Bio-insecticidal Activity of *Bacillus thuringiensis* Isolates on the Tomato Borer, *Tuta absoluta* (Meyrick) and their Molecular Identification

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ABSTRACT

Twelve bacterial isolates were isolated from dead larvae of *Tuta absoluta* (4th instar) infested tomato cultivated fields at Fayoum Governorate, Egypt. All isolates were preliminarily identified as members of the genus *Bacillus*, based on their morphological and biochemical characteristics. According to the results of the pathogenicity of *Bacillus* isolates against different larval instars of *T. absoluta*, the 12 isolates revealed varying efficiencies and the isolates B1, B_2, B_3 and B_4 showed high mortality rates of 93.3, 90, 86.7 and 80% on day7, respectively on the 4th instar larvae. Also, protecto (*Bacillus thuringiensis* subsp. *kurstaki*) recorded high mortality rate, when the 4th instar larvae were treated by 2 gm /2 liter of water (96.7% on 5th day post treatment). Isolate B_{12} recorded the lowest percentage of mortality (13.3%) of the 4th instar larvae. In addition, there was a significant decrease in egg hatching percentage reached 44.12 %, compared with the control. Meanwhile, adult emergence was decreased after treatment when only 38 and 30 adults emerged from the cages contained tomato plants infested by eggs with B_1 and protecto as compared with the control (253 adults). Further genetic identification of 12 isolates was performed using randomly amplified poylmorphic DNA (RAPD) markers to determine their genetic diversity pattern. Different random primers were used for RAPD amplification, which generated a total of 52 fragments; of these 42 were polymorphic and 10 monomorphic. The primers OPA02, OPA04, and OPA07 produced 100% polymorphic fragments, whereas primers OPA1, OPA3, OPA05, OPA06, OPA08 and OPA09 produced 1, 3, 1, 2, 1 and 2 monomorphic fragments, respectively. When the RAPD banding pattern data was subjected to dendrogram construction, the 4 isolates fell into two separate clusters, cluster I and cluster II, which includes 1 and 3 B. *thuringiensis* isolates, respectively. The RAPD technique was shown to be effective in differentiating closely related isolates and applied to confirm the identification of *Bacillus* isolates by API system to reveal the phylogenetic relationships among the isolates.

Key words: *Bacillus thuringiensis*, bioinsecticide, RAPD-PCR-, *Tuta absoluta*, tomato, Egypt.

INTRODUCTION

The tomato borer *Tuta absoluta* (Meyriek) (Lepidoptera: Gelechiidae) is one of the most important lepidopteran pests associated with tomato plants. It has a high reproductive potential, a single female can lay a total of about 260-300 eggs during its lifetime on aerial parts of its host plants and it has 10-12 generations per year (EPPO, 2005). *T. absoluta* is considered as a key pest of tomato both in the field and under protected conditions. Both yield and fruit quality is significantly reduced by direct feeding of the pest and invasion of secondary pathogens which may then enter through the wounds made by the pest. Severely attacked tomato fruits lose their commercial value. 60–100% losses have been reported on tomato (Cristina *et al.*, 2008).

T. absoluta larvae attack tomato plants during all growth stages, producing large galleries in leaves, burrowing stalks, and apical buds, green and ripe fruits (IAN, 1994). They feed on the mesophyell tissues and make irregular mines on leaf surface. Damage may reach 100%. Continuous use of chemical insecticides in agriculture, forestry and horticultural crop plants, has led to deleterious effects on the environment. Pest species have developed resistance to insecticides. Resistance to some insecticides has been reported in several countries, for example to abamectin, cartap and permethrin in Brazil (Siqueira *et al.*, 2000). *Bacillus thuringiensis* (*Bt*), an entomopathogenic bacterium, has also been used for the control of tomato plant pests (Marques and Alves, 1996). Biopesticides based on *B. thuringiensis* are used as an alternative strategy to control pests. *Bt* is a rod-shaped, gram-positive, endospore-forming bacterium, characterized by its ability to synthesize delta endotoxins as protein inclusion crystals (or Cry proteins) during sporulation. *B. thuringiensis* isolates are distinguished than other closely related *Bacillus* spp. such as; *B. cereus, B. anthracis* and *B. mycoides* that show toxicity against certain insect orders and mammals (Hofte and Whiteley 1989).

Native *B. thuringiensis* isolates were subjected to randomly amplified polymorphic DNA (RAPD) marker-based analysis for characterization of their genetic diversity. Various techniques, rely on different nucleic acid pattern and discriminate at genetic level have been developed to gain information about the genetic diversity and genetic relationship between different organisms (Caetano-Anolles *et al.*, 1991 and Sikora *et al.*, 1997). The RAPD marker based analysis was found to be an easy, quick and reliable technique to assess the

diversity of different types of organisms (Welsh and McClelland, 1990 and Williams *et al.*, 1990) and this technology was successfully applied to characterize the genetic diversity for various *B. thuringiensis* isolates (Brousseau *et al.*, 1993). RAPD analysis revealed that DNA characteristic fingerprints of different bacterial strains have been generated and even individual strains within the same serotype could be distinguished (Hansen *et al.*, 1998).

The objective of this study was to evaluate the efficacy of some isolates of *B. thuringiensis* and the most commonly used *B. thuringiensis* based formulates to control *T. absoluta* under laboratory conditions. In addition, RAPD-PCR technique was applied to confirm the identification of some *Bacillus* isolates by API system and to study the phylogenetic relationships among isolates.

MATERIALS AND METHODS

Insect rearing

The colony of *T. absoluta,* used in the laboratory assays, was established from larvae and pupae collected from infested tomato fields at Fayoum Governorate, Egypt. Larvae were reared on tomato plants in a climatic chamber at 27±2°C and 55% RH. Tomato plants were placed in the chamber weekly for feeding and egg lying. Adults were collected using a mechanical aspirator.

Isolation of *Bacillus thuringiensis* from dead larvae

Dead larvae of *T. absoluta* (4th instar) were collected from infested tomato fruits, at tomato farm, Fayoum Governorate, Egypt. Each dead larva was picked using sterile forceps and placed in a sterile plastic screw-top bottle and crushed in sterile crucible and added to a tube containing 9 Ml of sterile phosphate buffered saline (PBS). After completed homogenization, 1 mL aliquot was taken and heated at 80°C for 15 min in a pre-warmed 6 mL glass test tube to kill or inactivate all the vegetative forms, then spam for 5 min at 8000 rpm. The heat shocked aliquots were serially diluted to 10^{-7} and plated on nutrient agar then incubated overnight at 30°C. *Bacillus*-like colonies were randomly picked, sub cultured on nutrient agar and maintained for further investigation.

Morphological identification of the isolates

After incubation period, cells from *Bacillus* colonies were randomly selected and vegetative cell morphology observation were examined at 1000X magnification by phase contrast microscopy for shape of cells, presence of chains, spore formation, reaction with gram stain. Motility of *B. thuringiensis* isolates were tested by the growth pattern on nutrient agar plates. The isolates were streak-inoculated onto the middle of the agar plate from top to bottom and incubated overnight at 30°C. If a colony spread out from the inoculation site, the strain was scored as motile; otherwise it was scored as non-motile (Frederiksen *et al.*, 2006).

Biochemical identification of the isolates

Bacillus isolates were tested by API 50CH and API 20E systems (BioMerieux, Marcyle Etoile, France) according to the manufacturer instructions. *Bacillus* isolates were divided into biochemical types based on hydrolysis of esculin, urea or lecithin, and acid production from sucrose, or salicin. Lecithinase activity of *Bacillus* isolates was tested on nutrient agar containing 10% egg yolk and then incubated at 37°C overnight (Aramideh *et al.*, 2010). Identification according to the biochemical tests was based on comparison with the test results by dichotomous keys. API kits were used according to manufacturer's instructions and identification was done with API-web program.

Commercial products

The commercial bioinsecticide, protecto (*Bacillus thuringiensis* subsp. *kurstaki)* was selected for the assays. Toxicity of protecto, as a wettable powder, was evaluated on different larval instars (1st, 2nd, 3th and 4th) of *T. absoluta*. Protecto was obtained from the Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt. Serial dilution of protecto was prepared, using 1, 1.5 and 2 gram of the wettable powder and dissolved in 200ml of water.

Toxicity assay

Twelve treatments, each with three replicates of ten larvae were used. In order to assess the toxicity of *Bacillus* isolates against the 4th instar larvae of *T. absoluta*, a bacterial spore suspension of $8.9x10^9$ spore mL^{-1} was used according to Amin *et al.* (2008). Distilled water containing 0.05% Tweeen 40 was used as a control. Tomato leaf discs (2 cm diameter) were dipped into each suspension, and allowed to dry at room temperature. After evaporation of the excess water, the leaf discs were placed in Petri plates (6.5 cm x 2.5 cm) lined with filter paper. *T. absoluta* larvae were then placed on the leaf discs. Leaf discs of the same plants dipped into water were used as control. The treated discs were used only once at the beginning of the bioassay. After 24 hr., the larvae were fed on untreated leaves. The larval mortality was evaluated daily for 7 days to reveal variations on the efficacies of different isolates at 30 ± 1°C.

Efficacy of *Bacillus* isolates and commercial bioinsecticide

On several *T. absoluta* instars in laboratory assay

Four groups (different larval instars of 1st, 2nd, 3th and 4th) of three replicates with ten larvae were used.

Larvae were fed on tomato leaf discs (2 cm diameter), were dipped into each suspension (8.9 x 10^9 spore ml^{-1} of *Bacillus* isolates) of the highest four isolates mortality. The other group was sprayed by water and used as control. The larval mortality was evaluated at 4, 5 and 7 days for 1st, 2nd, 3th and 4th instars, respectively. While the alive ones were followed until the appearance of the next generation.

The commercial biocide formulation, protecto was used in the treatments, at three concentrations of 1.0, 1.5 and 2g/liter of water. The leaf was dipped in suspension of protecto and then offered to (different larval instars of 1st, 2nd, 3th and 4th of *T. absoluta*). Three treatments, each with three replicates of ten larvae, were tested. In addition, the untreated larvae were fed on tomato leaf dipped in water as a control. Larval mortalities were recorded daily for 5 days. Larvae survived after the 5th day of treatment were observed until adult stage.

Laboratory assay of *T. absoluta* eggs

T. absoluta adults emerged from the larvae survived after treatment with different isolates of the *Bt* (B_1, B_2, B_3 and B_4) and the biocide, protecto to the different larval instars (1st, 2nd, 3th and 4th of *T. absoluta*) were observed. Eighteen tomato plants (approximately 30 cm in height) were placed singly inside screened cages (30 x 30 x 45 cm). The plants were randomly grouped into six groups of three plants each. Four groups were sprayed by 8.9 x10^9 spore/ ml of *Bt* of highest isolation mortality (B_1, B_2, B_3 and B_4); the 5th group was sprayed by 1.5 gm /2 liter of water (approximately 25 ml per plant) of the commercial biocide protecto. Biocide was applied using a tigger operated hand sprayer. Sixth group was used as a control and was sprayed by water. The plants were air dried and replaced in the same cages and each was infested by placing three *T. absoluta* couples (resulted as delayed treatment) inside each cage for 4 days. Then, *T. absoluta* adults were removed and number of laid eggs on the plants was counted. Remained adults that emerged in the cages at the end of the experiment were also recorded.

Statistical analysis

Analysis of variances of obtained data was computed using the General Linear Model (GLM) procedure according to SPSS, 17.0 (2008). Significant differences among means were evaluated using Duncan's multiple range test (Duncan, 1955).

Isolation of bacterial genomic DNA

Isolation of genomic DNA was carried out by the standard protocol (Hoffman and Winston, 1987). Single colony was inoculated in nutrient broth and grown for overnight at 30°C. Cells were harvested from 5 mL of the culture and to this 100µL of lysozyme was added and incubated at RT for 30 min, followed by the addition of 700 µL of cell lysis buffer (6.06 g/L Tris, pH, 7.5;7.44g/L EDTA with 200 ml of a 10% SDS).The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. 700 µL of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA occurred. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50µL of 1X TE buffer. Quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 µg/µL).

Amplified polymorphic DNA (RAPD) analysis

A total of nine random primers from OPA Kit A (Operon Technologies Inc, Alameda, Calif., USA) was used (Table 1). PCR was carried out in a reduced volume of a 25 µl reaction mixture containing 1X PCR buffer (10 mM Tris HCl (pH 8); 50 mM KCl, 3.5 mM $MgCl_2$, 0.3mM (dNTP), 2 µM primer, 1 U of Taq DNA polymerase and 2 µl genomic DNA. The amplification started with an initial step of 40°C for 1 min and 72°C for 1min and final extension 72°C for 10 min. PCR products were analyzed in 1.5% agarose gel in Tris Borate EDTA (TBE) with ethidium bromide and compared with the molecular markers (lkb DNA ladder) and visualized under UV light.

Table (1): Nucleotide sequence of random primers used for RAPD analysis of *B. thuringiensis* isolates

No.	Primer name	Primer sequence	G+C %
1	OPA01	5′ - CAGGCCCTTC-3′	70
2	OPA02	5′ -TGCCGAGCTG-3′	70
3	OPA03	5′ -AGTCAGCCAC-3′	60
4	OPA04	5′ -AATCGGGCTC-3′	60
5	OPA05	5′ -AGGGGTCTTG-3′	60
6	OPA06	5′ -GCTCCCTGAC-3′	70
7	OPA07	5′ -GAAACGGGTG-3′	60
8	OPA08	5′ -GTGACGTAGG-3′	60
9	OPA09	5′ -GGGTAACGCC-3′	70

RAPD data analysis

Obtained data from amplification products by primers were used to estimate genetic similarity among different isolates on the basis of shared amplification products using RAPD distance software package, version 1.4 (Armstrong *et al.*, 1994). Patterns, on the basis of presence (1) or absence (0) of bands for each of the primer were recorded. Pair-wise comparisons of the strains, based on the presence or absence of unique and shared bands, were used to generate similarity coefficients (Excoffier *et al.*, 1992) .The strains were then clustered using the unweight pair-group method with arithmetic average (UPGMA). A dendrogram was generated from the similarity data following the method of (Sokal and Sneath, 1963).

RESULTS AND DISCUSSION

Morphological and biochemical characterization of isolates

Twelve *Bt* isolates were isolated from dead *T. absoluta* larvae. The different *B. thuringiensis* isolates (shape, gram staining and the presence of spores) were confirmed on the basis of the method described by Travers *et al.* (1987). All isolates were rod-shaped, gram-positive, and spores were seen inside the spored bacterium. Obtained data of sugar utilization using the API CH50 system for each of the twelve *Bacillus* isolates were processed by the provided kit's software. The software results revealed that all the tested isolates were identified by a possibility of over > 90% as *B. thuringiensis*. Furthermore, each of the 12 *Bacillus* isolates was also examined by API E20 system for relevant biochemical reactions to shed more light on the phenotypic characteristics and to help determining the possible biochemical types. The results obtained by API CH50 and API E20 biochemical systems revealed that some biochemical reactions (BRs) were found to be positive in general with production of gelatinase and assimilation of fructose, glycogen, trehalose, ribose, and N-acetyle- glucose-amine. Contrastingly, ONPG test, H_2S production, and assimilation of xylose, fucose, lactose, and galactose were negative in general with all *Bacillus* isolates (Table. 2).

Table (2): Morphological and physio-biochemical characteristics of *Bacillus* isolates

Characteristics	Observation
Morphological colony	Cream, large and spreading
Gram's stain	Gram positive
Cell shape	Rods
Motility	+
Catalase reaction	+
Oxidase	-
Indol	-
Methyl red	-
Starch hydrolysis	+
Tween 80	-
Citrate utilization	+
Casine utilization	+
Glucose	+
Galactose	+
Arabinose	+
Maltose	+
Xylose	+
Fructose	+
Sucrose	+
Mannose	+
Raffinose	-

+ positive - negative

Toxicity

Results presented in table (3) showed cumulative larval mortality percentages due to the treatment of the 4th instar larvae of *T. absoluta* with $8.9x10^9$ spore's m^{-1} of different isolates. Highest mortality was obtained when the 4th instar larvae were treated by B_1, B_2, $B_3$and B_4 (93.3, 90, 86.7 and 80%) on day 7, respectively, while the lowest mortality was obtained by B_{12} (13.3%).

Efficacy of *Bacillus* isolates and protecto on larval instars of *T. absoluta*

As shown in table (4), cumulative mortality percentages of *T. absoluta* were recorded daily. Highest mortality percentages were found for isolates $B_1$and B_2, with a gradual significant increase over the inspection period with different larval instars. The first instar larvae had percentage mortalities of 46.6, 33.3, 26.7 and 13.3% on the 4th day, due to feeding larvae on tomato treated leaves with different isolates of B_1, B_2, B_3 and B_4, respectively. The second larval instars recorded 60, 53.3, 46.7 and 30% mortality on the 5th day after the four treatments, respectively. The mortality was significantly increased as compared to the 3rd and 4th instars, being 80, 76.7, 66.7 and 63.3% in the 3rd and 93.3, 90, 86.7 and 80% in the 4th instars; 7 days post treatment with the isolates of B_1, B_2, B_3 and B_4, respectively.

Obtained results revealed high significant mortality rates of *T. absoluta* for the 1st, 2nd, 3rd and 4th larval instars to *Bt.* toxin expressed by *Bacillus* species. Applied *Bacillus* survived for a period of 45 days on the tomato leaf surface (Theoduloz *et al.*, 2003). *B. thuringiensis* proved to be highly significantly efficient in reducing the damage produced by 1st, 2nd, 3rd and 4th *T. absoluta* larval instars. However, there were differences in the mortality shown by each larval stage. Fourth instar larvae recorded the highest mortality rate, while mortality was lower in 1st and 2nd instar larvae. Several *T. absoluta* instars were found to be susceptible to *B. thuringiensis*, though to a different extent (Giustolin *et al.*, 2001). The higher mortality of the later instars than the first ones could be due to feeding behavior differences. Normally, 1st and 2nd larval instars penetrated directly the leaves without much feeding and were therefore exposed to a lower dose of bacterial spores and toxins. Older instars were more susceptible to treatments than younger ones, as a result of their longer stadia, beside their more sensitive integument and internal organs before and at the time of mortality. Larvae moved in and out of the mines and galleries several times during their development and at that moment they ingest the bacteria (Harizanova *et al.*, 2009). On the other hand, larvae of 1st and 2nd instars remained in the leaves. When they reached to later instars there

Table (3): Cumulative mortality % of 4th instar *Tuta absoluta* larvae recorded at different periods after treatment with *Bacillus* isolates

Bacillus isolate	Mortality % (Mean± S.E.)						
	Period after treatment (days)						
	1	2	3	4	5	6	7
B_1	33.33±6.67 ^{NOPQ}	53.33±6.67 ^{IJK}	66.67±8.92 ^{FGHI}	76.67±3.33 ^{CDEF}	80.00±5.77 ^{BCDE}	86.67±3.33 ^{ABC}	93.33 ±3.33 ^{A}
B_2	26.67±3.33 ^{PQRS}	43.33±3.33 ^{KLMN}	50.00±5.77 ^{JKL}	70.00±5.77 ^{EFG}	73.3±5.77 ^{DEFG}	83.33±3.33 ^{ABCD}	90.00±3.33 ^{AB}
B_3	20.00±5.77 ^{RSTU}	33.33±3.33 ^{NOPQ}	46.67±3.33 ^{JKLM}	56.67±3.33 ^{HIJ}	70.00±0.00 ^{EFG}	76.67±3.33 ^{CDEF}	86.67±3.33 ^{ABC}
B_4	10.00±5.77 ^{UVWX}	20.00±5.77 ^{RSTU}	33.33±6.67 ^{NOPQ}	43.33±3.3 ^{KLMN}	63.33±6.67 ^{GHIJ}	70.00±5.77 ^{EFG}	80.00±0.00 ^{BCDE}
B_5	10.00±5.77 ^{UVWX}	16.67±3.33 ^{STUV}	23.33±3.33 ^{QRST}	33.33±3.33 ^{NOPQ}	40.00±5.77 ^{LMNO}	53.33±3.3 ^{IJK}	70.00±0.00 ^{EFG}
B_6	0.00±0.00 ^{X}	3.33±3.33 ^{WX}	13.33±3.33 ^{TUVW}	26.67±3.33 ^{PQRS}	33.33±3.33 ^{NOPQ}	36.67±3.33 ^{MNOP}	56.67±3.33 ^{HIJ}
B_7	0.00±0.00 ^{X}	0.00±0.00 ^{X}	10.00±0.00 ^{UVWX}	13.33±3.33 ^{TUVW}	23.33±3.33 ^{QRST}	30.00±0.00 ^{OPQR}	43.33±3.33 ^{KLMN}
B_8	0.00±0.00 ^{X}	3.33±3.33 ^{WX}	6.67±3.33 ^{VWX}	10.00±0.00 ^{UVWX}	13.33±3.33 ^{TUVX}	16.67±3.33 ^{STUV}	26.67±3.33 ^{PQRS}
B_9	0.00±0.00 ^{X}	0.00±0.00 ^{X}	6.67±3.33 ^{VWX}	6.67±3.33 ^{VWX}	10.00±0.00 ^{UVWX}	16.67±3.33 ^{STUV}	23.33±6.67 ^{QRST}
B_{10}	0.00±0.00 ^{X}	0.00±0.00 ^{X}	3.33±3.33 ^{WX}	6.67±3.33 ^{VWX}	13.33±3.33 ^{TUVW}	13.33 ±3.33 ^{TUVW}	20.00±0.00 ^{RSTU}
B_{11}	0.00±0.00 ^{X}	0.00±0.00 ^{X}	3.33±3.33 ^{WX}	6.67±3.33 ^{VWX}	6.67±3.33 ^{VWX}	10.00±0.00 ^{UVWX}	16.67±3.33 ^{STUV}
B_{12}	0.00±0.00 ^{X}	0.00±0.00 ^{X}	3.33±3.33 ^{WX}	3.33±3.33 ^{WX}	6.67±3.33 ^{VWX}	10.00±0.00 ^{UVWX}	13.33±3.33 ^{TUVW}

A to X means highly significantly different (P≤ 0.001)

Table (4): Cumulative mortality % of *Tuta absoluta* larvae when fed on tomato leaves treated with different *B. thuringiensis*

Age of instars	Post treatment (day)	Mortality % (Mean± S.E.)				
		Treatments				
		Isolates of *Bacillus thuringiensis*				
		B_1	B_2	B_3	B_4	Control
1	1	3.33±3.33 ^{U}	0.00±0.00 ^{U}	0.00 ±0.00 ^{U}	0.00 ±0.00 ^{U}	0.00 ±0.00 ^{U}
	2	23.33±3.33 ^{RST}	13.33±3.33 ^{STU}	10.00±0.00 ^{STU}	3.33±3.33 ^{U}	0.00 ±0.00 ^{U}
	3	40.00±5.78 ^{MOPQ}	26.67 ±3.33 ^{QRS}	20.00 ±0.00 ^{RST}	10.00±5.78 ^{STU}	3.33 ±3.33 ^{U}
	4	46.00±3.33 ^{LMNO}	33.33±3.33 ^{OPQR}	26.67±3.34 ^{QRS}	13.33±3.33 ^{STU}	3.33 ±3.33 ^{U}
2	1	13.33±3.33 ^{STU}	10.00±5.78 ^{STU}	3.33±3.33 ^{U}	0.00 ±0.00 ^{U}	0.00 ±0.00 ^{U}
	2	30.00±5.78 ^{PQR}	26.67±3.33 ^{QRS}	20.00±5.78 ^{RST}	10.00±0.00 ^{STU}	0.00 ±0.00 ^{U}
	3	43.33±3.33 ^{MNOP}	33.33±6.67 ^{OPQR}	30.00±5.78 ^{PQR}	13.33±3.33 ^{STU}	0.00 ±0.00 ^{U}
	4	50.00±5.78 ^{KLMN}	40.00±5.78 ^{NOPQ}	33.33±3.33 ^{OPQR}	20.00±0.00 ^{RST}	0.00 ±0.00 ^{U}
	5	60.00±10.01 ^{GHIJK}	53.33±6.67 ^{IJKLM}	46.00±6.67 ^{LMNO}	30.00±5.78 ^{QR}	0.00 ±0.00 ^{U}
3	1	30.00±5.78 ^{PQR}	20.00±5.78 ^{RST}	13.33±6.67 ^{STU}	3.33±3.33 ^{U}	0.00 ±0.00 ^{U}
	2	46.00±3.33 ^{LMNO}	33.33±6.67 ^{OPQR}	23.33±6.67 ^{RST}	13.33±3.33 ^{STU}	0.00 ±0.00 ^{U}
	3	53.33±3.33 ^{IJKLM}	46.67±3.33 ^{LMNO}	43.33±3.33 ^{MNOP}	30.00±5.78 ^{PQR}	0.00 ±0.00 ^{U}
	4	60.00±5.78 ^{GHIJK}	53.33±3.33 ^{IJKLM}	50.00±5.78 ^{KLMN}	40.00±5.78 ^{NOPQ}	0.00 ±0.00 ^{U}
	5	66.67±6.67 ^{EFGHI}	60.00±5.78 ^{GHIJK}	53.33±6.67 ^{IJKLM}	43.33±3.33 ^{MNOP}	3.33 ±3.33 ^{U}
	6	73.33±3.34 ^{CDEFG}	70.00±0.00 ^{DEFGH}	66.70±0.00 ^{DEFGH}	50.00±5.78 ^{KLMN}	3.33 ±3.33 ^{U}
	7	80.00±5.78 ^{ABCDE}	76.67±3.33 ^{BCDEF}	66.70±0.00 ^{DEFGH}	63.33±3.33 ^{FGHIJ}	3.33 ±3.33 ^{U}
4	1	33.33±6.67 ^{OPQR}	26.67±3.33 ^{QRS}	20.00±5.78 ^{RST}	10.00±5.78 ^{STU}	0.00 ±0.00 ^{U}
	2	53.33±6.67 ^{IJKLM}	43.33±3.33 ^{MNOP}	33.33±3.33 ^{OPQR}	20.00±5.78 ^{RST}	0.00 ±0.00 ^{U}
	3	66.67±8.83 ^{EFGHI}	50.00±5.78 ^{KLMN}	46.67±3.33 ^{LMNO}	33.33±6.67 ^{OPQR}	3.33 ±3.33 ^{U}
	4	76.67±3.33 ^{BCDEF}	70.00±5.78 ^{DEFGH}	56.67±3.33 ^{HIJKL}	43.33±3.33 ^{MNOP}	3.33 ±3.33 ^{U}
	5	80.00±5.78 ^{ABCDE}	73.33±3.33 ^{CDEFG}	70.00±0.00 ^{DEFGH}	63.33±6.67 ^{FGHIJ}	3.33 ±3.33 ^{U}
	6	86.67±3.33 ^{ABC}	83.33±3.33 ^{ABCD}	76.67±3.33 ^{BCDEF}	70.00±5.78 ^{DEFGH}	3.33 ±3.33 ^{U}
	7	93.33±3.33 ^{A}	90.00±0.00 ^{AB}	86.67±3.33 ^{ABC}	80.00±0.00 ^{0ABCD}	3.33 ±3.33 ^{U}

A to U means highly significantly different (P≤ 0.001)

Table(5): Cumulative mortality percentages of *Tuta absoluta* larvae when fed on tomato leaves treated with bacterial biocide (Protecto) *B. thuringiensis* subsp. *Kurstaki*

Age of instar	Post Treatment (day)	Mortality % (Mean ± SE) Treatments bacterial biocide (Protecto concentration) g/2L 1	1.5	2	Control
1	1	20.00 ± 5.77^{O}	26.67 ± 3.33^{NO}	53.33 ± 3.33^{KL}	0.00 ± 0.00^{P}
	2	33.33 ± 6.67^{MN}	43.33 ± 3.33^{LM}	70.00 ± 0.00^{GHI}	0.00 ± 0.00^{P}
	3	56.67 ± 8.82^{JK}	66.67 ± 3.33^{HIF}	76.67 ± 3.33^{EFJH}	3.33 ± 3.33^{P}
	4	70.00 ± 5.77^{GHI}	80.00 ± 0.33^{DEFG}	90.00 ± 0.00^{ABCD}	6.670 ± 3.33^{P}
	5	73.33 ± 3.33^{FGH}	83.33 ± 3.33^{CDEF}	96.67 ± 3.33^{AB}	3.30 ± 3.33^{P}
2	1	26.67 ± 3.33^{NO}	33.33 ± 6.67^{MN}	73.33 ± 3.33^{FGH}	0.00 ± 0.00^{P}
	2	43.33 ± 3.33^{LM}	73.33 ± 6.67^{FGH}	76.67 ± 3.33^{EFGH}	0.00 ± 0.00^{P}
	3	66.67 ± 3.33^{HIF}	86.67 ± 6.67^{BCDE}	90.00 ± 0.00^{ABCD}	0.00 ± 0.00^{P}
	4	76.67 ± 3.33^{EFGH}	93.33 ± 3.33^{ABC}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}
	5	80.00 ± 0.00^{DEFG}	96.67 ± 3.33^{AB}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}
3	1	40.00 ± 5.77^{M}	53.33 ± 8.82^{KL}	83.33 ± 3.33^{CDEF}	0.00 ± 0.00^{P}
	2	60.00 ± 5.77^{HIF}	83.33 ± 8.82^{CDEF}	96.67 ± 3.33^{AB}	0.00 ± 0.00^{P}
	3	73.33 ± 3.33^{FGH}	90.00 ± 5.77^{ABCD}	100.00 ± 0.00^{A}	3.30 ± 0.00^{P}
	4	83.33 ± 3.33^{CDEF}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	3.30 ± 0.00^{P}
	5	90.00 ± 5.77^{ABCD}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	3.30 ± 0.00^{P}
4	1	66.70 ± 6.67^{HIJ}	83.33 ± 6.67^{CDEF}	93.33 ± 3.33^{ABC}	0.00 ± 0.00^{P}
	2	76.67 ± 3.33^{EFJH}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}
	3	83.33 ± 3.33^{CDEF}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}
	4	96.6 ± 3.337^{AB}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}
	5	96.67 ± 3.33^{AB}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	0.00 ± 0.00^{P}

A to P means highly significantly different ($P \leq 0.001$)

was more competition for food, and the larvae needed to spread over the tomato plants. Giustolin *et al.* (2001) reported that the progressive increase of mortality of older larvae that were fed on *Bt* treated leaves probably occurred due to the increasing period of time that larvae were exposed. Also, for the later instar larvae, high mortality was probably due to great leaf consumption, since this instar consumed the entire treated leaf disc, consequently ingested a higher dose of the pathogen and its toxin.

Table (5) presents the effect of protecto at different concentrations (1, 1.5 and 2 g /2 liter of water) on the mortality of different *T. absoluta* larval instars fed on treated tomato leaves. Within larval instars of *T. absoluta* (1st, 2nd, 3rd and 4th), mortality progressively increased as the concentration of protecto increased. On the other hand, the late instars suffered from higher mortality compared to the early instars. The potential of protecto for controlling pests of economic importance is well known as a key part of Integrated Pest Management Programs (Roh *et al.* 2007). These results are in agreement with those obtained by Cabello *et al.* (2009) who reported that the effect of *B. thuringiensis* subsp. *kurstaki* on all larval instars have exhibited satisfactory efficacy against *T. absoluta* larval infestations.

Protecto, tested in laboratory bioassays showed a highly significant efficacy in reducing the damage caused by different larval instars (1st, 2nd, 3rd and 4th) of *T. absoluta* at different concentrations, compared to the control. Generally, obtained results showed that there was a delay in killing effect due to latent period of protecto. Treated larvae were weakened, as a result of the action of the entomopathogens used (Abd El-Kareem *et al.* 2010).

Based on the data in table (6), there was a significance decrease in total number of eggs and adult emergence to each bacterial isolate and protecto. The lowest number of eggs and adults was reduced after protecto treatment (106 no. of egg/ female and 30 emerged adults) and B_1 (130 no. of egg/ female and 38 emerged adults). Results agree with that of (Alwan *et al.* 2012) who reported a significant decrease in the percentage of egg hatching for *T. absoluta* treated with *Bt* filtrate (33.36%), compared with the control (86.74%). Use of biocide and bacterial isolates interfered with egg formation or development and consequently led to a reduction in the number of laid eggs. This phenomenon reveals that some larvae could accept slight infection and need a longer period to affect gut cells and appearance of infection symptoms, where the symptoms could appear lately on the larvae progressive stages (pupae and adults).

Random amplified polymorphic DNA (RAPD)

Nine random primers were used to identify the four isolates of *B. thuringiensis*. These primers generated reproducible and easily securable

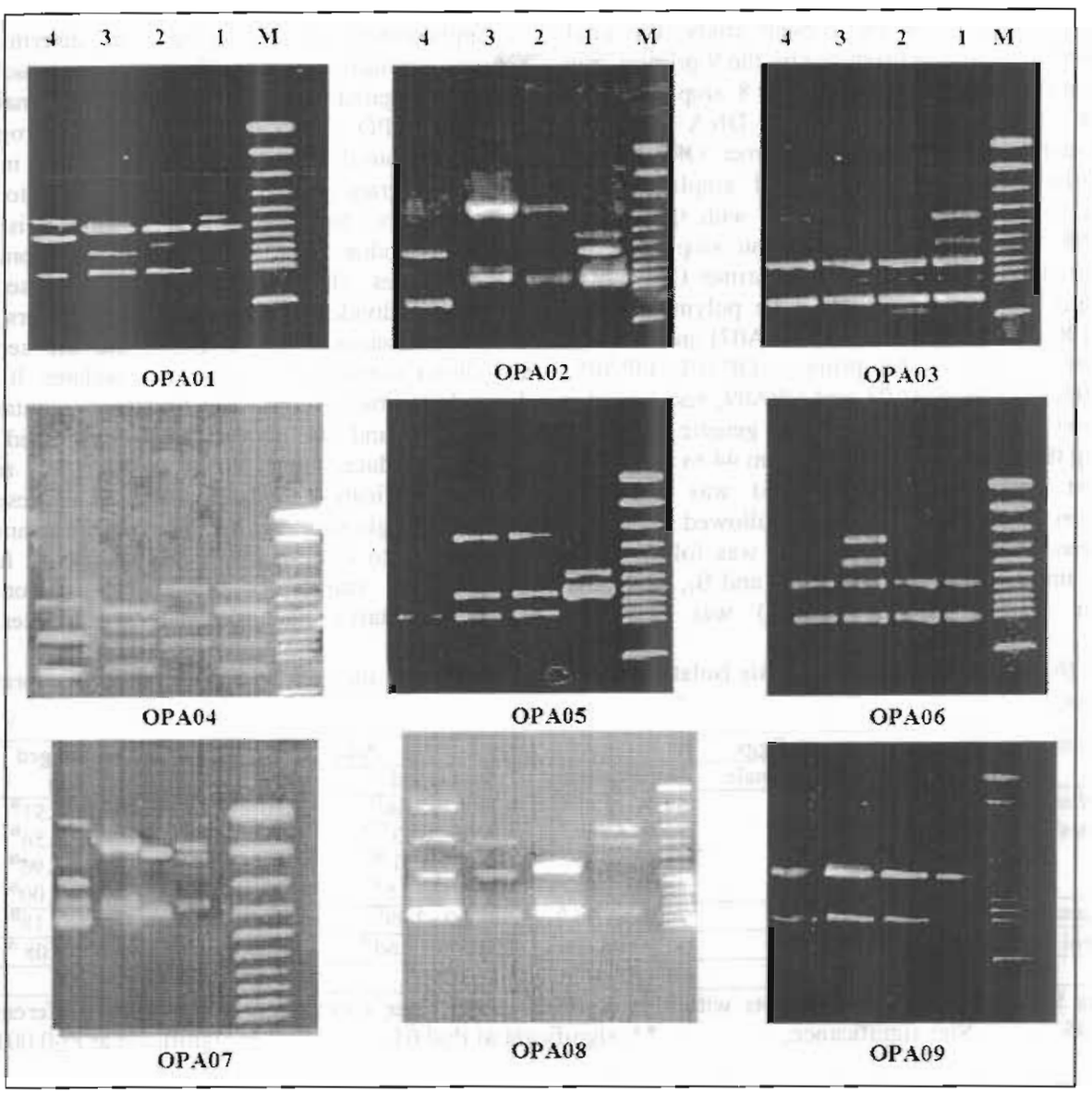

Fig. (1): RAPD profiles of 4 *B. thuringiensis* isolates amplified with RAPD primers (OPA01, OPA02. OPA03, OPA04, OPA05, OPA06, OPA07. OPA08 and OPA09. M: molecular weight marker (1 kb DNA ladder) lanes from 1 to 4 represent: isolates B_1, B_2, B_3, and B4 respectively.

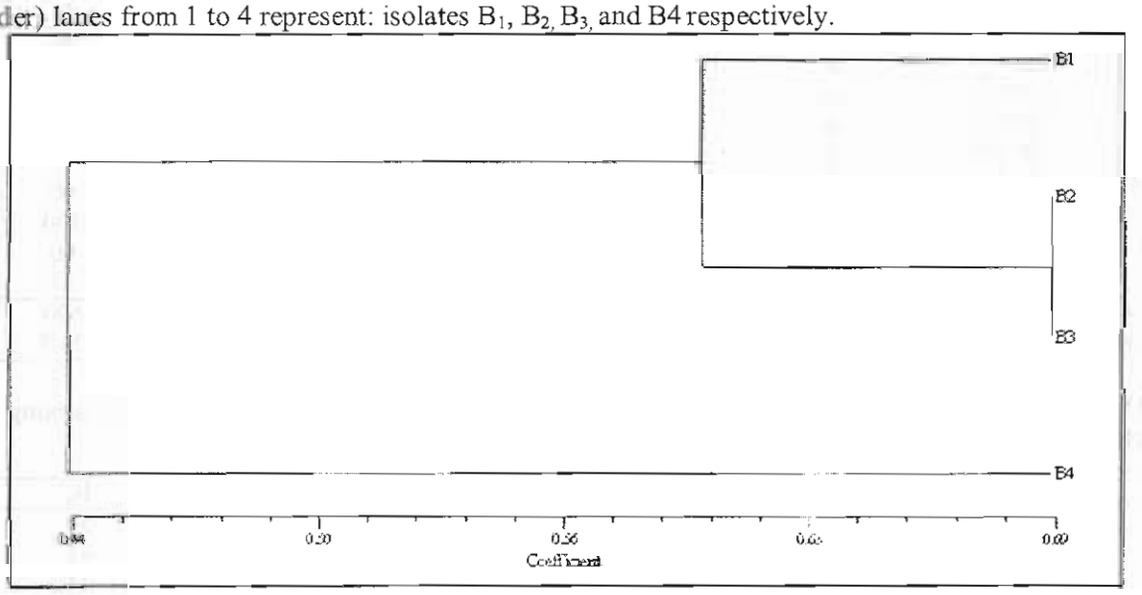

Fig. (2): Dendrogram for four *B. thuringiensis* isolates from RAPD's data using UPGMA and similarity matrices.

RAPD profiles (Fig. 1) with a number of amplified DNA fragments ranged from 3 to 8 amplicons per primer (Table 7). In the present study, the total number of fragments produced by the 9 primers was 52, with an average number of 5.8 amplicons per primer. The number of amplified DNA fragments was scored for each primer. Primer OPA04 was amplified the highest number of amplicons (8), while the lowest number was 3, with the primer OPA09. The number of polymorphic amplicons per primer ranged from 1 amplicons (primer OPA09) to 8 amplicons, (primer OPA04).The polymorphic % was 100 (OPA02, OPA04 and OPA07) and 86, 75, 60, 60, 50 and 33 for primers, OPA01, OPA05, OPA06, OPA08, OPA03 and OPA09, respectively. As shown in table (8), the level of genetic similarity among the four isolates ranged from 44 to 80%. The highest genetic similarity (80%) was observed between B_2 and B_3 isolates, followed by 68% between isolates B_1 and B_3. This was followed by 65 % similarity index between B_1 and B_2, while the lowest genetic similarity (44%) was observed between isolates B_3 and B_4.

Applicability of the method for determining genome similarities among *B. thuringiensis* isolates was investigated by performing cluster analysis on the RAPD data. The UPGMA dendrogram generated from the similarity values is shown in fig. (2). Dendrogram grouped the four isolates into two main clusters; the first cluster contained isolate B_4. On the other hand, the second cluster contains three isolates (B_1, B_2 and B_3). This second cluster was divided into two main sub clusters; the first one contained isolate B_1, while the second sub-cluster contained the other two isolates. It was divided into two groups. The first group contained isolates B_2 and the second group contained B_3. RAPD, introduced by Williams *et al.* (1990), relies on the amplification of fragments with the presence only of a single short primer. The RAPD technique was applied to solve many problems in both fungi and bacteria mainly in the characterization of complex habitats or the differentiation of isolates.

Table (6): Effect of *B. thuringiensis* isolates and formulate Protecto on eggs of *T. absoluta* in laboratory assay

Treatment		Eggs		Adult		% emerged adult
		Total no. of eggs/ female	% reduction	Emerged	% reduction	
Bacillus isolates	B_1	130.00±15.18 ^{C}	64.17±2.89 ^{A}	37.67± 5.36 ^{D}	85.16±1.64 ^{A}	28.81±0.91 ^{B}
	B_2	172.67± 19.75 ^{B}	52.98±5.94 ^{B}	50.00±6.03 ^{CD}	79.32±2.59 ^{AB}	٣٠.٠٢±5.56 ^{B}
	B_3	187.33 ±9.55 ^{B}	48.18 ±0.47 ^{B}	70.00 ±6.81 ^{BC}	72.10±3.43 ^{BC}	٣٧.٧٢±4.96 ^{B}
	B_4	202.00±6.11 ^{B}	44.12 ±1.07 ^{B}	77.00 ±8.15 ^{B}	69.28±4.15 ^{C}	٣٨.٣٦±5.00 ^{B}
Protecto		106.67 ±2.90 ^{C}	70.61 ±1.76 ^{A}	30.00 ±2.89 ^{D}	88.02±1.55 ^{A}	٢٨.٠٢±3.18 ^{B}
control		361.33 ±16.18 ^{A}	0.00	252.67± 11.60 ^{A}	0.00	٦٩.٩٢±0.08 ^{A}
Sig.		***	**	***	**	***

Means having different superscripts within each effect in the same column are significantly different at P≤ 0.05 Sig: significance, **: significant at P≤0.01, **: significant at P≤0.001

Table (7): Random primers showing polymorphism among native isolates of *B. thuringiensis*

No.	Primer name	No. of isolates amplified	Total bands obtained	Polymorphic bands	Monomorphic bands	Polymorphism %
1	OPA01	4	7	6	1	86
2	OPA02	4	5	5	0	100
3	OPA03	4	6	3	3	50
4	OPA04	4	8	8	0	100
5	OPA05	4	4	3	1	75
6	OPA06	4	5	3	2	60
7	OPA07	4	7	7	0	100
8	OPA08	4	7	6	1	60
9	OPA09	4	3	1	2	33
Total			52	42	10	690
Average			5.8	4.7	1.1	76.7

Table (8): Genetic similarity matrices computed according to Dice coefficient percentage among four isolates of *B. thurigiensis* on RAPD-PCR

Bacillus isolates	B_1	B_2	B_3	B_4
B_1	100	65	68	50
B_2		100	80	46
B_3			100	44
B_4				100

The RAPD analysis could effectively distinguish the different native isolates of *B. thuringiensis* isolated from dead larvae of *T. absoluta*. RAPD analysis was considered an important molecular biology technique, which was used for the identification of indigenous *B. thuringiensis* isolates. In comparison to other molecular typing methods, RAPD is faster, less labor-intensive and eliminates the need for pure DNA. Only a small amount of template DNA was required for amplification reaction (Sikora *et al.*, 1997). The present study showed the usefulness of this technique to characterize the *B. thuringiensis* isolates and accordingly new strains of *B. thuringiensis* can be identified and used as source for new genes. These could possibly have a broad insecticidal spectrum against insects of different orders.

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