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ISOLATION AND DIAGNOSTIC *BACILLUS THURINGIENSIS TENEBRIONIS* PATHOGENESIS FOR INSECTS FROM DATE PALM STEM BORER LARVA (*JEBUSAEA HAMMERSCHMIDT* :COLEOPTERA :CEARMBYICIDAE)

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Abstract

The aim of this study to isolate and diagnose pathogen bacteria that caused date palm stem borer larva.economic important for date palm in Iraq. The process of diagnostic depend on morphological properties and biochemical tests in addition it biophysical specialties and polymerase chain reaction (PCR) for Cry3 gene, the pictures when taken by transmission electron microscope (TEM). The results of different tests when fourel this bacteria responsible for larvae death *Bacillus thuringiensis* subsp. *tenebrionis.* through compatibility of nucleotide based sequences in 99% between molecular diagnostic results and Cry gene analysis *Bacillus thuringiensis serovar morrisoni* BBSC 4AA1 which NCBI bank genes. The isolated bacteria *B. t. tenebrionis* caused 100% mortality after 2 day in the first and second larvae instare of date palm stem borer *Jebusaea hammerschmidt* and 100% mortality in the last instar of the larvae after 4 days.This bacteria *B. t. tenebrionis* was recorded the first time on larvae of date palm stem borer.

Key words : *Bacillus thuringiensis tenebrionis*, insects, palm.

Introduction

More than one million of insects species in the one fauna (Vilmos and Kurucz, 1998 includes coleopteran (Beetles) this important pest infest stored seeds, field crops, ornamental plants and forest trees (Sezen *et al.*, 2008). Relation with a large number of microorganism specific of main pathogenic bacteria infect insects, they are found four *Bacillus* species including *B. popilliae*, *B. lentimorbus*, *B. larvae* and *B. thuringiensis* (DeBarjac, 1981). *B. thuringiensis* was gram positive stain produce toxic crystal protein during spore formation stag (Qiuling *et al.,* 2015) that highly toxic effect on different instar of the larva Diptera and Coleopteran this is alternative of chemical pesticide application in agriculture and forestry (Schnepf *et al.,* 1998) the appearance of resistance properties against chemical pesticides with increased anxiety for refers effects on non-organism target of public health, that induce to find insects pathogen alternative factors (Beron and Salerno, 2006).

B. t first reported in 1901 and commercial product at the first time in 1939 (Beegle and Yamamoto, 1992) following few years new strain of *Bacillus* was isolation named *B.t. var. tenebrionis,* found its toxicity on Coleopteran (Krieg *et al.,* 1983) reported strain *Bacillus* in (BGSC) under 4AA1 icon and its crystal toxin Cry3Aa icon. This toxin is three dimcutonal the backbone of *B.thuringinsis* when identified (Li *et al.,* 1991 *B.t. var. tenebrionis* isolated from *Xyleborus dispar* diagnosed based on physical and biochemical theratical characteristics and molecular specifies that causes 100% kill larvae of *Agelastica alni* (Coleoptera: Chrysomelidae) and 100% for *Amphimallon solstitiale* (Coleoptera: Scarabaeidae) larvae, therefore, consider the important value for Coleopteran pests (Sezen *et al.,* 2008).

The aim of study of bacteria caused mortality of larvae isolation and diagnoses in molecular and biochemical technique as a result of found a large number at date palm stem borer.

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Materials and Method

Bacterial isolation from died larva during the program of sample collection and cutting date palm to small parts to take stages of date palm stem borer was found some of the dry died larva which after of symptoms of bacteria as cured of larva and take black color (Fig. 1-a). Isolation and diagnostic this bacterium that causes the pathogenic according to Thomas-George (1978) by using the following steps:

1. Sterilization of dead and infected larva using 70% ethanol for 2 sec.
2. Using 5% NaHCl (Sodium hypochlorite).
3. Sterilization by 105 Sodiuthiosulfate for 3-5 min.
4. Steeping in three-time change of sterile water.

Prepare 9×1.5 cm petri dishes for group blood agar culture media, while other groups for nutrient agar, inoculation of larva steeping solution for both types of culture media, then the plates incubation at 30^{0}C for 24-48 hrs. Other bacteria growth sub-cultured to harvest pure colonies (Fig. 1-b) by using streaking several times at 30 for 24 hrs. Incubation.

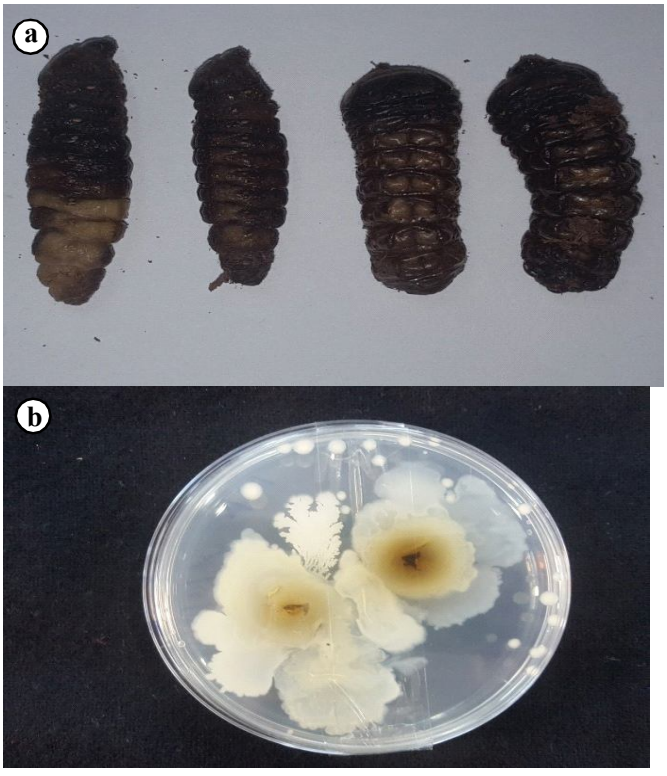

Fig. 1: Symptoms of bacteria infection on stem borer larvae and the growth bacteria on nutrient agar.

To confirm bacterial pathogenesis with Kocu postulate application, prepared healthing larva in 10×25×10 cm plastic cubs containing crushed food from date palm parts, the mixture of containing cubs inoculated with isolated pathogenic bacteria with daily monitoring to notice any significant disease that appear on larva and compare with larva sores of isolated bacteria.

Diagnosis of bacteria

Morphological properties diagnosis

They are think the independent colonies similar to *B.thuringinsis* by the white color, irregular surround colonies, and fast growth and stained its crystal protein by basic faxin.

Biochemical teste

The biochemical test based on (Washington *et al.,* 2006) including (Fructose, Sucrose, Glucose, Mannose, Indole production, Celibiose, Citrate utilization, Vogues proskauer's, H_2S, TSI, Oxidase, Phenyl alanine, Methyl red, Catalase) of bacteria which isolated in laboratory of department of plant protection and compare with partial diagnosis of standard *B. thuringiensis tenebrions* provided by (Al-Waeily, 2018) and using Vitek 2 version technique.

Spore formulation properties

By Schaeffer Fulton procedure, using growth at

Table 1: Material for amplification of the Cry gene.

Finduaium µl	Volium µl	Component test
20	5	DNA extract of isolated bacteria
	2	Forward primer 10pmol
	2	Reverse primer 10pmol
	11	Free nuclease water

Table 2: PCR amplification of the Cry gene of the local bacteria isolated.

Steps	Temperature C°	Time (min)	No. of cycles
Predenaturation	94	5	1
Denaturation	94	1	30
Annealing	55	1	
Extension	72	1:40	
Final extension	72	5	
Cooling	4	"	1

Table 3: Mrphological and physiological characteristics of *B.t. tenebrions.*

Lj.2	Test
Rod	Shape of bacteria
Chain	Cell pooling
+	Gram stain
+	Endospore formation
Cream	Colony color
Cream	Colony on blood agar
Cream	Colony on N.B

Table 4: Biochemical characteristics of *B.t. tenebrions* by Vitek.

Well	Test	Mnemonic	Amount/WELL	Res 1	Res 2
2	Ala Phe Pro Arylamidase	APPD	0.0384mg	-	-
3	Adonitol	ADO	0.1875mg	+	+
4	L-Pyrrolyl-Arylmidase	PyrA	0.018mg	-	-
5	L-Arabitol	LARl	0.3mg	-	-
6	D-Cellobiose	DCEL	0.5mg	+	+
7	Beta Galactosidase	BGAL	0.036mg	+	+
8	H2S Production	H2S	0.0024mg	-	-
9	Betan Acetyl Clucosamindase	BNAC	0.0408mg	+	+
10	Glutamyl Arylamidase pNA	AGLTp	0.0324mg	-	-
11	D GLUCOSE	dGLU	0.3mg	+	+
12	Gamma Clutamyl Transferase	GCT	0.0228mg	-	-
13	Fermantion Glucose	OFF	0.45mg	+	+
14	Beta Glucosidase	BGLL	0.036mg	-	-
15	D Maltose	dMAL	0.3mg	+	+
16	D Mannitol	dMAN	0.1875	+	+
17	D Mannnose	Dmne	0.3mg	+	+
18	Beta Xylosidase	BXYL	0.0324mg	+	+
19	Beta Alanine anylamidase Pna	BALap	0.0174mg	-	-
20	L Proline ARYLAMIDASE	ProA	0.0234mg	-	-
21	LIPASE	LiP	0.0192mg	-	-
22	PALATINOSE	PLE	0.3mg	+	+
23	Tyrosine ARYLAMIDASE	TytA	0.0276	+	+
24	UREASE	URE	0.15mg	-	-
25	D SORBITOL	dSOR	0.1875mg	+	+
26	SACCHAROSESL CROSE	SAC	0.3mg	+	+
27	D TACATOSE	dTAC	0.3mg	-	-
28	D TREHALOSE	dTRE	0.3mg	+	+
29	CITRATE SODUM	CIT	0.054mg	+	+
30	MALONATE	MNT	0.15mg	+	+
31	L LACTATE alkalinsation	ILATK	0.15	+	+
32	5 KETO D GLUCONATE	5KG	0.3mg	-	-
33	ALPHA CLUCOSIDASE	ACLU	0.036mg	-	-
34	SUCCINATE alkalinsation	SUCT	0.15mg	-	+
35	Beta N ACETYL GALACTOSAMINDASE	NACA	0.0306mg	+	+
36	ALPHA CALACTOSIDASE	ACAL	0.036mg	+	+
37	PHOSPHATASE	PHOS	0.0405mg	+	+
38	Glycine ARYLAMIDSE	GlyA	0.012mg	-	-
39	ORNTHINE DECARBXYLASE	ODC	0.3mg	-	+
40	LYSINE DECARBOXYLASE	LDC	0.15mg	+	-
41	L HISTIDINE assimilation	lHSa	0.087mg	-	-
42	COUMARATE	CMT	0.126mg	-	-
43	BETA GLUCORONIDASE	BGLR	0.0387mg-	-	-
44	O 129 RESISTANCE (comp vitria)	O129R	0.0105mg	-	-
45	Glu Gly ARYLAMIDASE	GCAA	0.0576mg	-	-
46	L MALATE assimilation	lMLTa	0.024mg	-	-
47	ELLMAN	ELLM	0.3mg	-	-
48	L LACTATE assimilation	lLATa	0.186	-	-

Table 5: Biochemical characteristics of *B.t. tenebrions* .

Biochemical reaction	Isolated strain	Standard strain
Fructose	+	+
Sucrose	+	+
Glucose	+	+
Mannose	+	+
Celibiose	+	+
Indol production	-	-
Citrate utilization	-	-
Voges proskauer's	-	-
H_2S	-	-
TSI	AA	AA
Oxidase	+	+
Phenyl alanine	-	-
Methyl red	+	+
Catalase	+	+

bacteria on nutrient agar at 30^0C. for 5 day including both spores and vegetation form. Dry slide preparation by exposure to air cover with filter paper, then added malachite green stain, the slide was put on water path to exposure to steam for 3 min. added additional drops of malachite green.

Remove the filter paper carefully after 3 min. and washing by water to remove of remaining of malachite green, immersion the slide containing bacteria in safranin stain for 1 min to remove reaming stain by washing with water and dry the slide.

Microscopically examiner at the first by (10x) then turner to (40x) end finally by oil immersion in (100x).

PCR (Polymerase Chain Reaction)

a. Nucleic acid isolation

Extraction of *B.thuringiensis* DNA isolated from date palm stem borer died larva diagnostic by morphological and biochemical properties using presto mini Gdna bacteria kit (Al- Waeily, 2018).

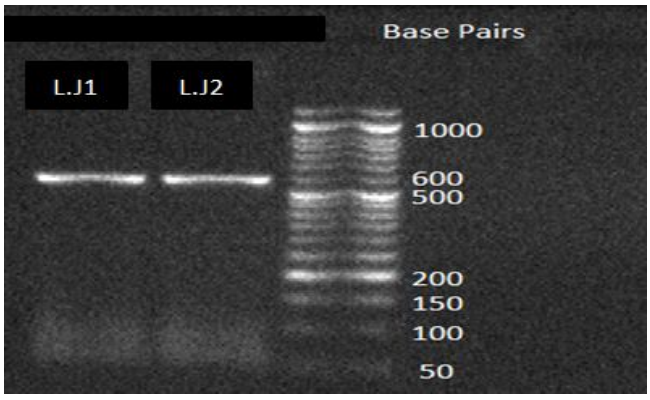

Fig. 2: PCR amplified product from Lj.2 isolate for 16s RNA gene.

Biological Fluids

Transfer 1 ml of biological fluid to a 1.5 ml micro centrifuge tube. Centrifuge for 5 minutes at 14-16,000 × g then discard the supernatant. Add 200 µl of GT Buffer then re-suspend the pellet by vortex or pipette. Add 20 µl of Proteinase K then mix by vortex. Incubate at 60ºC for at least 10 minutes. During incubation invert the tube every 3 minutes.

Lysis

Add 200 µl of GB Buffer to the sample and mix by vortex for 10 seconds. Incubate at 70ºC for at least 10 minutes to ensure the sample lysate is clear. During incubation, invert the tube every 3 minutes. At this time, pre-heat the required Elution Buffer to 70ºC.

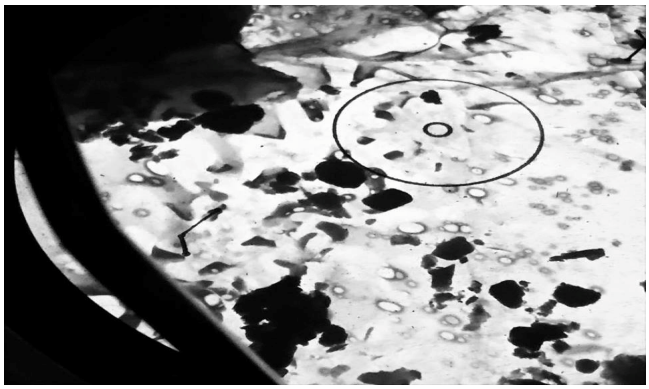

Fig. 3: PCR amplified product from Lj.2 isolate for 16s RNA gene.

Optional RNA Removal Step

Add 5 µl of RNase a to the clear lysate then shake vigorously. Incubate at room temperature for 5 minutes.

DNA Binding

Add 200 µl of absolute ethanol to the sample lysate and mix immediately by shaking vigorously. If precipitate appears, break it up as much as possible with a pipette. Place a GD Column in a 2 ml Collection Tube. Transfer mixture.

To the GD Column then centrifuge at 14-16,000 × g for 2 minutes. Discard the 2 ml Collection Tube containing the flow-through then place the GD Column in a new 2 ml Collection Tube.

Wash

Add 400 µl of W1 Buffer to the GD Column. Centrifuge at 14-16,000 × g for 30 seconds then discard the flow-through. Place the GD Column back in the 2 ml Collection Tube. Add 600 µl of Wash Buffer (make sure ethanol was added) to the GD Column. Centrifuge at14-16,000 × g for 30 seconds then discard the flow-through. Place the GD Column back in the 2 ml Collection Tube.

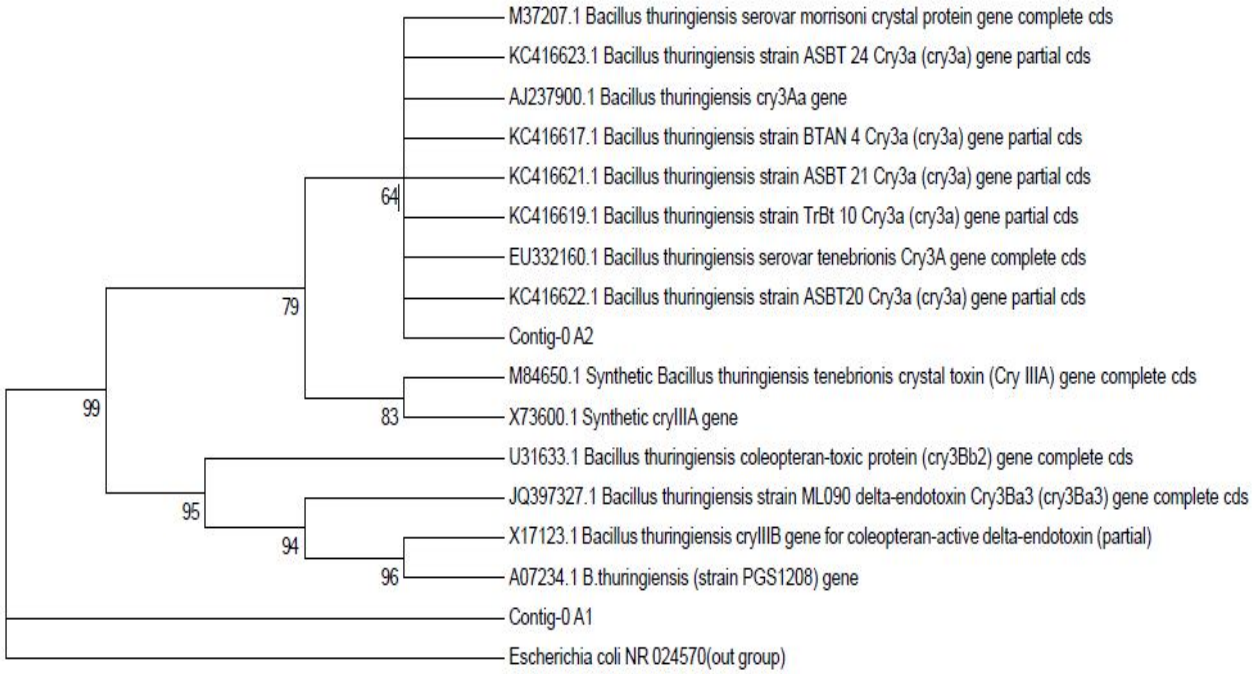

Fig. 3: Phylogenetic analysis of L.J1 based on 16s rRNA gene sequence.

Centrifuge again for 3 minutes at 14-16,000 × g to dry the column matrix.

Elution

Standard elution volume is 100 µl. If less sample is to be used, reduce the elution volume (30-50 µl) to increase DNA concentration. If higher DNA yield is required, repeat the DNA elution step to increase DNA recovery and the total elution volume to approximately 200 µl. Transfer the dried GD Column to a clean 1.5 ml micro centrifuge tube. Add 100 µl of pre-heated Elution Buffer, TE Buffer or water into the center of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer or water to be completely absorbed. Centrifuge at 14-16,000 × g for 30 seconds to elute the purified DNA.

b. Gen amplification

For the amplification of Cry3 gene of the local isolate, forward (26) 5-CGTTATCGCAGAGAGATGACAT TAAC-3 (and reverse primers (23) CATCTG TTGTTTCTGGAGGCAAT-3 (Al- Waeily, 2018). Provided Pioneer Company / South Korea it used, were designed according to the nucleotide sequence of Cry3 of *B.t. tenebrions*. by add 20 µl of the muster max (test constituents) (Table 1).

Amplification was with 1 cycle predenaturation at 94^{0}C for 5 min, and 35 – cycle programme (each cycle consisting of denaturation at 94^{0}C for 60 annealing at 55^{0}C for 60 s, and extension at 72^{0}C for 1.40 min), following by a final extension step at 72^{0}C for 5 min (Table 2).

PCR products were analyzed by add 5 ml sample extraction to gel electrophoreses included agarose at 50-55^{0}C. and Put the comb and remove coffer hardening the gel which covered by TBE Buffer, the UV light tranzillminator were used for band detection offer put the gel mold inside the volumes of bands selected by using (DND 2000pb).

The primer and DNA extraction were sent to confirm nucleotide base sequences using Bio Edit software (Thomas, 1999) and similar the compatibility of the nucleotide sequences at the reported species in Genbank and other species by using MEGA 7 software (Koichiro *et al*., 2011).

Imaging and Electron microscope (TEM)

Electron microscope (Philips CM-10) and negative staining of phosphotenestic acid were used, purification of bacteria cutler by filter paper (pore size 0.45 µl) to remove any impurities and drying of purified bacteria by four different concentration (50, 75, 95, 100) of ethanol alcohol squinty to isolate and diagnose crystal protein.

Results and Discussion

The morphological and biophysical characterization of isolated bacteria table 3 in addition to diagnostic by Vitek 2 version table 4 and biochemical teste table 5 08.01 equipment, all of their confirmed pathogen bacteria for date palm stem borer.

Table 6: Details of *Bacillus thuringiensis homologous* sequences selected from GenBank

Accession	Identity	Strain	Nucleotide
CP010582	99%	*Bacillus thuringiensis serovar morrisoni* BGSC 4AA1 plasmid Pbmb51 complete sequence	CGTAATGGTCTGTAGCATCTCTCGTGTTTCCGGACGC ACAAAAGCTTGTGAGAAGAGGTACAGGTG AATGCGTGAATACCCCTAAATTGCACGTT TGGAACGCCTGTAAACGCTTCGGACGGTAAC TCCCCAAGATTTGCCTTTGCTCCGGCTTTATA GACTTTTTCTCCATTAAATTCTAAATTTTGTAC AGGTTCCCTGGATTTATTTCCATAGAATGGAG ATGTGATTATATCATTTGATCCTATGCTTGCCC TAGTTGAAACATAATTCCCGGACCATTAATTG AAAGAG TCATTTCCATAATATCCTGGTTGGAACCGCG TGTGAACTTGAATTCTATGCAGAGAGTCAAATAG ATGTGGTTTTCTAATATAATTTTCTATATTATAA AAAGTTGTTCCATACCCCCTAAGGTTGTTG CCCCGTCCACTGGATCTGTTAAAACGTCTCTTGTT AATTCCCTTTTAACTTCTTTTGGGAATAACCCA ACATCACACAGCTGAATTAGCGCAATTAAATAAA ATACAGGTTTTGTCATCTCTCTGCGATAACGAT TGCCTCCAGAAACAACAGATGATTGCCTGCAGA AACAACAGATGATTGCCTCCAGAACACAGA TGATTGCCTCAAGATACAACAGATGA TTGCCGCCGAAAAAATTCACGTTTCAAAAAGAGAT
EU332160	99%	*B.thuringiensis serovar tenebrionis* Cry3A gene complete cds	
Y0042O	99%	*B.thuringiensis (var tenebrionis)* ot13 gene for crystai protein	
AJ237900	99%	*B.thuringiensis* cry3Aa gene	
M30503	99%	*B.thuringiensis* coleopteran -specific insect control protein gene complete cds	
KC416623	99%	*B.thuringiensis* strain ASBT 24 Cry3a (cry3a) gene partial cds	

The tests by electrophoreses (Fig. 2) using both forward and reverse prime with 600pb length. Found that similar with local isolated *B.thuringinsis* and this agreement with the same finds (Al-Waeily, 20018; Zothansaga, 2011).

The nucleated sequences for Cry gene of the local bacteria strain L.j2 (fig 2) and reported strain in NCBI, was found the same Cry gene nucleated sequence of *Bacillus thuringiensis serovar morrisoni* BGSC 4AA1 in 99% the finding suggest *Bacillus thuringiensis* subsp. *tenebrionis* (Table 6 and Fig. 3).

Electron microscope photography of crystals were found square shape this agreement with (Khanna and Babita, 2018). The crystals were used as microbian pesticide certified by Environmental Protection Agency Colorado beetle larva on potato (Schnepf *et al*., 1998). specifically at Cry3b and Cry3a toxins for Coleoptera (Li *et al*., 1991; Grouchulski *et al*., 1995 ; Morse, 2001) The recent control to prevent spread date palm stem borer and decrease its effects on date palm induce to find active alterative pesticide, that suitable with nature of life cycle of insect.

According to this finding *Bacillus thuringiensis* subsp. *tenebrionis* have efficacy in 100% killing of one and two instar larva at 2 day treatment for date palm stem borer, also efficacy in 100% of killing at the final larva instar after 4 days treatment, *Bacillus thuringiensis* subsp. *tenebrionis* is important to develop to propose a pioneer pesticide as a commercial preparation.

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