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Encapsulation of *Metarhizium anisopliae* spores with zeolite nanoparticles and magnesium silicate nanoparticles against mortality and lethal times of *Crocidolomia pavonana* larvae

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ABSTRACT

Aims: Cruciferae crops failure such as cabbage generally due to heavy attacks by of *Crocidolomia pavonana* larvae. *Metarhizium anisopliae* is a species of fungus that can infect more than 200 insect pests including *C. pavonana* larvae. However, the direct application of *M. anisopliae* spore in the field is susceptible to UV rays from the sun, which can decrease the efficiency of *M. anisopliae*. Thus, this study aims to investigate the encapsulation performance of *M. anisopliae* spore with zeolite and magnesium silicate nanoparticles in terms of mortality and lethal time against 3rd instar *C. pavonana* larvae.
Methodology and results: Zeolite and magnesium silicate nanoparticles were chosen because they are non-toxic, environmentally friendly and able to maintain moisture, so that they are best used as protective materials for spores. The experiment was designed by Randomized Block Design (RBD) with a single factor. The obtained results showed that encapsulation of *M. anisopliae* spores with zeolite nanoparticles coating agent increased the mortality rate to 92.5% and accelerated the lethal time up to 1.075 days compared to only spore correspondingly 27.5% and 2.235 days. The *M. anisopliae* spores encapsulated with magnesium silicate nanoparticles also increased the mortality to 85.0% and accelerated the larval lethal time up to 1.150 days.
Conclusion, significance and impact of study: *Metarhizium anisopliae* spores that encapsulated with zeolite nanoparticles coating received higher mortality and faster lethal time to *C. pavonana* compared to those encapsulated with magnesium silicate nanoparticles. The encapsulation formulation of these two coatings can be used as bioinsecticide in controlling *C. pavonana* larvae.

Keywords: Effectiveness, *Crocidolomia pavonana*, *Metarhizium anisopliae* spores, magnesium silicate nanoparticles, zeolite nanoparticles

INTRODUCTION

Cabbage head caterpillar (*Crocidolomia pavonana*) is pest insects that damage cabbage plants. The attack causes holes in the leaves with a diameter range of up to 0.5 cm, the worst damage is often ended with crop failure. Farmers usually control C. *pavonana* using synthetic insecticides. However, the use of synthetic insecticides creates negative impacts such as increased resistance of pests, decreasing the population of natural enemies (parasitoids and predators), and environmental pollution (Suresh *et al.*, 2018).

Many researchers have tried to use biocontrol agents as a solution to reduce the impact of environmental pollution instead of using synthetic pesticides. Biocontrol agents obtain from entomopathogenic microorganisms such as viruses, bacteria, fungi, and nematodes (Shapiro -Ilan *et al.*, 2012). Since the *Metarhizium anisopliae* is one of entomopathogenic microorganism possess a wider host range, it is well-known pest that able to infect more than 200 types of insects and various arthropods (Lacey *et al*., 2011). Aryo *et al.* (2017) found that the application of fungal spores directly (without encapsulant) in the field caused the effectiveness of spores to infect pest insects

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decreased. This occurred might due to environmental factors such as direct exposure to sunlight (UV light), which causing the spores to become inactive.

To prevent these effects, the spores usually encapsulated with a non-toxic carrier to protect them against environmental influences. Encapsulation is the process of coating a material to the biocontrol agents with the aim to control or slow release of the biocontrol agents (i.e. spore). Encapsulation techniques can be used to release encapsulants at certain points and prevent degradation due to direct sunlight exposure. Almost all material can be protected with this technique including being able to encapsulate microorganisms (Risch, 1995).

Zeolite is one of the common materials used as a delivery system in biocontrol (Puterka *et al*., 2000). Zeolite is an excellent encapsulate due to the properties such as adsorbents, molecular filters, catalysts, and ion exchangers. In agriculture, the surface active properties of zeolites that are most often utilized are adsorption and cation exchange properties because of its crystalline structure and porosity are very high. Zeolite also provide adequate adsorbed water, so it keeps moist during the dry season. This advantage makes zeolites often used as carriers of agricultural pesticides (Polat *et al.*, 2004). It is also reported that the fungus spores of *Beauveria bassiana* mixed with zeolite carriers infect and caused the death of *Aphis glycines* aphids on soybean plants within 3 days after treatment (Mandasari *et al.*, 2015). Another carrier or bioinsecticide coating agent is magnesium silicate. Magnesium silicate mixed with *B. bassiana* able to control *Macrosiphoniella sanborni* aphids and *Thrips parvispinus* (karny) thrips correspondingly with mortality of 54.37% and 97% (Yusuf *et al.*, 2010; Yusuf *et al.*, 2011).

The use of nanotechnology such as reducing the size of coating material into nano-sized particles is an alternative to enhance the performance of the encapsulation of fungal spores (Chowdappa and Gowda, 2013). Nano-sized material is known to have a higher surface area compared to the original size consequently improving on the reactivity (Perez de and Hermosin, 2013). Nanoparticles have been applied in various fields such as cell labeling, diagnostics, antimicrobial agents, disinfectants, mosquito control and drug delivery (Elangovan *et al.*, 2015; Benelli, 2016). The application of nanoparticles as encapsulations of biocontrol is believed to improve their performance. Therefore, the aim of this study is to investigate the encapsulation performance of *M. anisopliae* spore with zeolite and magnesium silicate nanoparticles in terms of mortality and lethal time against 3rd instar *C. pavonana* larvae.

MATERIALS AND METHODS

Experimental design and analysis

The experiment was designed following the method of a single factor Randomized Block Design (RBD) with 6 treatments, namely: control, spores, zeolite nanoparticles, magnesium silicate nanoparticles, spores + zeolite nanoparticles and spores + magnesium silicate nanoparticles with 4 replications of each treatment. The obtained data were analyzed using analysis of variance (ANOVA) with a significant level of 5%.

Rearing of *Crocidolomia pavonana*

Crocidolomia pavonana larvae was obtained from a broodstock of the Indonesian Vegetable Research Institute (IVRI). *C. pavonana* imago of 6-7 pairs was placed in a nesting box where cabbage leaves were available as feed. Imago was then transferred and maintained in the "rearing cabinet". The substitution of feed was done every 24 h until the adult *C. pavonana* was formed. The obtained 3rd instar larvae were used as bioassay tests for all treatments.

Metarhizium anisopliae spore culture

The initial population of the fungus *Metarhizium anisopliae* was received from a collection of microbiology laboratories at Padjadjaran University. The media for cultured the fungus was prepared from potato dextrose agar (PDA) added with 5 wt.% of shrimp shell powder. The shrimp shell powder was prepared manually by drying processes consecutively under the sunlight for two days and then drying in the oven for 2 h at temperature 50 °C. The fungus was planted on the surface of the media and incubated for 4 days at room temperature 27 °C.

Preparation and analysis of zeolite and magnesium silicate nanoparticles

The zeolite and magnesium silicate were purchased from commercially available materials correspondingly from PT. Global Air Teknologi and PT. Global Karya, Indonesia. Zeolite and magnesium silicate nanoparticles were prepared by using the top-down method by breaking or milling the initial size of zeolites and magnesium silicates into nanoscale using the beads milling technique. Briefly, the initial zeolite and magnesium silicate were ball-milling up to -400 mesh. Then, the zeolite and magnesium silicate were separately suspended into the water with a concentration of 0.5 wt.%. This suspension was stirred for 30 min using a magnetic stirrer and subjected to a beads-milling process for about 2 h (Joni *et al.*, 2010). The size and size distribution of zeolite and magnesium silicate nanoparticles before and after beads milling were observed using Particle Size Analysis (PSA, Horiba Scientific SZ-100). The morphology of particles was observed using a Scanning Electron Microscope (SEM, JEOL Jsm-6360LA).

Spore encapsulation phase with zeolite and magnesium silicate nanoparticles

The encapsulation of *Metarhizium anisopliae* spores with zeolite and magnesium silicate nanoparticle coatings was made by mixing 1 mL (with 10^5 spore/mL) of each spore with 99 mL of zeolite nanoparticles (0.5 wt.%) and 99 mL of magnesium silicate nanoparticles (0.5 wt.%)

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respectively. The surface morphology of the encapsulated zeolite and magnesium silicate nanoparticles were observed by Scanning Electron Microscope (SEM, JEOL Jsm-6360 LA).

Stage of larval infection

The stage of larval infection was carried out by using 3rd instar larvae which leave without feeding for 3 h prior experiments. Cabbage leaves are cut in a circular diameter of 5 cm and dipped with *M. anisopliae* fungal spore formulated with either zeolite or magnesium silicate nanoparticles. As a comparison, the investigation also carried out by using only 0.5 wt.% zeolite, 0.5 wt.% magnesium silicate nanoparticles suspension, and only spore (100 mL contained 10^5 spore/mL) and control (without treatment). The total larvae used in this study was 240 larvae for 24 plots. The observation was conducted with 10 larvae inserted into each treatment of the plot and was observed every 24 h for 7 days.

RESULTS AND DISCUSSION

Particle size analysis of zeolite and magnesium silicate nanoparticles

Figure 1 showed the size distribution of suspension before and after beads milling of zeolite and magnesium silicate particles. The average size of initial zeolite was 375.1 nm (Figure 1a) and after beads milling the average size was 143.7 nm (Figure 1b). The polydispersity index of the zeolite suspension was reduced from 1.626 to 0.776, indicated by sharp peaks observed at around 143.7 nm. While the average size of the initial magnesium silicate suspension was 3842.7 nm with two peaks at around 6802.5 nm and 487.5 nm (Figure 1c-d). This indicated that the particles of magnesium silicate suspension were mostly agglomerated. After beads milling, the size distribution was showed a very sharped peaks with an average size of 195.3 nm with a low polydispersity index (0.96), which means that the size of the particles was homogenous in the suspension.

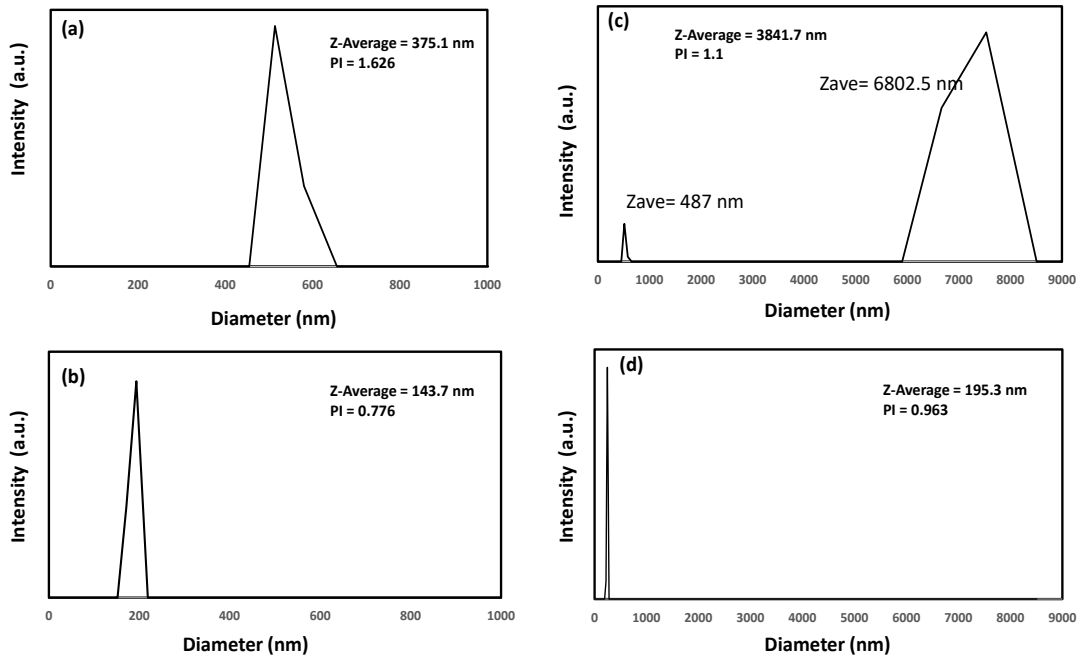

Figure 1: The particles size distribution of zeolite and magnesium silicate before and after beads milling: (a) initial zeolite suspension, (b) zeolite after beads milling, (c) initial magnesium silicate suspension and (d) magnesium silicate suspension after beads milling.

Encapsulation of *Metarhizium anisopliae* spores with zeolite and magnesium silicate nanoparticles

Figure 2 showed the SEM images of the before and after encapsulation of *Metarhizium anisopliae* spore with zeolite and magnesium silicate nanoparticles. The morphology of fungus spores of *M. anisopliae* was elliptical shapes with a rather rough surface as highlighted in a dotted circle as shown in Figure 2a. The morphology of obtained zeolite nanoparticles was flaky shaped (Figure 2b) and similarly for magnesium silicate nanoparticles as shown in Figure 2d. Figure 2c highlighted with the dotted circle indicated the encapsulation morphology of *M. anisopliae* with zeolite nanoparticle. The surface of encapsulated *M. anisopliae* with zeolite nanoparticle appeared smoother compared to the spores without the coating process. Similarly, for the

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encapsulation of *M. anisopliae* with magnesium silicate, it also appeared smoother compared to spores without the coating process (Figure 2e). It was observed that encapsulation of *M. anisopliae* with magnesium silicate covered almost the whole body of the spore compared with zeolite encapsulation. This result indicated that both zeolite and magnesium silicate successfully encapsulated fungal spores as expected to ensure their capability to protect the spores from the environment hindrance (Bansode *et al.*, 2010). Qiu *et al.* (2019) reported the successful encapsulation of *M. anisopliae* with gum arabic (GA) and gelatin (GE) type A against *Solenopsis invicta* and found that those both materials able to protect *M. anisopliae* fungal spores from UV light. However, it is essential to evaluate their performance on the pest control due to the improvement of encapsulation. Therefore, it is important to investigate the ability of the encapsulation to enhance the mortality and accelerate the lethal time against *C. pavonana* larvae in the following section.

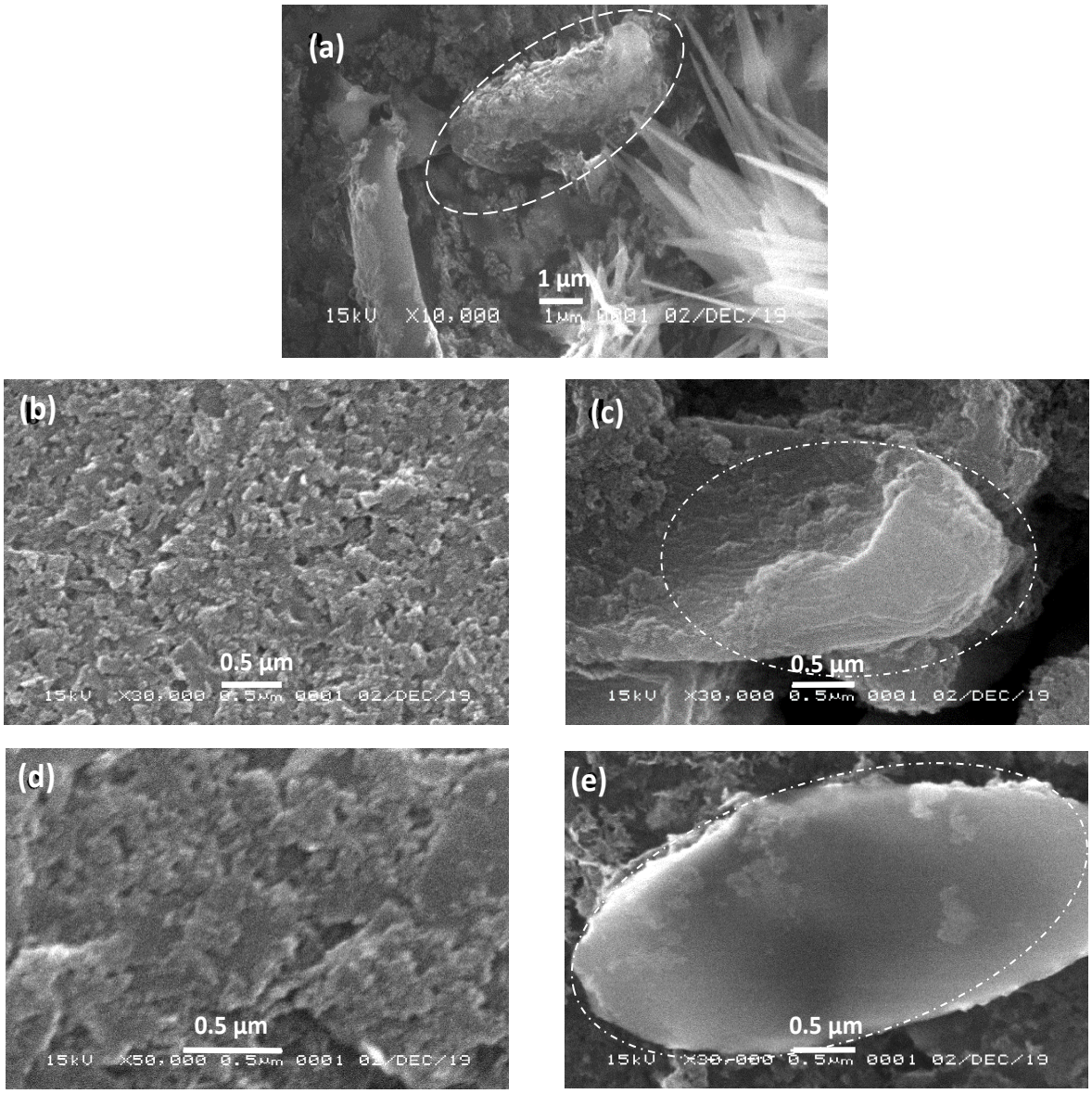

Figure 2: SEM images of (a) *Metarhizium anisopliae* spores (magnification: 10000×) (b) Zeolite nanoparticles (magnification: 30000×) (c) Spores encapsulation with zeolite nanoparticle (magnification: 30000×) (d) magnesium silicate nanoparticles (magnification: 50000×) and (e) Spores encapsulation with magnesium silicate nanoparticles (magnification: 30000×).

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Mortality of *Crocidolomia pavonana* larvae

Table 1 showed mortality of larvae infected by fungal spores with various encapsulation in comparison with spore and only zeolite nanoparticles and magnesium silicate nanoparticles observed for 24 h. The result indicated that all treatments were significant over the control. The treatment with only zeolite nanoparticles was increased the mortality significantly (75.5%) compare to control (without any treatment) and with of the only spore (27.5%). Similarly, the mortality for the treatment using magnesium silicate (72.5%), however, did not significantly different compared to the only zeolite treatment (75.5%). The mortality obtained from the encapsulation of spore with zeolite nanoparticles showed significant enhancement (92.5%) compare to the treatment of only zeolite nanoparticle (75.5%). Similarly, the mortality of larvae which treated with encapsulated spore with magnesium silicate nanoparticles showed significant enhancement (85 %) compared to those treated only with magnesium silicate nanoparticles (72.5%). Therefore, it was found that there was a synergetic performance between spore and both nanoparticles (either zeolite or magnesium silicate). However, there was no significant different in the mortality of larvae between encapsulation of spore with zeolite and encapsulation of spore with magnesium silicate nanoparticles.

We justify that the high standard deviation might due to inhomogeneous of larvae healthy (viability), even we have treated with similar rearing system. Thus, increasing number of samples might statistically resolve the health inhomogeneity of larvae that caused high standard deviation.

Table 1: The mortality of larvae infected by *M. anisopliae* spores encapsulated with zeolite and magnesium silicate nanoparticles for 24 h.

Treatment	Mortality (%)
Control	0.0 ± 0.0^{a}
Spores	27.5 ± 9.6^{b}
Zeolite nanoparticles	75.5 ± 5.8^{c}
Magnesium silicate nanoparticles	72.5 ± 5.0^{c}
Spores + zeolite nanoparticles	92.5 ± 5.0^{d}
Spores + magnesium silicate nanoparicles	85.0 ± 5.8^{d}

The numbers followed by different letter in the same column is significantly difference at α= 5% by Duncan's Multiple Range Test.

Lethal time of *Crocidolomia pavonana* larvae

Table 2 showed the data of the lethal time of larvae due to the encapsulation of spores with zeolite nanoparticles and magnesium silicate nanoparticles in comparison with control and only zeolite and magnesium silicate nanoparticles. The result showed that all treatments were significant decreased the lethal time of the larvae compare to control. While other treatments were significantly decreased the lethal time of the larvae compare to those treated with only spores. However, there was no significant difference in the lethal time of larvae among these treatments. It was highlighted that the encapsulation of spore with zeolite nanoparticles received the fastest lethal time of larvae.

Table 2: The lethal time of larvae infected by *M. anisopliae* spores encapsulated with zeolite and magnesium silicate nanoparticles within 7 days of test period.

Treatment	Lethal time (days)
Control	7.000 ± 0.00^{a}*
Spores	2.325 ± 0.17^{b}
Zeolite nanoparticles	1.250 ± 0.05^{c}
Magnesium silicate nanoparticles	1.275 ± 0.05^{c}
Spores + zeolite nanoparticles	1.075 ± 0.05^{c}
Spores + magnesium silicate nanoparicles	1.150 ± 0.05^{c}

The numbers followed by different letter in the same column is significantly difference at α= 5% by Duncan's Multiple Range Test.
*All larvae were surviving (viable 100%) during the period of test (7 days).

On the investigation of both mortality and lethal time of the larvae, it was found that treatment with only zeolite and magnesium silicate nanoparticles were quite effective method to infect the larvae. However, additional spores encapsulated with nanoparticles did not significantly decrease the lethal time of the larvae compare to the only nanoparticles. The lethal time of the larvae was relatively similar might due to relatively similar infection mechanism. This result indicated that the spore and zeolite nanoparticles demonstrated a synergetic performance. In the case of spores, infective spores attached to surface layer of the cuticle of the larvae (Wang and St. Leger, 2005). While, the zeolite nanoparticles might be able to damage the outer cuticles of larvae through abrasion that absorbs or interferes with epicuticles, which allowed epicuticular lipid adsorption, which caused the larvae to lack liquid and caused shrinking and dried of infected body of the larvae as shown in Figure 3 (Puterka *et al.*, 2000).

In this study, spores that encapsulated with nanoparticles significantly improved the mortality of the larvae compared with only nanoparticles. The effectivity of the performance might due to the homogenous distribution in nanosized-particles and successful encapsulation of spore with the nanoparticles at the surface as observed in Figures 1 and 2 (Rhodes, 2014; Setiawan *et al.*, 2018; Li *et al.*, 2019). It was emphasized that the encapsulation of spores with zeolite nanoparticles received the highest performance. This might due to zeolite in nano-sized not only encapsulated spores, but also acted as toxic to the larvae.

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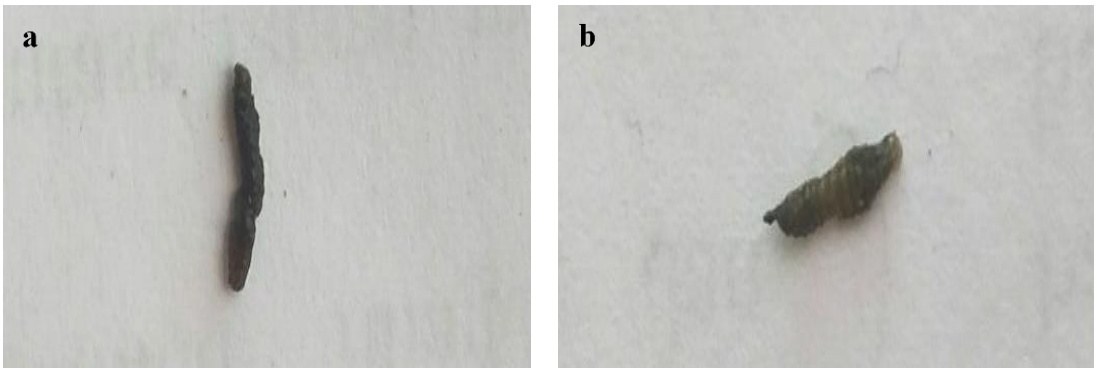

Figure 3: Photo images: (a) larvae infected by spore encapsulated with zeolite nanoparticles and (b) larvae infected by spore encapsulated with magnesium silicate nanoparticles.

CONCLUSION

It was concluded that the encapsulation of *M. anisopliae* spores with zeolite nanoparticles increased the mortality of *C. pavonana* larvae to 92.5% and accelerate lethal time of to 1.075 days compared to only spore correspondingly 27.5% and 2.235 days. Encapsulation of spores with magnesium silicate nanoparticles also increased the mortality of larvae to 85.0% and accelerate larval lethal time to 1.150 days compared to only spore. It was highlighted that encapsulation with zeolite nanoparticle received the highest performance in the bioassay test.

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