Bacillus thuringiensis (Bt) toxin for the Control of Citrus Trees *snails.*

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Abstract: Land snails become the most important agricultural pest in the world. It caused large damage to the different crops this by feeding on its roots, stems, leaves and fruits. ***Bacillus thuringiensis* (Bt)** causing 100% mortality to *Monacha cartusiana* (*M. cartusiana*)in laboratory treatment. Using the morphological and biochemical tests *B. thuringiensis* was identified as *B. thuringiensis* Subsp. *Kurstaki* (ES^+, Sa^+, Le^+, Su^-) on whole isolate. Parasporal bodies of *B. thuringiensis* isolate had biological activity when assayed against *M. cartusiana* snails. The protein composition of parasporal bodies 140 Kilo Dalton (KDa) parasporal bodies caused mortality reached 100% to *M. cartusiana* snails by concentration 7.8 mg/ml. Field application of *B. thuringiensis* as toxic spray on citrus trees of infected parts with adults of *M. cartusiana* snails by using 2 x 10^3 c.f.u/ml showed that mortality reached 89% of snails within 21 day.
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1. Introduction

Land snails are dangerous agricultural pests which classified under phylum Mollusca, Class Gastropoda. In recent years, these snails have increased rapidly causing economic damage in the field crops, vegetables as well as horticultural crops. Moreover they are fungal feeders; consume a variety of fungi as *Agaricus bisporus* and *Pleurotus ostreatus* **(Puslednik, 2002)**.

Bacillus thuringiensis is an entomopathogenic bacterium which forms a parasporal crystal during Sporulation. *B. thuringiensis* is used commercially as an insecticide, and is marketed under several trade names as Dipel.

B. thuringiensis and *Bacillus cereus* are very closely related species. The species have been compared by several methods including biochemical clustering studies, comparing the utilization of sugars, DNA homology, and agglutination testing to determine the degree of relatedness. From the results of DNA homology studies, *B. thuringiensis* is considered a variant of *B. cereus* **(Berkley and Good Fellow, 1968).**

B. thuringiensis subspecies, the biological basis of toxic action has been established only for *B. thuringiensis* subs.*Israelensis*. The initial effect of parasporal bodies when they are fed to mosquito larvae is on the midgut epithelium. On exposure to toxins solubilized from the parasporal body by proteolytic alkaline gut juices, the cells swell, become vacuolated and disorganized, and lyse. This cell lysis is though to result primarily from the action of the toxins on membrane phosoholipids.in addition to its activity against midgut cells, the solubilized parasporal body of *B. thuringiensis* subsp.*israelensis* is also toxic and cytolytic to dipteran, lepidopteran, and vertebrate cells. In *B. thuringiensis* subsp.*israelensis* the parasporal body contains a series of proteins ranging from 28 to 230 kilodaltons (KDa). Much of the biological activity of this subspecies appears to be due to the 28- KDa protein which has been reported to be both cytolytic and mosquitocidal. **(El-Kersh *et al.,* 2012)**

2. Material and Methods

(I)Material:

(1) Collection of snails*:*

The adult snails of *M. cartusiana* snails and juveniles were collected from citrus trees in Fakos, El-Salhia, Abohammad, Meniet Elkamh and Belbies districts, Sharkia Governorate. These snails were kept in a muslin bags, transferred to the laboratory and provided with fresh cabbage leaves.

(2) Culture media:

The media used during this study were prepared according to **Gams *et al.*, (1998).**

(II)Methods:

2) Isolation of B. thuringiensis according to **(Padua et al., 1980)**

Approximately 0.5 g of *M. cartusiana* was suspended in 10 ml of sterile distilled water, and the preparations were mixed vigorously by vortexing for 1 min. After mixing, homogenized of *M. cartusiana* was allowed to settle out for 2 min, and then 1 ml of the supernatant was pasteurized at 80°C for 3 min in prewarmed 20-ml glass universal bottles to kill most non-spore-forming organisms. Samples were plated at two concentrations (undiluted and 10 dilution) on

to nutrient agar (Oxoid) containing extra Technical No. 3 agar (Oxoid) so that the final agar concentration was 2%. The plates were incubated at 30°C for 48 h and examined for colonies having *B. thuringiensis* morphology. The numbers of such colonies that grew from any one sample varied from zero to many hundreds, and it was not considered feasible to examine *B. thuringiensis* colony obtained from every sample. Therefore, routinely, a maximum of 30, *B. thuringiensis* colonies from any one sample were selected and subcultured on Sporulation agar following incubation for 48 h at 30°C. The isolates were examined for the presence of parasporal crystal by phase-contrast microscopy. A random selection of isolates classified as *B. thuringiensis* were treated with Sudan Black, a stain that is specific for poly-p-hydroxybutyrate granules (a common storage polymer that is not specific to *B. thuringiensis*, in order to ensure that isolates which produced poly-p-hydroxybutyrate crystals but not γ-endotoxin crystals were unlikely to have been classified as *B. thuringiensis.* If necessary, following the first subculture, isolates were purified by further sub culturing on nutrient agar. To minimize cross-contamination of *B. thuringiensis* from different samples, careful aseptic techniques were used throughout. In addition, the laboratory in which the isolations were performed is located in a separate building that is some distance from other laboratories in which *B. thuringiensis* is routinely handled.

Table (1): Biochemical types of *B. thuringiensis (B.t)* strains. Biotyping according to Martin and Travers, (1989)

Biochemical type (described subsp)	Biochemical and physiological test			
	Hydrolysis of		Utilization of	
	Esculin	Lecithin	Sucrose	Salicin
Thuringiensis	+	+	+	+
Kurstaki	+	+	-	+
Indiana	+	-	+	+
Galleriae	+	-	-	+
Aotto	+	+	+	-
Dendrolimus	+	+	-	-
Morrisori	+	-	+	-
Darmstadiensis	+	-	-	-
Ostriniae	-	-	-	+
Israelensis	-	+	-	-

3) Identification of B.t isolates according to (Lecadet et al, 1999).

5) Identification of B. thuringiensis isolates and causing death of M. cartusiana according to **(Lecadet et al., 1999).**

6) Isolation and solubilization of parasporal bodies isolated from B. thuringiensis:

B. thuringiensis isolate was grown in peptonized milk-yeast extract-glucose-salt (10g of peptonized milk) [BBL Microbiology System Cockeysvile Md]; 10g of glucose [Difeco Laboratory]; 2gm of yeast extract [Difeco]; 0.3 gm of $MgSO_4.7H_2O$ per liter of medium in 2.8 liter Ferbach flasks at 30°C for 48hrs.

The spores, parasporal bodies and cell bodies were harvested by centrifugation (12.000 x g 10min.) and pellet was washed 3 times in cold 0.5 M Na Br. Parasporal bodies isolated in linear discontinuous NaBr (20 to 40%) according to **Ang and Nickerson (1978)** were collected, washed with cold double distilled water and stored at – 20°C for weight determination then parasporal bodies preparations were dried and weighted on microbalance.

The insoluble material was removed by centrifugation (12000xg, 10 min.). The supernatant was brought to a pH of (3.9) with 1N HCl concentrated with ultrafiltration unit with 10000 molecule weight cutoff and stored at -20°C until needed. Protein concentration was determined by methods described by **Lowery *et al.*, (1951)**.

SDS-PAGE and immunoblot. Molecular masses of solubilized proteins and parasporal bodies were determined by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE 12%, 1.5mm) as described by **Laemmli, (1970).**

Proteins were visulized by coomassie staining. For immunoblot analysis, the separate proteins were transferred electrophoretically to nitro cellulose **(Towbin et al., 1979).** The electroblotted protein was probed first with primary antibodies (immunoglobulin IgG) fractions that were raized in rabbits against 25 KDa Protein and 140 KDa proteins of *B. thuringiensis.*

7) The toxicity of purified parasporal bodies against *M. cartusiana* snails:

Freshly isolated parasporal bodies (0.2mg/ml) were solubilized in 50mM Na_2CO_3.HCl (pH 10.5) for 1 h at 37°C.

Plastic boxes (¾kg capacity) were used, each containing ½ kg sterilized clay soil and 10 adult snails of *M. cartusiana.* Water holding capacity of the

soil in boxes was adjusted and ten disces of fresh cabbage leaves were introduced in each box and this was repeated at 3 days intervals during the experiment. Protein concentration of *B. thuringiensis* of each concentration was sprayed on the soil and cabbage disces in the boxes. Three replicates of boxes, each containing ten adult snails of *M. cartusiana* were used for each concentration. All boxes were placed in the incubator at 24°C. Mortality percentages were recorded at intervals of three days for 21 days.

8) Histological Technique: according to (Lee, 1965)

3. Results

1) Isolation and Identification of *B. thuringiensis*:

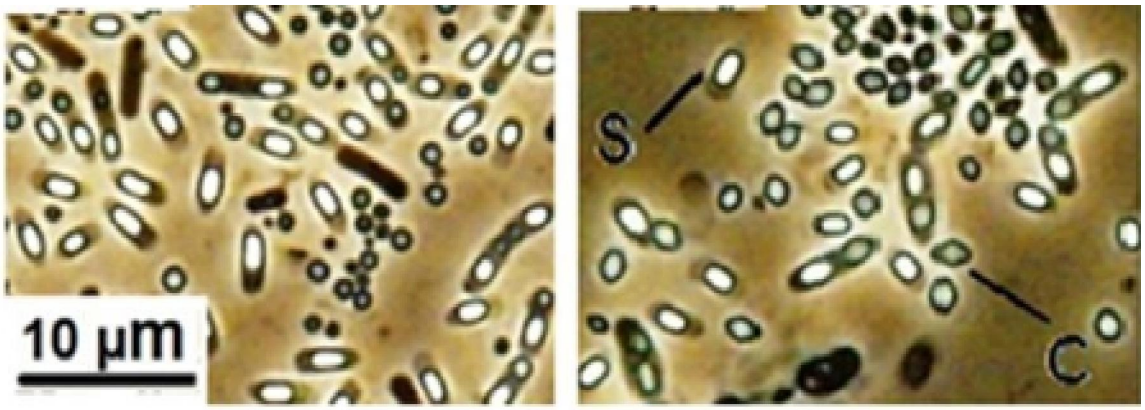
B. thuringiensis on nutrient agar (2%) media produced flat, dry, white colonies with uneven borders.

B. thuringiensis is gram positive soil-dwelling, spore-forming rod shaped bacteria. It produces a diamond shaped crystal from its crystal protein (Cry proteins).

The main criterion for *B. thuringiensis* differentiation from other spore- forming bacteria was crystal production by Sporulation culture.

2) Crystal morphology:

Most strains produce spherical and bipyramidal crystals. Only a low percentage of strain (16%) formed atypical crystals often heterogeneous in size and shape. The protein profiles of spherical and bipyramidal crystals consist of poorly defined component which could be a source of novel insecticidal prosperities (Fig. 1a, b).



a) b)

Figure (1 a, b): Phase contrast micrographs of *Bt* isolates showing spore (S) and crystal shapes ©.

3) Biochemical typing:

Using the biochemical typing method, all the *B. thuringiensis* strain were divided 4 biochemical types. Based on biochemical typing, *B. thuringiensis* Subsp. *Kurstaki* (Es^+ Sa^+, Le^+ and Su^-) of the whole isolates as shown in table (2).

(Es^+ Sa^+, Le^+ and Su^-) of the whole isolates as shown in table (2).

4) Determination of Parasporal bodies Cry- type of local B.t isolates:

Parasporal bodies of *B. thuringiensis* isolate had biological activities when assayed against *M. cartusiana* snails.

The estimated concentration of parasporal bodies killed 50% of *M. cartusiana* snails for *B. thuringiensis* isolates was 3.9 mg/ml.

The protein compositions of the parasporal bodies in *B.t* isolate were 140 kilo Dalton (KDa) as shown in (Fig.2).

140 KDa protein solubilization in Na_2Co_3 - HCl buffer. In the experiment, the soils of *M. cartusiana* apparently healthy were transferred into plastic boxes (¾kg capacity) containing 0.5 kg sterilized clay soil.

Result in table (3) indicated that the highest mortality 100% occurred to *M. cartusiana* by concentration 7.8 mg/ml of parasporal bodies, the second effective concentration was 3.9 mg/ml, while 1.8 was caused mortality *M. cartusiana* snails 10%.

Data presented in table (4) illustrated the efficacy of *B. thuringiensis* isolated on juveniles and adult of *M. cartusiana* snails under laboratory condition. Results revealed that none of the tested *B. thuringiensis* exhibit any bacteriological activity one day post treatment. Mortality percentage increased gradually to reaches in maximum after 21 days where it gave highest effect 100%.

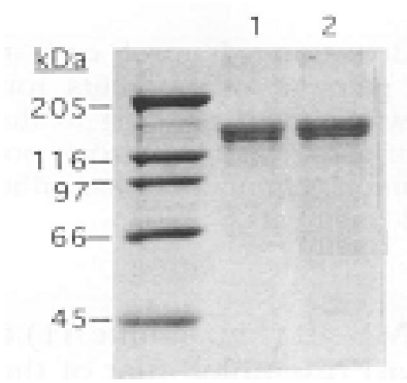


Figure (2): Cry protein profiles of *B.t* isolates on SDS- 7.5% PAGE (lane 1 and 2).

Table (2): *B. thuringensis* isolated from shell, gastropod and juvenile of *M. cartusiana* snail and typing from different districts of Sharkia Governorate

	No.	Isolation			Biochemical typing
		Shell	Gastropod	Juveniles	
Abohammad	2	- ve	+ ve	+ve	*B.thuringiensis Var. Kurstaki*
El-Salhia	3	+ ve	+ ve	- ve	*B.thuringiensis Var. Kurstaki*
Meniet Elkamh	2	+ ve	+ ve	+ve	*B.thuringiensis Var. Kurstaki*
Fakos	1	- ve	+ ve	+ve	*B.thuringiensis Var. Kurstaki*
Belbies	1	- ve	+ ve	+ve	*B.thuringiensis Var. Kurstaki*
Aboukabeer	1	- ve	+ ve	+ve	*B.thuringiensis Var. Kurstaki*

Table (3): Effect of parasporal bodies, (140 KDa) of *B. thuringensis* on mortality of juveniles and adult of *M. cartusiana* snails after 21 days.

Concentration of 140KDa	Juveniles		Adult stage	
	No.	%	No.	%
1.8 mg/ml	1	10	1	10
3.9 mg/ml	6	60	5	50
7.8 mg/ml	10	100	10	100
11.8 mg/ml	10	100	10	100

Table (4): Efficacy of different concentration *in vitro* of *B. thuringiensis* as toxic spray of juveniles and adult stage of *M. cartusiana snails*

Concentration	Reduction percentage juveniles				Reduction percentage adult			
	1 day	7 days	14 days	21 days	1 day	7 days	14 days	21 days
2 x 10^2 c.f.u/ml	0	4	4	10	0	3	20	30
2 x 10^3 c.f.u/ml	0	20	30	100	0	30	50	100
2 x 10^5 c.f.u/ml	0	30	50	100	0	40	60	100

5) Histopathological results:

Histopathological tubule of digestive gland of infected ***M. cartusiana*** showed atrophoid acini with distributed architecture acini and all acini narrow lumen after 48h with treated Bt toxin (fig3).

The morphology of kidney of M. cartusiana revealed it is yellowish white but infected snails, it turns dark yellow or slightly brownish. It lies immediately be health the mental cavity.

Histological, the renal lamellae are formed in rows of well defined renal cells which are characterized by thick wall and large nuclei, (fig. 4).

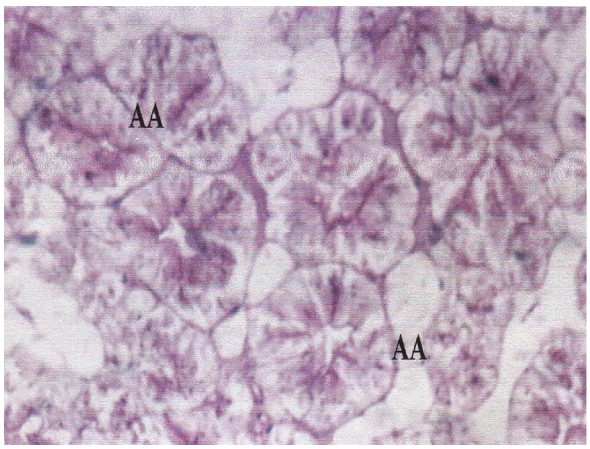


Fig. (3): Photomicrograph through tubules of the digestive gland of M. car snails treated by *B. thuringensis* showing atrophoid acini (AA) with disturbed architecture acini. All acini exhibited narrow lumen post 48h.

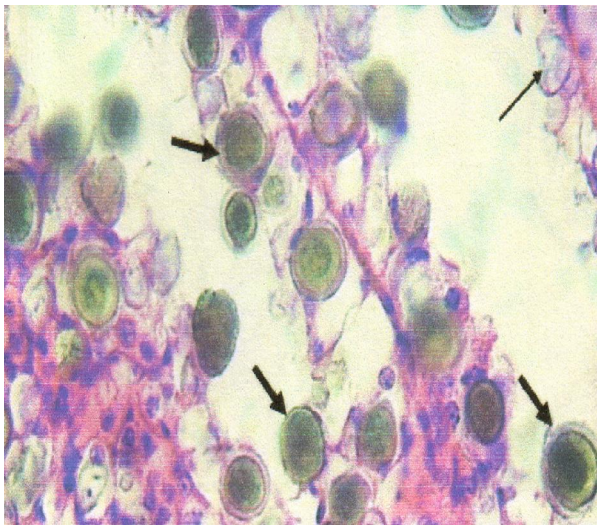


Fig. (4): Revealing magnified renal lamellae with renal cell (small arrows) and wondering brown cells (large arrows). Note these cells are collecting wastes from various body compartments to renal parenchyma (H & E x 150)

4. Discussion

Land snails have become increasingly important as crop pests in agriculture and vector. Land snails that cause plant damage have been the subject of intensive study and control measures.

Bacillus thuringiensis was found effective against land snails *M. cartusiana*. The mortality rate reached 100% according to variety of bacteria and the method of pathogen application and spraying method was find highly effective application.

Isolation and identification of *B. thuringensis* have been found to colonize many different habitats **(Heimpel, 1967; Goldberg and Margalit, 1977 and Meadows *et al.*, 1992)** but its normal habitat is the soil **(Dulmage and Aizawa, 1982).**

On specific media, the putative *B. thuringiensis* isolates produced flat, dry, white colonies with uneven border. The main criterion for *B. thuringiensis* is differentiation from other spore forming bacteria was crystal production by sporulating culture **(Lecadet *et al.*, 1999)** for further confirmation a number of biochemical tests was also used. *B. thuringiensis* biochemically tests could occasionally by observed by **Lecadet *et al.*, (1999).**

Most strains produced a typical crystals after heterogenous in size and shape. Abundance of heterogenous crystal of *B. thuringiensis* strain has already been reported by **Lecadet *et al.*, (1999)** who found more than 50% of *B. thuringiensis* strains produce irregular or heterogenic crystal. The protein profiles of heterogenic crystals consist of many poorly defined components which could be a source of novel insecticidal properties **(Juarez-Oerez *et al., 1994*; Burtseva *et al., 1995* and Chaufaux *et al.*, 1997*).***

Using the biochemical typing method, all B. thuringiensis isolates were typing as *Bacillus Thuringiensis Var. Kurstaki*. This system is based on the biochemical tests which have been identified by **De Barjac, (1981)** and have been used for *B. thuringiensis* classification in many investigations **(Dow and Lone 1999 and Elliot *et al.*, 2002)**.

Using oligoprimers, we identified five profiles of cry-type genes including $cryl_A$, $Cryl_A$ (b) Cryl C, Cryl D9c) and Cryl D type genes from *B. thuringiensis* isolate observed that the distribution on geography. Similar observations were reported by **Martin and Travers (1989) and Ohba and Aizawa, (1986)**.

In our results *B. thuringiensis* isolates containing either Cryl A(a), Cryl A(b) and Cryl (C) gene profile exhibited conferred efficacy against snailcidal agreed with result of **Visser *et al.*, (1990)** were discovered the Cryl (c)-type genes insecticidal activity against spodoptera exigua.

The protein pattern of parasporal bodies of our isolates determined by SDS-PAGE was essentially as reported by **Ibarra and Federici (1986)**. We have demonstrated that snails can be target of single cloned Bt. Cry toxin and the *B.t* toxicity can be studied in *M. cartusiana* snails where the toxin produced in *Bt* isolate ingestion of toxin damaged intestine.

There are scattering of previous studies involving *B.t* toxin and nematodes. In some studies the nematocidal was lepidopteran and dipteran-specific *B.t* strain that potentially express multiple toxins were used **(Ignoffo and Dropking, 1976; Zuckerman *et al.*, 1993 and Borgonie *et al.*, 1996)**.

Another study developed assays for testing potential snailcidal properties of *Bt* snailcidal *Bt* toxin expressed transgenically in appropriate root and stem tissue, might provide an effective strategy to control plant-parasitic, a major class of agriculture pest that cause billion of dollars in crop damage per year in United States alone **(Sasser and Freckman, 1987)**. These results indicate that Cryl A could be useful for managing *Monacha cartusiana* snails.

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