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In Vivo Production of *Nosema bombycis* Spores and their Efficacies against Diamondback Moth and Beet Armyworm Larvae in Laboratory Conditions

(Penghasilan Spora *Nosema bombycis* Secara *In Vivo* dan Keberkesanannya terhadap Larva Rama-rama Belakang Intan dan Ulat Ratus dalam Makmal)

SYARAFINA RAMLI*, B.A.H. ZAINAL-ABIDIN & A.B. IDRIS

ABSTRACT

Diamondback moth (DBM), Plutella xylostella and beet armyworm (BAW), Spodoptera exigua are major insect pests of crucifer crops found worldwide. Since these pests are highly resistant to many chemical insecticides, using biological control agent(s) or biopesticides might be a good choice to overcome this problem. Nosema bombycis is a microsporidial pathogen with the potential to control insect pests. This study was aimed at producing N. bombycis spores in vivo and to test their efficacies against DBM and BAW in laboratory conditions. Production of this pathogen was carried out by feeding both DBM and BAW larval instars with artificial diet inoculated with viable spores of N. bombycis. Spore concentrations in the larvae were determined and accumulated. If a concentration of 1.56×10^8 spores/mL was obtained, the production was continued. For laboratory efficacy tests, artificial diets with different spore concentrations namely 1×10^4, 1×10^5 and 1×10^6 spores/µL were fed to different larval instar. Mortality rates were analysed using the Two-way ANOVA test. For both DBM and BAW, third instar showed the highest mean mortality rates and the mortality in each instars were significantly different ($P<0.05$). The concentration 1×10^6 spores/µL caused the highest mean mortality rates and each concentration resulted in significantly different mortality ($P<0.05$). However, there was no interaction between both factors, where a combination of spore concentrations and instars did not show any significant differences ($P>0.05$). These results indicated that third instar larvae for both species and 1×10^6 spores/µL treatments demonstrated the highest efficacy rates.

Keywords: Nosema bombycis; Plutella xylostella; Spodoptera exigua

ABSTRAK

Rama-rama belakang intan atau diamondback moth (DBM), Plutella xylostella dan ulat ratus atau beet armyworm (BAW), Spodoptera sp merupakan serangga perosak utama tanaman kubis di seluruh dunia. Masalah kerintangan serangga perosak ini terhadap insektisid kimia mendorong penggunaan biopestisid sebagai jalan penyelesaian. Nosema bombycis merupakan patogen mikrosporidia yang berpotensi untuk mengawal serangga perosak. Objektif kajian ini ialah menghasilkan spora Nosema bombycis secara in vivo dan mengkaji keberkesanannya mengawal DBM dan BAW dalam keadaan makmal. Bagi penghasilan spora, larva DBM dan BAW dijangkitkan dengan Nosema bombycis melalui diet buatan. Kepekatan spora dalam larva ditentukan dan hasil yang diperolehi adalah 1.56×10^8 spora/mL, seterusnya penghasilan spora itu diteruskan. Keberkesanannya dalam makmal dikaji dengan menjangkitkan kepekatan spora yang berbeza, iaitu 1×10^4, 1×10^5 dan 1×10^6 spora/µL kepada larva instar berbeza melalui diet buatan. Kadar mortaliti dianalisis menggunakan ujian ANOVA 2 Hala. Bagi kedua-dua DBM dan BAW, min kadar mortaliti yang tertinggi ditunjukkan oleh instar ketiga dan terdapat perbezaan kadar mortaliti yang beerti antara instar berlainan ($P<0.05$). Kepekatan 1×10^6 spora/µL menyebabkan min kadar mortaliti yang tertinggi dan perbezaan beerti ditunjukkan pada kadar mortaliti kepekatan spora berbeza ($P<0.05$). Walau bagaimanapun, tiada interaksi antara kedua-dua faktor tersebut di mana gabungan kepekatan dan instar tidak menunjukkan perbezaan yang beerti ($P>0.05$). Hasil kajian tersebut menunjukkan instar ketiga kedua-dua spesies dan kepekatan spora 1×10^6 spora/µL menyebabkan kadar keberkesanan yang paling tinggi.

Kata kunci: Nosema bombycis; Plutella xylostella; Spodoptera exigua

INTRODUCTION

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae) is a major insect pest of crucifer crops worldwide. It was first reported in Malaysia in 1925 and was found in Fraser Hill. Since 1941, DBM has caused huge economical loss in crucifer crops grown in Cameron Highlands (Ooi 1986). Beet armyworm (BAW), *Spodoptera exigua* (Lepidoptera: Noctuidae) originally a native of southern China, is now distributed widely in farms and greenhouses all over Asia, Europe and America (Idris et al. 2006). Since 1986, *S. exigua* has become the main pest in eastern province and central China, especially in Huanghuai and Jianghuai. In 1999, this pest has damaged 30 000 km^2 of crops in Henan and Shandong (Wang et al. 2006). *S. exigua* was reported found in Malaysia during early 1990 at an onion farm in Perak (Idris et al. 2006).

According to Tsai et al. (2003), this species is one of five most important pests that causes huge economic loss in Taiwan.

Microsporidia *Nosema bombycis* is an agent that caused infection on silkworm (*Bombyx mori* L.), also known as Pebrine disease. This parasite is usually found in insect hosts especially from the order Lepidoptera. *N. bombycis* infects all stages in the host lifecycle from egg, larvae, pupae and adult (Lian 1991). This parasite reproduces through vegetative duplication and the lifecycle is divided into two phase, named merogony and sporogony. Merogony refers to the process where spore cells splits while sporogony is the process of spore formation (Didier et al. 2000). The complete lifecycle for this species takes around 24 h after infection, and then reproduce itself by forming merons which duplicates into large quantity of spores (Ann & Peter 1999). *N. bombycis* infects the host through infected eggs or through oral while feeding. (Hoch et al. 2000). The virulent spores are released to the environment together with the feces and silk, or when the host died the spore is reproduced in the host cells (Brian & Joseph 1996).

The majority of farmers throughout the world have used conventional chemical insecticides for controlling DBM and BAW. However, the wide usage of insecticide has caused more problems, where pests developed high resistant towards the chemical content. There are also other side effects such as pollution, food poisoning and residue in crops, also threat to other organisms (Loke et al. 1997; Ooi 1986). Because of that, an alternative technique to control the population of pests is very crucial. The solution to this problem is the application of microbial control, the usage of pathogen like protozoa, bacteria, virus and fungi to control pest. DBM field populations in Malaysia have been naturally infected by several insect pathogens including *Nosema bombycis* but the impact does not seem to reduce chemical insecticide use for controlling DBM. This is probably because of its slow killing actions on the host and the low concentration of spores in natural infection (Idris et al. 2001).

Different types of pathogen has been used as a biological control agent such as microsporidia (Tsai et al. 2003), bacteria *Bacillus thuringiensis* (Rajagopal et al. 2002) and nucleopolihedrosis virus (NPV) (Takatsuka et al. 2003). There are few species from genus *Nosema* being used as biopesticide. For example *N. locustae* to control grasshopper (Debach & Rosen 1991), *N. melolonthae* for cockchafer (Bosch et al. 1982), *N. algerae* for larvae of mosquito *Aedes aegypti* (Huffaker & Mesenger 1989), *N. whitei* for fleas (Fuxa & Tanada 1987), and *N. pyrausta* for cornborer (Sajap & Lewis 1992). *N. locustae* has been commercialised and registered as microbial insecticide by Environment Protection Agency (EPA) in America (Debach & Rosen 1991; Koul & Dhaliwal 2002). This study was aimed at producing *N. bombycis* spores *in vivo* and to test their efficacies against DBM and BAW in laboratory conditions. *N. bombycis* has the potential to be used as a biological control agent like the other members of this genus.

Materials and Methods

In vivo production of *Nosema bombycis* spores

Samplings were done in various cabbage fields in Cameron Highlands, Pahang to get DBM adults which are naturally infected with *Nosema bombycis*. The DBM caught using insect nets were kept in universal bottles containing 70% alcohol to be preserved. Then they were crushed using a homogeniser and filtered with muslin cloth to get rid of skin and wings. They were centrifuged three times at 3000 rpm, 10°C for 20 min to purify the spores. The purified spores were kept in liquid nitrogen tank as stock.

DBM larvae were provided by MARDI which are free from infection and were starved from any food for six hours to ensure that they finish the nosema-infected artificial diet. Then each larva was fed with 0.5×0.5×0.5 cm^3 artificial diet inoculated with viable spores of *N. bombycis*. After ten days, spore concentrations in the larvae were determined by crushing and centrifuging as mention earlier. Ten micro liter of the spore solution were pipetted and placed on haemocytometer for counting. The spores were counted under light microscope with 40× magnification using the Cantwell formula (1970). Then the spores were kept in liquid nitrogen for further use.

Efficacy test of *Nosema bombycis* infection against DBM and BAW larvae in laboratory conditions

Different spore concentrations namely 1×10^4, 1×10^5 and 1×10^6 spores/μL were prepared by diluting spores from the stock. Second, third and fourth instar DBM larvae and second, third, fourth and fifth instar BAW larvae provided by MARDI which are free from infection, were fed with 0.5×0.5×0.5 cm^3 artificial diet inoculated with viable spores of *N. bombycis*. A group of 12 larvae for each instar were infected with different spore concentrations and arranged as Complete Random Design with four replicates. Distilled water was used as control treatment. The number of dead larvae was recorded every 24 h for five days. Abbott's Formula (1925) was used to determine the corrected mortality percentage and then analysed with Two-way ANOVA test using MINITAB 14.

Results and Discussion

In vivo production of *Nosema bombycis* spores

Spore concentration obtained from *in vivo* production ten days after infection showed a total of 1.56×10^8 spores/mL. However, the production was continued in few more batches in order to get the sufficient amount for the next test.

As different instars were used in this *in vivo* production, the spore concentration of each larva also varies according to larva stages. During the early stage second instar, the larvae were less active in consuming the infected diet so it takes more time for the diet to be finished. This also caused less spores entering the body. The

larvae size represents the larvae body mass where small larvae at early stage might contain less spores (Blaser & Schmid-Hempel 2005). Larvae third instar might produce a higher spore concentration based on the active feeding activities observed. However, in late stages fourth instar, spore concentration might be less as the insects' immune system had developed. Jung and Kim (2006) stated that larvae resistant improve as it mature into late instars.

EFFICACIES TEST OF *NOSEMA BOMBYCIS* INFECTION AGAINST DBM AND BAW LARVAE IN LABORATORY CONDITIONS

Based on Two-way ANOVA analysis for DBM, the instar with the highest mean mortality rate was third instar (46.55% ± 3.53) and the highest mean mortality rate (46.16% ± 4.48) was caused by the highest spore concentration (1×10^6 spores/μL) (Table 1). Mortality rates caused by different spore concentrations were significantly different from one another (F=11.46, P=0.00) and so did the mortality rates in different instars (F=32.10, P=0.00). However, there was no interaction between both factors, where a combination of spore concentrations and instars did not show any significant differences (F=0.70, P=0.598). This means that the two factors worked independently and does not have to be a combination of certain instar with a certain concentration.

TABLE 1. Mean mortality rate for DBM and BAW according to instars and spore concentrations

Instar	Mean of mortality (%)	
	DBM	BAW
2	44.21 ± 2.89 a	25.32 ± 2.61 a
3	46.55 ± 3.53 a	40.98 ± 2.61 b
4	23.62 ± 1.73 b	25.69 ± 1.91 a
5	X	16.68 ± 1.78 c
Spore concentration (spores/μL)	Mean of mortality (%)	
	DBM	BAW
1×10^4	31.20 ± 2.75 a	21.94 ± 2.25 a
1×10^5	37.01 ± 3.84 a	26.66 ± 2.95 ab
1×10^6	46.16 ± 4.48 b	32.91 ± 2.96 b

*Means in column with same letters are not significantly different (P > 0.05)

The highest mean of mortality rate for BAW was in third instar (40.98% ± 2.61) and the concentration 1×10^6 spores/μL also caused the highest mean of mortality (32.91% ± 2.96). Mortality rates caused by the different spore concentrations were significantly different from one another (F=10.80, P=0.00) and also did the mortality rates in different instars (F=27.29, P=0.00). However, there was no interaction between both factors, where a combination of spore concentrations and instars did not show any significant differences (F=0.38, P=0.884). This means that the two factors worked individually and does not have to be a combination of certain instar with a certain concentration.

In Figure 1, the highest mortality rate was shown by third instar DBM larvae in all concentrations. However, the standard error (SE) value for third instar mortality which is overlapped among all three concentrations showed that the differences are not significant. On the contrary, SE values for second instar are not attach to each other showed significant differences between different concentrations. For fourth instar, the SE values indicated that mortality caused by 1×10^6 spores/μL is significantly different from 1×10^5 and 1×10^4 spores/μL.

In Figure 2, the highest mortality rate was shown by third instar BAW larvae in all concentrations. The SE value for third instar which is overlapped between 1×10^6 and 1×10^5 spores/μL showed that the difference among the two concentrations was not significant. However, 1×10^4 spores/μL caused a significantly lower mortality in third instar. In fourth instar, 1×10^6 spores/μL caused a significantly higher mortality compared to 1×10^5 and 1×10^4 spores/μL. Both second and fifth instar had overlapped SE value which indicated that the differences are not significant among all concentrations.

The mortality rate of DBM larvae infected with different spores concentrations increased every 24 h, except in fourth instar larvae (Figure 3). The horizontal line from

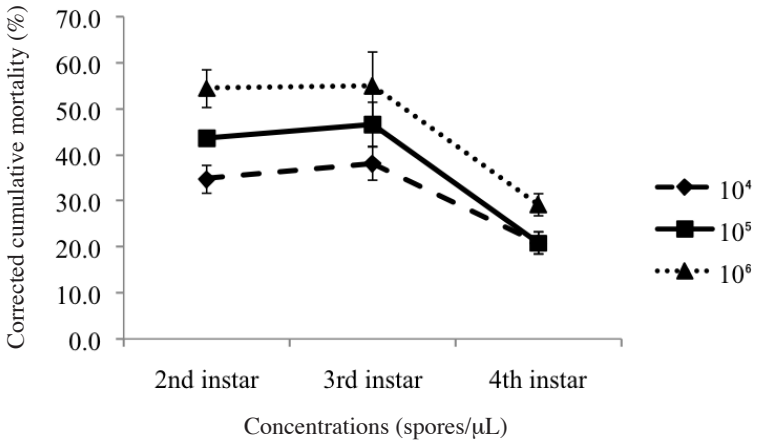

FIGURE 1. Corrected cumulative mortality of second, third and fourth instars of DBM larvae five days after infected with different spore concentrations

96 h to 120 h showed that there was no death of fourth instar larva infected with 1×10^5 spores/μL 120 hours after infection (Figure 3(a)). There was no significant difference between groups of larvae where the SE value overlapped. However, some of the mortality percentage had no SE value because of the same rate in all four replicates.

The mortality rate of second and third instar BAW larvae infected with different spores concentrations increased every 24 hours (Figure 4). However in fourth instar there are two horizontal lines indicating no increase of mortality rate (96 to 120 hours after infected with 1×10^5 spores/μL and 72 to 120 hours after infected with 1×10^4 spores/μL). There was no mortality at all in fifth instar after 72 hours. Overlapped SE value indicated no significant difference between two groups of larvae. However, some of the mortality percentage had no SE value because of the same rate in all four replicates.

The high mortality rate in third instar for both DBM and BAW larvae is caused by many factors. One of the

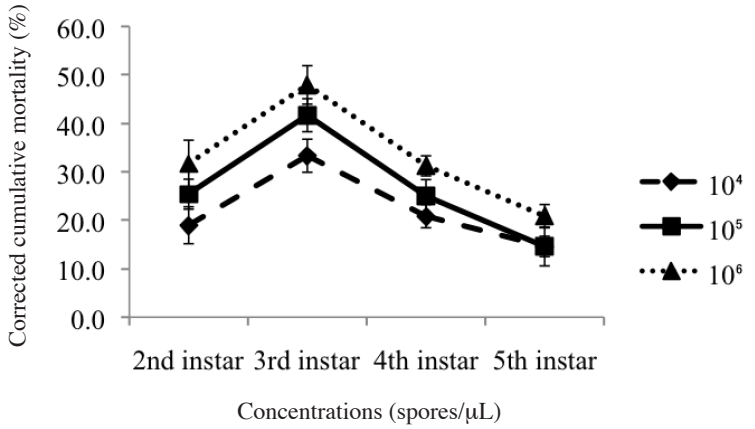

FIGURE 2. Corrected cumulative mortality of second, third, fourth and fifth instar BAW larvae five days after infected with different spore concentrations

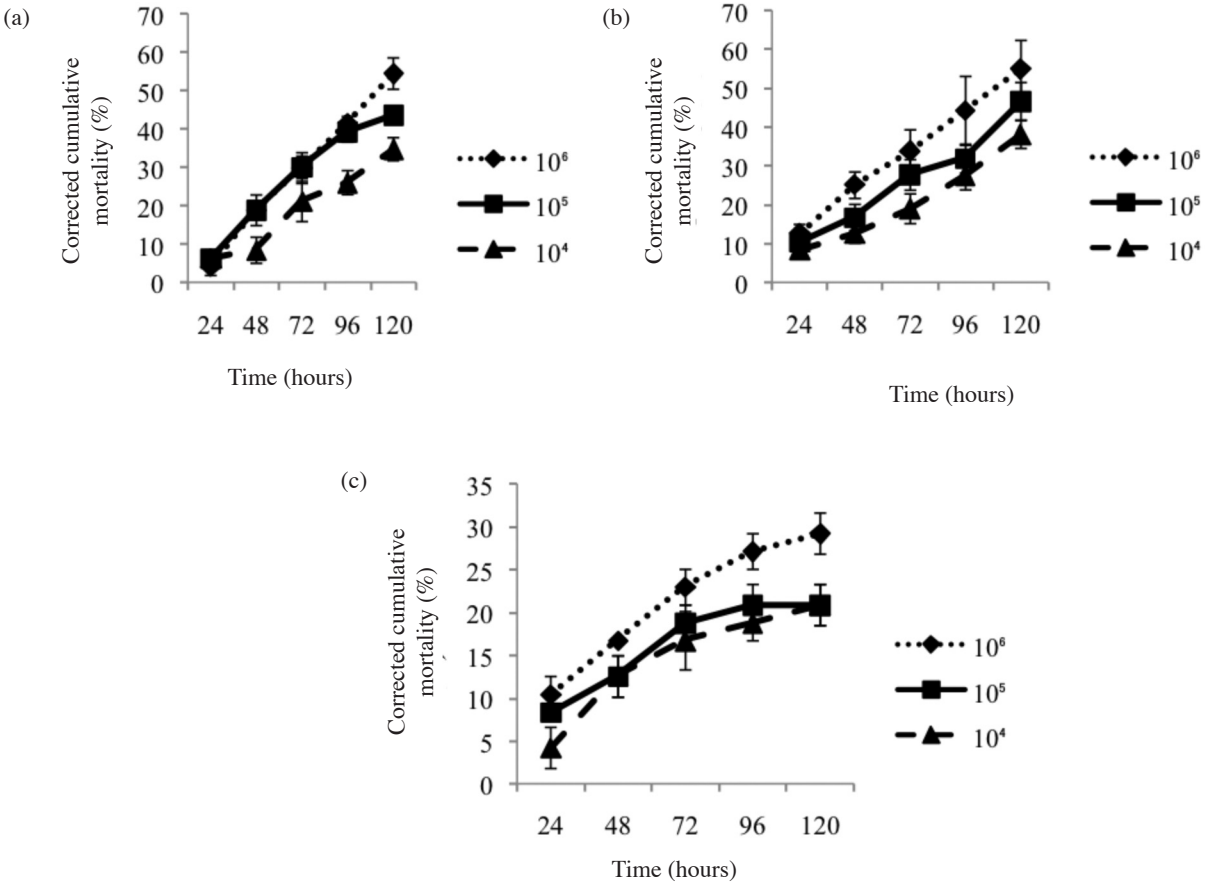

FIGURE 3. Corrected cumulative mortality of (a) second, (b) third and (c) fourth instars DBM larvae at every 24 h after infected with different *Nosema* spore concentrations

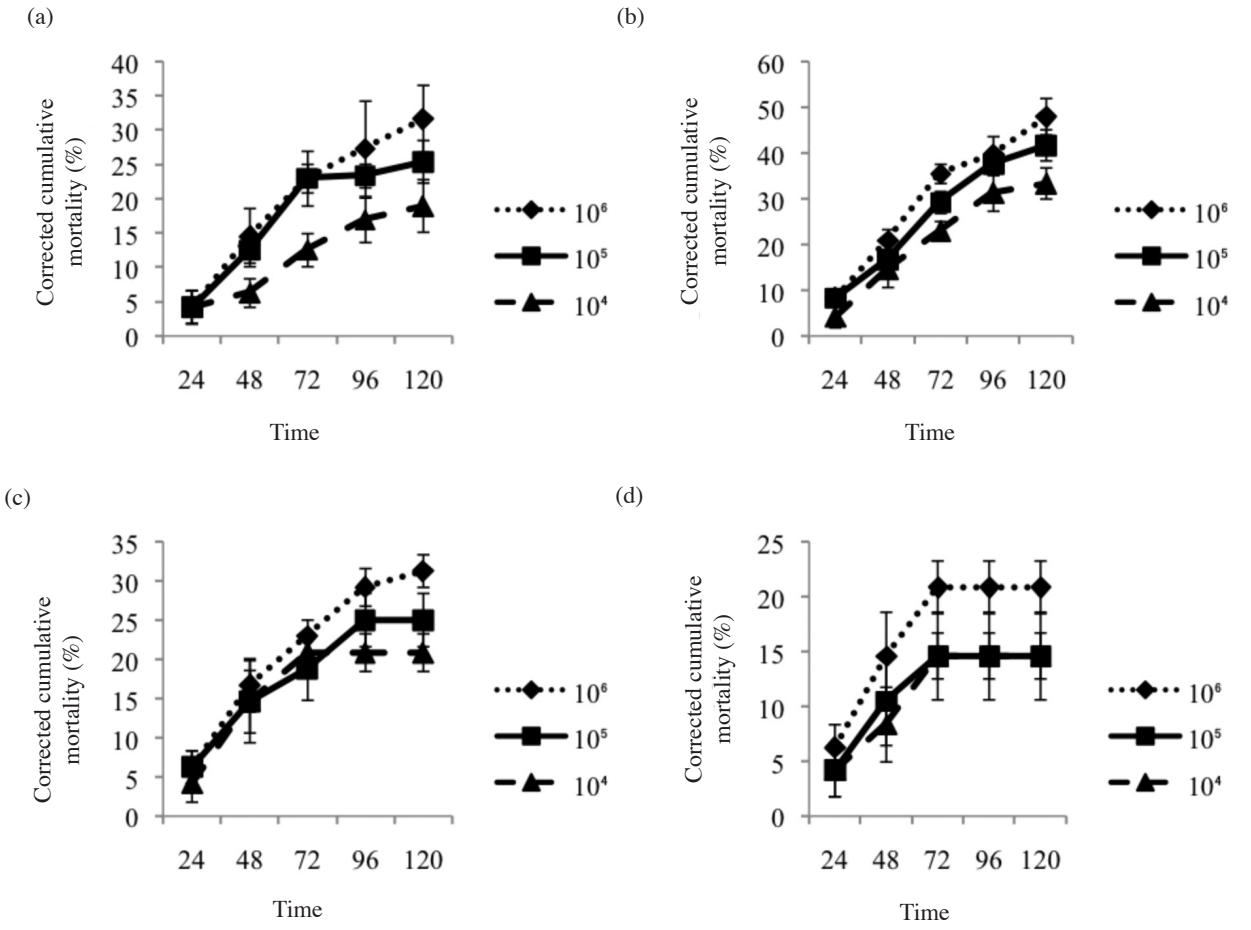

FIGURE 4. Corrected cumulative mortality of (a) second, (b) third, (c) fourth and (d) fifth instars of BAW larvae at every 24 h after infected with different *Nosema* spore concentrations

factors is eating habit. Burges (1981) stated that larvae are exposed the most to pathogen because it is the stage where insect eats the most. Among all larvae stages, third instar larvae ate more frequent compared to other instars. Second instar larvae take longer time to finish the infected diet because of the small size. After third instar, the larvae started to reduce the eating activity as a preparation to become pupae where the morphology changes to short rounder shape.

The immune system also affects the mortality caused by pathogen. Tanada and Kaya (1993) stated that lower spore content could result in high mortality in second instar larvae because of the weak immune system and susceptibility to infection. The larvae resistance against infection and disease become stronger as they develop into late instars (Jung & Kim 2006). Bianchi et al. (2002) also stated that the best control can be obtain when larvae is infected as soon as they hatch from the eggs.

According to Burges & Hussey (1971), the effects of protozoa on host are slower compared to virus. The slow action causes damage in aspects other than death. Usually important functions in the host are disturbed quickly such as infertility (reduced mating frequency and low number of eggs), lifecycle is shortened, reduced movements and less response towards stimulant. These criteria could be beneficial in pest control if exploited properly. Protozoan distribution and infection in host is the best in a continuous disease. This provides a source of inoculums, rather than drastic death of host which destroys the pathogen. Basically, the infection disappears once the host died. So transferring the spores to eggs is a more consistent technique in order to make the disease last longer in the pest population. Burges & Hussey (1971) also stated that microsporidia is transferred to progeny more often compared to other protozoa, which reduces the resistance towards weather, insecticides, predator and parasitism.

CONCLUSIONS

Our results indicated that a total of 1.56×10^8 spores/mL *N. bombycis* spores were obtained through *in vivo* production in ten days. The spores produced have caused a higher mean mortality in DBM (38.12 ± 2.35) than BAW (27.17 ± 1.68). Third instar larvae for both DBM and BAW, with concentration of 1×10^6 spores/µL treatments demonstrated the highest efficacy rates. However, the two factors, instar stage and spore concentrations does not interact between each other and caused mortality separately.

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Syarafina Ramli* & B.A.H. Zainal-Abidin
School of Biosciences and Biotechnology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 Bangi, Selangor
Malaysia

A.B. Idris
School of Environmental Science and Natural Resources
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 Bangi, Selangor
Malaysia

*Corresponding author; e-mail: eina_syara_fina@yahoo.com
syarafinaramli@gmail.com

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