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Bioinsecticide activity of *Bacillus thuringiensis* isolates on tomato borer, *Tuta absoluta* (Meyrick) and their molecular identification

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Twelve (12) bacterial isolates were isolated from dead larvae of *Tuta absoluta* (4th instar) from tomato cultivated fields at Fayoum Governorate, Egypt. All isolates were preliminarily identified as members of the genus *Bacillus* based on 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 B_1, B_2, B_3 and B_4 showed high mortality of 93.3, 90, 86.7 and 80% on day 7, respectively, on the 4th instar larvae. Also, protecto (*Bacillus thuringiensis* subsp. *kurstaki*) recorded the highest mortality when the 4th instar larvae were treated with 2 g / 2 liter of water (96.7%) on the 5th day post treatment. Isolate B_{12} recorded the lowest percentage mortality of the 4th instar larvae (13.3%). In addition, there was a significant decrease in egg hatching percentage reaching 44.12% compared with the control, meanwhile, the adult emergence decreased after treatment and only 38 and 30 adults emerged from the cages containing tomato plants infested by eggs with B_1 and protecto compared with the control which gave 253 adults that emerged. 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, clusters I 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 which was used to reveal the phylogenetic relationships between the isolates.

Key words: *Bacillus thuringiensis*, bioinsecticide, randomly amplified poylmorphic DNA-polymerase chain reaction (RAPD-PCR), instar larvae, *Tuta absoluta*.

INTRODUCTION

The tomato borer *Tuta absoluta* (Meyriek) (Lepidoptera; Gelechiidae) is one of the most important lepidopteran pests associated with the tomato crops. *T. absoluta* has a high reproductive potential, females lay eggs on aerial parts of their host plants and a single female can lay a total of about 260-300 eggs during its lifetime and there

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may be 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 can be significantly reduced by the direct feeding of the pest and the secondary pathogens which may then enter through the wounds made by the pest. Severely attacked tomato fruits lose their commercial value. 60 to 100% losses have been reported on tomato (Cristina et al., 2008). The larvae instar which is the source of infection was difficult to control because of its presence inside the plant. Resistance to some insecticides has been reported in several countries, for example to abamectin, cartap and permethrin in Brazil (Siqueira et al., 2000).

Larvae can damage tomato plants during all growth stages, producing large galleries in their leaves, burrowing stalks, apical buds, green and ripe fruits (Caceres, 1992; IAN, 1994). It can cause a substantial loss of tomato production regions under diverse production systems (Benavent et al., 1978; Caceres, 1992). The larvae feed on mesophyell tissues and make irregular mines on leaf surface. Damage can reach to 100% which occurs throughout the entire growing cycle of tomatoes. *Bacillus thuringiensis*, an entomopathogenic bacterium, has also been used in the control of tomato plant pests (Prada and Gutierrez, 1974; Souza and Reis, 1992; Marques and Alves, 1996). Continuous use of chemical insecticides in agriculture, forestry and horticultural crop plants, leads to deleterious effects on the environment. Pest species have developed resistance to insecticides. Biopesticides based on *B. thuringiensis* are used as an alternative strategy to control pests. *B. thuringiensis* 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* are distinguished from other closely related *Bacillus* such as *Bacillus cereus, Bacillus anthracis* and *Bacillus mycoides*, that show toxicity against certain insect orders (Hofte and Whiteley, 1989). For more than 50 years, *B. thuringiensis* has been used to control various insect pests due to its ecofriendly nature, safety and target specificity. Delta endotoxin protein in *B. thuringiensis* is ingested by insect larvae. The protoxin in the parasporal bodies are dissolved and activated under alkaline conditions in the midgut of target insects, thereby releasing the active peptides that bind to specific receptors in the insect's midgut epithelial cells and creating pores in the epithelial membrane. Soon the insect stops feeding and ultimately dies due to starvation (Charles et al., 1996).

Native *B. thuringiensis* isolates were subjected to randomly amplified polymorphic DNA (RAPD) marker-based analysis for characterization of their genetic diversity. Various techniques that 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; 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; 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 can be distinguished (Hansen et al., 1998).

Therefore, 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, as well as to establish, if possible, the basis for success in controlling this pest in commercial tomato plantation. In addition, RAPD-PCR technique was applied to confirm the identification of some *Bacillus* isolates by API system and study the phylogenetic relationships between the isolates.

MATERIALS AND METHODS

Insect rearing

The colony of *T. absoluta* used in our laboratory assays were established from larvae and pupae collected from infested tomato fields at Fayoum Governorate, Egypt. They 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 infected tomato fruits, at tomato farm, Fayoum Governorate, Egypt. Each dead larva was collected 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 complete 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 (Quesnel, 1971). 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 was 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 were 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

Commercial bioinsecticide protecto (*B. thuringiensis* subsp. *kurstaki)* was selected for our assay to control the lepidopteran pests. The toxicity of protecto as wettable powder and was evaluated on the different larval instars (1^{st}, 2^{nd}, 3^{th} and 4^{th}) of tomato borer, *T. absoluta*. The bioagent was obtained from Plant Protection Research Institute Biopesticide, Egypt. Serial dilution of protecto was prepared using 1, 1.5 and 2 g of the wettable powder and dissolved in 200 ml of water.

Insect toxicity assay

Twelve treatments, each with three replicates of ten larvae were used. In order to assess the toxicity of *Bacillus* isolates against the 4^{th} 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 of the beginning of the bioassay. After 24 h, the larvae were fed on untreated leaves. The larval mortality was evaluated daily for 7 days to reveal variations on the efficacies with 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 1^{st}, 2^{nd}, 3^{th} and 4^{th}) of three replicates with ten larvae were used. Larvae fed on tomato leaf discs (2 cm diameter) were dipped into each suspension (8.9×10^9 spore ml^{-1} of *Bacillus* isolates) of the highest four isolates mortality. The other group were sprayed with water and used as controls. The larval mortality was evaluated daily at 4, 5 and 7 days for 1^{st}, 2^{nd}, 3^{th} and 4^{th} instars, respectively, while the alive ones were followed until the appearance of the next generation.

Commercial biocide, protecto (*Bacillus thuringiensis* subsp. *kurstaki)* and at three concentrations of 1.0, 1.5 and 2 g/liter of water was used in treatments. The leaf was dipped in suspension of protecto and then offered to different larval instars of 1^{st}, 2^{nd}, 3^{th} and 4^{th} 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 fivedays. arval that survived after the 5^{th} day of treatment were followed until adult stage.

On *T. absoluta* eggs in laboratory assay

T. absoluta adults emerged from larvae survived after treatment with different isolates of *B. thuringiensis* (B_1, B_2, B_3 and B_4) and biocide; protecto to different larval instars of 1^{st}, 2^{nd}, 3^{th} and 4^{th} of *T. absoluta* were followed. Eighteen tomato plants of 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 with 8.9×10^9 spore/ml of *B. thuringensis* of *highest* isolation mortality (B_1, B_2, B_3 and B_4); the fifth group was sprayed with 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. The six group was used as a control and sprayed with the water. The plants were air dried and replaced in the same cages, and each was infested by placing three *T. absoluta* couples inside each cage for 4 days. Then *T. absoluta* adults were removed and the plants were checked to count the eggs. The adults that emerged from the cages at the end of the experiment were recorded.

Statistical analysis

Analysis of variance for 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 overnight at 30°C. Cells were harvested from 5 mL of the culture and to this 100 µL 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 were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50 µL of 1X TE buffer. The 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) were 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 1 min 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.

RAPD data analysis

The data obtained from amplification products by primers were

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

S/N	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

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). Pattern is base on the presence (1) or absence (0) of band for each of the primer. 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 unweighted 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

In the present study, twelve *Bacillu*s isolates were isolated from died *T. absoluta* larvae. The different *B. thuringiensis* isolates were confirmed on the basis of the method described by Travers et al. (1987), shape, gram staining and the presence of spores. All isolates were rod -shaped, gram-positive, and spores were seen inside the spored bacterium. The 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 tested isolates were identified with a possibility of ove r90% 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 determine 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).

Toxicity

Results presented in Table 3 show cumulative larval mortality percentages due to treatment of the 4th instar larvae of *T. absoluta* with 8.9X 10^9 spore's m^{-1} of different isolates. The highest mortality was obtained when the 4th instar larvae were treated with 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 with B_{12} (13.3%).

Table 2. Morphological and physio-biochemical characteristics of *Bacillus* isolates.

Characteristic	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.

Efficacy of *Bacillus* isolates and commercial bioinsecticide on several *T. absoluta* larval instars in laboratory assay

Different larval instars of *T. absoluta* (1st, 2nd, 3rd and 4th) were fed on tomato leaf discs (2 cm diameter) previously dipped into each suspension of bacterial isolates, 8.9x 10^9 spores ml^{-1} (the highest four bacterial isolates mortality). Similar treatment by dipping in distilled water was used as a control. As shown in Table 4, cumulative mortality percentages of *T. absoluta* were recorded daily. The 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 B_1, B_2, B_3 and B_4, respectively. The second larval instars were recorded 60, 53.3, 46.7 and 30% mortality on 5th day after

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

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

A to X means highly significant different (P≤ 0.001).

Table 4. Cumulative mortality percentages of *Tuta absoluta* larvae fed on tomato leaves treated with different *B. thuringiensis*.

Age of instar	Periods after treatment (day)	Treatment				
		Isolates of *Bacillus thuringensis*				
		B_1	B_2	B_3	B_4	control
1	1	3.33^{U}	0.00^{U}	0.00^{U}	0.00^{U}	0.00^{U}
	2	23.33^{RST}	13.33^{STU}	10.00^{STU}	3.33^{U}	0.00^{U}
	3	40.00^{MOPQ}	26.67^{QRS}	20.00^{RST}	10.00^{STU}	3.33^{U}
	4	46.00^{LMNO}	33.33^{OPQR}	26.67^{QRS}	13.33^{STU}	3.33^{U}
2	1	13.33^{STU}	10.00^{STU}	3.33^{U}	$0.00\,0^{U}$	0.00^{U}
	2	30.00^{PQR}	26.67^{QRS}	20.00^{RST}	10.00^{STU}	0.00^{U}
	3	43.33^{MNOP}	33.33^{OPQR}	30.00^{PQR}	13.33^{STU}	0.00^{U}
	4	50.00^{KLMN}	40.00^{NOPQ}	33.33^{OPQR}	20.00^{RST}	0.00^{U}
	5	60.00^{GHIJK}	53.33^{IJKLM}	46.00^{LMNO}	30.00^{QR}	0.00^{U}
3	1	30.00^{PQR}	20.00^{RST}	13.33^{STU}	3.33^{U}	0.00^{U}
	2	46.00^{LMNO}	33.33^{OPQR}	23.33^{RST}	13.33^{STU}	0.00^{U}
	3	53.33^{IJKLM}	46.67^{LMNO}	43.33^{MNOP}	30.00^{PQR}	0.00^{U}
	4	60.00^{GHIJK}	53.33^{IJKLM}	50.00^{KLMN}	40.00^{NOPQ}	0.00^{U}
	5	66.67^{EFGHI}	60.00^{GHIJK}	53.33^{IJKLM}	43.33^{MNOP}	3.33^{U}
	6	73.33^{CDEFG}	70.00^{DEFGH}	66.70^{DEFGH}	50.00^{KLMN}	3.33^{U}
	7	80.00^{ABCDE}	76.67^{BCDEF}	66.70^{DEFGH}	63.33^{FGHIJ}	3.33^{U}
4	1	33.33^{OPQR}	26.67^{QRS}	20.00^{RST}	10.00^{STU}	0.00^{U}
	2	53.33^{IJKLM}	43.33^{MNOP}	33.33^{OPQR}	20.00^{RST}	0.00^{U}
	3	66.67^{EFGHI}	50.00^{KLMN}	46.67^{LMNO}	33.33^{OPQR}	3.33^{U}
	4	76.67^{BCDEF}	70.00^{DEFGH}	56.67^{HIJKL}	43.33^{MNOP}	3.33^{U}
	5	80.00^{ABCDE}	73.33^{CDEFG}	70.00^{DEFGH}	63.33^{FGHIJ}	3.33^{U}
	6	86.67^{ABC}	83.33^{ABCD}	76.67^{BCDEF}	70.00^{DEFGH}	3.33^{U}
	7	93.33^{A}	90.00^{AB}	86.67^{ABC}	80.00^{ABCD}	$3.33.^{U}$

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

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

Age of instar	Periods after treatment (day)	Treatment			
		bacterial biocide (Protecto concentration) (g/2)L			Control
		1	1.5	2	
1	1	20.00^{O}	26.67^{NO}	53.33^{KL}	0.00^{P}
	2	33.33^{MN}	43.33^{LM}	70.00^{GHI}	0.00^{P}
	3	56.67^{JK}	66.67^{HIF}	76.67^{EFJH}	3.33^{P}
	4	70.00^{GHI}	80.00^{DEFG}	90.00^{ABCD}	6.67^{P}
	5	73.33^{FGH}	83.33^{CDEF}	96.67^{AB}	3.30^{P}
2	1	26.67^{NO}	33.33^{MN}	73.33^{FGH}	0.00^{P}
	2	43.33^{LM}	73.33^{FGH}	76.67^{EFGH}	0.00^{P}
	3	66.67^{HIF}	86.67^{BCDE}	90.00^{ABCD}	0.00^{P}
	4	76.67^{EFGH}	93.33^{ABC}	100.00^{A}	0.00^{P}
	5	80.00^{DEFG}	96.67^{AB}	100.00^{A}	0.00^{P}
3	1	40.00^{M}	53.33^{KL}	83.33^{CDEF}	0.00^{P}
	2	60.00^{HIF}	83.33^{CDEF}	96.67^{AB}	0.00^{P}
	3	73.33^{FGH}	90.00^{ABCD}	100.00^{A}	3.30^{P}
	4	83.33^{CDEF}	100.00^{A}	100.00^{A}	3.30^{P}
	5	90.00^{ABCD}	100.00^{A}	100.00^{A}	3.30^{P}
4	1	66.70^{HIJ}	83.33^{CDEF}	93.33^{ABC}	0.00^{P}
	2	76.67^{EFJH}	100.00^{A}	100.00^{A}	0.00^{P}
	3	83.33^{CDEF}	100.00^{A}	100.00^{A}	0.00^{P}
	4	96.60^{AB}	100.00^{A}	100.00^{A}	0.00^{P}
	5	96.67^{AB}	100.00^{A}	100.00^{A}	0.00^{P}

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

the four treatments, respectively. The mortality was significantly increased as compared to the third and fourth instars being 80, 76.7, 66.7 and 63.3% in the third instar and 93.3, 90, 86.7 and 80% in the fourth instar seven days after treatment with the isolates of B_1, B_2, B_3 and B_4, respectively.

The obtained results revealed high significant mortality of *T. absoluta* for the first, second, third and fourth larval instars to *B. thuringiensis*. Toxins expressed by *Bacillus* species naturally colonize the phylloplane of tomato plants. Transformed *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 1^{st}, 2^{nd}, 3^{rd} and 4^{th} *T. absoluta* larval instars. However, there were differences in the mortality shown by each larval stage. Fourth instar larvae recorded the highest mortality, while mortality was lower in first and second 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 first larvae can be explained by feeding behavior differences. Normally, first and second larval instars penetrate directly the leaves without much feeding and are therefore exposed to a lower dose of bacterial spores and toxins. Older instars were more susceptible to treatments than younger ones, as result of their longer stadia, beside their more sensitive integument and internal organs before and at the time of mortality. Larvae move in and out of the mines and galleries several times during their development and at that moment they are very vulnerable to infection by the bacteria (Harizanova et al., 2009). On the other hand, larvae of first and second instars remain in the leaf where oviposition took place. When they reach to the later instars there is more competition for food, and the larvae need to spread over the tomato plants. Giustolin et al. (2001) reported that the progressive increase of mortality of older larvae that were fed *B. thuringiensis* 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 ingesting a higher dose of the pathogen and its toxin.

Table 5 presents the effect of protecto (*B. thuringiensis* subsp. *kurstaki)* at different concentrations (1, 1.5 and 2 g

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 ^{C}	64.17 ^{A}	37.67^{D}	85.16^{A}	28.81^{B}
	B_2	172.67^{B}	52.98 ^{B}	50.00 ^{CD}	79.32^{AB}	30.0**2**^{B}
	B_3	187.33 ^{B}	48.18 ^{B}	70.00 ^{BC}	72.10^{C}	**37.73**^{B}
	B_4	202.00^{B}	44.12 ^{B}	77.00 ^{B}	69.28^{C}	**38.36**^{B}
Protecto		106.67 ^{C}	70.61 ^{A}	30.00 ^{D}	88.02^{A}	**28.52**^{B}
control		361.33 ^{A}	0.00	252.67^{A}	0.00	**69.92**^{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

/2 l of water) on the mortality of different *T. absoluta* larval instars fed on treated tomato leaves. Within an instars larval 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 *B. thuringiensis* subsp *kurstaki* commercial biocide in 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.

Commercial biocide tested in laboratory bioassays showed high significant efficacy in reducing the damage caused by different larval instars (1st, 2nd, 3rd and 4th) of *T. absoluta* at different concentrations compared to non-treated once as a control. Generally, the obtained results show that there was a delay in the killing effect due to latent period of the tested biocide (Protecto). The 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 significant decrease in total number of eggs and adult emergence to each bacterial isolation 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). Our result is in agreement with the study of Alwan et al. (2012) who found a significant decrease in the percentage of egg hatching for *T. absoluta* treated with *B. thuringiensis* filtrate (33.36%) compared with control (86.74%). *B. thuringiensis* excreted (α- β and ♁) exotoxins which affected and killed the insects. Mohamed et al. (2000) showed that, a significant decrease in hatching egg percentage of *Spodoptera littoralis* treated with *B. thuringiensis* reached 52.5% and the histological studies showed the presence of voids in the cells of the ovary and contraction in the feeder cells and decrees in adult yolk. Spoonam et al. (2002) showed a significant decrease in the percentage of laid and hatching eggs of *Culex quinque fasciatus* treated with *B. thuringiensis* suspension.

Mohamed et al. (2000) found that, *B. thuringiensis* led to a reduction in the number of hatched eggs of *S. littoralis* but without significant differences compared to control. While the results found by El-Emara (2009) showed a positive effect in the reduction of hatched eggs of *Trogoderma granariumin*. The use of biocide and bacterial isolates interfered with egg formation or development and consequently, led to reduction in the number of laid eggs. This phenomenon reveals that some larvae could accept slight infection which need an extension period to attack the stomach 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 in the present study to identify the four isolates of *B. thuringiensis*. These primers generated reproducible and easily securable RAPD profiles (Figure 1) with a number of amplified DNA fragments ranging from three to eight amplicons per primer (Table 7). In the present study, the total number of fragments produced by the nine 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 with 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

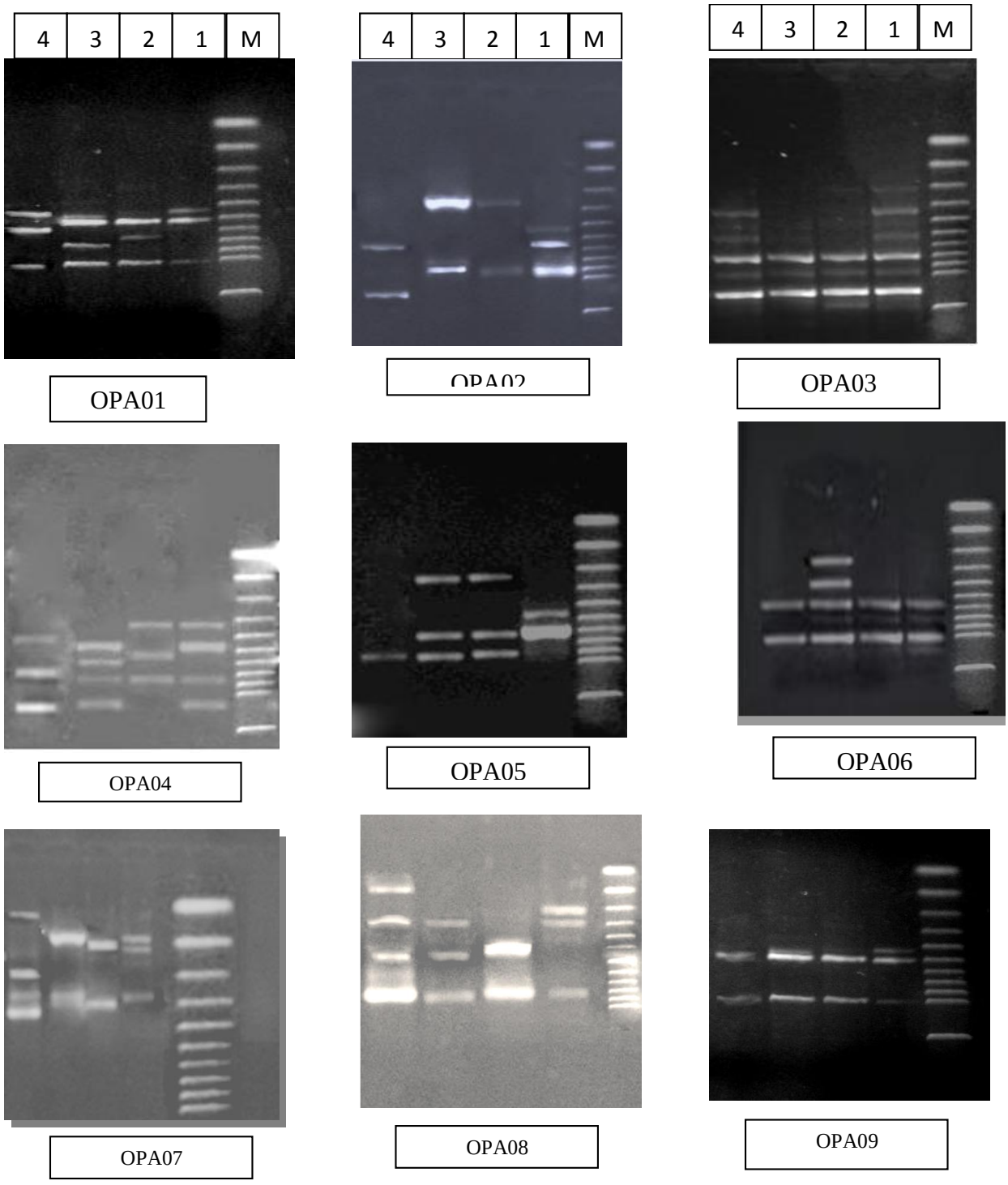

Figure 1. RAPD profiles of 4 *B. thuringiensis* isolates amplified with RAPD primers (OPA01, OPA02. OPA03, OPA04, OPA05, OPA06, OPA07. OPA08 and OPA09). M, Molecular weight marker(1 kb DNA ladder); lanes 1 to 4 represent isolates B_1, B_2, B_3, B_4, respectively.

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.

The 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

Table 7. Random primers showing polymorphism among native isolates of *B. thurigiensis*.

S/N	Primer name	No. of isolates amplified	Total band obtained	Polymorphic band	Monomorphic band	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 isolate	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

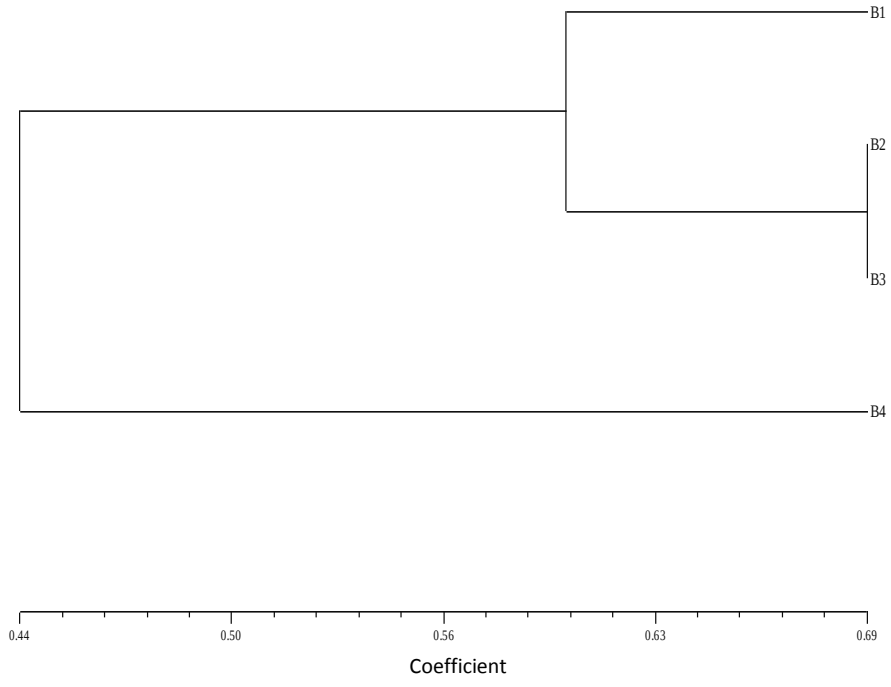

Figure 2. Dendrogram for four *B. thuringiensis* isolates from RAPD's data using UPGMA and similarity matrices.

values is shown in Figure 2. The 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 subcluster contained the other two isolates. It was divided into two groups. The first group contained isolate 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.

The RAPD analysis could effectively distinguish the different native isolates of *B. thuringiensis* isolated from dead larvae of *T. absoluta*. RAPD analysis is considered an important molecular biology technique, which is 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 is 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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