Isolation and Identification of locally isolated bacterial strains effective against cotton leafworm *Spodoptera littoralis* (Boisd.)

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Abstract

Insecticides derived from the commen soil bacterium *Bacillus thuringensis*are becoming increasingly important for pest management. The biological activities of bacterial strains effective against the cotton leafworm, *Spodoptera littoralis*,larvae (2nd and 4th instar), were isolated from tomato cultived fields at Fayoum Governorate, Giza, Egypt. Of 72 isolates, 12 with the most morphologically distinct- looking bacterial colonies were selected and named A1, A2, A3, A6, A7, A9, A12, A13, A107, B37, B45 and B100. All isolates were preliminarily identified as members of the genus *Bacillus* based on morphological, physiological, and biochemical characteristics. When tested for their pathogenicity against *S.littoralis*, the 12 isolates revealed varying efficiencies with isolates A1 and A9 being superior, exhibiting maximum mortality of 90.0 and 83.33% on day 7 respectively. Isolate A7 recorded the lowest percentages at 20.0%. Further genetic characterization of the 12 isolates was performed using Inter simple sequence repeat (ISSR), randomly amplified polymorphic DNA (RAPD) and 16S rDNA gene sequencing analysis. RAPD and ISSR results confirmed each other. The combined ISSR and RAPD phylogenetic tree showed two major clusters. With 16S rRNA gene analysis, isolate A1 and A12 sequences recorded 100% identity with *B. thuringiensis*, while isolates A7 and B100 showed 95.7% and 95.6% identity with *B. cereus* and *B. sphaericus*, respectively. Work in progress in order to optimize the cultural, nutritional, environmental and operational parameters needed for a successful large-scale production of the most effective isolated *Bacillus* strain(s).

Key Words: *Bacillus sp*; molecular markers; ISSR; RAPD; biological control agents, *spodoptera littoralis*

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Introduction

The cotton leafworm, *Spodoptera littoralis* (Boisd.) is a major pests of economic importance for different crops, in different countries. An effective biological control agent as part of an integrated

control program would serve as an important contribution to *Spodoptera* control, in addition to limiting the use of toxic chemicals hazardous to the environment **Sneh *et al*.,(1981).Frederiksen *et al*.,(2006) and Hanafi *et al*., (2007)** reported that *Bacillus* species were among the most effective microorganism against various agricultural pests. *Bacillus* that possesses a parasporal crystalline protein that is highly toxic to wide- range of pest insects especially Lepidopteran, Dipteran and Coleopteran insects **Vettori *et al*.,(2003) and Mansour *et al*.,(2012).** More recently, **Valenzuela-Soto et al (2010)** found that root inoculation of tomato (*Solanum lycopersicum*) plants with a *B. subtilis* strain BEB-DN (BsDN) isolated from the rhizosphere of potato plants was able to promote growth and generate an induced systemic resistance (ISR) response against virus free *B. tabaci*. Full identification of isolated microbial strains applied as biological control agents using microbiological, biochemical, physiological and genetic analysis is of crucial importance before their effective use in large- scale commercial applications.

This study aims to analyze the genetic diversity of locally isolated and partially identified *Bacillus* strains effective against *S.littoralis* using inter simple sequence repeat (ISSR), randomly amplified polymorphic DNA (RAPD) techniques. 16S RNA gene sequencing will also complete further identification of the isolates to species level.

Materials and Methods

Isolation and preliminary characterization of bacterial isolates

Soil samples were collected from the rhizosphere of infected tomato plants, over one week, at tomato farm, Fayoum Governorate, Egypt. Samples were thoroughly mixed, and a representative sample was taken. A 2.0 g aliquot from each sample was added to 98 ml of sterile saline solution (9.0 g $NaCl.l^{-1}$) Each sample was serially diluted to 10^{-8}, plated into nutrient agar (NA) medium and incubated overnight at 30°C. Developing colonies were randomly selected and vegetative cell morphology observations were examined at x1000 magnification using phase contrast microscopy, for shape of cells, presence of chains, spore formation, reaction with gram stain and indol test (Quesnel 1971). To determine motility, strains were grown on slopes of NA and after 6 h, or as soon as growth appeared thereafter, a loopful of the liquid at the base of the slope was examined at x1000 magnification, by phase contrast microscopy. The ability to grow on different carbon sources was determined by monitoring the optical density at 600 nm of cultures grown in nutrient broth supplied with glucose, xylose, maltose, sucrose, cellobiose, galactose, starch, mannitol or Tween 80 as a carbon source. The catalase activity of bacterial isolates was detected by resuspension of a

colony in a 3% solution of hydrogen peroxide (Sigma). Positive results were indicated by bubble formation. Gram positive spore- forming rods sharing other characteristics (Table 1) were selected and subcultured into the same medium and named isolates A1, A2, A3, A6, A7, A9, A12, A13, A107, B37, B45 and B100. They were further used in toxicity and identification experiments.

Pathogenicity assessment

Stock culture of Spodoptera littoralis

Spodoptera littoralis eggs were obtained from Agriculture Research Centre, Giza, Egypt, and were reared under laboratory conditions(30°C and 55-85% relative humidity),for two generations before experimental starting. Insects were fed on the leaves of castor oil plants *(Ricinus communis*).

Toxicity

Twenty four treatments each with three replicates of 10 individuals larvae were used. In order to assess the toxicity of *Bacillus* isolates against the 2nd and 4th instar larvae of S. littoralis, a bacterial spore suspension of $9.6x10^7$ spore.ml^{-1} **Amin *et al.*,(2008)** was used. Distilled water contained 0.05% Tween 40 was used as control.

Newly emerged adults of *B. tabaci* were collected from the stock culture and introduced onto oviposition cages (twenty pairs/cage) for 4 h. The cages were supplied with castor seedlings in pots covered with chimney glasses. The upper openings of the cages were covered with muslin held in position by rubber bands. Newly deposited egg masses were inspected daily to the 2nd nymphal instar. The obtained instars were used for pathological experiments. In order to assess *Bacillus* isolate toxicity against *B. tabaci*, a bacterial spore suspension of $9.6x10^{10}$ spore.ml^{-1} (Amin et al. 2008) was used. Distilled water contained 0.05% Tween 40 was used as control. Five pots (each contained a castor seedling with 5 leaves and infested with certain numbers of the 2nd nymphal instar of *B. tabaci*) were chosen and sprayed completely with the bacterial spore preparation and were left to dry at room temperature. The treated nymphs fluctuated in number between 75-198. At 48 h regular intervals and over 10 days, leaves were examined under binocular stereo-microscope where dead and alive nymphs were counted and the mean values were used in order to calculate percentages of mortality.

Isolation and purification of genomic DNA from Bacillus isolates

Bacterial cells were grown overnight at 30°C in 5mL of Luria Bertani (LB) broth containing ($g.l^{-1}$): 1.0 tryptone, 0.5 yeast extract and 1.0 NaCl. DNA extraction was performed using the instructions of DNeasy Mini Kit protocol (QIAGEN), (Helsen et al. 2007).

RAPD and ISSR analysis

A total of 10 random primers and 4 ISSR primers were used (Table 1). The amplification performed in a 25 µl reaction volume containing about 3µl ($10ng.\mu l^{-1}$) genomic DNA, 3 µl primer (Operon Technologies Inc.) and 19µl master mix (Promega). The PCR temperature profile was applied through a Gene Amp® PCR System 9700 (Perkin Elmer, England).The thermal cycler was programmed with an initial step of 5 min at 94°C; the amplification reaction was carried out using 40 cycles of 40 s at 94°C, an annealing step of 1 min at 36° C, and an elongation step of 1min at 72°C; and finally a 7 min extension at 72°C. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide ($0.5\mu g.ml^{-1}$) in 1X Tris-borate-EDETA (TBE) buffer containing: 89 mM Tris base, 89 mM boric acid and 2 mM EDETA at 95 volts. Ph x 174 Marker cutting by *hae*III (OP-A07 and OP-O02) and 100 bp DNA ladder were used. PCR products were visualized by UV light and photographed using a gel documentation system (Bio-Rad® Gel Doc-2000). Each band produced in the RAPD and ISSR reactions were considered as an individual character (present or absent).

The 16S rRNA gene analysis

PCR amplification of Bacillus 16S rRNA gene

A 200bp fragment corresponding to the 16S rRNA gene, of different 12 Bacilli isolates was PCR-amplified using two specific primers, *Bacillus* forward: (5'GGGTCATTGGAAACTGGGGGA-3') and Bacillus reverse: (5'-GGAAACCCTCTAACACTTAGCACT-3'). 16S rRNA was amplified from the obtained DNA in a reaction mixture of PCR conditions were as follows: 10X *Taq* buffer, 1.25 U AmpliTaq Gold DNA Polymerase, 2mM premixed solution containing sodium salts of Datp, dCTP, Dgtp and dTTP each at 10 mM in water (dNTP mixture), 25 mM $MgCl_2$, 0.7 µg DNA, double-distilled water mixed in a final volume of 50 µl. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and extension at 72°C for 7 min. Amplifiction was done using Perkin Elmer GeneAmp PCR system 2400. Amplicons were visualized by electrophoresis on 1% agarose gel after staining with ethidium bromide.

Sequencing of PCR products of some Bacillus isolates

16S rRNA gene PCR products of isolates A1, A7, A12 and B100 were extracted from gel using gel extraction kit QIAquick Qiagen and cloned using TA cloning kit (invitrogen, SanDiego, Calif.) as recommended by the manfacturer. DNA sequencing was conducted using ABI Prism BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit according to instructions of manufacturer (PE Applied Biosystems) ABI PrismTM 377XL DNA Sequencer (Perkin Elmer). The obtained 16S rDNA sequences of isolates A1, A7, A12 and B100 (Accession numbers: JQ314094, JQ314096, JQ314097 and JQ314095 respectively) were aligned with 16S rRNA gene sequences of *B. thuringiensis*, *B. sphaericus*, *B. cereus* and *E. coli*, by using CLUSTALW software (Thompson et al. 1994). Gaps at the 5'and 3' ends of the alignment were omitted from further analyses. The sequences were integrated into the database with the automatic alignment tool.

Data scoring and statistical analysis

To ensure the absence of artifacts, bands were carefully selected from replicated amplifications. Amplified bands designated by their primer code and their size in base pairs. Data recorded as discrete variables: 1 for the presence and 0 for the absence of a similar band. Only intense and reproducible bands appearing on the gel were scored. Comparison of genotypes was carried out based on the presence or absence of fragment produced by RAPD PCR amplification. '1' was designated for presence of fragments and '0' for the absence of fragments. Pair wise Jaccard's similarity coefficients (Jaccard 1908) were calculated. Cluster analysis was performed for the molecular data based on the unweighted pair-group method with arithmetic mean (UPGMA) method (Sneath and Sokal 1973).

Results and discussion

Isolation and partial identification of isolated strains

Seventy two microbial strains representing different colony morphologies observed with the ability to grow on NA medium were isolated and microscopically examined. These include 50 (69.4%) isolates as non-spore forming bacteria, 10 (13.9%) as yeasts and 12 (16.7%) as spore forming bacteria. Non-spore forming bacteria and yeast isolates were not further used. This study was therefore limited to examination of the spore forming isolates as biological control agents against *B. tabaci*.

Based on tabulated data in Table (2), the twelve selected spore formers were aerobic, motile, gram positive rods, catalase positive, indol negative, and can utilize glucose, xylose, maltose, sucrose, cellobiose, galactose, starch and mannitol as carbon and energy sources but they can not utilize Tween 80. This partial identification characterizes them as members of the genus *bacillus* (Krieg and Holt 1984). They were named A1, A2, A3, A6, A7, A9, A12, A13, A107, B37, B45 and B100.. These results are in good agreement with those obtained by Frederiksen et al. (2006) and Hanafi et al. (2007) who reported that Bacilli species were among the most effective microorganisms against various agricultural pest.

Insecticide activity of bacterial isolates against B. tabaci

Nymphs of *B. tabaci*, in the 2nd instar, were sprayed with the bacterial isolates; $9.6X10^7$ spores/ml each. Similar treatment, sprayed with distilled water, was used as a control for correcting mortality values. Cumulative mortality percentages were recorded after 2,4,6,8 and 10 days of treatment. As shown in Table (3), recorded mortality percentages were correlated with type of isolate. The highest percentages were found for isolates A1 and A9 with a gradual increase over inspection period recording the highest value of 92.2% on day 10 for isolate A1 and 90.2% for isolate A9. On the contrary, isolate A7 recorded the lowest percentages being 18.3%. Values in-between were observed for the other isolates with descending order for isolates A2, A12, A3, A13, B100, B37, A6, A107, B45 and A7. Such outcome was in agreement with the results of Hanafi et al. (2007) who found that incubation of tomato plant with *Bacillus subtilis* (strain PGPR) was shown to lead to a significantly lower survival of *B. tabaci*'s nymphs and pupae. More recently, Valenzuela-Soto et al. (2010) proposed that *B. subtilis*, strain BEB-DN, generates an induced systemic resistance (ISR) response that retarded the nymphal development of virus free *B. tabaci*, which appeared to be a combination of the well-known jasmonic acid (JA) dependent and JA-independent responses (Zarate et al. 2007).

RAPD-PCR fingerprinting of Bacillus isolates

The DNA of twelve *Bacillus* isolates was used as templates for four RAPD primers. The RAPD-PCR results, using primer OP-A07 are illustrated in Figure (1-A). The molecular size of 9 PCR products ranged from 100 - 800bp, and showed 100% polymorphism among the 12 *Bacillus* isolates. A3 and A107 isolates showed a unique PCR fragment of 500bp. By using primer (OP-A15), a total of eleven bands among the 12 used Bacilli isolates were found (Figure 1-B and Table 2). The molecular size of the 11 PCR products ranged from 300 - 2000bp. 100% of the fragments

has shown polymorphism among the 12 isolates. This primer has showed a specific PCR product of 2000bp with the A13 isolate. A total of fourteen bands in the 12 isolates with primers OP-B03 are shown in Figure (1-C). The molecular size of 14 PCR products ranged from 100 - 1000bp fragments. Two common bands were observed in all Bacilli isolates, and exhibited almost 14% monomorphism, while the other 12 fragments have shown around 86% polymorphism among isolates. This primer has produced a 1000bp fragment specific to isolate A7.

The RAPD-PCR reaction with primer OP-B15 is depicted in Figure (1-D). Fourteen PCR products ranged from 50 - 1000bp and exhibiting 100% polymorphism were found. This primer is specific to A45 isolate. Among 13 PCR products with molecular size ranged between 200 – 2100bp produced by primer OP-B16 (Figure 1-E). Among them, a 300bp fragment is specific to isolate A 107. Figure (1-F) shows 15 PCR fragments ranging between 100-2000bp with 100% polymorphism produced by primer OP-B17. With primer OP-B18, 20 PCR fragments were produced with molecular size ranging between 100 – 1600bp (Figure 1-G). This primer has shown specific product for each of isolate B45 and B100. Primer OP-C11 produced 11 PCR fragments with molecular sizes between 300-1600bp (Figure 1-H). One out of the 11 fragments was common among the isolates and has shown more than 9 % monomorphism, while the other 10 fragments have recorded around 90% polymorphism. Figure (1-I) shows PCR fragments ranged from 300 – 1000bp and produced by primer OP-O02 which is specific to isolate B-45. Finally, PCR fragments with molecular size ranged from 300 – 700bp and produced by primer OP-O16 are illustrated in Figure (1-J). It was found that 100% of PCR fragments with the two primers were monomorphic.

ISSR-PCR fingerprinting of Bacillus isolates

The twelve *Bacillus* isolates were used as templates for four ISSR primers. The ISSR-PCR results, using primer (IS-1) are illustrated in Figure (2-A). The molecular size of ten PCR products ranged from 150 - 1100bp. All the produced fragments were polymorphic among the twelve isolates. Reaction of primer (IS-3) showed a total of seven bands among the twelve *Bacillus* isolates, at molecular size ranging between 250 - 1200bp are illustrated in Figure (2-B). Four out of the 7 fragments were polymorphic among the twelve isolates, revealing 57% polymorphism. This primer was very important in our study, because isolates A7 and A9, exhibited a unique product with molecular size 1200bp. The results of ISSR-PCR with primer IS-4 are depicted in Figure (2-C). The molecular size of these products ranged from 250 - 900bp. The primers gave five polymorphic bands, recording 100% polymorphism among the isolates. This primer was important in our study, because it showed a specific product for A3 and B100 isolates. Nine bands in the used isolates with

primer IS-5 are illustrated in Figure (2-D), at molecular size ranging between 200 - 900bp. Seven out of the nine fragments were polymorphic among the isolates, exhibiting 78% polymorphism.

Genetic relationships as revealed by ISSR and RAPD markers

The ISSRs in this study showed a genetic similarity ranged from 51 - 96 %. The highest genetic similarity revealed by the ISSR analysis was 96% between *Bacillus* isolates A3 and A13. On the other hand, the lowest genetic similarity was 51% between isolates A107 and A13. The RAPD's product showed a genetic similarity ranged from 45 - 91%. The highest genetic similarity revealed by the RAPD analysis was 91% between *Bacillus* isolates A3 and A13. On the other hand, the lowest genetic similarity was 45% between isolates A107 and A13. The RAPD's data have confirmed the ISSR's data, when both data have combined together. A genetic similarity ranged from 48 - 94% (Table 3).The highest genetic similarity was 94% between Bacilli isolates A3 and A13. On the other hand, the lowest genetic similarity was 48% between isolates A107 and A13.

Cluster analysis as revealed by combined RAPD and ISSR markers

Molecular methods have emerged as the most rapid reliable and simple alternatives to characterize and differentiate microorganisms (Rademarker et al 2005). Genotyping of bacteria using PCR based methods, such as RAPD analysis is a commonly used approach for strain typing because it is economic, fast and simple (Vilas-Bôas and Lemos 2004). These methods also allow distinguishing isolates from commercial strains of *B. thuringiensis* (Frederiksen et al. 2006). The combination of classical and molecular methods helps us to group the isolates and give more details than a single classification method. The bioinformatic analysis of the RAPD fingerprints using the most discriminatory primers helped to differentiate the native isolates from the used control strains. The genetic diversity between the isolates is evident from the dendrogram generated using the UPGMA method (Sneath, and Sokal, 1973) (Figure 3). The combined ISSR and RAPD data has generated a phylogenetic tree with two clusters, at 53 % similarity level, separating the strong antagonists (cluster I) from the rest of the isolates, sharing a similarity coefficient around 58 %. This cluster contains three sub clusters with genetic similarity of more than 60%. The 1st sub cluster contains four bacterial isolates A1, A2, A9, and A12 sharing a similarity around 73 %. The second sub cluster contains three isolates A3, A13 and B100 sharing a similarity coefficient of 84 %, and the 3rd contains two isolates A6 and B37 with similarity of around 62 %. Two *Bacillus* isolates, A7 and B45 with a high similarity level of 81% found to be cluster II. Finally the *Bacillus* isolate A107 was falling outside the two major clusters with dissimilarities of 55 %.

16S rRNA expression in different Bacillus isolates

In this analysis, we have aligned the sequences of different *Bacillus* species and *E. coli* 16S rRNA genes together, in order to design a pair of primers only specific to different *Bacillus* species 16S rRNA gene. The genomic DNA of the twelve isolates A1; A2; A3; A6; A7; A9; A12; A13; A107; B37; B45 and B100 has been used as a template for the *Bacillus* specific primers. This experiment was designed in order to make sure that the twelve isolates belong to the same Bacillus genus. All the twelve isolates showed a reproducible fragment of 200 bp belong to16S rRNA gene (Figure 4). A negative PCR reaction contains *E. coli* DNA as a template. This experiment was repeated twice to confirm the results.

From the biological control results, four different *Bacilli* isolates (A1, A7, A12 and B100), with varying efficacies were selected in order to confirm the phylogenetic tree analysis. 16S rDNA sequence of A1 isolate showed 100% identity with *B. thuringiensis*, while that of A7 isolate showed 95.7% identity with *B. cereus*. Also B100 isolate recorded 95.6% identity with *B. sphaericus*. Moreover, A12 isolate recorded 100% identity with *B. thuringiensis*. The 16S rDNA sequence analysis of different Bacillus isolates A1, A7, A12 and B100 confirmed both biological control results and phylogenetic analysis, because isolates A1 and A12 sequences showed high mortality percentage and they were falling in the same subcluster. At the same time both isolates recorded 100% identity with *B. thuringiensis*. Moreover, isolates B100 and A7 showed moderate and lower mortality percentage, as expected from *B. sphaericus* and *B. cereus*, respectively.

Conclusion:

It was found that sequencing analysis of four of the most effective bacterial isolates, A1, A2, A9 and A12, against whitefly *B. tabaci* have recorded high percent of identity with different Bacillus species, and therefore confirmed the biological control experiment and phylogenetic tree analysis. Analysis of the data revealed that *Bacillus* thuringiensis was the most predominant species in the region under study followed by *Bacillus sphaericus* and *Bacillus cereus.* Work in progress in order to optimize cultural, nutritional, environmental and operational parameters needed for a successful large-scale production of the most effective isolated *Bacillus* strain(s).

Table 1. RAPD and ISSR primers.

No	Primer	Sequences
1	OP-A07	5’-GAAACGGGTG-3’
2	OP-A15	5’-TTCCGAACCC-3’
3	OP-B03	5’-CATCCCCCTG-3’
4	OP-B15	5’-GGAGGGTGTT-3’
5	OP-B16	5’-TTTGCCCGGA-3’
6	OP-B17	5’-AGGGAACGAG-3’
7	OP-B18	5’-CCACAGCAGT-3’
8	OP-C11	5’-AAAGCTGCGG-3’
9	OP-O02	5’-ACGTAGCGTC-3’
10	OP-O16	5’-TCGGCGGTTC-3’
1	IS-1	5’-TAT$(CA)_7$C-3’
2	IS-3	5’-TTT$(TCC)_5$-3’
3	IS-4	5’-CAT$(CA)_7$T-3’
4	IS-5	5’-$(GA)_8$CG-3’

Table 2. Morphological and biochemical characteristics of local bacterial isolates.

Characteristics	Results	characteristics	Results
A-Morphological		C-Utilization of:	
Colony	Cream, big and spreading.	- Glucose	+
Gram stain	Positive rods.	- Mannose	+
Mortality	Extreme motile.	- Xylose	+
		- Cellobiose	+
B-Biochemical		- Galactose	+
Catalase reaction	+	- Starch	+
Indol	-	- Sucrose	+
		- Maltose	+
		- Mannitol	+
		- Tween 80	-

Table (3): Cumulative mortality percentages of *S. littoralis* larvae (2nd and 4th instars) recorded at different periods after treatment with *Bacillus* isolates

Age of instars	Post-treatment (Days)	*Bacillus* isolates Mortality %											
		A_1	A_2	A_3	A_6	A_7	A_9	A_{12}	A_{13}	A_{107}	B_{37}	B_{45}	B_{100}
2nd	1	13.3	6.67	3.33	0.0	0.0	16.67	10.	6.67	0.0	0.0	3.3	6.67
	2	23.3	10	6..67	3.33	3.33	20	13.33	10	6.67	3.33	6.67	20
	3	53.3	30	26.67	16.67	10	46.67	23.33	20	13.33	10	10	40
	4	66.67	43.3	40	20	13.3	63.3	33.33	36.67	16.67	16.67	13.33	43.33
	5	76.67	60	53.3	23.33	16.67	73.33	46.67	43.33	20	20	20	53.33
	6	83.83	66.67	56.67	26.67	16.67	76.67	63.33	53.33	23.33	26.67	20	53.33
	7	90	73.3	63.33	26.67	20	83.33	70	60	26.7	30	23.3	56.67
4th	1	6.67	0.0	0.0	0.0	0.0	6.67	3.33	0.0	0.0	0.0	0.0	3.3
	2	13.3	3.33	6.67	0.0	0.0	10	6.67	3.33	0.0	3.33	0.0	10
	3	40	23.3	20	3.33	3.33	33.33	16.67	10	6.67	6.67	3.33	26.67
	4	46.67	36.67	30	16.67	6.67	56.67	20	30	10	10	6.67	30
	5	63.33	46.67	43.33	16.67	10	60	36.67	33.33	16.67	13.33	10	40
	6	73.33	60	50	20	13.3	66.67	40	36.67	16.67	20	13.33	43.33
	7	80	60	53.33	20	13.3	70	56.67	43.33	20	23	16.7	46.67

Table (4): Effect of bacterial isolates A_1 and A_9 on fecundity and longevity of *S. littoralis* adults.

Treatment	Pre-oviposition period	Day	No. of eggs/female (Mean± SE)	Reduction%	Longevity
Control	1.2±0.379	1	150±0.189	0.00	11.6±1.07
		2	209±1.93		
		3	266±0.07		
		4	178±0.79		
		5	191±1.91		
		6	163±0.77		
		7	150±0.07		
		8	133.6±0.89		
		9	58.5±1.30		
		10	00.0±0		
A_1	2.9±0.107*	1	66.6±0.861**	55.6±1.32**	6.0±0.958**
		2	93.3±0.99**	55.36±1.20**	
		3	110±0.94**	58.86±0.85**	
		4	Dead	Dead	
A_9	2.2±0.081*	1	101±0.321**	32.67±0.67**	7.3±0.99*
		2	160±0.99**	23.44±1.37**	
		3	150±0.95**	43.61±0.19**	
		4	90±0.861**	49.44±1.89**	
		5	113±0.25**	40.84±0.951**	
		6	Dead	Dead	

**Highly significant

*Significant

Table (5): Effect of bacterial isolates A_1 and A_9 on the development of *S. littoralis* larvae.

Treatment	Larval instars	Day	Mortality% (Mean± SE)
Control	1st	1	0.0
		2	
		3	
	2nd	1	0.0
		2	
		3	
	3rd	1	0.0
		2	
	4th	1	0.0
		2	
	5th	1	0.0
		2	
	6th	1	0.0
		2	
A_1	1st	1	0.0
		2	6.7±0.81*
		3	13.3±0.35*
		4	20. ±1.02*
	2nd	1	24.4±1.89*
		2	36.9±0.915*
		3	63.3±1.19**
		4	66.7 ±0.69**
		5	73.3±1.171**
		6	80.8±± 0.97**
		7	86.67±1.071**
		8	Dead
A_9	1st	1	0.0
		2	0.0
		3	6.7±0.12*
	2nd	1	10.0 ±0.25*
		2	16.7±0.85*
		3	30.0 ±0.55*
		4	43.3±0.18**
		5	46.7±1.77**
		6	63.3±0.90**
	3rd	1	66.7±0.85**
		2	70.0 ±0.07**
		3	90.0 ±0.95**
		4	93.3±0.96***
		5	Dead

**Highly significant

*Significant

Table 6. Genetic similarity (GS) matrices computed according to Dice coefficient from the combined RAPD and ISSR marker of the twelve *bacillus* isolates (A1, A2, A3, A6, A7, A9, A12, A13, A107, B37, B45 AND B100).

	A1	A2	A3	A6	A7	A9	A12	A13	A107	B37	B45	B100
A1	100	0.88	0.92	0.62	0.68	0.74	0.73	0.79	0.54	0.53	0.68	0.80
A2		100	0.91	0.61	0.67	0.75	0.74	0.78	0.53	0.51	0.67	0.79
A3			100	0.58	0.69	0.72	0.80	0.94	0.49	0.50	0.57	0.84
A6				100	0.70	0.62	0.63	0.75	0.58	0.63	0.51	0.51
A7					100	0.73	0.62	0.72	0.64	0.70	0.81	0.57
A9						100	0.76	0.73	0.60	0.67	0.58	0.74
A12							100	0.71	.59	0.61	0.56	0.75
A13								100	0.48	0.78	0.58	0.84
A107									100	0.72	0.54	0.53
B37										100	0.61	0.66
B45											100	0.72
B100												100

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