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Genetic Improvement of Some Microorganisms That Naturally Colonize of Tomato Plants to Increase the Effect of Bio-Control on *Tuta absoluta*.

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ABSTRACT

Four Bacillus bacterial strains were used in this study. One strain of *Bacillus thuringienesis* strain (Bt) were used as a cry 1Ab donor. Two bacillus strains that naturally colonized on tomato plant were used as recipients; *Bacillus Subtillus subsp. subtilis* strain (Bs1) and *Bacillus licheniforms* strain (Bl). Genetic marker test was carried using eleven antibiotics on the previous strains. Two attempts of protoplast fusion technique were done, the first was (Bt::Bs1) and the second (Bt::Bl). Fifty sex of fusants products were obtained, 26 and 30 for the first and second attempt, respectively. Twenty strongest growths (10 of each attempt) out of 56 fusants products were chosen for bioassay treatments. For mortality under lab conditions, obtained results showed as follow; for the first attempt, the best Fusan time was 40 min that was registered high mortality percentage of tomato leaf miner ranged from 74 to 100% for fusants product F7, F8 and F9 but fusants products of F10 was recorded down the same results of the Bt parent 65% larval mortality. For second attempt, the accumulative larval mortality were notation the highest percentage reach to 100 in case of treatment by fusants product F19 follow by F20, F13 were reported 87 and 72% larval mortality compare to their parent Bt and Bl were recorded 65 and 57 % larval mortality respectively. Under semi-filed conditions, the obtained results showed that all the chosen fusants products able to tolerate sunray from two to three days and caused mortality to tomato leaf miner larvae reached to more than 80% compare to their parent.

Keywords: *B. thuringiensis*, *Tuta absoluta*, Protoplast fusion, mortality, persistence

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INTRODUCTION

In recent years, the need for environmentally safe pesticides has encouraged the replacement of these chemicals with biological approaches, which are friendlier to the environment. Strategies are being developed to control a variety of phytopathogenic agents including the development of transgenic plants expressing d-endotoxin of B. thuringiensis. This has led to the production of insect resistant

Bt-transformed lines of tobacco, cotton, corn, potatoes, maize, tomatoes and others [1]. Cry genes have been transferred into other plant-associated microorganisms to improve stability and efficacy, obtaining maximum insect control on aerial and subterranean surfaces of plants [2,3]

The tomato borer, *Tutaabsoluta* (Meyrick), is a neotropical oligophagous insect, which attacks solanaceous crops. Since the 1960s it has become one of the key pests of tomato crop in many South American countries [4,5 and6]and recorded in Egypt in the middle of 2009.

Economic importance of Tomato moth, *Tuta absoluta* (Meyrick):

Tomato crop considered one of the most important horticultural crops in the world. About 130 million tons of tomatoes were produced in the world in 2008. China was the largest producer by about 34 million tons while Egypt comes in the fifth order by about 10 million tons. (FAO, 2010 from free Wikipedia) In Egypt, about seventy percent of the production destination is for consumption and the rest is industrialized.

consumption brings the highest gross financial return to the farmers in Egypt *Tuta absoluta* is a very harmful leaf mining moth with a strong preference for tomatoes. It also occurs on eggplants, sweet peppers as well as potatoes and various other cultivated plants. It also occurs on weeds of Solanaceae family (Solanum nigrum, Datura spp.). Larvae can damage tomato plants during all growth stages, producing large galleries in their leaves, burrowing stalks, apical buds, green and ripe fruits. [7,6] .It can cause important yield losses in different production regions and under diverse production systems severely attacked tomato fruits lose their commercial value. 50–100% losses have been reported on tomato (mainly under low rainfall) and its presence may also limit the export of the product to several destinations [7].

The main host of *T. absoluta* is tomato, but potato is also reported as a host [8] together with *Lycopersicon hirsutum* ,*Solanum lyratum* and various wild solanaceous species such as *Solanum nigrum* ,*Solanum elaeagnifolium* , *Solanum puberulum* , *Datura stramonium* , *Datura ferox* and *Nicotiana glauca* . In laboratory studies [9], aubergine was reported as a potential host (with other solanaceous species), but there are no references to its importance in the field. There is an old record of tobacco being attacked in Argentina [10].

The *tuta absoluta* tomato borer (Meyrick) (Lepidoptera:Gelechiidae) is originally from South America, where it is considered one of the most devastating pests of tomato crops [11]. This pest is present throughout the crop growing cycle. Larvae can infest leaves, flowers, stems and fruits, causing important losses in tomatoes [12]. From the end of 2006 to the end of 2009 *Tutaabsoluta* was detected in the Mediterranean Basin, the most common control practice has been based on the use of chemical insecticides [13].

However, these treatments may disrupt the existing integrated pest management programs [14] in since pesticides may have effects on natural enemies [15] and they may lead to resistance [13], as

documented in the area of origin of this pest [16].Therefore, implementation of environmentally safe measures to manage *T. absoluta* while limiting the use of chemical insecticides is urgently necessary.

As an alternative to chemicals, commercial formulations based on *Bacillus thuringiensis* (Berliner) have been used to control insect pests for decades. These formulations are used because they are environmentally friendly, harmless to humans and other vertebrates, and highly compatible with the use of natural enemies [17,18]. Furthermore, they are recommended for pre-harvest treatments and in cases where the insect populations have developed resistance to other products. However, few studies have evaluated the efficacy of *B. thuringiensis* treatments on *T. absoluta* and most of these studies were performed in the region of origin of this pest [19,20 and 21]. In Spain, commercially available formulations based on *B. thuringiensis* have been tested against *T. absoluta* in laboratory, greenhouse and open staked tomato field experiments [21]. These studies demonstrated that *B. thuringiensis* can be highly efficient in controlling *T. absoluta*, with a reduction in damage closeto 90% when sprayed regularly at 90.4 MIU l-1 (millions of international units per liter). First-instar larvae were the most susceptible [23].

The previous studies have demonstrated that first instar larvae of *T.absoluta* are highly susceptible to *Bacillus thuringiensis* (Bt) treatments. Hence, the main objective of this work is to increase the effectiveness of biological control against the lepidoptera insects specially *Tuta absoluta*up for the production of toxins resistance to these pests damned using the methods of biotechnology to produce ge not ypesnew from some bacteria that grow on the outer surface of the shoots and roots of the plants under study and have the ability to produce toxins with the same efficiency of the original bacteria [24].

This study aims is to increase the effectiveness of biological control against the lepidoptera insects specially; *Tuta absoluta, S. littoralis and Phthorimaea operculella.* Up for the production of toxins resistance to these pests damned using the methods of biotechnology to produce genotypes new from some bacteria that grow on the outer surface of the shoots and roots of the plants under study and have the ability to produce toxins with the same efficiency of the original bacteria.

MATERIALS AND METHODS

a-Strains:

Four bacillus bacterial strains were used in this work as follow:

1-*Bacillus thuringienesis*strain (Bt)was obtained from Agriculture and Genetic Engineering Research Institute (AGERI), Giza, Egypt.
2-*Bacillus Subtillus subsp. subtilis* strain (Bs1)was obtained from American Type Collection Center (ATCC), Pro Bio Gem laboratory, University of Lille France.
3-*Bacillus licheniforms* strain (Bl) was obtained from Microbial Genetic dept. type collection center, National Research Center, Dokky, Giza, Egypt.
4- *Bacillus subtilis subsp. spizizenii* strain (Bs2) was obtained from Genetic Engineering Center, Genetic Dept., Fac. of Agric., Ain Shams Univ.
Definition and molecular characterization were done for the previous strains [25].

b- Antibiotics:

Eleven antibiotics with the following concentrations (µg/ml) were used when required (Table 1).

Table1: The antibiotics used in this study and their concentrations.

No	Antibiotic	Final concentration (µg/ml)
1	Chloramphenicol (Cm)	35µg
2	Streptomycin (Sm)	200µg
3	Ampicillin (Amp)	100µg
4	Rifampicin (Rif)	100µg
5	Kanamycin (Km)	40µg
6	Tetracycline (Tc)	15µg
7	Neomycin (Nm)	40µg
8	Erythromycin (Erm)	20µg
9	Amikacine (Amk)	15µg
10	Polymyxin (Pol)	50µg
11	Gentamicin (Gm)	15µg

Antimicrobial susceptibility:

The four bacterial strains were grown on LB medium at 300C overnight with shaking and their antimicrobial susceptibility to the eleven antibiotics used in this study (Table 1) were tested using National Committee for Clinical Laboratory [26].

Protoplast fusion technique:

Protoplast fusion of four parent strains; Bt(main parent) and each of Bs1, Bl and Bs2 were carried out according to the method described[27,28].

Protoplast preparation:

Cultivation of the four parent strains were carried out in 500 ml flasks containing 70 ml of LB medium. Flasks were incubated for 24 hr. at 30 °C with shaking at 120 rpm. Cells were harvested at the mid- point of the log phase by centrifugation at 5000 rpm for 10 min and washed once with 1% N-laurylsarcosine.

This was followed by washing three times with osmotic stabilizer buffer. The bacterial cells were then pelleted by centrifugation. Lysozyme was dissolved in osmotic stabilizer buffer to a final concentration of 15 mg/ml, sterilized by 0.2µm Millipore filter.

Lysozyme was then, and added to the cell pellets at final concentration of 1/10th the volume and mixed thoroughly to make the suspension. The resulting mixture was incubated at 37oC for 4h. The viable protoplast was counted by spreading appropriate dilution onto LB medium solidified by adding 2% agar, where all in viable protoplasts were lysed and only the intact protoplast will grow after incubation.

Regeneration of protoplast:

The protoplasts in the reaction mixture were collected by centrifugation at 3000 rpm for 10 min. The precipitate was washed with Tris-HCl buffer with an osmotic stabilizer and the resulting precipitate was re-suspended in the same buffer. Protoplast suspension was diluted and overlaid on the LB medium solidified by adding 2% agar.

Protoplast fusion:

Aliquots of the parental protoplasts (1.0 ml each) were mixed in the presence of 25% PEG 6000 and 100 mM CaCl2 [29] and incubated at 30 °C. Aliquots of 100 µl from the mixture were taken at 10 min intervals and diluted 10 times in osmotic stabilizer. A sample of 100 µl was added to selective-soft–agar medium and over layed on the same selective basal medium to screen the fusants.

Bioassay treatments:

Insect rearing:

The culture was provided by infested tomato leaves harboring *T. absoluta* pre-imagine stages which collected from the field for isolation of eggs or outer larvae and pupae to maintain it. Newly emerged adults were collected and transferred to another glass cage (50x50x50 cm^3) containing untreated plastic pots of tomato after that, the adult stages were collected by Aspirator and released on other glass cages in which plastic pot of planted tomato was placed. The deposited eggs and hatched larvae were maintained on tomato leaves. The experiments were carried out on the 1st generation of tomato leaf miner [30].

The effect of new bacterial strains on 1st instar larvae of *T. absoluta*:

To evaluate efficacy of new bacterial strains, fusants products, the experiments were carried at two levels of conditions;

Under laboratory conditions:

The tomato leaflets were collected from tomato plants and washing well; after that they were separately dunked inside prepared suspension solution of bacterial strains and then left to dryness in air for 15secand. Each treatment was placed in Petri dish (15 cm.) which provided with wet filter paper. In each Petri dish was liberated 6 individuals from 1st instar larvae. Each test was replicated twenty time and calculated % mortality of larval stage (after 3 day and 7 days), and percentage of pupal and adult malformation. The mortality percent in treatments was corrected according to Abbott's formula [31].

Mortality %= (T-C)/(100-C) X100

Under semi-field condition:

To test efficacy of new bacterial strains in open area, the tomato sapling was plantation on pots (20cm.) at isolated area in National Research Center (Fig.1). After two weeks of plantation was sprayed tomato leaflets with prepared suspension solution of bacteria. Periodically (1, 2, 3….one week, etc.) was taken leaflets from planted tomatoes and placed in Petri dishes (15 cm.) and liberated 6 individuals from 1st instar larvae. Each test was replicated twenty time and calculated % mortality of larval stage.

Fig 1: semi-field experiment to test persistence efficacy of new bacterial strains

RESULTS & DISSECTION

Bacillus thuringiensis (Bt) is a microbial pesticide widely used to control crop pests. Its strains have good biocontrol activity against crop insect pest, but lack some desirable characteristics that are found in *Bacillus subtilis*.

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1- If all strains were wiled type?

The four parent strains which used in this study; *Bt* (main parent) and each of *Bs1, Bl and Bs2* growing (plating) on minimal media (Table 2).

Table2: The four wiled type strains plating on minimal media (mm)

Strain	Bt	Bs1	Bl	Bs2
Plating on mm	+	+	+	+

2- Antibiotic resistance patterns of the four parent strains

The antibiotic resistance patterns of the Bt(main parent) and each of Bs1, Bl and Bs2 are presented in Table (3). All the four strains were resistant to Streptomycin (Sm) while, they are sensitive to three different antibiotics; Kanamycin (Km), Neomycin (Nm) and Gentamic in (Gm). The main parent Bt was resistant to Polymyxin (Pol), while the other three parent strains sensitive to it. On the contrary, the main parent Bt was sensitive to Polymyxin (Pol), while the other three parent strains resistant to it. The two strains; Bt and Bs were resistant to Rifampicin (Rif) and Amikacine (Amk), while the other two parents; Bl and Bs2 sensitive to them.

The four parent strains were sensitive to Erythromycin (Erm) except strain Bs, while they resist to Ampicillin (Amp) except strain Bs2. The two strains; Bs1 and Bl were resisting to Tetracycline (Tc), while the other strains; Bt and Bs2 sensitive to sensitive to the rest of the tested antibiotics (Table 3).

Table3: Antibiotic resistance patterns of the four strains; *Bt* (main parent) and each of *Bs1, Bl and Bs2.*

Strain	Tc	Km	Amk	Cm	Sm	Erm	Rif	Amp	Nm	Pol	Gm
Bt	-	-	+	-	+	-	+	+	-	+	-
Bs1	+	-	+	+	+	+	+	+	-	-	-
Bl	+	-	-	+	+	-	-	+	-	-	-
Bs2	-	-	-	+	+	-	-	-	-	-	-

+ Resistant - Sensitive

Antibiotic resistance patterns were done to draw genetic marker map of the four parent strains. Antibiotic resistance pattern was used as selectable markers [32], who used a medium containing rifampicin and tetracycline to select the recombinants of the fused protoplasts of *B. thuringiensis* var. *galleriae* and *B. cereus* carrying the plasmid pBC16 responsible for resistance to tetracycline.

In the same manner, fusion between *Bacillus cereus* protoplasts resulted in isolation of hybrid cells having acquired Tcr phenotype (harboring pBC16) with high frequencies [33].

3- Genetic construction of new strain(s), Protoplast fusion technique:

An attempts has been made to combine those desirable characteristics; we used a highly effective biocontrol strain of *B. thuringiensis* in protoplast fusions with a strain of *B. subtilis*. The fusants were identified through cell culture and stained with crystal violet. The Bt and *B. subtilis* protoplasts were induced to fuse by PEG 6000.Protoplast fusion technique used to produce genetically modified microorganisms (new strains) produce cry proteins and can naturally colonize the phylloplane of host plants to increase the effect of bio-control on pests.

Three attempts of protoplast fusion technique used between the four selected parent strains. Strain *Bt* used as a main parent (one side) and the rest three parent strains; Bs1, Bl and Bs2 (other side).

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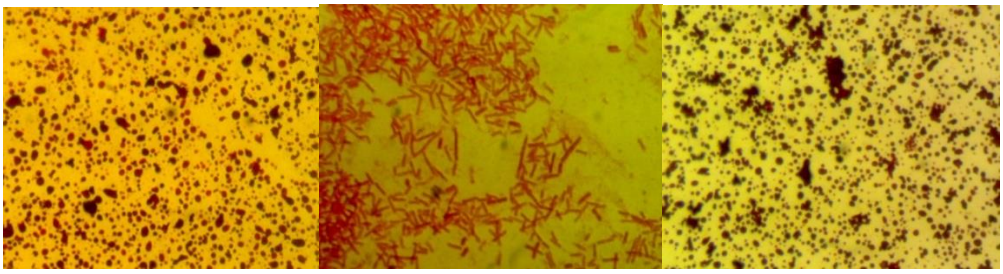
3.1-First attempt, Protoplast fusion between (*Bt*) and(*Bs1*) strains:

The two strains; *Bt* and *Bs1* were selected for first protoplast fusion attempt according to their differences in their antibiotic resistance patterns. The *Bt* strains were resistant to Amk^r, & Pol^r and sensitive to Tc^s, Cm^s and Erm^s. While the *Bs1* strain was resistant to Tc^r, Cm^r& Erm^r and sensitive to Amk^s& Pol^s as shown in Table (3). Such difference in the antibiotic selectable markers between the two parental strains *Bt* and *Bs1* were used (with illustrated concentrations in Table 1) to select the desirable fusants by adding the above five antibiotics to the selective medium. Only the fusant strains combined the antibiotic resistance patterns of their corresponding two parental strains.

Protoplast formation of the two selected strains; *Bt* and *Bs1* were observed periodically by microscopic examinations shown in Fig (2). Aliquots (1.0 ml each) of the parental protoplasts were mixed in the presence of 25% PEG 6000 and 100 mM $CaCl_2$ [29] and incubated at 30 °C. Aliquots of 100 µl from the mixture were taken at 10 min intervals and diluted 10 times in protoplast buffer. During the two days of fusion time, a total of 26 (Bt::Bs) fusants were produced on the selective medium (LB medium supplemented with the previous five antibiotics; Tc, Amk, Cm, Rif and Pol. Fourteen out of theses fusants were produced during the first day at 10min intervals. Three fusants were produced after 10 min, five fusants after 20 min, two fusants after 40 min, 1 fusants after 50min and 3 fusants after 60min. The remaining fusants (12 fusants) were produced next day (after 16h). 5 fusants were produced after 10 min, 3 fusants after 20 min, as shown in Tables (6 and 7) 2 fusants after 30 min and 2 fusants after 40 min.



A-B **A1** **A2**

Fig2: Photomicrographs showing the protoplast of the two parental strains; *B. thuringienesis kuristaki*, before and after protoplasting (A1 and 2) and *B subtillussubsp.subtillus*I68 (B) as revealed by the light microscope

3.2- Second attempt, Protoplast fusion between (*Bt*) and (*Bl*) strains

The two strains; *Bt* and *Bs* were selected for second protoplast fusion attempt according to their differences in their antibiotic resistance patterns. The *Bt* strains were resistant to Amk^r, Rif^r& Pol^r and sensitive to Tc^s& Cm^s. While the *Bl* strain was resistant to Tc^r& Cm^r and sensitive to Amk^s, Rif^s, Pol^s& Gm^s as shown in Table (3). Such difference in the antibiotic selectable markers between the two parental strains *Bt* and *Bl* were used (with illustrated concentrations in Table 1) to select the desirable fusants by adding the above five antibiotics to the selective medium. Only the fusants strains combined the antibiotic resistance patterns of their corresponding two parental strains.

Protoplast formation of the two selected strains, *Bt* and *Bl* were observed periodically by microscopic examination(Fig.3). Aliquots (1.0 ml each) of the parental protoplasts were mixed in the presence of 25% PEG 6000 and 100 mM $CaCl_2$ (Hopwood *et al.,* 1985) and incubated at 30 °C. Aliquots of 100 µl from the mixture were taken at 10 min intervals and diluted 10 times in protoplasting buffer. During the two days of fusion time, a total of 30 Bt::Bl fusants were produced on selective medium containing the previous five antibiotics; TC, Amk, Cm, Pol and Rif. These fusants were produced during the first day at 10min intervals. Of these, 11 fusants were produced after 10 min, 5 fusants after 20 min, 3 fusants after 30min and 2 fusants after 60min. The residual fusants (9 fusants) were produced next day (after 16h) , where, four fusants was produced after 30 min, two fusants after 40 min and 3 fusants after 60 min.

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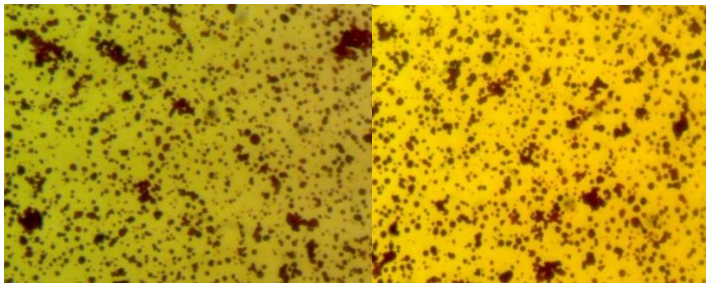

(A) *B. licheniformus* **(B) *B. thuringienesis kuristaki***

Fig3: Photomicrographs showing the protoplast of the two parental strains; *B. licheniformus*(A) and *B. thuringienesis kuristaki* (B) as revealed by the light microscope.

In conclusion, the obtained results agreed with the results described before [34], who successfully integrated into two genes of *cyt1Aa* and *cry11Aa* using the transposon Tn917 of the *Bacillus thuringiensis* strain S184 that was toxic against the third instar larvae of *Spodoptera littoralis*, yielding the primary engineered strain TnX. The strain TnX revealed high toxicity to the third instar larvae of *C. pipiensfatigans* 1.82-fold higher than that of *B. thuringiensis* subsp. *israelensis*, but expressed low toxicicity to Lepidopteran larvae. By using protoplast fusion of the strain TnX and the strain S184-Tet^r (resistant to tetracycline), the target engineered strain TnY was obtained. This strain (TnY) showed increased toxicity against the third instar larvae of *S. littoralis* by 2.08-fold in comparison with the parental strain S184. Meanwhile, the comparison of δ-endotoxin concentration between *B. thuringiensis* spp. *israelensis* (H14) fusion and the wild type strains showed that the new fusants had 1.48 time more toxins than the wild type [27].

4 Bioassay treatment.

During the last five decades, chemical synthetic pesticides have been used extensively to kill pests and protect crops. Use of synthetic pesticide has caused some unfortunate consequences like environmental pollution, pest resistance and toxicity to other non-target organisms. Due to hazardous effects of chemical residues to human and animal health, several studies have been carried out to determine the most effective control methods without using insecticides [35]. One of the latest discovered methods is the use of the natural enemies of the insects such as bacteria virus and fungi to biocontrol insect pest [36,37]. Entomopathogenic bacteria have unique insecticidal properties and it is mainly due to the production during sporulation of larvicidal proteins that accumulate as parasporal crystalline inclusions within the cell. The insecticidal bacterium Bacillus thuringiensis (Bt) has been widely used in agriculture for the control of pest insects in crops [38].

Insecticidal proteins from Bt are routinely fabricated on a large scale for commercial purposes [39]. The selected Bt strains were effectively against insects like Spodoptera frugiperda, Anticarsia gemmatalis (Hübner) and Anthonomus grandis (Boheman), and also the Bacillus spp. and its by-products are effective to plant pathogenic diseases [40,41 and42]. All the Bt strains characterised were exposed in the presence of cry genes. [43,44].

In this part of this work the influence of the effect of fusion time on the efficiency of fusants products caused the mortality on the first generation on life span of *Tuta absoluta* insect in each of the three protoplast fusion attempts has been studied. Thirty-three strongest growth (10, 10 and 13 for of first, second and third attempt, respectively) out of 58 fusan products were chosen for bioassay treatments.

Bioassays were performed with first instars of *Tuta absoluta* larvae using leaves amended with one concentration and three replicates of fusion. Control leaves received distilled water only. Deformities in the larvae, pupae and emerged adults were recorded. Mortality was recorded every day and final mortality was recorded after7 days. The percentage of mortality was calculated by using the formula as follow:

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$$\text{Percentage of mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

The percent mortality data after corrections (Abbott 1925) were subjected to probity analysis [45]. to calculate the mean of replicates.

Under laboratory condition

1-Effect of fusion products of first protoplast (Bt and Bs1) attempt on 1st instar larvae of *Tuta absoluta* under lab condition:

Results in table (4) indicated that new bacterial strain which resulted from fusion *B*. *thurningensis* and *Bs1*caused gradual mortality to tomato leaf miner after feeding on treatment leaflets but the highest mortality percentage was recorded in case of treatment by fusion product F9 (100%) followed by F8 (97%), while their parents recorded 73% and 0% for Bt and Bs1, respectively. After 3 days of larval feeding on treatment leaflets, high percentage of mortality was observed to fusion F5 that recorded 52% larval mortality compare to their parent BT and Bs1 was registered 15 and 13 % larval mortality. On the contrary, F4 and F6 showed lowest percentage of mortality 10% for both.

After 7 days of treatment, the mortality percentage of remain larvae was recorded different degree of death whateverF7, F8 and F9 caused high mortality percentage ranged between 63 to 82% compared to their parent Bt was recorded 58% larval mortality while Bs1 wasn't recorded. F3 recorded lowest mortality percentage 3%.

Table4: Efficacy of fusion products of first protoplast (Bt::Bs1) under lab conditions on first instars larvae of *Tuta absoluta*.

ISSN: 0975-8585

Parental and fusion strains / Replicates			% Mortality of tomato leaf miner larvae								Correct % of accumu-lative mortality
			After 3day %				After 7 days %				
			R1	R2	R3	% mean	R1	R2	R3	% mean	
Parental strains Bt::Bs1	Bt		10	15	20	15	65	60	48	58	65
	Bs1		10	10	20	13	-	-	-	-	0
Fusion Time	Fusion Products										
	Names	Code									
20min	Bt::Bs1-F1	F1	30	20	20	23	15	15	15	15	21
	Bt::Bs1-F2	F2	20	35	35	30	15	5	5	8	21
	Bt::Bs1-F3	F3	20	45	40	35	10	-	-	3	21
30min	Bt::Bs1-F4	F4	10	5	15	10	70	45	45	53	52
	Bt::Bs1-F5	F5	40	55	60	52	10	15	-	8	49
	Bt::Bs1-F6	F6	10	10	10	10	50	55	60	55	55
40min	Bt::Bs1-F7	F7	20	20	10	17	60	40	90	63	74
	Bt::Bs1-F8	F8	30	23	15	27	70	67	85	74	96
	Bt::Bs1-F9	F9	15	25	15	18	85	75	85	82	100
	Bt::Bs1-F10	F10	30	35	30	32	30	45	50	42	65
control	-----		20	25	20	21.7	-	-	-	-	---

ISSN: 0975-8585

2-Effect of fusion products of second protoplast (Bt and Bl) on mortality percentage of *Tuta absoluta* larvae:

Table(5) cleared that feeding 1st instar larvae of *T. absoluta* on leaflets treated by product of fusion Bt::Bl brought out high mortality percentage to treatment larvae after three day more than seven days, the same observation was noticed in case treatment by Bl parent. After three day of treatment by fusion products F12, F20 and F19 were given rise to mortality larval percentage ranged from 73 to 100% respectivly, compare to their parent Bt and Bl caused 15 and 57% larval mortality. Here after 7 days, the most of remain larvae were able to continue and few percentages of mortality ranged from 35 to 1% were noticed in case of treatment by fusion products F18, F13,F15, F11 and F17 compare to Bt parent occasioned 58% larval mortality while other parent Bl wasn't. Ordinarily, the accumulative larval mortality was notation the highest percentage reach to 100 in case of treatment by fusion product F19 follow by F20, F13 were reported 87 and 72% larval mortality compare to their parent Bt and Bl were recorded 65 and 57 % larval mortality respectively.

Table5: Efficacy of fusion products of first protoplast (Bt::Bl) under lab conditions on first instars larvae of *Tuta absolu*ta.

Parental and fusion strains / Replicates			% Mortality of tomato leaf miner larvae								Correct % of accumu-lative mortality
			After 3 day %				After 7 days %				
			R1	R2	R3	% mean	R1	R2	R3	% mean	
Parental strains Bt::Bl	Bt		10	15	20	15	65	60	48	58	65
	Bl		50	55	65	57	-	-	-	-	45
Fusion time	Fusant products										
	Names	Code									
50min	Bt::Bl-F 11	F11	65	60	50	58	5	-	-	2	49
	Bt::Bl-F 12	F12	65	70	85	73	-	-	-	-	65
	Bt::Bl-F 13	F13	80	45	80	68	-	30	-	10	72
	Bt::Bl-F 14	F14	45	55	65	55	-	-	-	-	42
60min	Bt::Bl-F 15	F15	60	30	50	47	-	20	10	10	45
	Bt::Bl-F 16	F16	50	65	70	62	-	-	-	-	51
	Bt::Bl-F 17	F17	60	50	55	55	3	-	-	1	44
	Bt::Bl-F 18	F18	50	40	30	40	30	30	45	35	68
70min	Bt::Bl-F 19	F19	100	100	100	100	-	-	-	-	100
	Bt::Bl-F 20	F20	80	100	90	90	-	-	-	-	87
control	-----		20	25	20	21.7	-	-	-	-	---

Under semi field condition:

1- Measurement efficacy of new bacterial strains after exposure to sun ray toward the percentage of larval mortality of *Tuta absoluta*:

The data at table (6) described those new bacterial strains which resulted from fusion Bt with three other bacterial strains (Bs1 and Bl) able to tolerate sun ray from two to three days and caused mortality to tomato leaf miner larvae reached to more than 80% compare to their parent. In case of fusion products the two attempts (Bt::Bs1 and Bt::Bl) had ability to cause the percentage of mortality ranged between 70.9-91.8% after one day and 65.7 – 81.7% mortality after two days under sun ray compare to their parent Bt or Bl were caused mortality to tomato leaf miner reached to 27.2 and 60% after one day, respectively. This results are agreement with the results which obtained before [46].

Under field condition:

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From the investigation the sprayed tomato pots noticed the increased the number of leaflets and reduction rate of infestation by tomato leaf miner as described in Fig (5) and table (7). There are negative correlation between number of tomato leaflets and percentage of infestation i.e. when the number of tomato leaflets increase the infestation percentage decreased.

Table6: effect of exposure period of different bacterial strains under sun ray on Percentage of larval mortality

Tested Bacterial strain		Exposure period of treatment leaflets							
		0 time*	One day *	Two day*	Three day*	Four day*	Five day*	Six day*	One week*
Parents	*Bt*	88.3	27.2	11.3	2.6	1.2	2.0	0.0	0.0
	Bs1	12.7	19.3	16.02	14.5	5.2	3.9	3.3	10.4
	Bl	71.8	60	59.3	45.6	32	34.1	36	24.1
Fusion products	(Bt::Bs1 F9)	70.3	70.9	65.7	59.3	11.5	3.7	2.5	0.3
	(Bt::Bl F19)	90	91.8	81.7	50.3	26.1	5.7	2.8	4.3

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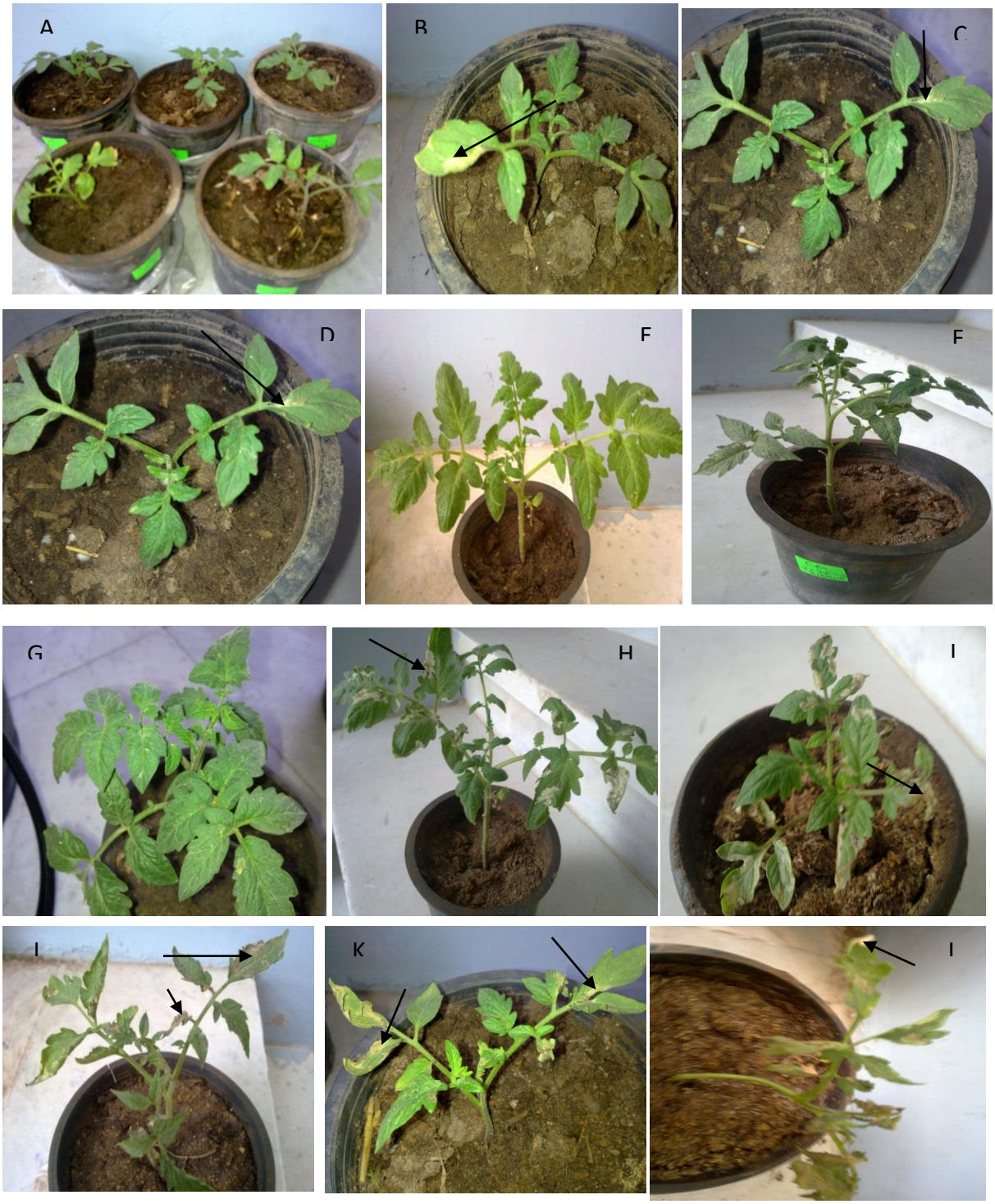

Fig (5): show the different between tomato plant before and after spray: (a) before treatment (b, c and d) after spray by fusion products (F9) (F19) (F32) and exposed to sun ray for one day and then to artificial infestation by tomato leaf miner; arrow indicated to dead larvae, respectively (e, f and g) after spray by fusion products (F9) (F19) (F32) and exposed to sun ray for two day and then to artificial infestation by tomato leaf miner arrow indicated to dead larvae, resp.(h, I and g) treatment with three parent; Bt, Bs1 and Bl only after one day of exposure to sun ray and then to artificial infestation**. respectively**

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Table (7): efficacy of spraying different bacterial strains on rate of plant growth and infestation percentage by *Tuta absoluta* under field condition

Treatment		Investigation After one week of spraying			Investigation After one month of spraying			Investigation After two months of spraying		
		Mean no. of tomato leaflets/3 plots (X±S.E.)	No. of larval tunnel	% of reduction in infestation	Mean no. of tomato leaflets/3 plots (X±S.E.)	No. of larval tunnel/ 300 leaflets	% of reduction in infestation	Mean no. of tomato leaflets/3 plots (X±S.E.)	No. of larval tunnel/300 leaflets	% of reduction in infestation
Parent	Bt	178±6,01 [a] (170-190)	12	48.1	287,3±1, 5 [c] (285-290)	39	62.9	415,6 ±12,9 [c] (390-430)	36	88
	Bs1	171,67±4,4 [a] (165-170)	12	48.1	250±5,8 [c] (240-260)	31	70.6	1070 ± 28,9 [c] (1020-1120)	32	96
	Bl	85±8,7 [a] (70-100)	3	51.6	215±8,667 [c] (200-230)	28	73.4	750 ± 86,6 [c] (600-900)	30	94
Fusion products	(Bt::Bs1 F9)	167,33±4,3 [a] (160-175)	12	48.1	375±14,4 [c] (350- 400)	8	92.3	1090 ±23,1 [c] (1050-1130)	40	95
	(Bt::Bl F19)	137,3±7,2 [a] (125-150)	4	48.1	257,3± 4,3 [c] (250-265)	2	98.3	875 ±72,2 [c] (750-1000)	-	100
Control		107,3±7,2 [a] (95-120)	23	--	45±2,9 [a] (40-50)	106	0.0	80±5,8 [a] (70-90)		-
LSD0.01		274.9			136.1			408.3		
LSD0.05		205.5			101.4			305.2		
Simple correlation		0.008			-0.625			-0.625		

From the obtained results, can be concluded that efficacy of Bt towered larvae of tomato leaf miner was recorded approximately 70% mortality while fusion with any of choice strain had ability to increase its' efficacy to reach to more than 80%, but the best fusion was (Bt::Bl) under lab condition. The results was introduced surprise when the experiment was elicited that *B.Lichinoformus* (one of tested parent) had ability to be caused mortality to tomato leaf worm as nearly that caused by Bt (the main target parent).

On other side, efficacy of *Bacillus thuringensis* alone can't continue more than one day but when fused with other bacterial strains increased its ability to more than two day, especially fusion products; F19 and F9.

This test was carried on other Lepidoptera species that agree with KannanRevathi *et al*., 2014 who tested *B. thuringiensis* in protoplast fusions with a strain of B. subtilis against first instar larvae of Spodopteralitoralis. They found that fusants were produced almost95% mortality in first instar larvae of *Spodoptera litura* when compared with Bt and B. subtilis individual treatment.

In 2007Sarawathy and others studied the insecticidal effect of a chimeric fusion gene of Bt cry 1 Ac and Vip fusion protein on three insect species (Helicoverpa armigera, Spodoptera littoralis and Plutella xylostella. They found that fusion protein is toxic to H. armigera and P. xylostella which is similar to cry 1Ac toxicity but partially lost that of Vip3 Aa14.

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