Pathogenicity of *Bacillus thuringiensis* against three important date palm insect pests

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Abstract

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The larval stage of the following three insect pests *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) and *Batrachedra amydraula* Meyrick (Lepidoptera: Batrachedridae) cause serious yield loss to stored date palm fruits that can reach 50 to 70%. This study investigated the pathogenic effect of the bacterium *Bacillus thuringiensis* (*Bt*) *kurstaki* on larval stage of the three moth species. The bacterial concentrations used in the bioassay were selected and prepared logarithmically spaced between the minimum dose of 10^7 CFU/ml and the maximum dose of 10^9 CFU/ml. Results showed that the pathogenic bacterial isolate had the ability to cause disease on the moth larva. The first symptoms observed were brown rot and black discoloration of larval cuticle. The external symptoms of the disease on larvae appeared 2 to 3 days after the death of larvae. The *Bt* LD_{50} on *B. amydraula*, *E. kuehniella* and *P. interpunctella* were 2.15×10^8, 5.18×10^8 and 2.71×10^8 CFU/ml, respectively. The shortest LT_{50} was 5.79 days when the concentration of 10^9 spores/ml of Bt was used against *B. amydraula*, and the longest was 11.57 days when the concentration of 10^8 spores/ml of *Bt* was used against *E. kuehniella*.

Keywords: Date moth, stored pests, *Bacillus thurengiensis kurstaki*

Introduction

Lepidopteran species are the most important pests of stored products throughout the world. Unfortunately, control of Lepidopteran pests worldwide is achieved almost entirely by synthetic insecticides. Larvae cause considerable damage by feeding and/or by contaminating stored food with dead bodies and their own products, e.g. excreta, webbing, silk and feces, while no direct damage from adults is obtained, as they feed on liquid food and/or do not feed at all, but their bodies can become undesirable. Yet, stored products pest control depends almost entirely on chemical fumigants. The two universally available fumigants for disinfestations of durable commodities are methyl bromide (MB) and phosphine (PH3), and each one of them has its own limitations. MB is being phased-out worldwide by the year 2015 under the terms of the Montreal Protocol (6). This dependence on insecticides has contributed to the development of insecticide resistance in many of the most serious pests. Heavy reliance and frequent indiscriminate use of pesticides has also resulted in pesticide residues in food and has had a significant negative impact on the environment. Of particular importance to agriculture is the destruction of crop pollinators and other beneficial insects, parasites and predators that maintain secondary pests under control (11).

Date palm, *Phoenix dactylifera* , is the most common cultivated tree throughout the Middle East and the kingdom of Saudi Arabia, which is one of the largest producers of dates in the world with dates production reached 986,000 tons in 2008 (4). Residual insecticides have been employed to control insect pests of stored date palm, but alternative control strategies are desirable because of the loss of insecticides due to pest resistance and consumer desire for pesticide free date fruits (17). The biggest impetus for the growth of biopesticides comes from the growing awareness by farmers of the value of integrated pest management as a more environmentally sound, economical, safer and a selective approach to crop protection (19).

Development of alternative tactics to the use of insecticides alone is therefore a major approach adopted by most local, national and international research organizations concerned with pest control. *Bacillus thuringiensis* (Berliner) (*Bt*) is a Gram-positive soil bacterium characterized by producing d-endotoxins that act as a powerful intestinal toxin for various insect hosts. The crystalline inclusions together with spores have a great potential to control insect pests (27). *Bt* was investigated in three different habitats (vegetable and crops-cultivated soils, phylloplanes and insect guts) of Bangladesh. A total of 61 *Bacillus cereus*-like isolates were obtained by selective methods and 57 of those were identified as *B. thuringiensis* isolates based on their hemolytic activity, presence of parasporal crystal proteins, plasmid profile and crystal protein profile. The prevalence of Bt was highest (60%) in soil samples followed by leaf and insects (5). BLB1 is a new *B. thuringiensis kurstaki* strain, isolated from a Tunisian soil sample, and has been considered as a strain of great interest and would allow the production of quantities of bioinsecticides at low cost (22). *B. thuringiensis* was detected in 12.5% of soil samples collected from different regions in Syria and 25 *B. thuringiensis* isolates were found to be highly toxic to larvae of *E. kuehniella*, *Phthorimaea operculella* Zeller, and *Cydia pomonella* L. A comparison of the LC_{50} values of the tested isolates with those of the reference strains *B. thuringiensis kurstaki* HD-1 and HD-73 showed that some of these isolates have a higher toxicity potential. Some of these isolates exhibit toxic potential and, therefore, could be adopted for future applications to control some important insect pests (3). The main objective of this research was to

evaluate the ability of *Bt* to control three important species of date palm pests.

Materials and methods

Preparation of *Bacillus thuringiensis krustaki* inoculum
Bacillus thuringiensis krustaki was used in this study. A pre-culture flask containing 500 mL of medium was incubated at 30 °C on an orbital shaker for 9 h and used to inoculate each of culture flasks. The composition of the pre-culture medium was: glucose (10 g/l); ammonium sulfate (1.5 g/l); yeast extract (2 g/l); K2HPO4 (1.5 g/l); KH2PO4 (1.5 g/l); CaCl2 (60 mg/l); MgSO4 (500 mg/l); MnSO4 (50 mg/l). The medium pH was adjusted to 7.0 by NaOH and autoclaved at 120 °C for 10 min (9).

Insect culture
E. kuehniella and *P. interpunctella* used in this study were reared on their artificial diet at 27 ºC with a photoperiod of 14:10 (L: D) h and 65 ± 5% RH in a rearing cabinet (6, 15). *E. kuehniella* larvae were reared on a mixture of wheat flour, glycerol, and wheat bran (65: 5: 30 by weight) (14, 15). *P. interpunctella* larvae were reared in clear glass jars containing a ratio of 2:1:0.25:0.50:0.25:0.25 mixture of rough wheat bran, corn flour, dry yeast, honey, milk powder and glycerin, respectively (21).

Mass rearing of *B. amydraula* was carried out on semi-artificial diet (400 g powder of dry "Sayer" date, 400 g whole wheat flour, 150 g honey, 25 g yeast and 120 mL glycerin) developed by Marouf *et al*. (18). Fifty unsexed adult moths were released on 35 g semi-artificial diet in a plexiglass box (14×8×4 cm) and the boxes were kept in a constant room temperature as described above. Before conducting experiments, insects were reared for one generation on date palm.

Bioassays
The *Bt krustaki* isolate used in this study was obtained from the Plant Protection Research Institute. The different concentrations of bacteria were selected and prepared at logarithmic intervals between the minimum of 10^7 CFU/ml and maximum dose of 10^9 CFU/ml. One gram of date palm fruits was ground and soaked into 1 ml of spore-suspension mixture solution and left to absorb the toxin for 20 min before drying at room temperature. The mixtures were then transferred into Petri plates together with 15 larvae (25 days old) and left in an acclimatization chamber at 27 ºC and 70 ±5% RH, with a photoperiod of 14:10 (L:D) h for 10 days (7, 8). A control group with three replicates was set up for each species.

Data analysis
Mortality rate values were arcsine transformed to normalize the data (12) after correcting for natural mortality (1); angular values were then subjected to analysis of variance using the ANOVA procedure of SAS (23). Lethal time and lethal concentration for 50% mortality (LT_{50} and LD_{50}) and LD_{90} mortality were estimated with repeated measures of logistic regression using generalized estimating equations (26). All analyses were carried out using GENMOD procedure of SAS (24).

Survival analysis of the moth species
Daily mortality rate of *B. amydraula*, *E. kuehniella* and *P. interpunctella* larvae was used to determine survival and hazard functions, after the LD_{50} was determined. Various models such as the Gompertz, exponential, Weibull, and logistics were used for simulating the survival and hazard functions in this study (10, 13, 16). Hazard and survival functions were used for life table analysis of infected larvae. The life table parameters were calculated as shown in Table 1.

Table 1. Life table parameters based on the hazard and survival functions.

Parameters	Definition	Calculating equation
Ti	Start time interval i	$n_i - 1 - w_i - 1 - d_i - 1 - \frac{w_i}{2}$
Di	The number of deaths during the i	$\frac{d_i}{n_i}$
Qi	The mortality rate in the study condition	$1 - q_i$
Pi	The survival rate in the study condition	$\prod_{j=0}^{i} p_j$
Si	The cumulative survival rate	$\frac{2q_i}{b_i(1+p_i)}$
Hi	The cumulative Hazard rate	$(t_j + t_i) + \frac{b_j(s_j - \frac{S_j}{2})}{s_j - s_j + 1}$
Ei	Mid-life capacity	$n_i - 1 - w_i - 1 - d_i - 1 - \frac{w_i}{2}$

j refers to the interval in which $\frac{s_j}{2}$ occur.

Results

Koch test results showed that the Bt was pathogenic on three species of moth larvae. First symptoms on infected larvae appeared as watery larvae with brown to black body cuticle. Two to three days after dying, the cuticle of the larval body broke down (Figure 1).

Pathogenicity
The *Bt* LD_{50} on *B. amydraula*, *E. kuehniella* and *P. interpunctella* were 2.15×10^8, 5.18×10^8 and 2.71×10^8 CFU/ml, respectively (Table 2).

The 50 percent mortality time (LT_{50}) of *Bt* isolate are shown in Table 2. LT_{50} values for the different concentrations of *Bt* ranged from 5.79 to 10.43 days for *B. amydraula*, from 7.76 to 11.57 days for *E. kuehniella* and from 6.16 to 11.1 days for *P. interpunctella*. The lowest LT_{50} (5.79 days) occurred at a concentration of 10^9 spores/ml of Bt for *B. amydraula*, and the highest (11.57

days) was at concentration of 10^8 spores/ml of *Bt* for *E. kuehniella*.

Survival life table of infected larvae

Survival function of the three species larvae were fitted by Weibull and their hazard function fit by exponential model for *B. amydraula* and *P. interpunctella* and by logistic model for *E. kuehniella*. The best fit of survival and hazard curves are represented in Figures 2 and 3.

The results in Table 3 showed that Si (probability of survival) has gradually decreased and hazard rate increased during 12 to 14 days of experiment period. Furthermore, Ei (life expectancy) as the combined outcome of the probability of survival parameters was reduced.

Based on the results of the survival analysis, the pathogen effectiveness varied as expressed by different coefficients of variation based on the different species of the infected larvae. The highest and lowest slope changes were observed in *B. amydraula* and *E. kuehniella*, respectively.

Discussion

Bioassays of some botanical extracts, granulosis virus, paraffin summer oil and bacterial toxin of *B. thuringiensis* and plant extracts (matrine) were used against the first generation of lesser date moth *B. amydaula* at several sites during two campaigns (2004 and 2005) in Saudi Arabia in order to assess their efficacy against this important pest in the region. Several trials with biopesticides were conducted in date palm areas (Bicha, Riyadh, Haer, Oyayna) using the following bio-products according to the infestation level: *B. thuringiensis* kurstaki (Condor) at 300-1000 g/hl; Spinosad (tracer 480) at 100 ml/hl; Carpovirisin: (CYD-X) at 500 ml/hl; Sunspray 98.8% (summer oil) at 1.0 l/hl; Herbal source 150 ml/hl. A trial protocol was adapted to each region according to its specificity. The application was made at the rate of 3 to 7 liters per tree, washing all bunches of the tree according to the size of trees and number of bunches. In each bioassay, samples of 400 to 500 fruits were analyzed from each plot weekly during one month after treatment. The results showed that when applied at the time corresponding with oviposition and hatching periods (a few days after fruit set), the biopesticides controlled the lesser date moth well. The protection period was important in plots treated with Matrine one, Sunspray 7E and *B. thuringiensis* Kurstaki, but was less important in the plots treated with Spinosad and Carpovirusin (25).

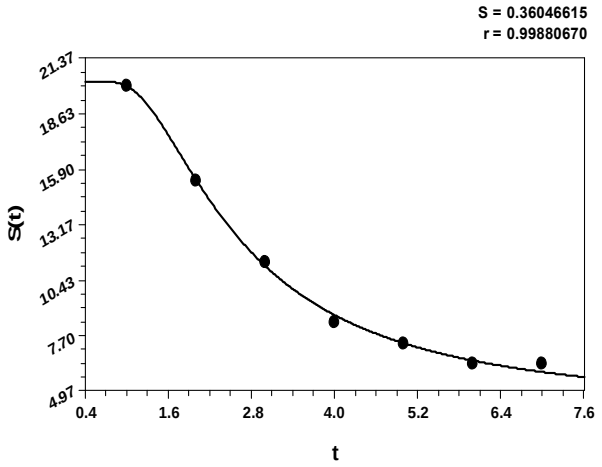
Figure 1. Black larvae body cuticle of *E. kuehniella* infected with *Bacillus thuringiensis*.

Table 2. Lethal concentrations abbreviated as LD_{50} values of *Bacillus thuringiensis* on the larvae of *B. amydraula*, *E. kuehniella* and *P. interpunctella*.

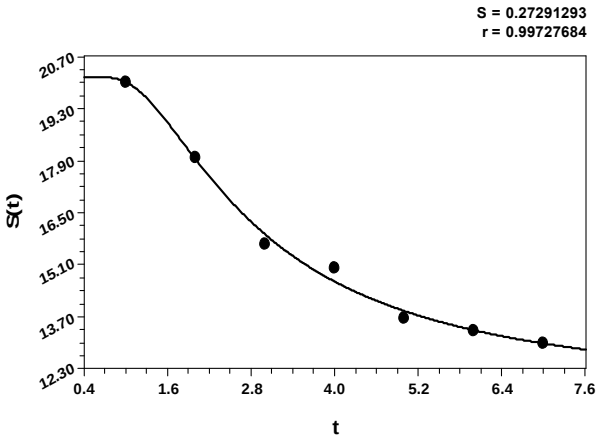
Moth species	LD_{50} (95% fiducial limits)	LD_{90}(95% fiducial limits)	Slope ± SE	X^2
B. amydraula	2.15×10^8(1.66-2.65)× 10^8	8.68×10^9(4.31-9.85)× 10^9	1.108 ±0.128	0.31
P. interpunctella	2.71×10^8(2.09-3.34)× 10^8	1.86×10^{10}(1.09-2.75)× 10^{10}	1.242 ±0.23	0.34
E. kuehniella	5.18×10^8(4.02-6.29)× 10^8	2.27×10^{10}(1.69-3.94)× 10^{10}	1.634 ±0.17	0.27

Table 3. LT_{50} mortality values of *Bacillus thuringiensis* on the larvae of *B. amydraula, E. kuehniella and P. interpunctella.*

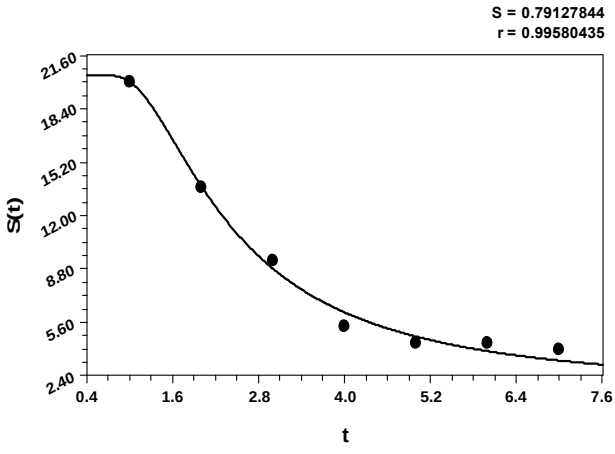
Moth species	Concentrations (conidia/ml)	(95% confidence limits) LT_{50}	Slope ± SE	X^2
B. amydraula	10^8	10.43 (9.86–11.02)	3.58 ± 0.14	3.82
	5 x 10^8	6.85 (6.44–7.25)	3.72 ± 0.43	4.07
	10^9	5.79 (4.41–6.19)	3.9 ± 0.72	5.21
P. interpunctella	10^8	11.1 (9.98–12.48)	3.41 ± 0.14	2.3
	5 x 10^8	8.1 (7.23–10.56)	3.78 ± 0.22	5.1
	10^9	6.16 (4.94–6.89)	3.91 ± 0.14	4.9
E. kuehniella	10^8	11.57 (10.89–12.32)	3.52 ± 0.23	4.62
	5 x10^8	9.34 (8.63–10.03)	3.87 ± 0.16	4.2
	10^9	7.67(5.82–8.02)	4.67 ± 0.42	4.7



B. amydraula

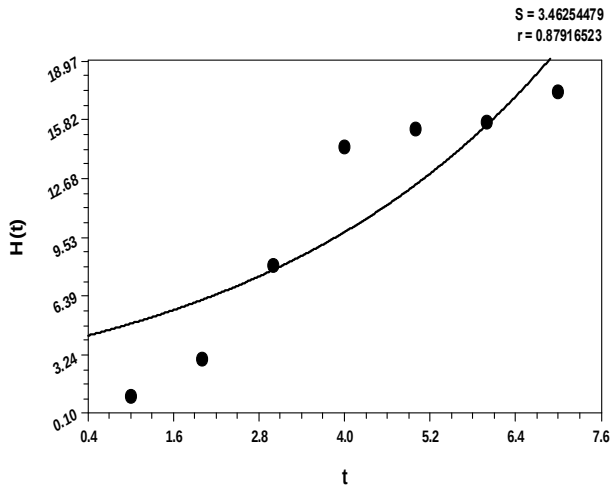


P. interpunctella

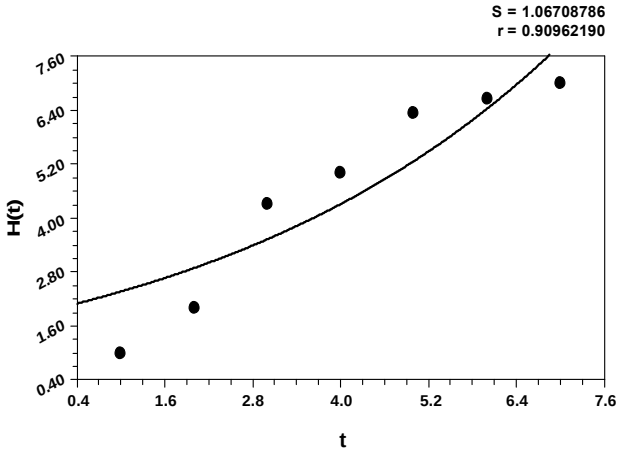


E. kuehniella

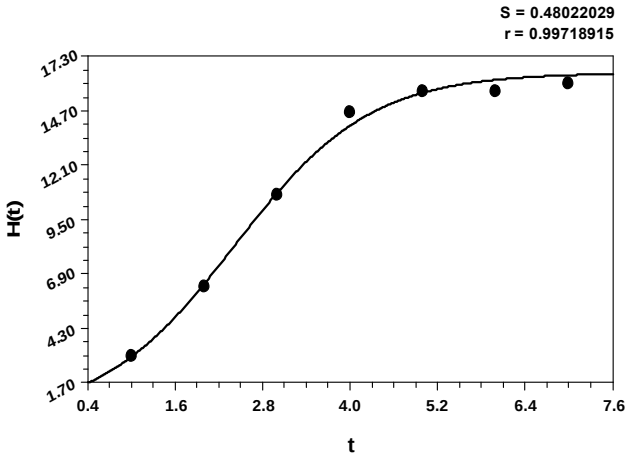
Figure 2. Survival curves of *B. amydraula*, *P. interpunctella* and *E. kuehniella* of Bt-infected larvae.



B. amydraula



P. interpunctella



E. kuehniella

Figure 3. Hazard curves of *B. amydraula*, *P. interpunctella* and *E. kuehniella* Bt-infected larvae.

Table 3. Survival table of *B. amydraula*, *P. interpunctella* and *E. kuehniella* infected larvae

Species	Ti	Di	Qi	Pi	Si	H i	Std.Err	Ei	Std.Err
B. amydraula	2	0	0.071	0.928	1.000	0.022	0.031	14.031	1.835
	4	0	0.071	0.928	0.928	0.022	0.031	11.045	1.704
	6	0	0.071	0.928	0.862	0.022	0.031	8.034	1.582
	8	2	0.285	0.714	0.80	0.100	0.069	5.000	1.469
	10	3	0.600	0.400	0.571	0.257	0.134	2.777	1.240
	12	1	0.500	0.500	0.228	0.200	0.188	1.666	2.357
P. interpunctella	2	0	0.071	0.928	1.000	0.022	0.031	12.92	1.835
	4	0	0.071	0.928	0.928	0.022	0.031	9.934	1.704
	6	0	0.071	0.928	0.862	0.022	0.031	7.051	2.374
	8	3	0428	0.571	0.800	0.163	0.090	4.166	2.204
	10	2	0.500	0.500	0.457	0.200	0.133	3.333	3.333
	12	1	0.500	0.500	0.228	0.200	0.188	2.770	2.357
E. kuehniella	2	0	0.071	0.928	1.000	0.022	0.031	14.03	1.835
	4	0	0.071	0.928	0.928	0.022	0.031	11.045	1.704
	6	0	0.071	0.928	0.862	0.022	0.031	8.034	1.582
	8	2	0.285	0.714	0.80	0.100	0.069	5.000	1.469
	10	3	0.600	0.400	0.571	0.257	0.134	2.777	1.240
	12	1	0.500	0.500	0.228	0.200	0.188	1.666	2.357

A 36.5-69% control level of lesser date moth was achieved earlier by using bacterial insecticide *Bt* at the rate of 150 g/100 L water in New Vally, Egypt (25). Annual report from Oman (4) revealed that the use of bacterial bio-pesticide *Bt* aizawai 10.3% gave 43.8% reduction in the infestation rate of lesser date moth seven days after treatment and 60.8%, 14 days after treatment. *Bt* at the rate of 1 kg/100 L water was also used successfully in Saudi Arabia, with a volume of 3-7 l/palm tree to control this pest (11). Efficacy of the bio-pesticide bacteria, *Bacillus thuringiensis kurstaki* (Btk) in controlling lesser date moth, *B. amydraula* infestations were studied during 2011 and 2012 seasons. The result of 2011 showed high reduction in infestation was achieved by Btk (78.65%) at a rate of 6 g Btk powder/kg talc powder. The result of 2012 showed that the highest control efficacy (58.78%) was achieved at the first time of the control process, using Btk at a rate of 6 g/kg talc powder. At the second time (after one week from the first application), the efficacy of Btk at the rate of 6g/kg talc powder was increased to 78.78%. Reapplication of the control treatment achieved an increase in efficacy of the control agents, such increase was significant for Btk at a rate of 6 g/kg talc powder (2). Among 201 new *B. thuringiensis* strains isolated from different countries, two strains (BLB249 and BLB384) showed higher toxicity than the commercial strain *B. thuringiensis kurstaki* HD1 against *E. kuehniella* larvae. Morphological, molecular and biochemical investigations revealed that these strains were similar to HD1 but presented different cry gene content. Additional bioassays revealed that only strain BLB249 displayed higher toxicity than HD1 against *P. interpunctella* larvae. The study of Cry protoxin activation by midgut proteases of *E. kuehniella* and *P. interpunctella* larvae supported that higher toxicity of BLB249 and BLB384 strains compared to HD1 was not due to differential protoxin activation (9). Eight serotypes with *B. thuringiensis israelensis* being the most common. Out of the twenty-six isolated strains, five strains (serotype: kenyae, kurstaki, kurstaki HD1 and thuringiensis) that produced bipyramid crystal proteins were toxic to the lepidoptera larvae of *E. kuehniella*. The SDS-PAGE protein profile analysis showed a relationship between the crystal protein shape and the toxicity to the larvae of the tested insect (20). In conclusion, the results of this investigation indicated that using Bt at the rate of 6 g/kg talc powder were a successful option to control the lesser date moth as an alternative strategy to methyl bromide fumigation.

Results obtained showed that the bacteria had a good application potential for the biological control of date stored product moths based on epizootiological perspectives. However, further studies are still required to validate the use of biological control of date pests under storage conditions.

الملخص

لطيفان، م و ج.ر. كاجبافقالا. 2015. القدرة الإمراضية للبكتيريا *Bacillus thuringiensis* إزاء ثلاث آفات حشرية على نخيل التمر. مجلة وقاية النبات العربية، 33(3): 323−329.

يسبب الطور اليرقي للآفات لبحشرية الثلاث التالية ,*Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)، *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) و *Batrachedra amydraula* Meyrick (Lepidoptera: Batrachedridae) خسائر في الغلة للتمور المخزونة يمكن أن تصل إلى 50−70%. بحثت هذه الدراسة الأثر الإمراضي للبكتيريا *Bacillus thuringiensis* (*Bt*) *kurstaki* في الطور اليرقي لعثات التمور الثلاث. انتخبت التراكيز المستخدمة في الاختبار الحيوي وتم تحضيرها لوغاريتميا بفاصل بين جرعة دنيا قدرها 10^7 وحدة مشكلة للمستعمرات/مل وجرعة عظمى قدرها 10^9 وحدة مشكلة للمستعمرات/مل. أظهرت النتائج أن للعزلة البكتيرية الممرضة المقدرة على إحداث المرض ليرقات العث. وكانت أولى الأعراض الملاحظة تعفن بني وتلون أسود للكيوتيكل اليرقي. ظهرت الأعراض الخارجية للمرض على اليرقات بع يومين إلى ثلاثة أيام من موت اليرقات. كانت الجرعة المميتة من *Bt* لـ 50% من يرقات *B. amydraula*، *E. kuehniella* و *P. interpunctella* هي 2.15×10^8، 5.18×10^8 و 2.71×10^8 وحدة مشكلة للمستعمرات/مل. كان أقصر وقت قاتلة نصفي 5.79 يوم عند استخدام التركيز 10^9 بوغ/مل من *Bt* إزاء *B. amydraula* وكان الوقت النصفي الأطول 11.57 يوماً عند استخدام التركيز 10^9 بوغ /مل إزاء *E. kuehniella*.

كلمات مفتاحية: عثة التمو، أفات مواد مخزونة، *Bacillus thurengiensis kurstaki*

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