Bioinsecticide activity of *Bacillus thuringiensis* strains on tomato borer, *Tuta absoluta* (Meyrick) and molecular identification using RAPD-PCR based fingerprinting.

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Abstract

Twelve isolates of *bacterial strains* were isolated from dead larvae of *Tuta. absoluta* (4^{th} 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* strains against different instar larvae of *T. absoluta,* the 12 isolates revealed varying efficiencies and isolates (B_1, B_2, B_3 and B_4)were showed a highly mortality of 93.3, 90, 86.7 and 80 % on day 7 respectively on the 4^{th} instar larvae. Also protecto(*Bacillus thuringiensis var kurstaki*) recorded the highest mortality when the 4^{th} instar larvae were treated with 2 gm /2 liter of water (96.7% on 5^{th} day post treatment. Isolate B_{12} was recorded the lowest percentage at 13.3% . In addition, there was a significance decrease in adult emergence, only 38 and 30 adults emerged from the cages containing tomato plants infested by eggs with B_1 and protecto. In contrast, 253 adults emerged from the control. Further genetics 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 42were 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 3 and 1 *B. thuringiensis* isolates, respectively. The RAPD technique was shown to be effective in differentiating closely related strains and applied to confirm the identification of *Bacillus* isolates by API system and study the phylogenetic relationships between the isolates.

Key words: *Bacillus thuringiensis*, Bioinsecticide, RAPD-PCR-, instar larvae, *Tuta. absoluta*

Introduction

The tomato borer *Tuta absoluta* (Meyriek) lepidopteron; Gelechudae)is one of the most important lepidopteron pests associated with the processing tomato crops. Where crop losses range from 60 to 100% (**Cristina et al 2008**). 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**), causing a substantial loss of tomato production regions and under diverse production systems **(Benavent et al 1978,** Caceres**, 1992).** The larvae feed on mesophyell tissues and make irregular mine on leaf surface. Damage can reach up to 100%. This pest damage occurs throughout the entire growing cycle of tomatoes. *Bacillus thuringiensis*, an entomopathogenic bacterium, has also been used in the control of tomato plant pests **(Prado and Gutierrez, 1974; Souza and Reis, 1992; Marques and Alves, 1996).** The continuous use of chemical insecticides to control insect pests of agriculture, forestry and horticultural crop plants, leads to deleterious effects on the environment. Some insects have developed resistance to insecticides. An alternative strategy used to control harmful insects is biopesticides based on *Bacillus thuringiensis*. It is a rod-shaped, gram-positive, endospore-forming bacterium, and distinguished from other closely related three *Bacillus spp*. viz. *B. cerus, B. anthracis*

and *B. mycoides*, because of its ability to synthesize delta endotoxins as protein inclusion crystals (or Cry proteins) during sporulation (**Hofte and Whiteley 1989)**. For more than 50 years, *B. thuringiensis* has been used to control various insects 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 create pores in the epithelial membrane. Soon the insect stops feeding and ultimately dies due to starvation **(Charles et al.1996)**. 3,000 species, belonging to the orders of insects, have been reported susceptible to *B. thuringiensis* **(Huang et al 2004).** Commercial formulated based on this bacterium have been used for decades to control insect pests as on alternative to chemicals. Such formulates are environmentally friendly, harmless to humans and other vertebrates **(Entwistle et al 1993, Mc Clintock et al l 1995; IPCS –WHO, 2000).**

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 and Williams et al 1990)** and this technology was successfully applied to characterize the genetic diversity in various *B. thuringiensis* isolates **(Brousseau et al 1993**). It has been found that using RAPD analysis ،characteristic fi ngerprints 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.

Material and Methods.

Isolation of *Bacillus thuringiensis* from dead larvae.

Dead larvae of *T. absoluta* (4^{th} 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 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 and incubated overnight at 30ºC. *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 examines 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 to 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. *Bacillu*s isolates were divided into biochemical types based on hydrolysis of esculin, urea or lecithin, and acid production from sucrose, or salicin. Lecithinase activity of *Bacillu*s 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 of the test results with dichotomous keys. API kits were used according to manufacturer's instructions and identification was done with API-web program.

Commercial products.

Commercial bioinsecticide protecto (*Bacillus thuringiensis var kurstaki)* was selected for our assay are among the most commonly used to control lepidopteran pests as wettable powder was evaluated for their toxicity on the different larval instar of tomato borer, *Tuta absoluta*. Bioagents was obtained from Plant Protection Research Institute Biopesticide , Egypt. Serial dilution of protecto was prepared using 1, 1.5 and 2 gram of the wettable powder and dissolved in 200ml of water.

Insect rearing.

The colony of **Tuta** *absoluta* ***used*** in our laboratory assays was established from individuals collected from infested tomato fields at Fayoum Governorate, Egypt. They were reared on tomato plants in a climatic chamber at 27 ± 2 C. 55% RH. Tomato plants were placed in the chamber weekly for feeding and egg lying. When required for our assays, adults were collected using a mechanical aspirator.

Insect toxicity assay.

Twelve treatments, each with three replicates of ten larvae were used. In order to assess *Bacillus* isolates toxicity against (4th instar) larvae of T .*absoluta*, a bacterial spore suspension of $8.9x10^9$ spore ml^{-1} according to **Amin et al. (2008**). Distelled water contained 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 used as control. The treated discs were only used once of the beginning of the bioassay. Afterward, the larvae were fed untreated leaves. The larval mortality was evaluated daily for 7 days to determine revealed varying efficiencies with different isolates.

Efficacy of *Bacillus* isolates and commercial bioinsecticide.

On several *T. absoluta* instars in laboratory assay.

Four groups (different instar larva 1^{st}, 2^{nd}, 3^{th} and 4^{th}) of three replicates with ten larvae were used. Larvae were fed on tomato leaf discs (2 cm diameter) were dipped into each suspension (8.9×10^9 spore ml^{-1} of *Bacillus* isolates) of the highest four isolates mortality. The other groups were used as the control and were sprayed

with water. The larval mortality was evaluated daily at 4, 5 and 7 days for 1st, 2nd, 3th and 4th instar respectively.

Commercial biocide was protecto (*Bacillus thuringiensis var kurstaki)* at three concentrations, 1.0, 1.5 and 2g/liter of water was used in the test. The leaf was dipped in solution of protecto and then offered to (different larval instar 1st, 2nd, 3th and 4th of *T. absoluta*) Three treatments each with three replicates of ten larvae for feeding . In addition, untreated larvae were fed on untreated tomato leaf as a control. Larval mortalities were recorded daily for 5 days.

On *T. absoluta* eggs in laboratory assay.

Fiveteen tomato plants of approximately 30 cm in height were placed singly inside screened cages (30 cm x 30 cm x 45 cm). The plants were randomly grouped into five groups of three plants each. Each group (four groups) was sprayed with 8.9 x 10^9 spore/ ml of *B. thuringensis* of *highest* isolation mortality (B1, 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 other group was used as a control and was 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 the cages for 24 h. Then *T. absoluta* adults were removed and the plants were checked daily to count the eggs. The adults that emerged from the cages at the end of the experiment were recorded.

Statistical analysis.

Analysis of variance 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 **(Duncan1995).**

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 37ºC. 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 were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50µL 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 (Table1). 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 40ºC for 1 min and 72ºC for 1min and final extension 72ºC 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 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 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 *T. absoluta* larval died. 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 and crystals. All isolates were rod -shaped, gram-positive, and spores and crystals were seen inside the 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 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 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; production of gelatinase and assimilation of fructose,

glycogen, trehalose, ribose, and N-acetyle glucoseamine. Contrastingly, ONPG test, H2S 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) showed cumulative larval mortality percentages due to treatment of the 4^{th} larval instars of *T. absoluta* with 8.9X 10^9 spore's m^{-1} of different isolates. The highest mortality was obtained when the 4^{th} 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%).

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

Different larval instars of *T. absoluta* (1^{st} , 2^{nd} , 3^{rd} and 4^{th}), were fed on tomato leaf discs(2 cm diameter) were dipped into each suspensions of bacterial isolates, 8.9x 10^9 spores m-1 (the highest four bacterial isolates mortality) . Similar treatment dipped with distilled water was used as a control for correcting mortality values. As shown in Table (4) cumulative mortality percentages of *T. absoluta* were recorded after different periods of day. The highest mortality percentages were found for isolation B1and B2 with a gradual significant increase over the inspection period with different larval instars. The first larval instar had percentage mortalities of (46.6, 33.3, 26.7and 13.3%) on 4 day due to feeding larvae on tomato leaves treated with different isolates B1, B2, B3 and B4 respectively. The second larval instar was recorded (60, 53.3, 46.7 and 30%) on 5 day after treatment. The mortality was significantly increased with 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 on 7days after treatment.

The results obtained here reveal the high significant mortility of *T. absoluta* first, second, third and fourth instar larvae to *Bacillus thuringiensis*. Toxins. Toxin expressed by *Bacillus* species that naturally colonize the phyllplane of tomato plants was as toxic as the natural one and transformed bacteria survived for extended periods of time (45 days) on the leaf surface (**Theoduloz et al.2003).** *Bacillus thuringiensis* proved to be highly significant 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 were recorded the highest mortality, while mortality was lower in first and second instar larvae. Several *T. absoluta instar* were found to be susceptible to *Bacillus 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 results of their longer stadia, beside their more sensitive integument and internal organs before and at the time of mortality. Also, the high later instars mortality could be due to dispersal of the caterpillars as they grow, which would increase their exposure to bacterial spores and toxins. Larvae move in and out of the mines and galleries several times during their development and at that moment they are very vulnerable to an infection by the bacteria **(Harizanova et al. 2009)**. On the other hand, larvae of first and second instars remain in the leaf where oviposition takes place when they reach the later instars there is more competition for food, and the larvae need to spread over the tomato plant. This result was agreement with **Giustolin et al. (2001)** who 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 instars 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.

In table (5) presented the effect of protecto (*B. thuringiensis var kurstaki)* of different concentrations (1, 1.5 and 2 g /2 liter of water) on different larval instars mortality *T. absoluta* fed on tomato leaves treated with this biocides. Within an instar, mortality progressively increased as the concentration of protecto increased. On the other hand, the late instars suffered from higher mortality compared with the early instars. The potential of B. *thuringiensis var kurstaki* commercial biocide in controlling pests of economic importance is well know (**Roh et al . 2007)** such biocide have been used for years as a key part of Integrated Pest Management Programs. These results are in good agreement with those obtained by **Cabello et al. (2009)** who reported that the *B. thuringiensis var kurstaki* effect on all larval instars and 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 (1^{st}, 2^{nd}, 3^{rd} and 4^{th}) of *T. absoluta* at different concentration compared with non- treated controls. Generally, the obtained results show that there was a delay in the killing effect due to latent period of the tested biocide (Protecto) and 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 significance decrease in total number of eggs and adult emergence to each bacterial isolation and Protecta. The lowest number was reduced with protecto(106 no. of egg / female and 30 emerged dults) and B1(130 no. of egg/ female and 38 emerged adults) . The used of biocide

and bacterial isolates interfered with egg formation or development and consequently, led to reduction in the number of laid eggs. Furthermore, it may be due to inability of the sperms to be transferred to the females during copulation, as suggested by Ismail (1980).

Random amplified polymorphic DNA (RAPD).

Nine random primers were used in the present study to identify the four strains of *Bacillus thuringiensis*. These primers generated reproducible and easily securable RAPD profiles (Fig. 1) with a number of amplified DNA fragments ranging 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), (100), (100), (86), (75), (60), (60), (50) and (33), primers OPA02, OPA04, OPA07, OPA01, OPA05, OPA06, OPA08, OPA03 and OPA09, respectively. As shown in Table (8), the level of genetic similarity among the four strains ranged from 44% to 80%. The highest genetic similarity 80% was observed between B2 and B3 strain, followed by 68% between strains B1 and B3. This was followed by 65 % similarity index between B1 and B2, while the lowest genetic similarity (44%) was observed between strains B3 and B4.

The applicability of the method for determining genome similarities among *Bacillus Thuringiensis* strains was investigated by performing cluster analysis on the RAPD data. The UPGMA dendrogram generated from the similarity values is shown in Fig. (2). This dendrogram grouped the four strains into two main clusters, the first

cluster contained strain B1. On the other hand, the second cluster contains three strains B1, B2 and B3. It was divided into two main sub clusters; the first one contained strain B1, while the second subcluster contained the other strains. It was divided into two groups. The first group contained strain B2 and the second group contained B3.

RAPD, first introduced by **Williams *et al.* (1990**), relies on the amplification of fragments with only a single short primer present. The RAPD technique was applied to many problems both in fungal and bacterial microbiology 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 characterize the *B. thuringiensis* isolates and so that new strains of *B. thuringiensis* can be identified and used as the source of new genes. These strains could possibly have a broad insecticidal spectrum against insects of different orders.

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

Sr.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

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

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

Bacillus species	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 fed on tomato leaves treated with different *Bacillus thuringiensis*

Mortality % (Mean± S.E.)						
Age of instar	**periods after treatment (day)**	**Treatments**				
		Isolates of *Bacillus thuringensis*				**control**
		B_1	**B_2**	**B_3**	**B_4**	
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 fed on tomato leaves treated with bacterial biocides(Protecto) *Bacillus thuringiensis* var *kurstaki*

Mortality % (Mean ± SE)					
Age of instar	periods after treatment (day)	Treatments			
		bacterial biocides (Protecto concentration) g/2L			control
		1	**1.5**	**2**	
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 ($P \leq 0.001$)

Table(6): Effect of *Bacillus thuringiensis* and formulate Protecto on eggs of *Tuta absoluta* in laboratory assay.

Treatment		Eggs		Adult		% emerged adult
		Total no. of eggs/ female	% reduction	Emerged	% reduction	
B. 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}**	**٣٧.٧٣±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}**	**٢٨.٥٢±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 *Bacillus thurigiensis*

Sr.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 strains of *Bacillus 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

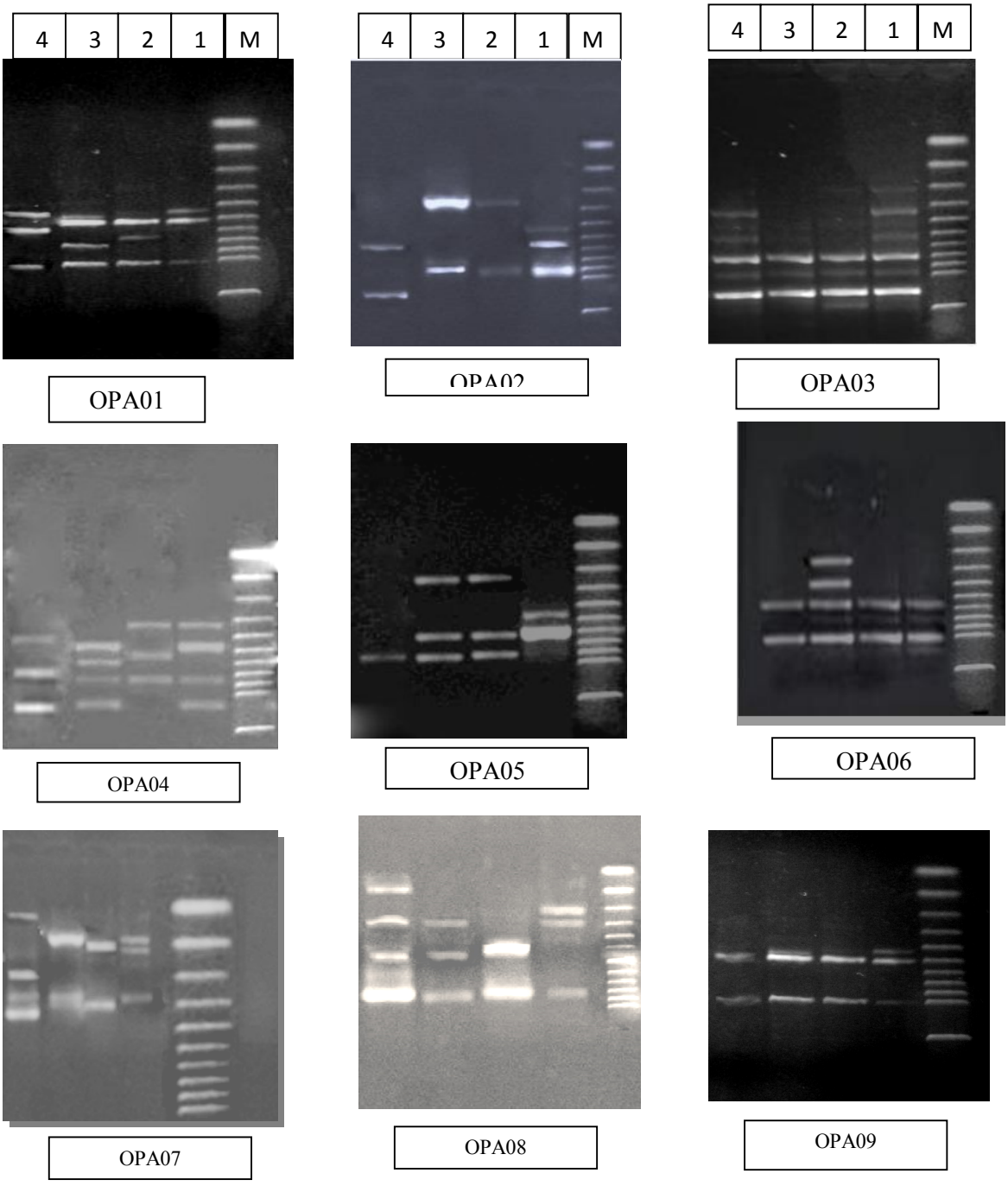

Figure (1) RAPD profiles of 4 *Bacillus thurigiensis* strains amplified with RAPD primers(OPA01, OPA02. OPA03, OPA04, OPA05, OPA06, OPA07. OPA08 and OPA09 . M: molecular weight marker(1 kb DNA ladder) lanes from 1 to 4 represent: B_1, B_2, B_3, B_4 respectively

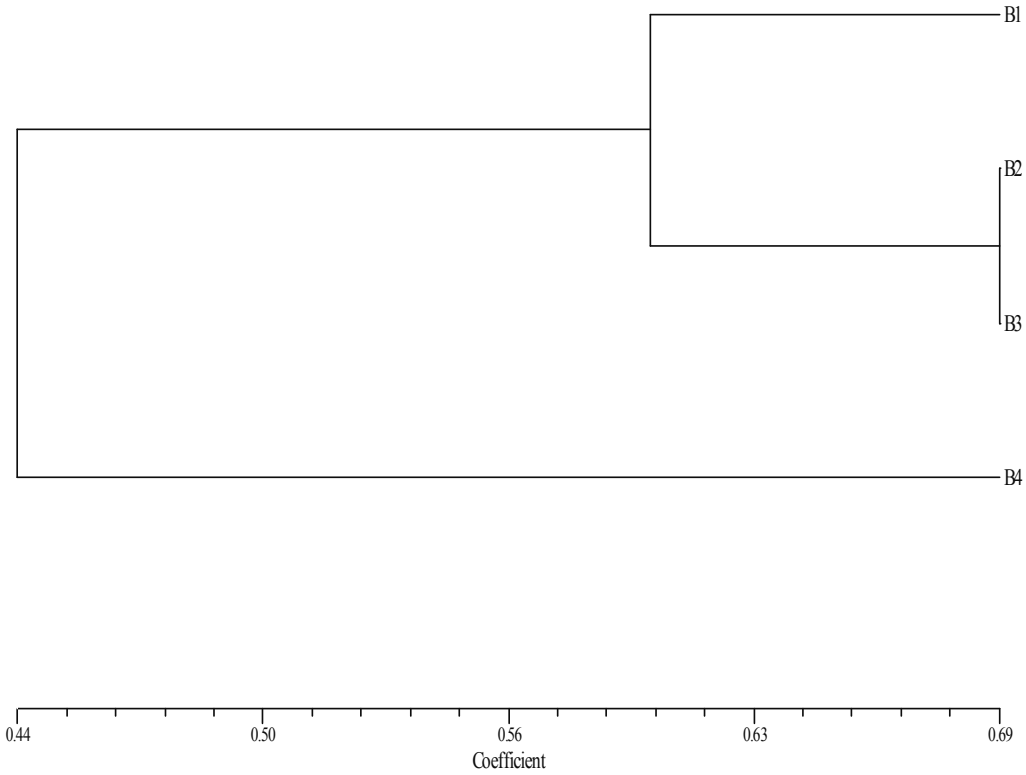

Figure (2). Dendrogram for four *Bacillus Thuringiensis* strains from RAPD's data using UPGMA and similarity matrices