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The Effect of Nanozeolite Concentration in a Delivery System of *Ha*NPV$_1$ to the Lethal Time against *Crocidolomia pavonana*

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ABSTRACT

The constraints on the effectiveness of nuclear polyhedrosis virus (NPV) as biocontrol are usually due to environmental factors such as temperature and ultraviolet (UV) exposure. Zeolite has been commonly used as a carrier or delivery system for nuclear polyhedrosis viruses. In this study, zeolite powder was reduced into nanosized particles by beads milling method and was investigated for the effect of its concentration in the delivery system of *Helicoverpa armigera* nuclear polyhedrosis virus (*Ha*NPV$_1$) on the lethal time against the larvae *Crocidolomia pavonana*. The formulation used three concentrations of nanozeolite suspension, 0.5, 1, 1.5, and 2 wt.% applied for each 4×10^7 of *Ha*NPV$_1$. A randomized block design (RBD) method was applied with 3 replications. The results showed that the scanning electron microscope (SEM) from nanozeolite was seen coating the entire surface of the *Ha*NPV$_1$ polyhedra and an increase of zeolite concentration caused acceleration of the lethal time of *C. pavonana* instar III. Thus, the fastest lethal time was 1.2 days receiving a concentration of 2 wt.%, which was significantly higher compared to without delivery (2.9 days). The increase of the zeolite concentration up to 2 wt.% in the delivery system for *Ha*NPV$_1$ improved their performance on lethal time

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and mortality against *C. pavonana*. It was concluded that nanozeolite as a delivery system enhanced and created a synergy in infecting *C. pavonana*.

Keywords: *Crocidolomia pavonana*, *Ha*NPV$_1$, nanoparticle, nanozeolite, pest control

INTRODUCTION

Crop caterpillars (*Crocidolomia pavonana*) is one of the highly harmful pests to cabbage (*Brassica oleracea* var. Capitata L). *Crocidolomia pavonana* frequently attacks cabbage plants at the early stage of crop growth, leaves holes, and may attack primordial tissues causing plants to stop growing. If there is no effort to control the pest, particularly in the dry season, it can cause harvesting failure (Yuliadhi et al., 2016). Therefore, it is important to apply integrated pest control (IPC) to deal with this insect. However, farmers usually use synthetic insecticides to control insects, which is harmful to the environment (Razak et al., 2014). Thus, it is important to have an alternative biocontrol to avoid the use of synthetic insecticides, i.e. microbial agents such as bacteria, fungi, and viruses.

One of the potential viruses usually used as a biocontrol is baculovirus which belongs to a family of entomopathogenic. This virus attacks arthropods, especially insects from the Lepidoptera order. Baculovirus is used as a microbial agent because it is safe, easily mass-produced, highly pathogenic to insects, and easily formulated and applied. One of the developed baculoviruses is nuclear polyhedrosis viruses (NPV), which is packaged in a protein matrix called "polyhedra" (Ompusungu et al., 2015). One strain of NPV is *Ha*NPV which is isolated from larvae *Helicoverpa armigera*. This virus is known to have infected the Lepidoptera order when polyhedra ingested by target pest insects (Govindaraju et al., 2011). Furthermore, to enhance the number of produced virus, the *Ha*NPV was sub-cultured and isolated in an alternate host of *Spodoptera litura,* named as *Ha*NPV$_1$ (Miranti et al., 2015).

Despite the capability of this virus to infect the targeted pest insect, the environmental factors influence the viability of this virus in the field application. To maintain the effectiveness and viability of this virus, zeolites were used as a carrier or delivery system to protect the virus from environmental constraint (Melanie et al., 2017). It was reported that the use of zeolite as a drug carrier enhanced solubility and effectively modulates drug (Karavasili et al., 2017). The zeolites were also reported applied in controlling the insect pest *Chironomus riparius*. It was found that zeolite concentration determined the effectiveness of their control (Lorenz et al., 2017). In addition, it is also expected that the delivery system (zeolite) is in synergy to support the virus-infected insect target. Some researchers tried to enhance the delivery system by reducing the size of the powder aims to obtain higher toxic effects due to the greater surface area (Wibowo & Putra, 2013). Therefore, this study

aimed to investigate the effectiveness of nanozeolite as a delivery system of *Ha*NPV$_1$ and investigated the effect of nanozeolite concentration on the lethal time against *C. pavonana*.

MATERIALS AND METHOD

Preparation *Ha*NPV$_1$ Suspension

The *Ha*NPV$_1$ was produced by infecting the *Ha*NPV$_1$ in *Spodoptera litura* as an alternate host. The virus was isolated after only 1 passage in *S. litura* larvae. *Spodoptera litura* third instar larval was infected by 4×10^5 OBs/mL of virus suspension. The infected larval was collected in a glass container and stored at 4°C. Then, the cadavers (40 larval) were crushed by mortar and mixed with 20 mL Tris buffer (1 mM, pH 7.6) solution and 20 mL 0.1% sodium dodecyl sulfate (SDS) solution. This mixture was stored at 4°C for 24 h (Miranti et al., 2015).

After storage, the mixture of the virus was filtered with two layers of the filter. Filtering using 2 layers of cotton cloth. The suspension of the virus was centrifuged at relative centrifuge force (RCF) 1,931 x *g* for 15 min at 4°C. The supernatant was suspended in 5 mL Tris buffer (1 mM, pH 7.6) solution and 5 mL 0.1% SDS solution and subsequently centrifuged at RCF 1, 931 x *g* for 15 min at 4°C. The centrifugation was conducted just for separating viruses from other debris. The last supernatant was suspended with mixed Tris buffer (1 mM, pH 7.6) solution and 0.1% SDS solution by adding 0.2% sodium azide to prevent the virus suspension from contaminant (Miranti et al., 2015).

To count the OBs numbers of a virus, 0.1 mL virus suspension was mixed by adding 0.9 mL of Tris buffer (1 mM, pH 7.6) and 0.1% SDS with a 1: 1 ratio. The suspension of the virus with concentration 4×10^7 OBs/mL in the liquid medium was used for bioassay.

Zeolite Beads Milling

Firstly, received zeolite was ball milled into -400 mesh. One hundred and fifty grams (150 g) of zeolite suspended into 2 liters of water and mixed using a stirrer at a speed of 2,000 rpm for 2 h. The suspension was then milled with a bead milling method for 3 h. This was a wet bead milling process utilized zirconia with 30 μm in size and detailed explain elsewhere (Joni et al., 2010; Rochima et al., 2018). The size and size distribution of the zeolite particles were performed using particle size analysis (PSA, HORIBA Scientifica SZ-100, HORIBA, Ltd. Japan) and their morphology observed with a scanning electron microscope (SEM, HITACHI SU3500. HITACHI High-tech GLOBAL, Japan).

*Ha*NPV$_1$ and Nanozeolite Formulation

The *Ha*NPV$_1$ formulation with nanozeolite carrier was obtained by mixing 1 mL of *Ha*NPV$_1$ suspension with a concentration of 4×10^9 OBs/mL with 99 mL of nanozeolite suspension at various concentration according to the treatment (i.e. 0.5, 1, 1.5, and 2 wt.%). This formula was used for bioassay testing against *C. pavonana*.

Bioassay Test

The vegetable as a host plant of *C. pavonana* was obtained from the Vegetable Research Institute (BALITSA) Lembang, Bandung, West Java, Indonesia. Test insects of *C. pavonana* were used at instar III. The observation of lethal time was conducted at various treatments including their control using only cabbage and in comparison, to the only *Ha*NPV$_1$. Thus, there were 8 levels of various treatment as follows:

C0 : Control (only cabbage)
C1 : *Ha*NPV$_1$
C2 : 0.5 wt.% Nanozeolite
C3 : 1 wt.% Nanozeolite
C4 : 1.5 wt.% Nanozeolite
C5 : 2 wt.% Nanozeolite
C6 : *Ha*NPV$_1$ + 0.5 wt.% Nanozeolite
C7 : *Ha*NPV$_1$ + 1 wt.% Nanozeolite
C8 : *Ha*NPV$_1$ + 1.5 wt.% Nanozeolite
C9 : *Ha*NPV$_1$ + 2 wt.% Nanozeolite

The *C. pavonana* larvae were placed 10 individual larvae in a plastic container for each treatment and subjected to acclimatization to ensure the health of larvae. Acclimation by way of the *C. pavonana* larvae was placed in condition without feed for 3 h before the applications of the formulation. The application means that the cabbage coated with the formulated biocontrol and was infected by means of oral ingestion. The application of formulations to larvae was carried out for 7 days of observation.

Data Analysis

This research is a biological test using descriptive exploratory methods in the laboratory. The research design used was a single factor randomized complete block design (RCBD). Each treatment was repeated three times based on the results of calculations using Federer's formula, namely (t-1) (n-1) ≥ 15. In this study, 30 experimental plots were obtained. The obtained data were analyzed using analysis of variance (ANOVA) with their significance were determined by Duncan's multiple distance test (DMRT) at a level of 5%. The average of lethal time was calculated using the formula in Equation (1) for 7 days observation (Tamimi et al., 2016).

$$W = \frac{\sum W_i \bullet Z_i}{Y} \quad (1)$$

where,

W = Average lethal time

W_i = Lethal time of test insects on the day i of infection

Z_i = Number of dead insects on the first day of infection

Y = Number of test insect death

RESULTS AND DISCUSSION

Particle Size Analysis (PSA)

Figure 1 shows the size distribution of zeolite before and after beads milling. The results showed that the initial size distribution of the zeolite particles with an average size of 499 nm with a high polydispersity index (1.626) means that zeolites were partially agglomerated. After beads milling, the

average size of zeolite particles was 175 nm with a very low polydispersity index (0.776) means that particles were relatively homogenous in size. The obtained zeolite suspension with homogenous in size ensures their affectivity on coating the virus.

Figure 2 shows the SEM images of zeolite before and after beads milling with magnification 20, 000. The result indicated that the morphology of zeolite was changed after beads milling. Before beads milling, the morphology of the zeolite particles was flaky and agglomerated as highlighted in a red circle as shown in Figure 2a. In contrast, the morphology of zeolite particles after beads milling was changed into smaller sized as highlighted in a red circle as shown in Figure 2b. Figure 2c shows the *Ha*NPV$_1$ with spherical in morphology highlighted in a dotted red circle. This is consistent with research conducted by Sudhakar and Mathavan (1999), in which the PIB *Ha*NPV$_1$ was spherical in morphology and some of them were irregular in shape. The nanozeolite has encapsulated the surface of the *Ha*NPV$_1$ as indicated in Figure 2d, which is highlighted with a dotted red circle. Nanozeolite can be used as a carrier material of *Ha*NPV$_1$ with visible nanozeolite covering the entire surface of the *Ha*NPV$_1$ polyhedra.

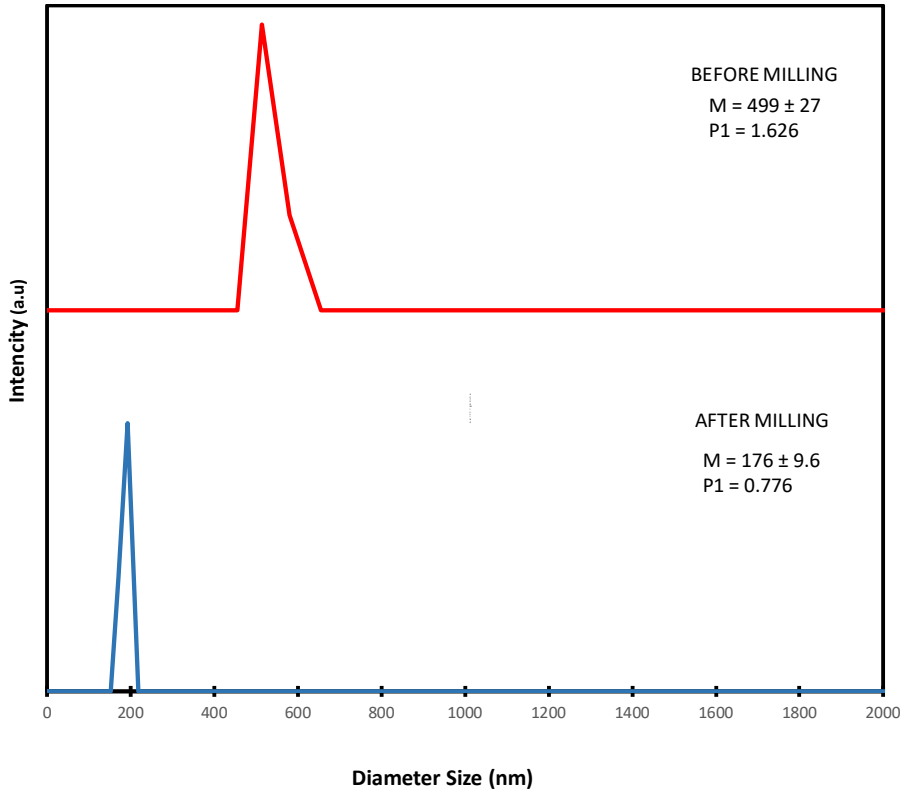

Figure 1. The size distribution of zeolite before and after beads milling

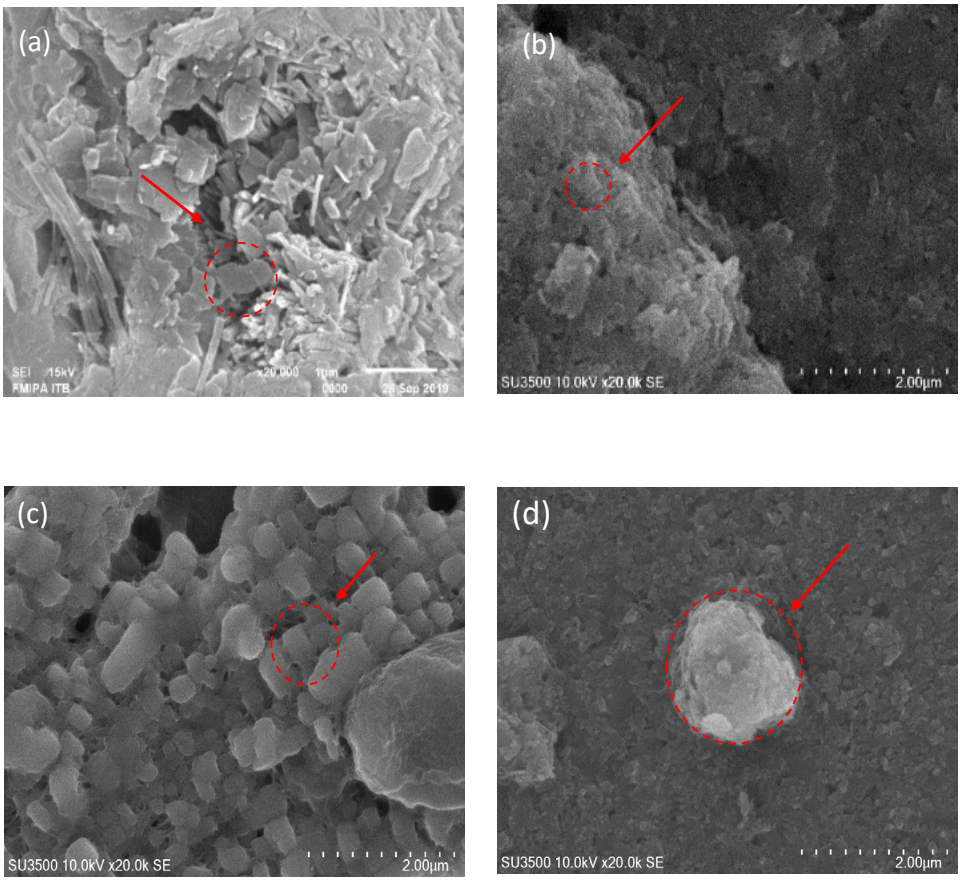

Figure 2. The SEM images of zeolite with magnification 20, 000 (a) before beads milling, (b) after beads milling, (c) *Ha*NPV$_1$, and (d) *Ha*NPV$_1$ with nanozeolite

Effect of Nanozeolite Concentration on Lethal Time and Mortality against *Crocidolomia pavonana*

The lethal time of the *C. pavonana* larvae in all treatments showed significant differences (Table 1). The use of *Ha*NPV$_1$ as control only produced a lethal time of 2.9 days. While the treatment with the only nanozeolite with a concentration of 0.5 wt.% decreased the lethal time up to 2.6 days and the significant difference compared to the treatment of only *Ha*NPV$_1$. However, the increase of nanozeolite concentration to 1 wt.% did not significantly improved the lethal time. The lethal time significantly improved when the concentration of zeolite 1.5 and 2 wt.% were used with a lethal time correspondingly 1.7 and 1.3 days. There were significant differences in the use of nanozeolites with concentrations of 0.5, 1, 1.5, and 2 wt.% with *Ha*NPV$_1$ compared with the use of nanozeolites alone. The results of the

analysis showed the fastest lethal time in the use of nanozeolite 2 wt.% with the fastest time of death, which was 1.2 days. These results are supported in Figure 3a showing *C. pavonana* larvae infected with *Ha*NPV$_1$ undergoing regurgitation within 2.9 days. whereas *C. pavonana* larvae infected with nanozeolite and *Ha*NPV$_1$ underwent regurgitation within 1.2 days. The fastest lethal time of larvae (1.2 days) was obtained from the application of nanozeolite 2 wt.% as a delivery system for *Ha*NPV$_1$. All treatment showed a significant deferent in the mortality of the larvae compared to the control. It was highlighted that the treatment with only *Ha*NPV$_1$ received quite lower mortality (86%) compared to other treatments (100%), however, it was not significantly different.

Figure 3 shows the photo images of larvae *C. pavonana* infected with *Ha*NPV$_1$, infected with nanozeolite, infected with *Ha*NPV$_1$ with the delivery system of nanozeolite. The larvae of *C. pavonana* was infected with the virus *Ha*NPV$_1$ appeared to be settled in the corners and on the sidelines of the cabbage crop leaves (Figure 3a). It was also observed that the larvae slowed their movements and tended to be settled with the body of the larvae becoming flabby and emitted a brown liquid. This phenomenon is in accordance with research reported by Rao et al. (2015), which stated that some of the common symptoms of attacked by a virus caused lethargy, skin discoloration, wet or very moist stools, and liquid regurgitation. The infected larva is generally characterized by reducing the ability to eat, slow motion,

Table 1

The lethal time and mortality of Crocidolomia pavonana *larvae*

Treatment	Average lethal time (day)	Average mortality (wt.%)
Control	0 ± 0^{a}	0 ± 0^{b}
*Ha*NPV$_1$	2.9 ± 0.65^{e}	86 ± 23^{a}
0.5 wt.% Nanozeolite	2.6 ± 0.30^{de}	100 ± 0^{a}
1 wt.% Nanozeolite	2.4 ± 0.47^{de}	100 ± 0^{a}
1.5 wt.% Nanozeolite	1.7 ± 0.20^{bc}	100 ± 0^{a}
2 wt.% Nanozeolite	1.3 ± 0.05^{b}	100 ± 0^{a}
0.5 wt.% Nanozeolite + *Ha*NPV$_1$	2.4 ± 0.02^{cd}	100 ± 0^{a}
1 wt.% Nanozeolite + *Ha*NPV$_1$	2.0 ± 0.25^{cd}	100 ± 0^{a}
1.5 wt.% Nanozeolite + *Ha*NPV$_1$	1.7 ± 0.55^{bc}	100 ± 0^{a}
2 wt.% Nanozeolite + *Ha*NPV$_1$	1.2 ± 0.10^{b}	100 ± 0^{a}

Note.
- Numbers in columns are average ± SD
- Numbers followed by a different letter in a column were significantly different according to Duncan's multiple range test $p < 0.05$

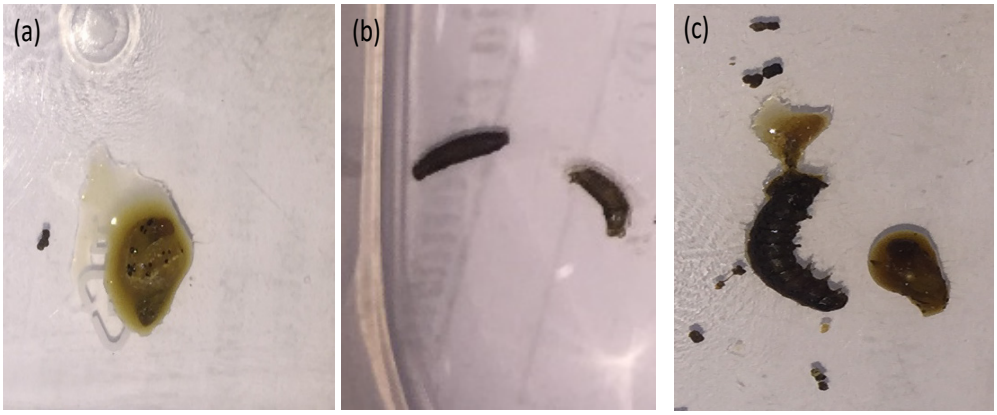

Figure 3. The photo images: (a) larvae *Crocidolomia pavonana* infected with *Ha*NPV$_1$, (b) larvae *C. pavonana* infected with only nanozeolite, and (c) larvae of *C. pavonana* infected *Ha*NPV$_1$ with nanozeolite

swollen body, due to the replication of the virus in the body (Bedjo, 2017). It is also the infected larvae that showed their body color turn pale, not actively moving, a larval body is flabby and secreted milk-brown liquid which contained polyhedra and reduced food activity (Arlita et al., 2014). This condition usually occurs 24 h after the larvae are infected. In line with the research reported by Sanjaya et al. (2011), which stated that the results of histological incision of the middle intestine of larvae *S. litura* within 24 h after treatment the damage occured to the outermost layer and the peritrophic membrane. Virion replicates or self-propagate in the cells of the insect's body so that eventually the insect dies because the whole body undergoes lysis. The infection is polyorganotrophic, which means the virus at the same time infects multiple tissues such as the epidermis, tracheal matrix, fat bodies, hemocytes, central nervous system cells, and pericardial (Das et al., 2019).

Figure 3b shows the death larvae with their bodies were dried out. This might be due to the absorption of liquid in the body of the larvae by nanozeolite. The absorption process by zeolites occurred because of its structure and also a high polarity of nanozeolite (Ginting et al., 2007). Figure 3c shows the death larvae infected with *Ha*NPV$_1$ with the delivery system of nanozeolite caused emitted a brown liquid and their bodies were dried out. This was an indicators for the synergy between *Ha*NPV$_1$ and nanozeolite infected the larvae.

Figure 4 shows the effect of treatment on the behavior of larvae consumption of cabbage. This behavior was investigated to know the important effect of additional nanozeolite 2 wt.% in the delivery system and also its role in the delivery system of *Ha*NPV$_1$. The *C. pavonana* larvae infected by only *Ha*NPV$_1$ were still able to consume cabbage before the death occurred and indicated by the existence of faces in the container (Figure 4a). This indicated that

Figure 4. The effect of treatment on the behavior of larvae consumption of cabbage: (a) cabbage leaves consumed by *Crocidolomia pavonana* infected with *Ha*NPV$_1$, (b) cabbage leaves consumed by *C. pavonana* infected with nanozeolite, (c) cabbage leaves consumed by *C. pavonana* infected with *Ha*NPV$_1$ with nanozeolite as a delivery system

the virus needs time to infected larvae (Sanjaya et al., 2011). In contrast, the cabbage leaves were partially consumed by the larvae when infected with only nanozeolite 2 wt.%, significantly less consumed compared to those infected only with *Ha*NPV$_1$ (Figure 4b). This might due to the larvae having suffered in the digestive tract because zeolite absorption leads to dry out the body of the larvae. It is also reported the similar phenomena that zeolite kills insects mainly by abrasive action or by absorption of epicuticular lipids from the insect exoskeleton causing excessive dehydration (Lu et al., 2017). Also, zeolites work by creating a barrier film by covering the leaves with a white powdery film, which adheres and irritates insects (De Smedt et al., 2016).

The cabbage leaves remained unconsumed by larvae infected by *Ha*NPV$_1$ with delivery system 2 wt.% of nanozeolite and no feces were found (Figure 4c). This is an indication of the synergetic effect of viruses and zeolite as a delivery system. The biocontrol formulated with *Ha*NPV$_1$ and nanozeolites as the delivery system was consumed from the leaves cabbage by the larvae. The consumed cabbage with the formulation was ingested by the larvae and firstly, the nanozeolite particles were absorbed by larval midgut and then the polyhedra directly entered the larval midgut lead to infection of the larval body cells. This phenomenon was also reported that the use of zeolites accelerated the lethal time in *Tuta absoluta* and as a result absorbed the liquid in the insect's body (De Smedt et al., 2016). In this study, the use of *Ha*NPV$_1$ tailored with a delivery system of nanozeolite was effective in accelerating the lethal time and significantly enhanced the mortality against *C. pavonana* larvae.

CONCLUSION

The increase of the zeolite concentration up to 2 wt.% in the delivery system for *Ha*NPV$_1$ improved their performance on lethal time and mortality against *Crocidolomia pavonana*. It was also found that nanozeolite as a delivery system enhanced and created a synergy in infecting *C. pavonana*. The virus

encapsulation with nanozeolite allowed the application of the formulation in the field since nanozeolite possible to protect the virus from UV exposure and other environmental factors. We also found that only nanozeolite received high performance as pest control.

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