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Molecular Screening and Bioactivity of Native *Bacillus thuringiensis* Isolates

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ABSTRACT

***Bacillus thuringiensis* strains were isolated from various soil samples collected from different regions of Kahramanmaraş in Turkey and were characterized by their colony morphology, PCR method with cryIAb/Ac, cryID, cry2 and cyt2 primers, and SDS-PAGE of crystal proteins with 130-140 and 60-70 kDa molecular weights. Plasmid profiles of selected isolates were also analyzed. Among the *cry* genes studied, *cry1Ab/Ac* was found to be the most common one (60%) in local isolates. Bioactivity tests were carried out under laboratory conditions to assess the efficacy of isolates against the third instar larvae of the stored product insects; *Anagasta kuehniella* and *Plodia interpunctella.* Toxicity values of the isolates varied between 20.00-53.33% for *A. kuehniella* and 20.00-55.56% for *P. interpunctella* at 1,000 µg g^{-1} concentration. Out of 55 isolates, ST13.1 was found to be the most effective against *A. kuehniella.* Also, ST7.2, ST7.3, and ST8.2 isolates yielded remarkable control against *P. interpunctella*. Further research is recommended to investigate the efficacy of these promising *Bt* isolates against other pest insect species.**

Keywords: *Bacillus thuringiensis*, Bioactivity, *Cry* gene, Stored product insects, Toxicity.

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive, facultative anaerobe and spore-forming bacterium used for biological control of insect pests and disease vectors (Santos *et al.,* 2010; Reddy *et al.,* 2013; Çakıcı *et al*., 2014). Biopesticides based on *Bt* products have been used for many years due to their insecticidal activity against target insect pests and environmentally friendly characteristics (Li and Bouwer, 2014). *Bt* produce crystal (Cry) proteins and hemolytic toxins (Cyt) during sporulation (Schnepf *et al.,* 1998) and these crystal proteins are solubilized in the insect midgut and bind specific receptor molecules of epithelial cells forming pores (de Maagd *et al.,* 2003). *Bt* has species-specific toxicity to insects and does not directly affect beneficial insects and non-target organisms (Akio *et al.,* 2004). These environmentally friendly bacteria can be isolated from diverse habitats such as soil, foliage, water, and storage grain from dead insects, annelids, crustaceans and insectivorous mammals (Raymond *et al.,* 2010). In this study, we isolated 55 *B. thuringiensis* isolates in Kahramanmaraş region and the presence of genes was determined using Polymerase Chain Reaction (PCR). Besides, plasmid and protein profile of isolates were obtained and insecticidal activities were tested on stored product pest insects. The purpose of this study was to screen native *Bt* isolates and search their effectiveness against stored product insects.

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MATERIALS AND METHODS

The analysis included: (i) *B. thuringiensis* isolation from soil samples to determine colony morphology, (ii) PCR analysis to identify *cry* and *cyt* genes, (iii) SDS-PAGE for determining the number and size of Cry proteins, (iv) plasmid analysis, and (v) bioassays against insect species to identify active isolates.

Isolation of Bacteria

Soil samples were collected from different locations of Kahramanmaraş, a region having fertile farm lands (Table1). Soil samples were used for *Bt* isolation according to the method of Travers *et al.* (1987). One gram of soil was added to 20 mL of LB Broth buffered with 0.25M sodium acetate (pH 6.8) and incubated at 37ºC for 4 hours in 100 mL flask with 200 rpm. One mL of sample was heated at 80ºC for 10 minutes. Then, 50 µL aliquot was spread on nutrient agar in Petri dishes and incubated overnight at 30ºC. *Bacillus*-like colonies were selected and examined for spore and parasporal crystals under a phase-contrast microscope. *Bt* var. *kurstaki* (*Btk*) HD125, *Bt* var. *aizawai* (*Bta*), *Bt* var. *israelensis* (*Bti*) and *Bt* var. *tenebrionis* (*Btt*) were used as references.

Characterization of Isolates

Isolates were grown in LB medium overnight, and then a loop of cells was placed into 400 µL sterile dH_2O. Having been mixed well, 2 µL were used as DNA templates. Properties of primers are given in Table 2. Each PCR reaction contained 0.5 µL of primers of each (100 nM), 0.1 µL taq DNA polymerase (5 u μL^{-1}), 1.4 µL $MgCl_2$ (25 mM), 1.5 µL taq buffer (10X), 0.5 µL

Table 1. *Bt* isolates and their GPS Location.

Soil sample	Location	GPS Position
1	Bölükçam/Pazarcık	37º26'56.39"K , 37º12'28.21"D, 674m
2	Narlı	37º23'49.45"K , 37º08'14.68"D, 605m
3	Yeşilgöz	37º53'25.34"K , 36º37'17.92"D, 1014m
4	Ahir Dağı	37º37'33.58"K , 36º57'41.40"D, 1224m
5	Güzelyurt	37º29'03.82"K , 37º01'43.48"D, 633m
6	Narlı Ormanlık	37º23'15.44"K , 37º08'09.90"D, 584m
7	Şekeroba/Türkoğlu	37º13'01.34"K , 36º46'15.64"D, 507m
8	Göksun Santral	38º02'58.03"K , 36º28'30.31"D, 1359m
9	Yeşilyurt/Andırın	37º34'55.76"K , 36º21'09.79"D, 1130m
10	Döngel Mağarası	37º51'29.11"K , 36º39'35.21"D, 1095m
11	Kayapınar/Göksun	38º09'36.84"K , 36º27'50.95"D, 1499m
12	Tekir	37º52'33.79"K , 36º37'27.09"D, 1014m
13	Göksun/ K.Maraş	37º57'11.72"K , 36º29'29.83"D, 1468m
14	Çınarlı Köyü/K.Maraş	37º27'40.07"K , 37º04'56.24"D, 691m
15	Türkoğlu	37º23'48.06"K , 36º52'03.39"D, 508m
16	Andırın/Merkez	37º35'33.44"K , 36º22'09.59"D, 1100m
17	Doğankonak/Göksun	38º15'01.93"K , 36º25'52.39"D, 1559m
18	Gölpınar Köyü/Göksun	38º14'27.58"K , 36º25'53.65"D, 1558m
19	Kireç Köyü/Göksun	38º00'09.99"K , 36º30'01.44"D, 1337m

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Table 2. Primer pairs and thier properties.

Primer pair	Product size (bp)	Sequences (5'-3')	Annealing temparature (°C)	References
Cry1Ab F	216	*AACAACTATCTGTTCTTGAC*	47	Ceron *et al.* (1994)
Cry 1Ac R		*CTCTTATTATACTTACACTAC*	42	
Cry1D F	290	*CTGCAGCAAGCTATCCAA*	53	Ceron *et al.* (1994)
Cry1D R		*ATTTGAATTGTCAAGGCCTG*	51	
Cry2 F	1556	*TAAAGAAAGTGGGGAGTCTT*	50	Sauka *et al.* (2005)
Cry2 R		*AACTCCATCGTTATTTGTAG*	47	
Cyt2gral F	355-356	*GAGTTTAATCGACAAGTAGATAATTT*	50.5	Iberra *et al.* (2003)
Cyt2gral R		*GGAAAAGAGAATATAAAAATGGCCAG*	55.2	

dNTP mix (10 mM each), 1.3 µL BSA (0.08 µg μL^{-1}), 7.2 µL sterile dH_2O and 2 µL (5 ng μL^{-1}) template DNA in a total volume of 15 µL. The amplification for *cry* genes was performed under the following conditions: initial denaturation at 95ºC for 2.5 minutes, followed by 30 cycles at 95ºC for 1 minute, 48ºC for 1 minute, 72ºC for 1 minute, and a final extension step at 72ºC for 5 minutes. The PCR products (15 mL) were electrophoresed (at 80V for 2 hours) on a 1X Tris acetic acid EDTA (TAE with ethidium bromide) buffer in 1% agarose gel.

Detection of Plasmid DNA

Plasmids were extracted by the method of Jensen *et al.* (1995) and Porcar *et al.* (1999). Isolates were grown in 5 mL LB broth at 30ºC and 200 rpm overnight and centrifuged at 10,000×g. Cells were re-suspended in 100 µL of TE buffer (40 mM Tris-HCI, 2 mM EDTA, pH 7.9) and lysed by adding 200 µL of freshly prepared alkaline solution (3% SDS, 15% sucrose, 50 mM Tris-hydroxide, pH 12.5) and incubated at 60ºC for 30 minutes. Later, two µL of proteinase K (20 mg mL^{-1}) was added to lysate and incubated at 41ºC for 90 minutes, followed by the addition of 1 mL of phenol-chloroform-isoamyl alcohol (25:24:1). Samples were inverted several times and centrifuged for 10 minutes at 6,000×g. The upper aqueous layer was transferred to a clean tube and 500 µL of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 6,000×g for 7 minutes and electrophoresed in 0.5% agarose gel at 65V for 12 hours at 4ºC.

Cry Protein Analysis

The lyophilized spore-crystal mixtures were re-suspended three times in 1 mL of 0.01% Triton X-100 solution (Valicente *et al.*, 2010). Mixture, solubilized in 500 µL buffer (0.01% Triton X-100, 10 mM NaCl and 50 mM Tris–HCl, pH 8.0) was centrifuged at 14,000×g for 5 minutes. Pellets were re-suspended in 500 µL of buffer (50 mM sodium bicarbonate and 10 mM β-mercaptoethanol, pH 10.5) and incubated at 37ºC for 3 hours. Samples were centrifuged at 14,000×g for 10 minutes, and then supernatant was transferred to new tubes. Pellets were re-suspended in 250 µL of 0.1M Tris, pH 8.0. Equal amounts of the supernatant and the re-suspended pellet were sampled and equal volume of the sample buffer (0.0625M Tris, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue, pH 6.8) was added and held in boiling water for 5-10 minutes. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed, using 12% running and 5% stacking gels. Molecular mass of proteins was determined by SM0431 protein marker (Fermentas). The gel was stained by 0.4% coomassie brilliant blue R250 described by Temizkan *et al.* (2004).

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Insect Rearing

The stored product insects ; *A. kuehniella* (Zeller) and *Plodia interpunctella* (Hübner) were reared on their artificial diet at 27±1ºC, with a photoperiod of 14:10 (L:D) hour and 65±5% RH (Ayvaz *et al.,* 2009 and 2010; Karabörklü *et al.,* 2010). *A. kuehniella* larvae were reared on a mixture of wheat flour, glycerol, and wheat bran (65+5+30 by weight) (Karabörklü *et al.,* 2011). *P. interpunctella* larvae were reared in a 2:1:0.25:0.50:0.25:0.25 mixtures of rough wheat bran, corn flour, dry yeast, honey, milk powder and glycerin (Ozkan *et al.,* 2006).

Bioassay

Bioactivity of isolates was tested against *A. kuehniella* and *P. interpunctella* larvae. Isolates were grown in T3 medium (3 g of tryptone L^{-1}, 2 g of tryptose L^{-1}, 1.5 g of yeast extract L^{-1}, 6 g of NaH_2PO_4 L^{-1}, 7.1 g of Na_2HPO_4 L^{-1}, 0.005 g of $MnCl_2$ L^{-1}) at 30ºC for 7 days at 200 rpm . Samples were centrifuged at 15,000×g for 10 minutes. Pellets were washed twice in 30 mL of dH_2O and centrifuged at 5,000×g for 5 minutes. Mixtures were freeze-dried and used in bioassays at 250, 500 and 1,000 µg g^{-1} concentrations. Insect food was soaked into 1 mL of spore/crystal mixture and let absorb for 20 minutes and allowed to dry. The mixture was then transferred into Petri dishes (90×15 mm) together with 10 third instar larvae and left at 27±1ºC and 60±5 RH with a photoperiod of 14:10 (L: D) hour for 10 days. Sterile dH_2O was used as a control and three replicates were set up for each treatment.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using SPSS for Windows and means were separated at the 5% significance level by the least significant difference (LSD) test. The Probit analyses were used to estimate LC_{50} and LC_{99} values for bioactivities (SPSS, 2001).

RESULTS

Bt Isolation

Fifty-five samples were processed for the isolation of *B. thuringiensis* by sodium acetate selective method. The colony morphology of the selected strains varied, *i.e*., off-white, light yellow, light pink color, smooth or slightly wrinkled margin, rounded, oval shape, 0.1-2.0 mm diameter.

Screening of native isolates was carried out by using PCR method for identifying *cry* and *cyt* gene contents. The profiles of all PCR products were compared with those of standard strains (Figure 1a-d). The majority of the isolates harbored *cry1Ab/Ac* (60%). Among the isolates two carried *cry1D* and *cyt2* (3.63%), and three carried *cry2* (5.45%) genes (Table 3). *CryIC, cryIB, cryIAc, cryIAd, cryIAa/Ad* and *cry3-7-8* genes were not observed in our isolates.

Plasmid Profile

The plasmids of the isolates were compared with those of *Bta* and *Btt.* Although some plasmid bands were shared among the isolates, their profiles were different and ranged from 1 to 6. The isolates ST19.3 and ST11.1 exhibited mega plasmids bigger than chromosomal DNA when compared to references and other isolates. Besides, these two isolates gave bands about 4.25 kb similar to *Bta*.

Cry Protein Profile

The SDS-PAGE profile of isolates is given in Figure 2. Our native isolates exhibited several protein bands with molecular masses ranging from 15 to 140 kDa. ST13.1 and ST4.4 produced 65 and 130 kDa major

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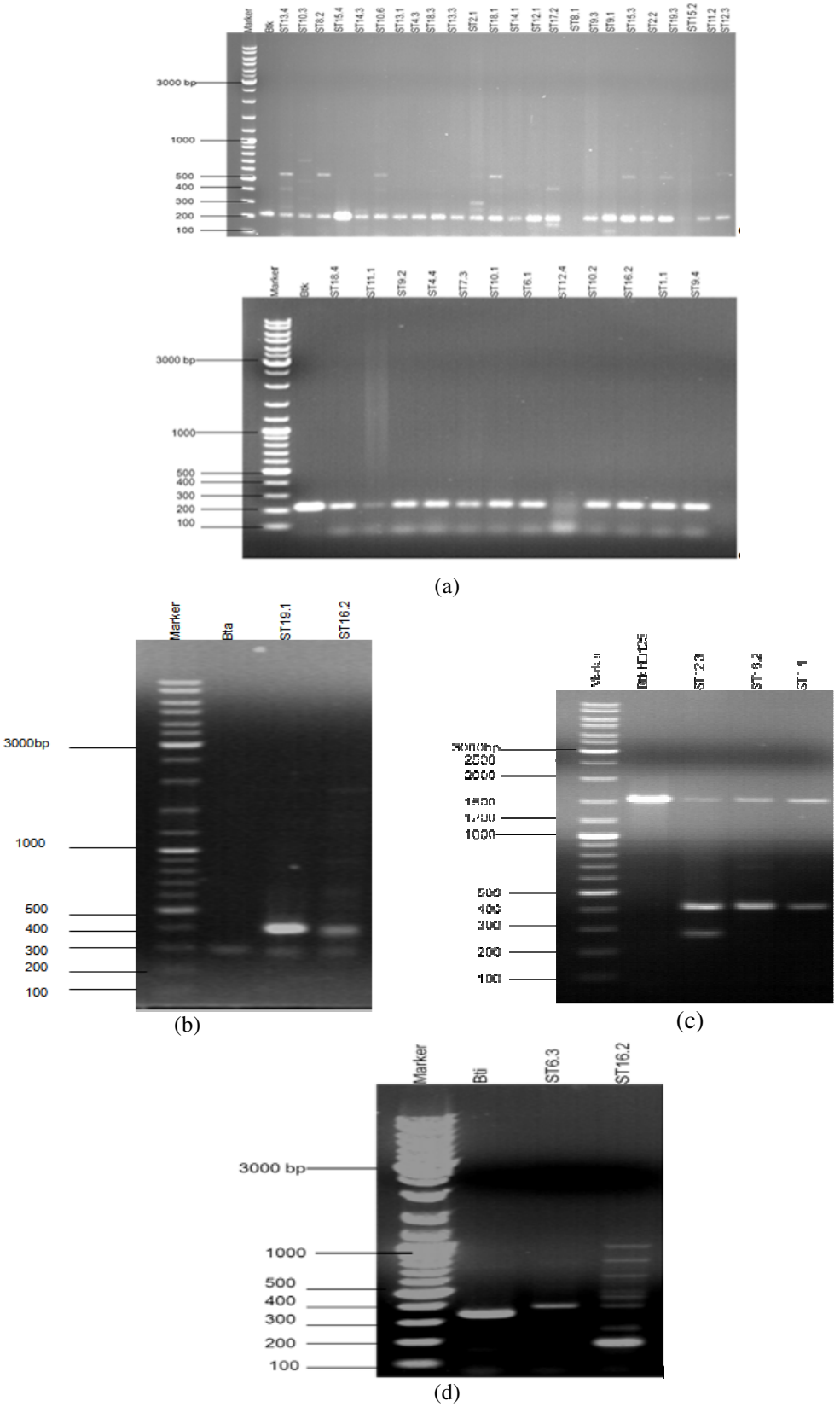

Figure 1. PCR analysis of *Bt* isolates with (a) cry1Ab/Ac (b) cry1D primers, (c) cry2 and (d) cyt2.

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Table 3. *Cry* and *cyt* genes carried by isolates, their Ferequency, spectrum and target insect orders of cry and cyt genes obtained from isolates.

Cry ve *cyt* genes	Insect order	The spectrum of *cry* and *cyt* genes of native isolates					Frequency (%)
CryIAb/Ac	Lepidoptera	ST13.4 ST10.3 ST8.2 ST15.4 ST14.3 ST10.6 ST13.1	ST4.3 ST18.3 ST13.3 ST2.1 ST18.1 ST14.1 ST12.1	ST17.2 ST9.3 ST9.1 ST15.3 ST2.2 ST19.3 ST11.2	ST12.3 ST18.4 ST11.1 ST9.2 ST4.4 ST7.3 ST10.1	ST6.1 ST10.2 ST16.2 ST1.1 ST9.4	60
CryID	Lepidoptera	ST19.1 ST16.2 ST12.3					3.63
Cry2	Lepidoptera Diptera	ST1.1 ST16.2					5.45
Cyt2	Diptera	ST6.3 ST16.2					3.63

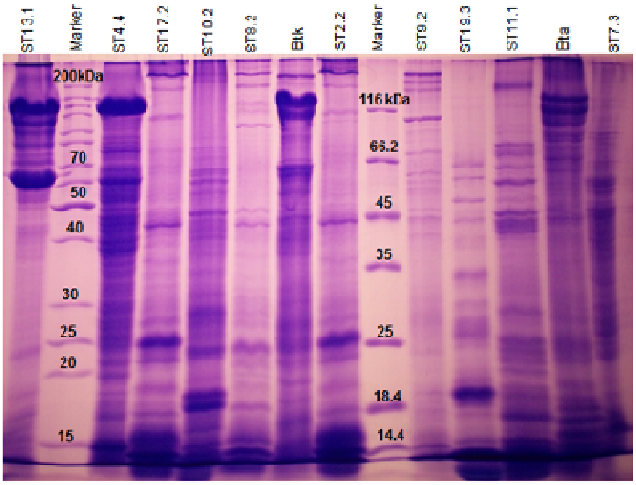

Figure 2. SDS–PAGE of spore/crystal from some native *Bt* strains.

insecticidal crystals as in *Btk* and *Bta*. St13.1, ST4.4 and ST7.3 isolates and *Bta* revealed 70 kDa crystal protein bands representing Cry2 protein.

Bioactivity

Toxicity of isolates and reference strains were tested on third instar larvae of *A. kuehniella* and *P. interpunctella* (Figure 3a-b). A concentration of 1,000 µg g^{-1} spore/crystal mixtures of *Btk*, ST13.1, ST7.3, ST17.2, and ST8.2 isolates caused 62.22, 53.33, 44.44, 44.44, and 40.00% mortalities for *A. kuehniella* larvae, respectively (Figure 3b). *Btk* displayed the highest insecticidal activity (73.33%) against *P. interpunctella* larvae at 1,000 µg g^{-1} concentrations as is the case for *A. kuehniella*. The insecticidal activity of local isolates was fairly low for *P. interpunctella* larvae at the same concentration. The most effective local isolate against *P. interpunctella* larvae were ST13.1, with a 55.56% mortality rate (Figure 3a). Lethal

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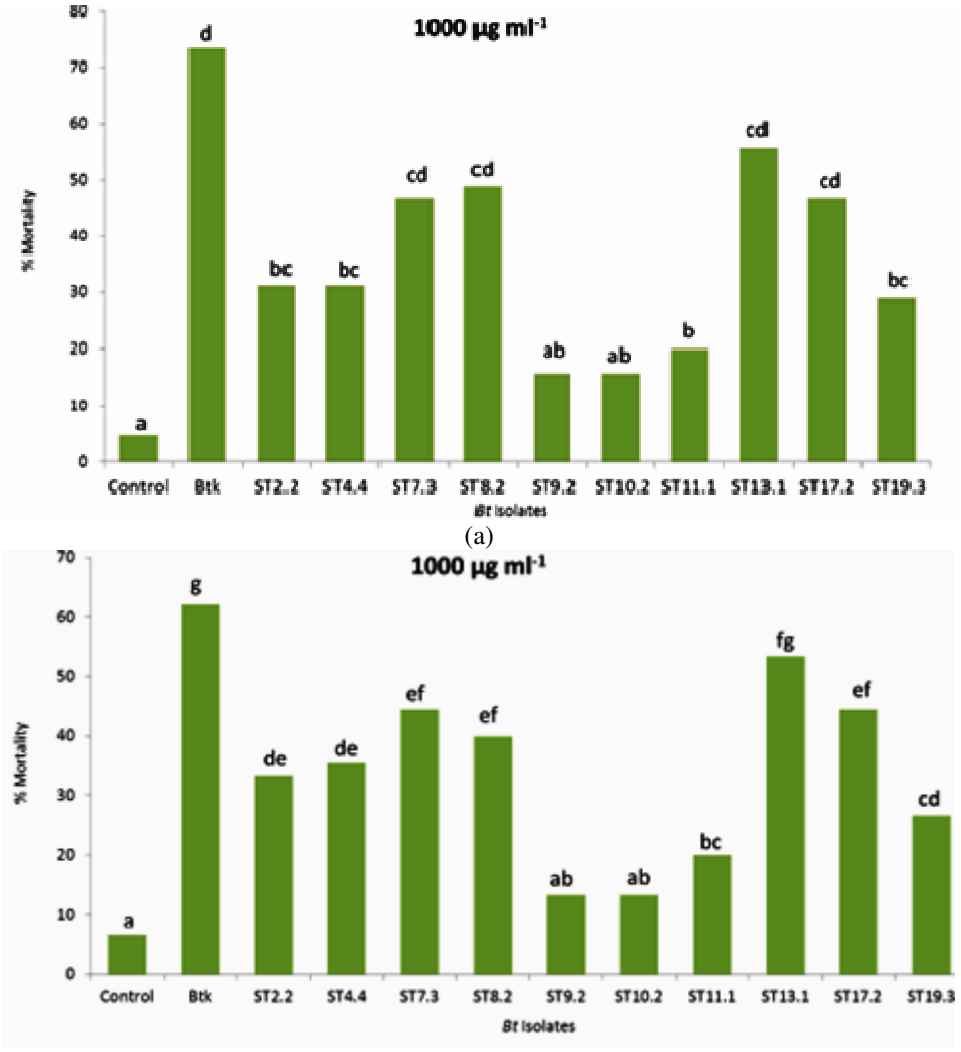

Figure 3. Percent mortality of (a) *P. interpunctella,* (b) *A. kuehniella*, larvae after exposure to spore/crystal mixture of *Bt* isolates. (ST stands for the name of a researcher).

concentrations (LC_{50} and LC_{99}) of local isolates are given in Tables 4 and 5.

DISCUSSION

The characterization of *Bt* isolates obtained from soil samples of different ecosystems in Kahramanmaraş, were conducted. This region was selected due to its productive and important farm lands. The large surface area, different climatic conditions, and rich variety of insects in this region prompted us to characterize native entomopathogenic bacteria. This characterization contributes to an understanding of *Bt* diversity in this region, where *Bt* characterization was not encountered previously. Screening the diversity of *Bt* and their *cry* gene content in unexplored areas will provide valuable information for biological control of pest insects from different orders. Some other researchers stressed the importance of isolating new *Bt* strains from different habitats and indicated differences in the

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Table 4. The LC_{50} and LC_{99} values of *Bt* isolates against *A. kuehniella* larvae.

Isolates [a]	LC_{50} (µg g^{-1})	LC_{99} (µg g^{-1})	χ^2	*df*	*P*
Btk	721.851	2649.818	0.232	1	0.01
ST2.2	1412.874	3744.096	0.175	1	0.011
ST4.4	1428.718	4748.711	2.143	1	0.059
ST7.3	1073.791	3378.968	1.973	1	0.006
ST8.2	1504.308	4997.198	0.522	1	0.072
ST9.2	4698.204	12506.642	0.024	1	0.512
ST10.2	4698.204	12506.642	0.024	1	0.512
ST11.1	2288.911	5899.785	0.051	1	0.132
ST13.1	928.118	2977.417	0.002	1	0.002
ST17.2	1167.692	3958.996	0.000	1	0.021
ST19.3	1575.278	3882.531	0.922	1	0.016

[a] ST stands for the name of a researcher.

Table 5. The LC_{50} and LC_{99} values of *Bt* isolates against *P. interpunctella* larvae**.**

Isolates [a]	LC_{50} (µg g^{-1})	LC_{99} (µg g^{-1})	χ^2	*df*	*P*
Btk	611.201	2081.292	0.019	1	0.000
ST2.2	1621.640	4802.853	0.648	1	0.057
ST4.4	1476.922	4096.046	0.024	1	0.022
ST7.3	1033.098	3614.084	1.290	1	0.012
ST8.2	985.710	3557.946	0.732	1	0.012
ST9.2	2174.126	4917.189	0.127	1	0.078
ST10.2	6952.677	25340.081	0.012	1	0.759
ST11.1	2134.061	5509.384	1.459	1	0.106
ST13.1	848.238	3181.479	0.057	1	0.005
ST17.2	1072.631	3752.175	0.190	1	0.016
ST19.3	1944.192	6019.849	0.132	1	0.137

[a] ST stands for the name of a researcher.

occurrence and diversity of *cry* genes (Chak *et al.,* 1994; Bravo *et al.,* 1998).

The isolates from this region were found to have *cry1Ab/Ac, cry1D, cry2* and *cyt2* genes. Of all, *cry1Ab/Ac* gene was the most common (60%). Wang *et al.* (2003) reported that *cry1* type genes were the most abundant among the *Bt* isolates from different regions and sources**.** Likewise, Yılmaz (2010) revealed that the frequency of *cry1Ab/Ac* was the highest (47.72%) of all the *cry* genes studied in isolates obtained from the neighbor region, Adana. However, frequency of *cry2* gene (5.45%) was lower than the result (31.82%) of the same researcher.

Bt has been known to have several circular/linear plasmids, and *cry* genes generally found in extra-chromosomal elements (Carlson *et al.,* 1996; Reyes-Ramirez and Ibarra, 2008). Plasmids, varying in number and size, are valuable tools to characterize strains. Plasmid profile of local isolates revealed large and smaller plasmid bands which were compared with those of *Bta* and *Btt*. Plasmid profiles of ST4.4, ST11.1, ST13.1 and ST19.3 isolates indicated unique plasmid patterns and variability as indicated by Noguera *et al.,* (2010). The isolates ST11.1, ST19.3 and *Bta* displayed a single co-migrating band indicating some degree of relationship among them. The isolates ST17.2, ST7.3, ST2.2, ST9.2, ST8.2 and ST10.2 gave identical plasmid patterns. However, due to their co-migration together with

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chromosomal DNA, it was difficult to distinguish among them. Reyes-Ramirez and Ibarra (2008) reported that it was difficult to distinguish between mega plasmids and chromosomal DNA and, therefore, comparison was focused only on those plasmids migrating below the chromosomal DNA. They also stated that mega plasmids were used as a secondary option to differentiate among plasmid patterns.

Crystal proteins of *Bt* isolates were further characterized by SDS-PAGE analysis. These local isolates exhibited protein bands with molecular masses in the range of 15 to 140 kDa. Most of the isolates contained two protein types, 130-140 and 60-70 kDa. It was reported that Cry proteins were generally either 60-80 or 130-150 kDa (Federici *et al.,* 2006; Haggag and Abou Yousef, 2010). The SDS-PAGE analysis also exhibited other proteins (approximately 15, 25, 45, and 100 kDa) in some of the isolates. Similarly, Lopez-Pazos *et al.* (2009) reported that great majority of their isolates revealed proteins with molecular masses between 35 and 135 kDa.

Bioactivity of the local *Bt* isolates was also tested against the two stored product pests *A. kuehniella* and *P. interpunctella* larvae, to evaluate their toxicity. But the efficacy of these isolates was lower than that of the *Btk*. Toxicity values of the isolates varied between 20-53.33% for *A. kuehniella* and 20-55.56% for *P. interpunctella* at the highest concentration applied. Although ST9.2 and ST10.2 carried *cry1Ab/Ac* gene and produced expected plasmid and protein bands, their toxicity values were insignificantly different from untreated control. Nevertheless, this result is only limited to the tested moth larvae. Local *Bt* isolations reported by other researchers also expresses that most of the isolates have weak or non-insecticidal activity against insect pests (Yılmaz, 2010; El-kersh *et al.,* 2012; Azizoglu, 2011).

A significant finding of this study is that spore-crystal mixture of local *Bt* isolates was toxic to third instar larvae of *A. kuehniella* and *P. interpunctella*. Although the results seem promising, further studies have to be conducted for more detailed characterization of the isolates to provide a more complete picture for their effectiveness on other insect pests.

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غربالگری ملکولی وفعالیت زیستی جدایه های بومی *Bacillus thuringiensis*

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چکیده

در این پژوهش، سویه های *Bacillus thuringiensis* از نمونه های خاک جمع آوری شده از مناطق مختلف کهرمان ماراش(Kahramanmaraş) تر کیه جدا شد و مشخصات آن ها با استفاده از موارد زیرمورد شناسایی قرار گرفت:مرفولوژی کلنی، روش PCR با آغازگرهای cryIAb/Ac, cryID, cry2 وcyt2 و SDD-PAGE کریستال پروتئین با وزن ملکولی 130-140 kDa و60-70 kDa . همچنین، پروفیل های پلاسمید جدایه های انتخابی هم تجزیه شد. در میان ژن های *cry* مطالعه شده،*cry1Ab/Ac* بیشتر از همه(۶۰٪) سویه های محلی بود. نیز برای ارزیابی موثر بودن جدایه ها علیه لارو سن سوم حشرات محصولات انباری شامل *Anagasta kuehniella* و *Plodia interpunctella* در شرایط آزمایشگاهی آزمون های فعالیت زیستی هم انجام شد. درجه سمیت جدایه ها در غلظت ۱۰۰۰µg/g بین 20.00-*53.33* ٪ برای *Anagasta kuehniella* و*20.00-55.56*٪ برای *Plodia interpunctella* تغییر می کرد. از میان ۵۵ سویه، *ST13.1*موثرترین آنها بر علیه *Anagasta kuehniella* بود در حالی که در شرایط آزمایشگاهی، سویه های *ST7.2*، *ST7.3* و *ST8.2* کنترل قابل ملاحظه ای روی*Plodia interpunctella* بروز دادند. در این زمینه انجام آزمون های بیشتر توصیه می شود تا موثر بودن این جدایه های *Bt* بر علیه گونه های دیگر حشرات آفات بررسی شود.

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