

Full Length Research Paper

Toxicity of local Malaysian *Bacillus thuringiensis* subspecies *Kurstaki* against *Plutella xylostella*

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The toxicity effect of *Bacillus thuringiensis* against *Plutella xylostella* is well established. However, effective *B. thuringiensis* strain especially local isolate is not well tested. In this study local strain *B. thuringiensis* subspecies *kurstaki*, SN5 was assessed for its effectiveness against *P. xylostella* 3rd instar larvae. Other factors such as spore germination, spore coat, L-alanine-adenosine (LAA) and streptomycin were evaluated with their possible effects on the toxicity of *B. thuringiensis* cry protein. The result of the study showed that SN5 spore exhibit higher toxicity than the commercial strain, HD-1. L-Alanine-adenosine not only improves rate of spore germination but also synergy effect of spore-crystal mix by increasing toxicity of the mixture. These results demonstrating the potential of local isolate in managing *P. xylostella* and its potential effect can be increase by adding LAA.

Key words: *Bacillus thuringiensis*, cry-like protein, L-alanine-adenosine, *Plutella xylostella*, streptomycin, toxicity.

INTRODUCTION

There are about 5600 ha of cruciferous vegetables land in Malaysia (Calderon and Hare, 1986) and 92% of this product is from Cameron Highlands (Batoto et al., 2010). Since 1940s, diamond back moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is considered as the major pest for crucifers planting in Cameron Highlands (Iqbal et al., 1996; Verkerk and Wright, 1997). It is reported that crop losses more than 90% during an outbreaks of *P. xylostella* (Verkerk and Wright, 1996). Heavy dependent and overuse of pesticides has eventually led to insecticide resistance and control failure. Sarfraz and Keddie (2005) reported that *P. xylostella* has shown significant resistance to almost every synthetic insecticide applied in the field even new chemicals like spinosyns, avermectins, neonicotinoids, pyrazoles and oxadiazines. Resistant populations of *P. xylostella* have also been found in Cameron Highlands (Iqbal et al., 1996; Verkerk and Wright, 1997). The usage of microbial insecticides

such as bacteria, fungi, viruses, protozoa, and nematodes is increasing because they are more target specific and has low environmental impacts.

The use of *Bacillus thuringiensis* (Berl.), a Gram-positive soil bacterium is the major success in microbial insecticides. During sporulation, *B. thuringiensis* (*Bt*) produce d-endotoxins or insecticidal crystal proteins that display a wide range of pathogenicity to insect orders (Bauer, 1995). More than 90% of microbial insecticides in the market currently have d-endotoxins as the active ingredients (Gassmann et al., 2009). *B. thuringiensis* subsp. *kurstaki* (*Btk*) is known to be toxic again Lepidoptera (Lambert et al., 1996). In the market currently, *Btk* HD-1 is the main active ingredient of the commercial available microbial insecticide such as Biolit[®] and Diple[®]. However, none of these products contain indigenous strains. Furthermore, resistant population of *P. xylostella* to application of *Btk* has been reported in the Philippines (Kirsch and Schmutterer, 1988), Hawaii (Tabashnik et al., 1990), Japan (Hama et al., 1992) and Florida (Shelton et al., 1993). In Cameron Highlands first resistance case to *Btk* was recorded in 1990 (Syed, 1992). Anitha et al. (2011) reported higher toxicity potential of local *Bt*

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isolates as compared to commercially available *Btk* HD-1 against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Hence, the present study was to assess local Malaysian *Btk* toxicity against *P. xylostella*.

MATERIALS AND METHODS

Organism culturing and harvesting

B. thuringiensis subspecies *kurstaki* strain HD-1, SN5 and 4D8 were cultured in phosphate-buffered growth medium (8 g Difco nutrient broth, 1 g KCl, 14 g K₂HPO₄, 6 g KH₂PO₄, 0.11 g MgSO₄ in 1 L distilled water, 1 ml 10⁻³ M FeSO₄·7H₂O, 1 ml 10⁻² M MnCl₂·2H₂O and 1 ml 0.5 M CaCl₂·2H₂O). Strain HD-1 is a commercial strain, SN5 is a soil isolate from Hutan Simpan Martin, Negeri Sembilan. Meanwhile, 4D8 is a mutant variety of strain HD-1 that does not produce crystal protein. Culturing condition was maintained at 30°C on continuous shaking at 200 rpm for 72 h before harvesting. The inoculums were obtained from 12 to 24 h cultures in glucose-yeast-sodium medium (Pigott and Ellar, 2007). Cry proteins and spores were harvested by centrifugation at 10,000 g for 10 min at 4°C in a Sorvall RC-5C centrifuge fitted with a GS-3 rotor and purified using a method described by Mahillon and Delcour (1984). The purity of the samples was observed under scanning electron microscope. Cry protein and spore samples were lyophilized and stored.

Extraction of spore coat

The spore coats were removed from the spores by suspending them in dithiothreitol-sodium dodecyl sulfate (DTT 0.05 M to SDS 0.5% at pH 8.5) solution and incubated for an hour at 37°C. Naked spores were recovered by 10 min by centrifugation at 27,000 g for 10 min at 4°C (Sorvall™ SS-34 rotor). Samples were rinsed six times with sterile distilled water prior to freeze-drying with Labconco Freeze Dryer System 7522900. Spore coat extractions were screened using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method (Laemmli, 1970). Naked spores were lyophilized and stored.

Spore germination

The germination of *Btk* spore was manipulated using germination inducing solution 4 mM L-alanine-adenosine (LAA, Sigma Chemical Co. at 4 mM) and spore suppressing antibiotic streptomycin (Sigma Chemical Co., 10 mg/L). The percentages of spore germination were evaluated using Optical-Density method (Gibbs, 1967). Spores were first suspended in 4.5 ml germination buffer (0.1 M Tris-HCl, pH 7.5) and subsequently 0.5 ml antibiotic, streptomycin (10 µg/ml) or 0.5 ml of spore germination inducing solution, L-alanine-adenosine or 0.5 ml of sterile distilled water as control were added to the solution. Differences between the first optical density reading at wavelength 600 nm (taken immediately after mixture) and the second optical density reading (20 min after mixture and incubated at room temperature) were calculated and the rate of spore germination for each treatment were determined.

Bioassay

The toxicity of crystals (cry protein) of the *Btk* SN5 and HD-1 strains were tested against *P. xylostella*. The combination of Cry protein

with wild type spores (SN5 spores and 4D8 spores), naked spores or mutant spores that are without Cry protein (4D8 spores) were also tested their toxicity levels. Third instar of *P. xylostella* larvae susceptible strain UPM generation 56 (SS UPM 56) were starved overnight and let to feed on an artificial diet (Masson et al., 1998). The diets were incorporated with different combination and concentration of cry proteins and spores solutions. Thirty larvae were placed on each diet cube. Bioassays and rearing were conducted at 25 ± 0.2°C with a photoperiod of 12 h of light and 12 h of dark. Mortality was recorded on the 4th day of assay. All possible combinations were replicated three times for three consecutive days.

Data analysis

Probit analysis was used to estimate 50% lethal concentrations (LC₅₀) for each bioassay of *Btk* crystals with or without spores, spore-crystal mixtures with or without streptomycin and spore-crystal mixtures with or without L-alanine-adenosine. LC_{50s} were considered significantly different if their 95% confidence intervals did not overlap (Payton et al., 2003). A model that assumed similar joint action of the component tested was used to estimate the level of synergy, an expected LC₅₀ value for the Cry-spore mixtures (Somerville et al., 1970). The equation used was:

$$LC_{50(ts)} = [R_{(t)}/LC_{50(t)} + R_{(s)}/LC_{50(s)}]^{-1} \quad (1)$$

Where, LC_{50(ts)} is the expected LC₅₀ of the toxin-spore mixture; LC_{50(t)} is the observed LC₅₀ for toxin; LC_{50(s)} is the observed LC₅₀ for spore; R_(t) is the ratio of toxin in the toxin-spore mixture and R_(s) is the ratio of spore in the toxin-spore mixture.

RESULTS

Spore germination

The percentage of spore germination varied between different types of spore and different treatments (Figure 1). The greatest percentage of spore germination was observed for 4D8 spores followed by SN5 spores and HD-1 spores. Results show that the addition of streptomycin can effectively suppress ($p > 0.05$) spore germination while LAA increased ($p > 0.05$) spore germination. Spores that had been chemically removed their spore coat also showed significantly ($p > 0.05$) improved germination rate.

Spore coat profile

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that both spore coat of HD-1 spores and SN5 spores contain Cry 1 and Cry 2 proteins (with molecular weight of 131-kDa for Cry 1 and 72-kDa for Cry 2) whereas spore coat of 4D8 spores have no Cry protein (Figure 2). 4D8 spores have 20.3 and 26 kDa proteins which were not found in spore coats of SN5 or HD1. There was also a 37 kDa protein found in both SN5 and HD-1 spores but not in spores of 4D8.

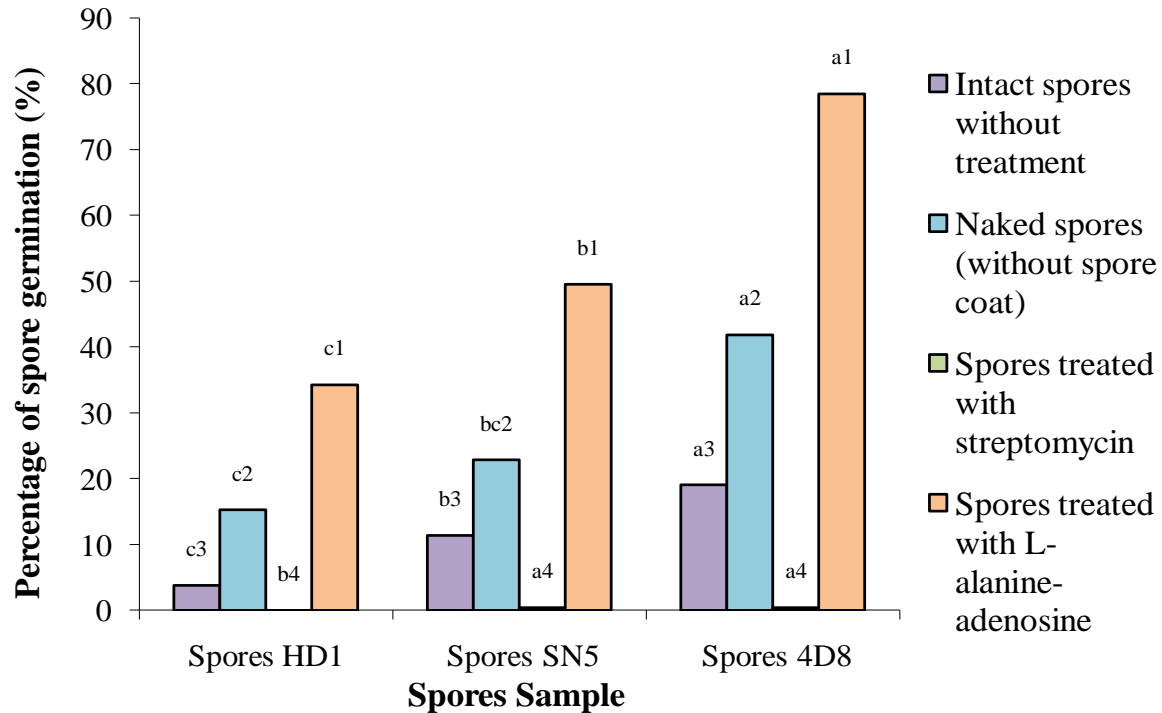


Figure 1. Percentage of spore germination determined with optical density technique at 600 nm. Means with the same alphabet (comparison between spore type) are not significantly different $p>0.05$, whilst means with the same number (comparison between treatment) are not significantly different $p>0.05$.

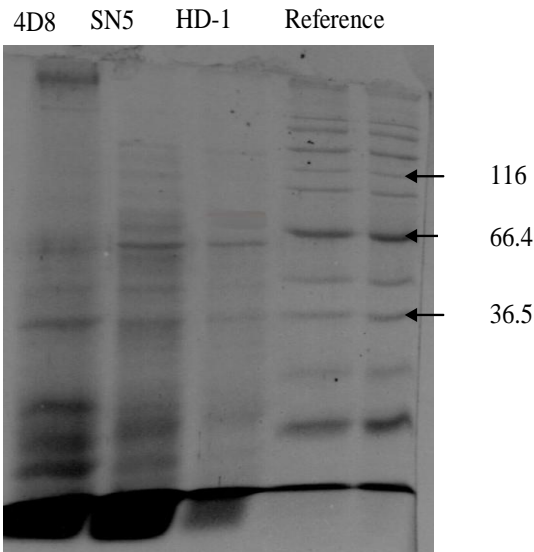


Figure 2. SDS-PAGE analysis.

Effect of adding L-alanine-adenosine and streptomycin

Adding 4 mM LAA significantly ($p>0.01$) improved LC_{50} of 4D8 spores against *P. xylostella* while significantly ($p>0.01$) lowered the effect of SN5 toxicity (Table 1).

Streptomycin (5 μ g), on the other hand, greatly ($p>0.01$) reduced the toxicity of all type of spores against *P. xylostella* (Table 1).

Effect of *Bacillus thuringiensis* subsp. *kurstaki*

Spore of SN5 have significantly ($p > 0.01$) high toxicity on *P. xylostella* larvae as compared to HD-1 and 4D8 spores (Table 1). There was little or no *P. xylostella* larvae mortality when treated with all type of naked spores (Table 1). Cry protein recorded significantly ($p>0.01$) high toxicity when compared with spore for both SN5 and HD-1 (Table 1). Mixture of spore and Cry protein greatly ($p>0.01$) increases toxicity effect as compared to spore alone for all type of spores (Table 2). Adding LAA into the mixture of spore and Cry protein significantly ($p > 0.01$) improved effect of toxicity of HD-1 spore and Cry protein mixture and HD-1 Cry protein and 4D8 spore mixture. All combination of spore and Cry protein with or without addition of LAA were found to be effectively synergized with each other and only HD-1 Cry protein and 4D8 spore mixture showed antagonistic effect (Table 2).

DISCUSSION

The result of this study showed that LAA helps to improve germination of different type of spores significantly.

Table 1. Toxicity of *Bacillus thuringiensis* subsp. *kurstaki*, L-alanine-adenosine and streptomycin against *Plutella xylostella*.

Sample [^]	LC ₅₀ [#] (µg/ml)
LAA	Lom
Strp	Lom
SN5 Cry	0.651 ^e
HD-1 Cry	0.725 ^e
SN5 spore	1.151 ^d
HD-1 spore	14.699 ^b
4D8 spore	25.406 ^a
SN5 naked spore	Lom
HD-1 naked spore	Lom
4D8 naked spore	Lom
SN5 spore with LAA	3.162 ^c
HD-1 spore with LAA	16.352 ^b
4D8 spore with LAA	4.739 ^c
SN5 spore with Strp	Lom
HD-1 spore with Strp	Lom
4D8 spore with Strp	Lom

[^]LAA: L-Alanine-adenosine, Strp: Streptomycin, LAA-treated, spores mixture added with L-alanine-adenosine; Strp-treated, spores mixture added with streptomycin. [#]Value represents mean of 3 replicates; lom, value cannot be determined as there were little or no mortality observed. Different alphabet indicate that values were statistically different $p > 0.01$ (as determined by the Tukey test).

Wilson and Benoit (1993) and Benoit et al. (1995) reported similar observation on the effect of LAA on *Btk* germination. While many findings reported that coatless spores have decreased rate of germination (Nakatani et al., 1985a; 1985b - *Bacillus megaterium*) or at a rate that comparable to coated spores (Stelma et al., 1978; Kutima and Foegeding, 1987 - *Bacillus cereus*), the current study showed opposite trend, where rate of germination was increased by removal of spore coat. These results made the influence of coat on germination remain unclear however it may be related to species.

There are as many as 84 *Bt* serotypes currently identified in the world (Roh et al., 2009) with loads of strains (Roh et al., 2007). Crickmore et al. (1998) reported that 492 *cry* gene has been sequenced. Cry 1 is a bipyramidal Cry protein with molecular weight of 131 kDa while Cry 2 with 72 kDa protein able to produce cuboidal inclusion. These two Cry proteins were found to exhibit toxicity against lepidoptera (Yamamoto and Powell, 1993; Obeidat et al., 2004). Both SN5 and HD-1 isolates have similar protein profile as shown through SDS-PAGE analysis confirming their genetic similarity. Obeidat et al. (2004) found that Jordanian isolate of *Btk* HD-1 recorded highest toxicity effect on *Euphestia kuehniella* as compared to other *Btk* isolate.

Addition of LAA significantly enhanced toxicity in 4D8 spores (LC₅₀ of 25.4 to 4.7). 4D8 spores lack Cry protein in their spore coat. Due to lacking of Cry protein that are important in promoting magnesium (Mg²⁺) dependent

cyclic adenosine monophosphate (cAMP) and activation of protein kinase signaling pathway (Zhang et al., 2005), pores or ion channels cannot be formed in the insect gut cell membrane (Schnepf et al., 1998), thus very little toxicity can be observed. However, by adding LAA that is believed to have some effect in promoting signal transduction pathway such as, cAMP (Aymerich et al., 2006), the toxicity of 4D8 spores were greatly enhanced. Adenosine in the LAA increased the adenosine monophosphate (AMP) intracellular concentration thus upregulates AMP-activated protein kinases. When the cAMP was promoted, pores formed in the insect gut, and exposed the hemolymph. These provided nutrient to 4D8 spores and help to provoke cytotoxicity in insect cells and may have caused cell death as suggested by Zhang et al. (2005).

The toxicity of local SN5 spore isolate was higher than that of HD-1 indicating the potential of local isolate in controlling the population of *P. xylostella*. Although removing spore coat has increased spore germination in *Btk* but failed to cause any or less mortality on *P. xylostella*, this proved that the importance of spore coat is causing insect death. Cry protein has significant better effect on mortality of *P. xylostella* as compared to spore. However when comparing SN5 and HD-1 Cry proteins, the toxicity effect was similar. Using mixture of spore and crystal in bioassay suspension and insecticidal formulation using *Bt* because they were found to be more effective causing mortality than either spore or crystal (Karamanlidau et al., 1991; Tang et al., 1996). These observations were different than current study. In the present study, although the LC₅₀ values of spore-crystal mix were lower than either spore or crystal but only significant when compared with spore. There were no significant different between crystal and spore-crystal mix.

Manipulating the spore germination rate by adding germination inducing LAA did significant improved the toxicity of spore-crystal of *Btk*. These were particularly true for HD-1 Cry protein mixed with either HD-1 or 4D8 spores. This indicates that accelerating spore germination rate has certain effect on *Btk* toxicity. Similar findings were reported by Miyasono et al. (1994) and Martinez-Ramirez et al. (1995) suggesting the importance of LAA in contributing towards spore and spore-crystal toxicities. The present result supported that spore germination have a role to play in *Btk* toxicity against *P. xylostella*. This is because little mortality of *P. xylostella* detected when streptomycin was mixed with spores indicating the suppressing effect on spore germination. Toxicity of Cry protein failed to exhibit with the presence of streptomycin indicates the spore germination is part of the mechanism causing the death of *P. xylostella*, probably through septicemia.

The spores germinate and propagate in the hemolymph of larvae and eventually cause death of insect. This is in consistence with the idea of Broderick et al. (2006) stating the importance of septicemia in *B. thuringiensis*

Table 2. Toxicity of *Bacillus thuringiensis* subsp. *kurstaki* spores, crystal protein and L-alanine-adenosine against *Plutella xylostella*.

Sample [^]	LC ₅₀ expected (µg/ml)	LC ₅₀ [#] (µg/ml)	Synergy factor ⁺
SN5 Cry	-	0.651 ^d	-
HD-1 Cry	-	0.725 ^d	-
SN5 spores	-	1.151 ^c	-
HD-1 spores	-	14.699 ^b	-
4D8 spores	-	25.406 ^a	-
SN5 Cry and SN5 spore	0.864	0.551 ^{de}	1.57
SN5 Cry and SN5 spore LAA-treated	0.864	0.496 ^{de}	1.75
SN5 Cry and HD-1 spore	1.098	0.642 ^d	1.72
SN5 Cry and HD-1 spore LAA-treated	1.098	0.582 ^d	1.88
SN5 Cry and 4D8 spore	1.228	0.653 ^d	1.87
SN5 Cry and 4D8 spore LAA-treated	1.228	0.580 ^d	2.12
HD-1 Cry and SN5 spore	0.928	0.492 ^{de}	1.89
HD-1 Cry and SN5 spore LAA-treated	0.928	0.436 ^e	2.13
HD-1 Cry and HD1 spore	1.175	0.562 ^d	2.09
HD-1 Cry and HD-1 spore LAA-treated	1.175	0.385 ^e	2.66
HD-1 Cry and 4D8 spore	1.344	1.418 ^c	0.95
HD-1 Cry and 4D8 spore LAA-treated	1.344	0.375 ^e	3.56

[^]LAA-treated, spores mixture added with L-alanine-adenosine. [#] Value represents mean of 3 replicates. Different alphabet indicate that values were statistically different $p > 0.01$ (as determined by the Tukey test). ⁺Synergy factor exceeding 1 indicates the presence of synergistic effect, synergy factor equal or close to 1 indicates additive effect whereas synergy factor less than 1 indicates presence of antagonism effect.

toxicity. In the current study, synergy occurred when spore was combined with Cry protein. Synergy factor was even higher when LAA was added into spore-crystal mix. Tang et al. (1996) made similar observation and suggested that the synergistic effect of spore-crystal mix resulted from spore being assisted into the hemolymph by crystal toxin attached to the midgut interface of insect. Miyasono et al. (1994) also reported synergy interaction between HD-1 spore and HD-1 Cry protein when tested on *P. xylostella*.

Conclusion

Local isolate, SN5 showed greater effect on mortality of *P. xylostella* compared to HD-1 spore indicating its potential utility of new formulation insecticidal. Adding LAA not only helps to improve germination rate of spore, toxicity of spore-crystal mix but also synergism effect between spore-crystal mixes.

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