Improving the efficiency of *Bacillus thuringiensis* against insects of different feeding habits by plasmid transfer technique

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Abstract: This study aimed to increase the efficiency of *Bacillus thuringiensis* (*Bt*) to become more effective against insects of different feeding habits by chitinase gene transfer from chitinolytic bacteria to *Bt* strain through conjugal transfer. Nineteen soil samples were collected from five locations in Qassim region, Saudi Arabia to isolate native strains of *B. thuringiensis* and chitinolytic bacterium (*Bacillus subtilis*). Chitinase gene was transmitted from B. *subtilis* (donor strain) to *B. thuringiensis* (recipient strain) by conjugal transfer. Five recombinants were selected from all appeared recombinants based on their chitinase activity. The selected recombinants and their parents were evaluated as bioinsecticides against *T. absoluta* (Lepidoptera) and *A. gossypii* (Hemiptera). In case of *T. absoluta*, *B. thuringiensis* was more effective than *B. subtilis*. The recombinants Tr5 and Tr10 were more effective than other treatments. While, in case of *A. gossypii*; *B. subtilis* was more effective than *B. thuringiensis*. Also, the recombinants Tr5and Tr10 were more effective than their parents and other recombinants.

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1. Introduction

Pesticides are applied in agricultural systems for protecting crops from damage by insects and disease. In general the amount of pesticides released into the environment has risen significantly that affect the natural balance between natural enemies and their hosts **(Cook, 1993**; **Herrera-Estrella and Chet, 1999**). Scientists always seek for an effective and environmentally friendly method for controlling pests and diseases (**Senthil-Nathan et al., 2005; Senthil-Nathan et al., 2009; El-Metwally et al., 2010).** Biological control agents holds great promise as an alternative to the use of chemicals. Secondary metabolites and crude enzymes from microorganisms have been used to control crop pest population **(Kramer et al., 1997).** Searching for new microbial agents to control pests is one of the most pressing needs. Therefor, isolation of more local entomopathogens that would be more adapted to the local environment and pests and possess insecticidal activities or broader host range is encouraged **(Abd-Elazim et al., 1991; Osman, 1992 and Keller, 1998)**. Several different habitats such as agricultural soil, stored product dust, insect cadavers and grains may contain a novel *Bt* strain, awaiting discovery, which has a toxic effect on a target insect group (**Chak et al.,1994; Theunis et al.,1998; Bravo et al., 1998; Uribe et al., 2003; Özgür et al., 2005**).

Bacillus thuringiensis (*Bt*) is a gram-positive, rod-shaped, motile, facultative anaerobic, spore-forming bacterium widely used as a biocontrol agent against pests (**Fernando et al., 2010**). *Bt* produces parasporal crystalline inclusion bodies constituted of highly specific insecticidal toxins. These toxins are mainly active against lepidopteran species and some also shows toxicity against dipteran and coleopteran species and other organisms (**Vidyarthi et al., 2002**; **Martin et al., 2010)**.

Chitin is a long unbranched polysaccharide of an amino sugar N-acetyl-b-D-glucosamine linked together by b-1,4-glycosidic linkages **(Chuan, 2006).** It is abundant in nature as a structural compound in cuticle and integument of animals, especially in insects **(Arakane and Muthukrishnan, 2009).** Chitin is metabolized by various chitinases that are found in insects, bacteria, fungi and higher plants (**Kramer and Muthukrishnan**, **1997; Saguez et al., 2005 and Nurdebyandaru et al., 2010).**Insect growth and development are strongly dependent on the construction and remodeling of chitin structures **(Merzendorfer and Zimoch, 2003).** Chitinase induced damage to the peritrophic membrane in the insect gut causes a significant reduction in nutrient utilization and consequently a decline in insect growth **(Terra and Ferreira, 2005).** Due to this, chitinase present in the insect diet can decrease insect growth **(Otsu et al., 2003 and Fitches et al., 2004).** Chitinolytic microorganisms have many potential applications as biocontrol agents **(Saguez et al., 2005; Wang et al., 2006 and Nurdebyandaru et al.,**

2010). Over-expression of a chitinase in an entomopathogenic organism can increase insect mortality **(Fan et al., 2007).**

Horizontal transmission of DNA between different species may have played an important role in evolutionary history. Gene transfer encoded by bacterial plasmids has occurred between distantly related bacterial species; it may have occurred between species of different kingdoms. Bacterial conjugation is one gene transfer mechanism that is important in the horizontal flow of genetic information (**reviewed in Mazodier and Davies, 1991**). Plasmids have been identified in a wide range of bacterial species, and transfer between distantly related bacteria has been demonstrated (**reviewed in Farrand, 1993)**.Conjugative transfer of bacterial plasmids is the most efficient way of horizontal gene spread, and it is, therefore, considered one of the major reasons for increasing the number of bacteria that exhibit multiple-antibiotic resistance **(Grohmann et al., 2003).** The objectives of the present study are the isolation of local isolates of *Bt* and chitinolytic bacteria, determination of parasporal crystal bodies and chitinase activities of the isolated bacteria, identification of isolated bacteria by API, plasmid transfer between the best chitinolytic bacterial isolate and *Bt* by conjugal transfer process and evaluation of the efficiency of recombinants isolates and their parental strains against *T. absoluta* (Lepidoptera: Gelechiidae) and *A. gossypii* (Hemiptera : Aphididae) which considered among the most serious economic pests in many agricultural crops (**Harris and Maramorosch, 1977; Apablaza, 1992**).

2. Material and Methods

Sample collection:

Nineteen soil samples were collected from five different locations of Qassim region, Saudi Arabia. Soil samples were taken from plant rhizosphere and placed in sterile plastic bags and transported to the microbiology laboratory for isolation of *B. thuringiensis* and chitinolytic bacteria on selective media.

Isolation of *B. thuringiensis* (*Bt*) from soil:

To isolate *B. thuringiensis*, a 10% soil suspension in 0.9% NaCl was preheated at 80°C for 10 min then, 100 µL of each samples was transferred to 900µl Mannitol-egg yolk-polymyxin (MYP) broth medium for enrichment of environmental samples and incubated overnight at 30°C. After incubation, the cultures were serially diluted and plated on MYP agar and incubated at 30°C for 24 hrs. Those which formed irregular white colonies with a pink background, similar to reference isolates were primarily identified as *Bacillus thuringiensis* then examined by light microscope. (**Braun, 2000**).

Isolation of chitinolytic bacteria from soil:

Soil samples were serially diluted with sterile water until the dilutions of 10^4, 10^5 and 10^6. Dilutions were inoculated on colloidal chitin agar medium (CCA) containing 0.5% colloidal chitin, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4$ $7H_2O$, 0.05% $CaCl_2$ $2H_2O$, 0.05% yeast extract and 2% agar, and incubated at 30°C for 3 days. Isolates exhibiting a clear zone (as a sign for chitin degradation) around the colony were picked and further purified on the same medium. **(Choi et al., 2004).**

Chitinase activity:

Chitinase activity was determined colorimetrically by detecting the amount of N-acetylglucosamine (GlcNAc) released from a colloidal chitin substrate that was prepared according to **Lee et al., 2006**. Flasks (250 ml) containing 100 ml chitin medium were inoculated with the bacterial isolates and incubated at 30ºC on a rotary shaker 180 rpm for 5 days; flasks were removed every 24 hours and the chitinase was measured colorimetrically. The assay mixture consisted of 0.05 mL of supernatant, 0.5mL of 0.5% colloidal chitin, and 0.45 mL of 50 mM sodium acetate buffer [pH 5.0] at 37°C for 1 h. The reaction was terminated by the addition of 200 µL of 1N NaOH. After centrifugation at 10, 000×*g* for 5min, 750 µL of supernatant was mixed with 1 mL of Schales' reagent and then heated in boiling water for 15 min. The amount of GlcNAc produced was measured at 585nm using a spectrophotometer. The activity was calculated from a standard curve based on known concentrations of GlcNAc. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of GlcNAc per h under the conditions of the study **(Lingappa and Lockwood, 1962)**

Identification of *Bt* and the best isolate of chitinolytic bacteria:

Identification of *Bt* strain was carried out by simple and gram stains and investigated under light microscope to determine cell shape and parasporal crystal toxin and gram reaction. Bacterial isolates that formed crystal toxin were defined by the use of API 20E and API 50CHB kits. In case of chitinolytic bacteria, the best isolate was selected according to its chitinase activity, then simple and gram stain tests were performed to determine the cell shape and gram reaction, then we used API 20E and API 50CHB for identification.

Plasmid Transfer by conjugation process:

B. thuringiensis and *B. subtilis* (best isolate of chitinolytic bacteria in the study) were grown in nutrient broth medium (NB) at 35°C for 48 hrs. Then

mix 1.0 ml of *B. subtilis* (donor strain) with 4.0 ml of *B. thuringiensis* (recipient strain) in a test tube and incubated at 35°C for 48hrs without shaking. After incubation, the mated cells were plated on selective plates of nutrient agar medium (NA) supplemented with antibiotics as genetic markers then incubated at35°Cfor 48 hrs. Single colonies of transconjugants which appeared on selective medium were picked up and tested for chitinase activity and the presence of parasporal crystals then stored on NA medium slants according to **Grinsted and Bennett (1990); Chassy and Rokaw (1981).**

Bacterial preparation for bioassay test:

Bacterial isolates were grown in colloidal chitin broth medium to prepare chitinase enzyme and in T3 medium for preparing spores and crystal toxin as follows:

Crude chitinase enzyme preparation:

For the crude enzyme preparation, *B. subtilis* and recombining isolates were cultured in 100 ml medium containing chitin, colloidal chitin 2 g/L; K_2HPO_4 1 g/L; NaCl 5 g/L: $MgSO_4$ $7H_2O$ 0.04 g/L; $CaCl_2$ 0.02 g/L and incubated at 30 ^{0}C under shaking for about 8 days. The incubated cultures were centrifuged at 10,000rpm for 20 min. The supernatant was allowed for ammonium sulphate precipitation, allowed to stand overnight. The pellet was centrifuged at 10,000rpm for 20 min. Then the precipitates were dissolved in a small amount of 20 mM citrate phosphate buffer (pH 7.8) and extensively dialyzed against the same buffer. The dialysate was used for bioassay test. **(Renwick et al., 1991).**

Preparation of spore/crystal (SC):

Bt and recombinants were grown in liquid T3 medium (**Yamagata et al.,1987**) on a rotary shaker (200 rpm) at 30°C for 72 h. The pellet (SC) was obtained by centrifugation at 8000 rpm for 15 min**,** and then the pellet was washed with sterile distilled water three times. Fifty mg of spore/crystal (SC) was dissolved in 20 ml of 100 mmol l^{-1} Na_2CO_3 (pH 9.5) aqueous solution supplemented with 10 mmol l^{-1} DTT (Dithiothreitol)and stirred for at least 2 h at room temperature and centrifuged at 15000 rpm for 15 min. The supernatant that contains the solubilized crystal protein (pro-toxin protein) was used in bioassay test. (**Hofmann et al., 1988**).

Bioassay:

The treatments in this study were usage crud enzyme for *B. subtilis*, crystal toxin for *B. thuringiensis* and crystal toxin plus crud enzyme for recombinants. All treatments were used with spores and without spores to control of *T. absoluta* and *A. gossypii* as follow:

***T. absoluta*:**

The stock culture of *T. absoluta* was prepared by obtaining insect larvae from the field. The larvae were put on seedlings of tomato inside large breading cages (60 X 50 X 40 cm). The cages containing the seedlings were maintained at 27±2°C and 70±5% relative humidity (RH). The pupa were collected and laid in glass jars which were lined with filter paper. Jars were covered with muslin fixed with a rubber band. When moths emerged, a piece of cotton wool soaked in a 10% honey solution was provided as a source of food for the moths in addition to new seedlings for egg laying. Newly deposited eggs were collected daily with its seedlings and maintained till reaching the second instar larvae.

Bioassay was performed on the second instar larval stage. Dipped leaves bioassay method was performed using whole tomato leaves. Control leaves received distilled water only. A group of 20 larvae were placed in a Petri-dish (15 cm in diameter) containing treated tomato leaves. The remained leaves were removed after 2 days and replaced with freshly treated leaves. For control test, fresh leaves were added. Each treatment was replicated five times. Deformities in the larvae, pupae and emerged adults were recorded. Mortality was recorded after 2, 4 and 6 days of treatment and corrected by Abbott's formula **(Abbott, 1925)**.

***A. gossypii*:**

Naturally infested egg-plant leaves with *A. gossypii* were obtained from untreated greenhouse with any pesticides located in the experimental farm of Faculty of Agriculture and Veterinary Medicine, Qassim University at Qassim region, Saudi Arabia. One hundred individuals of *A. gossypii* were counted on an infested leaf and then put in a Petri-dish (9 cm in diameter). Each plate was treated by spraying bioassay method (approximately 2 ml/Petri-dish). Control treatment was sprayed with distilled water only. Each treatment was replicated five times. Deformities and mortality were recorded after 2 and 4 days. Mortality percentages were corrected by Abbott's formula.

Statistical analysis:

The average of mortality percentages were corrected by using Abbott's formula (**Abbott, 1925**). Data were analyzed by using one way ANOVA. Means comparison was conducted according to Duncan's Multiple Range Test (LSD) at the probability of 5% **(CoHort Software, 2004).**

3. Results

Isolation, purification and identification of isolated bacteria:

***B. thuringiensis*:**

Six colonies of bacteria were found similar to a reference strain of *B. thuringiensis* (*Bt*) on Mannitol-egg yolk-polymyxin medium (MYP) during isolation from the nineteen agricultural soil samples. These

colonies were purified on the same medium then stored on nutrient agar slants. Parasporal crystal bodies were shown under microscope in one isolate only as shown in Table 1 and Fig. 1. This isolate was designated as Bt-4.2 and identified primarily by simple and gram stains then performed by API 20E and API 50CHB. The presented results in the API Kit profiling, were matched with those presented in API software. These characteristics suggest that isolate Bt-4.2 is *Bacillus thuringiensis* as shown in Table 3 and Fig. 3**.**

Table 1. Isolation of *Bt* on MYP medium from agricultural soil samples in Qassim area, KSA

Soil sample places (GPS)	Soil sample No.	Isolates no. similar *Bt* isolates on MYP medium	Isolate no. formed parasporal crystal bodies
N 28-20- 694 E 044- 04-991	3	1	0
N 26-20-706 E 044-04-991	4	2	1
N 26-20- 06 E 044-05-002	5	2	0
N 26-00-000 E 043-54-375	3	0	0
N 29-12-459 E 043-49-239	4	1	0

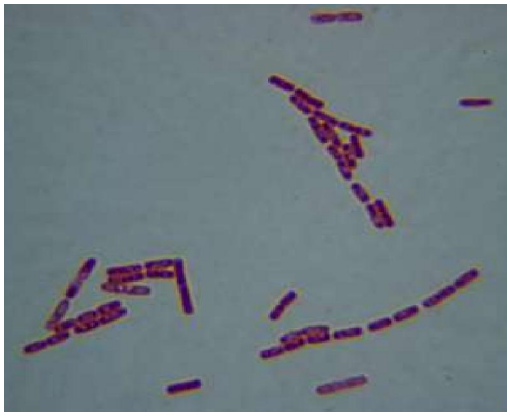

Fig. 1 light microscope photo of Bt cells. Note spores and parasporal crystal bodies

Chitinolytic bacteria:

Seventeen chitinolytic bacterial isolates were isolated on colloidal chitin agar medium (CCA) from nineteen soil samples in Qassim area. The Chitinase activity was tested qualitatively and quantitatively to determine the best isolate in chitinase activity. The best isolate caused high degradation of colloidal chitin in agar and broth medium compared with other isolates was selected and designated as Ch16 as shown in Table 2 and Fig.2. This isolate stained by simple and gram stains then identified by API 20E and 50CHB. The obtained results from API kits matched with API software. These results suggest that the isolate Ch16 is *Bacillus subtilis* as shown in Table 3 and Fig. 4**.**

Table 2. Isolation of chitinolytic bacteria from agricultural soil samples in Qassim area.

Soil sample places (GPS)	Soil samples No.	Isolates of chitinolytic bacteria	Diameter of clear zone (cm)	Chitinase activity in culture supernatant (U/ml)
N 28-20- 694 E 044- 04-991	3	Ch1	1.2	6.1
		Ch2	1.5	9.0
		-	-	-
N 26-20-706 E 044-04-991	4	Ch4	1.6	9.6
		-	-	-
		Ch6	1.8	10.8
		-	-	-
N 26-20- 06 E 044-05-002	5	Ch8	2.2	12.1
		-	-	-
		Ch10	2.0	12.0
		Ch11	2.3	12.2
		Ch12	1.5	9.0
N 26-00-000 E 043-54-375	3	Ch13	1.9	11.4
		Ch14	2.0	12.0
		Ch15	2.2	12.2
		Ch16	**3.5**	**24.3**
N 29-12-459 E 043-49-239	4	Ch17	3.0	19.0
		Ch18	1.4	8.4
		Ch19	2.5	15.0
		Ch20	1.2	6.3
		Ch21	1.3	7.8

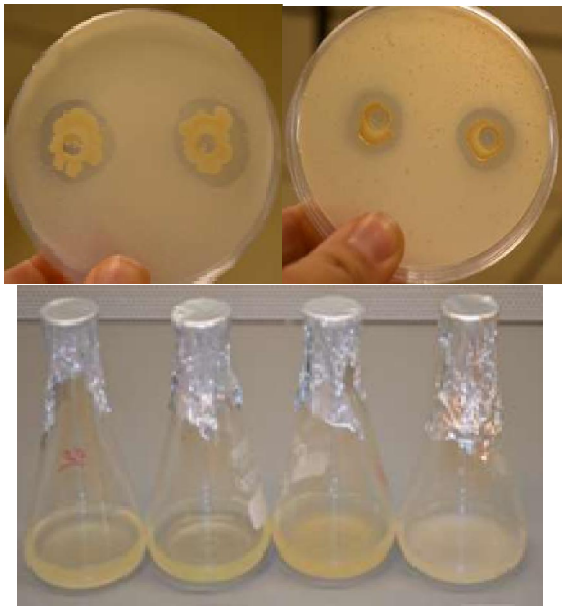

Fig.2 Colloidal chitin degradation by some chitinolytic bacterial isolates in this study

Table 3. Identification of isolated bacteria by API kits.

Isolate No	Cell shape	Spore forming	Gram stain	Forming of parasporal crystal	Chitinase production	Identification with API
Bt-4.2	Long rod	+	+	+	-	*Bacillus thuringiensis*
Ch16	Long rod	+	+	-	+	*Bacillus subtilis*

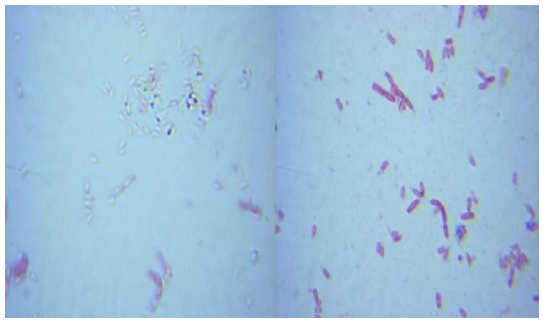

Fig. 3 light microscope photos of *B. thuringiensis*

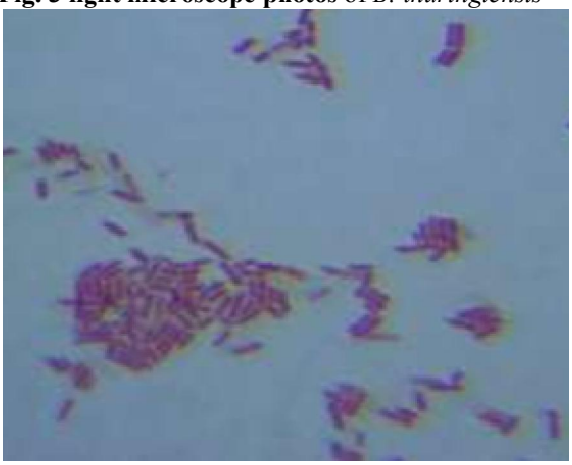

Fig. 4 light microscope photo of ***B. subtilis***

In the present study, chitinase gene transfer by conjugation process was carried out between chitinolytic bacterium, *B. subtilis* (donor strain) and *B. thuringiensis* (recipient strain) to improve the efficiency of *Bt* strain against insect pests that belong to different classification orders as shown in Table (4). Ten transconjugants (Tr) were taken from the mating at random and tested to chitinase activity and presence of parasporal bodies. Five transconjugants gave high chitinase activity and consist of parasporal bodies were selected to use it in bioassay test against cotton aphid (*Aphis gossypii*) and leafminer larvae (*Tuta absoluta)*. Five transconjugants; Tr2, Tr3, Tr5, Tr7 and Tr10 gave more chitinase activities than other recombinants reached to 21.4, 28.9, 29.6, 23.7 and 25.2 U/ml, respectively. Also, parasporal crystal bodies were found in all recombinants when investigated under light microscope.

Bioassay test:

Tuta absoluta

Data listed in Table 5 show the effect of five recombinants (Tr2, Tr3, Tr5, Tr7 and Tr10) and their parents (*B. thuringiensis*, P1 and *B. subtilis*, P2) on the 2nd instar larvae of *T. absoluta* under laboratory conditions. *B. thuringiensis* exhibited a significantly higher effect on *T. absoluta* in comparison with *B. subtilis*. Mortality percentages were 63.6 and 57.2 % after 6 days for *B. thuringiensis* and *B. subtilis*, respectively. With respect to recombinants, Tr5 and Tr10 proved to be the most effective treatments. Mortality percentages after 6 days were 87.6 and 91.6 % for tr5 and Tr10, respectively. Recombinants Tr2, Tr3 and Tr7 were less effective than Tr5 and Tr10 with no significant difference. In general, mortality percentages were more in case of the treatments with spores than without spores. Also, all of the tested recombinants exhibited significantly higher effects than those of their parents.

Table 4. Di-parental mating between chitinolytic bacterium, *B. subtilis* and *B. thuringiensis* that having the opposite genetic markers.

Mating	Relevant genotype	Designation recombinants	Recombinants genotype	Chitinase activity(U/ml)	Presence of parasporal crystal bodies
Bs x *Bt*	Chl^R , Amp^S x Chl^S , Amp^R	Tr1	Chl^R , Amp^R	18.7	+
		Tr2		**21.4**	+
		Tr3		**28.9**	+
		Tr4		17.3	+
		Tr5		**29.6**	+
		Tr6		-	+
		Tr7		**23.7**	+
		Tr8		17.8	+
		Tr9		19.7	+
		Tr10		**25.2**	+

Table 5. Mortality percentages of the second instar larvae of *T. absoluta* caused by recombinants and their parents under laboratory conditions.

Treatment	Mortality percentage (±SE) after treatment (days)								
	2			4			6		
	With spores	Without spores	LSD	With spores	Without spores	LSD	With spores	Without spores	LSD
P1 (C)	28.2±2.9	22.8±2.6	8.9	59.2±3.2	39.8±1.5	8.2	63.6±2.1	51.2±2.4	7.5
P2 (E)	17.0±1.0	17.0±1.0	3.4	51.8±2.0	33.4±2.1	6.8	57.2±2.4	45.2±3.0	9.1
Tr2 (C+E)	52.6±3.4	22.8±1.3	8.3	75.6±3.5	51.8±2.0	9.3	82.8±2.3	63.8±4.9	12.5
Tr3 (C+E)	52.6±3.4	40.6±2.5	9.7	75.6±0.2	59.2±2.2	5.1	81.6±2.1	67.4±1.8	6.4
Tr5 (C+E)	58.6±1.3	40.6±2.8	7.0	84.4±2.4	69.0±1.9	7.1	87.6±2.9	76.0±4.6	12.7
Tr7 (C+E)	52.6±4.5	31.8±1.0	10.6	64.0±2.9	58.0±2.9	9.3	75.6±3.4	63.8±2.2	9.5
Tr10 (C+E)	59.8±3.5	45.2±1.5	8.8	87.8±3.4	76.2±3.9	12.0	91.6±2.4	81.8±2.7	8.3
LSD*	8.9	5.7	---	7.9	7.2	---	7.4	9.6	---

Notes: (C) crystals and (E) enzyme
***LSD was calculated at probability of 5%**

Table 6. Mortality percentages of *A. gossypii* caused by recombinants and their parents under laboratory conditions.

Treatment	Mortality percentage (±SE) after treatment (days)					
	2			4		
	With Spores	Without Spores	LSD	With spores	Without spores	LSD
P1 (C)	48.4±2.5	33.6±1.5	6.7	63.8±1.7	54.8±1.2	4.7
P2 (E)	67.8±2.1	37.8±1.4	5.9	76.0±4.2	56.4±1.2	10.2
Tr2 (C+E)	78.2±3.5	48.2±1.3	8.6	84.8±3.0	70.0±3.3	10.3
Tr3 (C+E)	70.8±1.8	39.8±1.1	4.8	82.0±2.0	66.2±0.7	4.9
Tr5 (C+E)	85.0±1.2	64.0±3.0	7.5	90.4±2.5	90.2±1.7	6.9
Tr7 (C+E)	70.0±1.5	34.8±1.2	4.5	79.4±2.2	60.4±1.3	5.9
Tr10 (C+E)	85.6±1.2	48.6±2.0	5.4	92.8±1.0	76.0±1.8	4.8
LSD	6.1	5.1	---	7.4	5.2	---

Notes: (C) is crystals and (E) is enzyme & LSD was calculated at a probability of 5%

Aphis gossypii

With respect to *A. gossypii* (Table 6), *B. subtilis* caused higher mortality percentages in *A. gossypii* population than those caused by *B. thuringiensis*. Also, data showed that recombinants Tr5 and Tr10 exhibited the highest effects when treated with spores (mortality percentages were 90.4 and 92.8% after 4 days respectively). Tr2, Tr3 and Tr7 were the lowest effective recombinants on *A. gossypii*. Also, data listed in Table 6 showed that presence of spores in treatments significantly increased the activity of the treatment.

Figures 5 and 6 show the deformities and infection symptoms of *T. absoluta* and *A. gossypii* which resulted from the present treatments. As shown in these figures, the infected larvae of *T. absoluta* and *A. gossypii* individuals were greatly malformed (as a change in general shape) in comparison with the healthy ones.

4-Discussion

In this study *Bt* strain was isolated from soil samples, which were collected from some locations in Qassim area, Saudi Arabia. In the previous studies, more than 50,000 *Bt* strains have been isolated from different environments (**Sadder et al., 2006**). It has been reported that *Bt* can be present in several different habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, olive tree related habitats, different plant and aquatic environments (**Martin and Travers, 1989; Ben-Dov et al., 1997; Theunis et al., 1998; Bravo et al., 1998; Bel et al., 1997; Mizuki et al., 1999; Iriarte et al., 2000, Xavier et al., 2007**). In fact, each habitat may contain a novel *Bt* strain, awaiting discovery, which has a toxic effect on a target insect group (**Chak et al., 1994; Theunis et al., 1998; Bravo et al., 1998; Uribe et al., 2003; Özgür et al., 2005**).

a

b

c

Fig. 5 Deformities and infection symptoms of *T. absoluta* treated with recombinants and their parents
a- Healthy insect
b- Infection symptoms of larvae those were treated with crystal toxin
c- Infection symptoms of larvae those were treated with crystal toxin and chitinase enzyme

a

b

c

Fig. 6 Deformities and infection symptoms of *A. gossypii* treated with recombinants and their parents
a- Healthy insect
b- Infection symptoms of larvae those were treated with crystal toxin
c- Infection symptoms of larvae those were treated with crystal toxin and chitinase enzyme

In this study, chitinolytic bacteria were isolated from soil samples to transfer chitinase gene from these bacteria to local *Bt* strain to become more effective against insect pests. However, chitinase degrades the cuticle layer in insect pest that led to weaker insect and increase insect mortality. Besides, chitinase induced damage to the peritrophic membrane in the insect gut causes a significant reduction in nutrient utilization and consequently affect insect growth **(Terra and Ferreira, 2005).** Due to this, chitinase present in the insect diet can decrease insect growth **(Otsu et al., 2003; Fitches et al., 2004).**

Bacillus spp. are well-known as producers of extra-cellular enzymes such as chitinase and secondary metabolic products (**Schallmey et al., 2004 and Yang et al., 2009**). *B. subtilis* is non-pathogenic bacterium with notable chitinase activity **(Choi *et al.,* 2004**). *B. thuringiensis* is notable as a source of insect toxins (crystal protein) **(Kramer and Muthukrishnan, 1997).**

Gene transfer was conducted in this study from *B. subtilis* to *B. thuringiensis* by conjugation process to transfer chitinase gene to local *Bt* strain to become more toxic. Several articles reviewed that many insecticidal toxin genes are carried by large plasmids that can be transferred via conjugation between *B. thuringiensis* strains and other bacterial strains in broth cultures and in infected insect larvae. However, plasmid transfer frequencies using *B. thuringiensis* conjugative plasmids carrying selective markers have been rarely reported. Bacterial conjugation is one gene transfer mechanism that is important in the horizontal flow of genetic information (**reviewed in Mazodier and Davies, 1991**).Also, **Grohmann et al., (2003)** reported that Conjugative transfer of bacterial plasmids is the most efficient way of horizontal gene spread.

The present study showed that *B. thuringiensis* was more effective on *T. absoluta* larvae than *A. gossypii*. While, *B. subtilis* showed higher effect on *A. gossypii* in comparison with *T. absoluta* larvae. These results are attributed to variation of each bacterium effect and insect feeding habit. However, *Bt* produces toxin crystals which are mainly active in insect mid-gut; so, chewing insects (belong to lepidopteran, *i.e. T. absoluta*) are the mainly affected insects. Similar conclusion was reported by **Vidyarthi et al., (2002)** and **Martin et al.,(2010)** who mentioned that *Bt* is mainly active against lepidopterous species. On the other hand, *B. subtilis* produces chitinase enzyme which degrades cuticle layer of insect; so, *A. gossypii* (as a sucking insect) is affected by chitinase enzyme more than crystal toxins. **Saguez et al.,(2005); Nurdebyandaru et al.,(2010); Mubarik et al.; (2010)** mentioned that *Bacillus* sp. which produces chitinase enzyme demonstrated their ability to degrade exoskeleton chitin of aphids (*Myzus persicae* Sulzer and *A. gossypii*) and whitefly (*Bemisia tabaci* Genn.).

Data represented in this study explained that the tested recombinants exhibited significantly higher effects on both of the tested insects (*T. absoluta*; as cutting insectand *A. gossypii*; as sucking insect) than those of their parents. This may be attributed to that recombinants had the two modes of actions which presented in their parents. However, it produces crystal toxins and chitinase enzyme.

Bacillus thuringiensis, *B. subtilis* and their recombinants exhibited some morphological changes in *T. absoluta* larvae and *A. gossypii*. Also, previous researchers recorded malformations in lepidopterous (**El-Metwally et al., 2010**) and hemipterous insects (**Nurdebyandaru et al., 2010**) caused by *Bt* and *B. subtilis*. Generally, *B. thuringiensis*, *B. subtilis* and their recombinants proved to be entomopathogenic agents against the lepidopterous pest, *T. absoluta* and hemipterous pest, *A. gossypii*. Also, many authors recorded *B. thuringiensis* and *B. subtilis* as entomopathogenic agents against many agricultural pests (**Navon, 2000**; **Lacey et al., 2001**; **Collins and Jacobsen, 2003**; **Bravo et al., 2007**; **Huang et al., 2007** and **El-Metwally et al., 2010** and **Nurdebyandaru et al., 2010**). **Fernando et al., (2010)** added that *B. thuringiensis* is widely used as biocontrol agent against many pests. However, Bt produces parasporal crystal bodies consisted of highly specific insecticidal toxins which are mainly active against lepidopterous species **(Martin et al., 2010)**. Also, **Wang et al., 2006** mentioned that the chitinolytic organisms were used in biological control against many insect pests specially sucking insect pests. Where, these microorganisms able to degrade the cuticle layer in many insect pests by chitinase enzyme according to **Saguez et al., 2005; Nurdebyandaru et al., 2010 and Mubarik et al., 2010**.

5- Conclusion

The present study reveals that transferring chitinase gene from *B. subtilis* to *Bt* strain has improved the efficiency of *Bt* against both of *T. absoluta* and *A. gossypii* compared with the wild type of *Bt*. this can be attributed to that the modified *Bt* strain has two mode of actions against insect pests, the first one is through crystal toxin formation, which produce pores in insect's mid-gut causing insect death; and the second mechanism is chitinase enzyme production which degrade the cuticle layer in insect's

body resulting in insect dehydration then insect mortality.

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