافی در برابر اشعه UVA فراهم نکرد، بلکه در غیاب اشعه UV، موجب کاهش میزان OBs گردید. پس از ۸ ساعت، سدیم آلژینات (۳٪) به‌عنوان یک محافظ عالی در برابر اشعه UVA عمل کرد. در حالی‌که این ماده ۴۸ ساعت پس از اشعه دهی، پوشش کافی برای محافظت فراهم نکرد. سایر پلیمرهای بکار رفته پوشش متوسطی در برابر اشعه UVA ایجاد کردند. ۴۸ ساعت پس از اشعه دهی، چای سبز و قهوه (۳٪)، به‌طور قابل توجهی روی پایداری *HaNPV* در برابر اشعه UVA تاثیر گذار بودند. لیگنین در هر دو غلظت به‌کار رفته، محافظ مناسبی در برابر اشعه UVA پس از ۴۸ ساعت بود.

واژه‌های کلیدی: آفت‌کش‌های میکروبی، باکولوویروس‌ها، افزودنی، پایداری، اشعه ماوراء بنفش