Turkish Journal of Agriculture and Forestry

Volume 38 | Number 6 Article 14

1-1-2014

Distribution, occurrence of cry genes, and lepidopteran toxicity of native Bacillus thuringiensis isolated from fig tree environments in Aydın Province

MEHLİKA ALPER

HATİCE GÜNEŞ

ARZU TATLIPINAR

BEKİR ÇÖL

HASAN SUNGUR CİVELEK

See next page for additional authors

Distribution, occurrence of cry genes, and lepidopteran toxicity of native Bacillus thuringiensis isolated from fig tree environments in Aydın Province

Authors
MEHLİKA ALPER, HATİCE GÜNEŞ, ARZU TATLIPINAR, BEKİR ÇÖL, HASAN SUNGUR CİVELEK, CEM ÖZKAN, and BURCU POYRAZ

TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2014) 38: 898-907

doi:10.3906/tar-1402-27

Distribution, occurrence of *cry* genes, and lepidopteran toxicity of native *Bacillus thuringiensis* isolated from fig tree environments in Aydın Province

Mehlika ALPER[1], Hatice GÜNEŞ[1,*], Arzu TATLIPINAR[1], Bekir ÇÖL[1], Hasan Sungur CİVELEK[1], Cem ÖZKAN[2], Burcu POYRAZ[1]

[1]Department of Biology, Muğla Sıtkı Koçman University, Kötekli Campus, Muğla, Turkey
[2]Department of Plant Protection, Ankara University, Tandoğan Campus, Ankara, Turkey

Received: 10.02.2014 • **Accepted:** 25.06.2014 • **Published Online:** 31.10.2014 • **Printed:** 28.11.2014

Abstract: *Bacillus thuringiensis* (Bt) has a significant impact on biological pest control because of the insecticidal activity through its parasporal inclusion bodies (crystal proteins). Fig is an economically important plant in Turkey; agricultural pests result in a considerable economic loss in fig quality and cultivation. The aim of this work was to isolate, characterize, and determine the lepidopteran toxicity of Bt obtained from fig groves in Aydın Province. A total of 606 colonies (out of 1167) obtained from 380 samples were identified as Bt based on parasporal crystal formation. The highest Bt index of 0.60 was observed in the Kuyucak region. A total of 288 Bt isolates were characterized in terms of *cry* gene content by PCR analysis. It was found that the *cry1* plus *cry2* genotype was the most abundant (40%) in our collection. Bioactivity tests indicated that 6 isolates exhibited high mortalities against *Cadra cautella* and 3 isolates were found to exhibit high toxicity against *Carpophilus hemipterus*. Moreover, 13 Bt isolates exhibiting toxic activity against fig pests were further characterized based on specific *cry* gene content, protein profiles, and PCR-RFLP analysis. Among *cry1* genes, the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1C*, *cry1D*, and *cry1Ea* genes were the most frequent (100%). Protein profiles of isolates toxic to *C. cautella* were different from those of isolates toxic to *C. hemipterus*. PCR-RFLP analysis indicated that toxic isolates differed from the reference strain with respect to *cry1* type gene. Finally, it was concluded that Bt strains isolated from fig groves showed high level of toxicity against fig pests. These strains can serve as potential biopesticides for the control of *C. cautella* in the region as well as alternative biopesticides in the case of pesticide resistance in insects.

Key words: *Bacillus thuringiensis*, bioactivity, *cry* gene, fig groves

1. Introduction

Bacillus thuringiensis (Bt) has been known as a microbial biocontrol agent because of its ability to produce a large amount of insecticidal proteins during sporulation (Schnepf et al., 1998). Due to the crystal phenotype of the proteins, the genes encoding them were designated as *cry* and are usually located on large molecular weight plasmids (González et al., 1981). It was reported that Cry proteins showed a highly selective insecticidal activity against different insect orders (Höfte and Whiteley, 1989; Feitelson, 1992). To date, more than 200 *cry* gene sequences have been identified and classified into 72 groups according to amino acid sequence similarity (Crickmore et al., 2012)

Nowadays, harmful effects of chemical pesticides on human health and the environment have made biopesticides more attractive. Bt-based biopesticides are the main biopesticides used in agricultural pest control (Bravo et al., 2011). Worldwide isolation of Bt strains has been carried out to identify new *cry* genes and proteins with high toxic activity against the target insect species (Martin and Travers, 1989; Ben-Dov et al., 1997; Bravo et al., 1998; Uribe et al., 2003; Apaydin et al., 2005; Cinar et al., 2008; Seifinejad et al., 2008).

Fig trees are grown mainly in Aydın Province of the Aegean region in Turkey. *Cadra cautella* and *Carpophilus hemipterus* are among the main destructive pests of fig fruits (Akşit et al., 2003). They cause significant economic losses in fig quality and production. So far, commonly used pest control has been based on synthetic pesticides. As an alternative, the discovery of an environmentally friendly control mechanism is highly desirable. Bt-based biopesticides could be a promising control agent against the fig pests. Therefore, the aim of this study was to search for the occurrence of native Bt strains in the fig plantation area and to characterize *cry* gene content and bioactivity of candidate Bt strains. In this report, it is shown for the first time that some of the Bt strains isolated from Aydın Province of Turkey have high bioactivity against fig pests.

* Correspondence: haticegunes@mu.edu.tr

2. Materials and methods

2.1. Bacterial strains

Reference strains Bt subsp. *kurstaki* (BGSC 4D1), Bt subsp. *aizawai* (BGSC 4J3), and Bt biovar. *israelensis* (BGSC 4Q2) were kindly supplied by Prof Dr Zeigler (*Bacillus* Genetic Stock Center, Columbus, OH, USA).

2.2. Sample collection

A total of 380 samples were collected from fig-related areas in 8 different locations within Aydın Province. Samples for bacterial isolation included soil, fig fruit, and fig leaves. The source of these samples had not been previously treated with any Bt biopesticides. About 200 g of soil samples were collected with a sterile spatula at depths of 5–10 cm. Leaf samples were collected from trees and soil contamination was avoided. Dry figs were obtained from fig storage. Samples were placed in sterile plastic bags and stored at 4 °C until processed.

2.3. Isolation of Bt and analysis of parasporal crystal formation

Isolation of the bacteria was carried out according to the method of acetate selection (Travers et al., 1987) for soil samples and shaken-flask technique (Smith and Couche, 1991) for the leaf and fruit samples. After heat treatment at 80 °C for 10 min, the samples were plated on nutrient agar (NA) and allowed to grow overnight at 37 °C. Bt-like colonies described as cream-colored with a fried egg appearance on the plates were labeled and subcultured. Each pure subculture was grown on NA plates, dispersed in sterile water, and examined with a phase contrast microscope for crystal formation. Duplicate stock samples in 25% glycerol were kept at –80 °C.

2.4. Determination of *cry* gene

Polymerase chain reaction (PCR) was used to identify *cry* gene content. In total, 288 random isolates producing crystal inclusions were screened for 5 pairs of universal primers for the *cry1*, *cry2*, *cry3*, *cry4*, and *cry9* genes as described by Ben-Dov et al. (1997, 1999). *cry1*-positive strains exhibiting bioactivity against fig pests were subjected to further analysis for the *cry1* genotype. Gene-specific primers reported by Ceron et al. (1994, 1995) were used to detect the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1E*, *cry1F*, *cry1G*, and *cry1H* genes. Genomic DNA was isolated according to the method described by Ausebel et al. (1994) and the reference strains served as controls in PCR reactions. Each amplification process was carried out in a 50-µL reaction mixture containing 200 µM dNTP, 0.5 µM of each universal primer or 0.2–0.4 µM of each specific primer, 1.5 mM $MgCl_2$, and 2 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) in an Advanced Primus 96 Thermal Cycler (PeqLab, Erlangen, Germany). PCR conditions were as follows: initial denaturation step for 1 min at 95 °C, followed by 35 amplification cycles including denaturation at 95 °C for 1 min, annealing at 50 °C for *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1*, *cry1C*, *cry1G*, *cry3B*, and *cry3C* genes; 54 °C for *cry1*, *cry1Aa*, *cry1D*, *cry1Ea*, *cry1H*, *cry2Aa1* and *cry3A*; and 60 °C for *cry2*, *cry3*, *cry4*, *cry9*, *cry1F*, *cry2Ab2*, and *cry2Ac* genes for 1 min, and elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. After amplification, 10 µL of each PCR product was electrophoresed on 1% or 2% ethidium bromide agarose gel and DNA bands were visualized in a gel documentation system (Vilber Lourmat, Marne-la-Vallée, France).

2.5. SDS-PAGE analysis

Bt isolates were inoculated in 5 mL of nutrient broth and allowed to sporulate for 3–4 days at 30 °C with 200 rpm shaking conditions. After that, samples were centrifuged for 10 min at 10,000 rpm and supernatants were discarded. Pellets were resuspended in 1.4 mL of Tris EDTA buffer [10 mM Tris, 1 mM EDTA pH 8, and 1 mM phenyl-methane-sulfonyl-fluoride (PMSF)] and centrifuged for 10 min at 10,000 rpm at 4 °C. After washing the pellet twice with 0.5 M NaCl, the pellet was resuspended in ice-cold EDTA buffer and centrifuged for 10 min at 10,000 rpm at 4 °C. Pellet-containing spore–crystal mixture was dissolved in sterile H_2O with 1 mM PMSF and stored at –20 °C until used. The protein concentration of each sample was determined by Bradford assay. For electrophoresis, the sample (5 µg/lane) was mixed with an equal volume of sample buffer (0.15 M Tris/HCl pH 8.8, 375 mM EDTA, 0.75 M sucrose, 0.0075 M bromophenol blue, 2.5% SDS, and 7.4 mM dithiothreitol) and boiled for 10 min (Bel et al., 1997). Electrophoresis was carried out in 10% separating and 5% stacking gels under 35 mA as described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R250 (Sigma-Aldrich, Schnelldorf, Germany) and molecular masses of the crystal proteins were determined by using protein markers (Fermentas).

2.6. Bioassay

Second-instar larvae of *C. cautella* grown as described by Magana et al. (2007) were used for the investigation of toxic potency of Bt isolates. Spore–crystal mixtures were prepared from 102 Bt isolates harboring Cry1 proteins that are known to be toxic against lepidopteran insects (Bravo et al., 1998). A spore–crystal mixture was prepared according to the technique of Bravo et al. (1998). Briefly, Bt culture was grown in 100 mL of nutrient broth at 28 °C by shaking at 150 rpm for 3 days. Samples were centrifuged at 4 °C at 6000 rpm for 15 min and the pellets were washed twice with ice-cold 1 M NaCl and 3 times with sterile distilled water. Finally, the pellets were dried overnight at 37–40 °C, weighed, and stored as powder at –20 °C until used.

Spore–crystal powders were suspended in distilled water containing 0.1% Tween 80. Suspensions were mixed with a diet that included wheat bran/corn powder (3:1) at the concentration of 500 ppm (2500 µg of spore–crystal mixture in 5 g of compost) and dried. For some of the strains, 5 different doses prepared as serial dilutions (25, 50, 100, 250, and 500 ppm) were applied for probit analysis. Assays were carried out using 20 larvae per dose with 3 replicates. A diet without toxin served as a negative control. Toxicity tests were carried out at 25 °C and 70% relative humidity with 16:8 h L:D schedule; larval mortality was recorded after 4 days. The 50% lethal concentration (LC_{50}) and confidence limits (LC_{95}) of the most toxic isolates were determined by probit analysis with SPSS 11.0. The mortality data were corrected by Abbott's formula (1925).

Adult *C. hemipterus* specimens were collected from fig storage and grown on sliced orange and lemon in the laboratory. Second- and third-instar larvae were used for bioassay. Spore–crystal mixtures were prepared from the Bt isolates producing Cry3 and Cry9 proteins. Toxicity tests of the isolates were performed as described above.

2.7. PCR-RFLP analysis of cry1 gene of Bt isolates

In order to determine whether the *cry1* gene of the toxic Bt isolates differed from that of the reference strain Bt *kurstaki*, the *cry1* gene was amplified with K5un2F and K3un2R primers as described by Kuo and Chack (1996). After cleaning up the PCR product at around 1500–1600 bp with a gel extraction kit (QIAGEN GmbH, Hilden, Germany), PCR-RFLP analysis was carried out using different Fast Digest restriction enzymes (Fermentas). *cry1*-type genes listed on Neil Crickmore's web page (http://www.lifesci.sussex.ac.uk/Home/Neil-_Crickmore/Bt/) were aligned with the BioEdit program. After performing in silico digestion with different restriction enzymes, the sizes of DNA fragments were determined and compared with experimental results.

3. Results

3.1. Distribution of Bt in Aydın Province

A total of 380 samples consisting of 184 soil samples, 130 fig fruits, and 66 fig leaves collected from 8 different locations within Aydın Province were examined for the occurrence of Bt isolates (Table 1). According to colony morphology, 1167 isolates were obtained and 606 of them were identified as Bt based on parasporal crystal formation. Bt was found in 316 of the 380 samples, which corresponded to 83% of the total samples. The Bt index reflecting the ratio of Bt colonies in total colonies isolated ranged between 0.28 and 0.72 throughout the locations with an average value of 0.52 (Table 1). As compared to all locations, the Kuyucak region showed the highest Bt index of 0.60. The frequency of Bt occurrence among the sample types changed throughout the locations. For example, soil samples rendered the highest Bt index in the Kuyucak (0.72) and Nazilli (0.64) locations; however, the highest Bt index from the fig leaves was obtained in the Germencik (0.70) and İncirliova (0.69) regions.

3.2. Determination of the *cry* gene profile

PCR analysis was carried out with universal primers specific for the *cry1*, *cry2*, *cry3*, *cry4*, and *cry9* genes in order to identify the *cry* gene content of 288 randomly selected isolates. Of them, 222 isolates were found to be positive for at least 1 of the *cry* genes examined. PCR analysis indicated that 44% of the isolates had only 1 type of *cry* gene, as shown in Figure 1. The percentage of the isolates harboring 1 type of *cry* gene are 36% for *cry1*, 3% for *cry2*, 1% for *cry3*, 2% for *cry4*, and 2% for *cry9*. On the other hand, 56% of these 222 isolates possessed more than 1 type of *cry* gene. The percentage of the isolates containing both *cry1* and *cry2* genes was the highest (40%) as compared with the other *cry* gene combinations. As a result, 16 groups of *cry* genes were obtained (Figure 1).

3.3 Bioactivity of Bt isolates

Because the Cry1 protein is toxic to insects belonging to Lepidoptera, 102 Bt strains positive for the *cry1* gene were selected for bioassay analysis. Insecticidal toxicity of the selected isolates was evaluated against a lepidopteran species, *C. cautella*. It was found that only 1 Bt strain did not exhibit any toxicity, while the remaining strains showed various degrees of mortality (Table 2). Among those, it was observed that 6 Bt strains exhibited high levels of toxicity, causing mortality above 60%. The mortality obtained from the control strain Bt subsp. *kurstaki* was above 95% in all the experiments. Based on LC_{50} results and fiducial limits, isolates 13MY and 42MY were the most toxic native strains, causing 97% mortality as presented in Table 3.

Furthermore, 28 Bt isolates harboring *cry3* and/or *cry9* genes were examined for toxicity against second- and third-instar larvae of *C. hemipterus*. Among them, 26 showed variable degrees of toxicity, as shown in Table 4. Bt strains 69T, 71T, and 153T exhibited the highest mortality in the range of 50% to 65%.

3.4. Identification of *cry* type genes

Thirteen of the *cry1*-positive isolates with varying degrees of toxicity to *C. cautella* were further characterized by PCR analysis using gene-specific primers for the *cry1* genes: *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea*, *cry1F*, *cry1G*, and *cry1H*. Results indicated that all Bt isolates were positive for more than 1 type of *cry1* genes. The *cry1* gene content of 4 different *cry1* profiles ranged from 8 to 10, as presented in Table 5. Among these, *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1C*, *cry1D*, and *cry1Ea* genes detected in all isolates were the most frequent genes (100%), followed by the *cry1Ad* (94%), *cry1G* (78%), and *cry1H* (43%). However, the *cry1F* gene was not observed in the tested strains.

Table 1. Distribution of *B. thuringiensis* based on sample types and location.[a]

Location	Type of sample	No. of samples examined	No. of samples yielding Bt	No. of isolates obtained	No. of isolates producing crystals	No. of isolates screened by PCR	No. of isolates positive for *cry* genes	Bt Index
Bozdoğan/Aydın	Soil	25	19	74	43	18	12	0.58
	Fig	11	8	26	15	2	2	0.58
	Leaf	6	6	21	10	4	4	0.48
		42	33	121	68	24	18	0.56[b]
Germencik/Aydın	Soil	37	32	131	65	45	37	0. 50
	Fig	31	25	82	38	12	11	0. 46
	Leaf	18	16	57	40	14	12	0. 70
		86	73	270	143	71	60	0.52[b]
İncirliova/Aydın	Soil	38	31	116	60	34	18	0.52
	Fig	32	26	85	40	18	18	0.47
	Leaf	18	16	58	40	18	17	0.69
		88	73	259	140	70	53	0.54[b]
Köşk/Aydın	Soil	20	16	69	32	15	10	0.46
	Fig	11	7	34	16	4	3	0.47
	Leaf	5	4	21	6	2	2	0.29
		36	27	124	54	21	15	0.44[b]
Kuyucak/Aydın	Soil	16	16	43	31	12	8	0.72
	Fig	9	8	41	20	8	8	0.49
	Leaf	4	4	10	6	1	1	0.30
		29	28	94	57	21	17	0.60[b]
Nazilli/Aydın	Soil	19	18	61	39	23	12	0.64
	Fig	22	16	58	25	13	13	0.43
	Leaf	5	3	13	5	2	2	0.38
		46	37	132	69	38	27	0.52[b]
Sultanhisar/Aydın	Soil	17	15	55	29	17	12	0.53
	Fig	6	6	18	9	3	3	0.50
	Leaf	4	2	8	4	3	2	0.50
		27	23	81	42	23	17	0.52[b]
Center/Aydın	Soil	12	10	40	16	13	8	0.40
	Fig	8	7	32	9	3	3	0.28
	Leaf	6	5	14	8	4	4	0.57
		26	22	86	33	20	15	0.38[b]
	Total	380	316	1167	606	288	222	0.52

[a]: Isolates were examined by PC microscope for crystal inclusion. The *cry* gene content of crystal-positive isolates was screened by PCR. Bt index is the ratio of Bt isolates producing crystals to all isolates in each sample group.
[b]: Indicates the total Bt index in each geographical location.

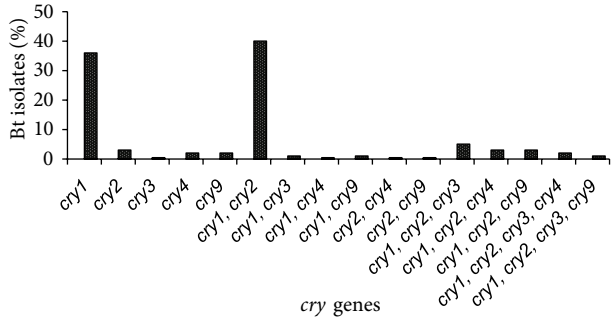

Figure 1. Distribution of *cry* genes in *B. thuringiensis* strains.

Table 2. Toxicity of native *B. thuringiensis* isolates to *Cadra cautella*.

Toxicity*	Name of Bt isolate	Percentage
Nontoxic	24T	0. 98 (1)[a]
≤15%	1T, 2T, 3T, 4T, 5T, 6T, 7T, 8T, 9T, 10T, 11T, 12T, 13T, 14T, 15T, 16T, 17T, 18T, 19T, 20T, 21T, 22T, 25T, 26T, 27T, 28T, 97T, 108T, 109T, 110T, 111T, 112T, 113T, 114T, 115T, 116T, 128T, 134T, 138T, 139T, 140T, 150T, 155T, 156T, 158T, 167T, 176T, 1MY, 2MY, 3MY, 4MY, 5MY, 6MY, 7MY, 8MY, 9MY, 10MY, 11MY, 12MY, 46MY, 49MY,50MY, 52MY, 75MY, 76MY, 77MY, 78MY, 79MY, 100MY, 101MY, 103MY	69.60 (71)[a]
15%–35%	33T, 98T, 124T, 125T, 145T, 169T,43MY, 44MY, 47MY, 48MY, 69MY, 70MY, 80MY, 81MY, 102MY, 105MY, 106MY	16.66(17)[a]
35%–60%	40T, 48T, 54T, 69T, 71T, 107T, 104MY	6. 86 (7)[a]
61%–80%	23T, 106T, 51MY	2. 94 (3)[a]
≥82%	13MY, 42MY, 45MY	2. 94 (3)[a]
Total		100.00 (102) [a]

*: One dose (500 ppm) assay against second-instar larvae of *C. cautella*.
[a]: Number of *B. thuringiensis* isolates.

Table 3. LC_{50} value of selected *B. thuringiensis* isolates toxic to *Cadra cautella*.

Isolate	Mortality (%)[a]	LC_{50} [b]	FL (95%)[c]
13MY	97	63	53–74
42MY	97	82	66–99
45MY	87	154	130–178
23T	72	176	147–216
106T	62	223	167–325
107T	52	449	388–541
Bt subsp. *kurstaki*	98	34	27–40

[a]: The highest mortality (%) observed in bioassay at 500 ppm toxin concentration.
[b]: Results are averages of 6 replicates and expressed as ppm of toxin per 5 g of compost; spore–crystal mixtures of isolates were assayed from 25 ppm to 500 ppm/5 g of compost.
[c]: FL_{95} min–max, 95% confidence limit.

Table 4. Toxicity of native *B. thuringiensis* isolates to *Carpophilus hemipterus*.

Toxicity*	Name of Bt isolate	Percentage
Nontoxic	49T, 96T	7.14 (2)[a]
≤10%	97T, 107T, 167T, 13MY, 42MY	17.85 (5)[a]
10%–30%	3T, 4T, 6T, 10T, 23T, 33T, 40T, 48T, 54T, 98T, 124T, 125T, 169T, 46MY	50.00 (14)[a]
30%–50%	106T, 158T, 145T, 48MY	14.28 (4)[a]
50%–65%	69T, 71T, 153T	10.71 (3)[a]
Total		100 (28)[a]

*: One dose (500 ppm) assay against second and third instar larvae of *C. hemipterus*.
[a]: Number of *B. thuringiensis* isolates.

Table 5. *cry1* gene profile of *B. thuringiensis* isolates.

Profile	*cry1* gene content	Name of isolate
1	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea*, *cry1G*	8T
2	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea*	71T, 153T, 44MY
3	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea*, *cry1G*	10T, 69T, 106T, 51MY
4	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea*, *cry1G*, *cry1H*	23T, 107T, 13MY, 42MY, 45MY

In addition, 10 *cry3*-positive isolates were further screened for the presence of the *cry3A*, *cry3B*, and *cry3C* genes. Isolates containing *cry3A* were the most abundant and represented 100% of the isolates, whereas *cry3B* was the least abundant and was found in 67% of the isolates, as shown in Table 6. The *cry3C* gene was not detected in any of the isolates.

3.5 Crystal protein composition

Protein profiles of some Bt isolates exhibiting toxicity against *C. cautella* or *C. hemipterus* were determined by SDS-PAGE along with the reference strain of Bt subsp. *kurstaki*. Isolates displayed proteins with molecular masses between 35 and 240 kDa, but the most common profiles contained proteins of around 65 kDa and 130 kDa in size (Figure 2, lanes 1 to 6). The protein profiles of the isolates toxic to *C. hemipterus* were observed to be different from those of the isolates toxic to *C. cautella* (Figure 2, lanes 7 to 11). Even though the isolates toxic to *C. hemipterus* carry *cry1* and *cry2* genes, the intensity of Cry1 and Cry2 protein bands at 65 kDa and 130 kDa was not as strong as that of protein bands of the isolates toxic for *C. cautella* (Figure 2).

3.6. PCR-RFLP analysis of *cry1* gene

In order to observe if a difference exists among the isolates with respect to *cry1* gene restriction profile, PCR products for the *cry1* gene of the most toxic isolates (13MY, 45MY, 23T 106T, and 107T) were digested with various restriction enzymes: *Xba*I, *Pst*I, *Dde*I, *Eco*RI, *Hind*III, and *Cla*I. The resulting DNA fragments were compared with those of in silico digest. All isolates exhibited the same RFLP pattern; however, the reference strain Bt subsp. *kurstaki* produced a different RFLP pattern. For example, the *Xba*I restriction enzyme produced 2 DNA fragments of approximately 1000 bp and 500 bp in size for the reference strain, indicating the presence of the *cry1Aa* gene. However, 4 DNA fragments of approximately 600, 500, 250, and 150 bp were obtained for the toxic isolates, indicating the presence of the *cry1Ea* gene (Figure 3A). In addition, *Pst*I restriction profiles of PCR products from the reference

Table 6. *cry3* gene profile of *B. thuringiensis* isolates.

Profile	*cry3* gene content	Name of isolates
1	*cry3A*, *cry3B*	23T, 69T, 71T, 106T, 107T, 45MY, 51MY
2	*cry3A*	13MY, 42MY, 153T

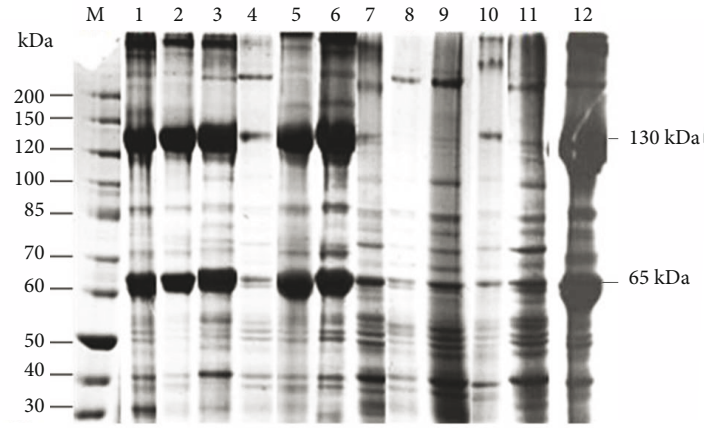

Figure 2. SDS-PAGE analysis of spore–crystal mixture from toxic Bt isolates. M, molecular weight marker (Fermentas); lane 1, 13MY; lane 2, 42MY; lane 3, 45MY; lane 4, 51MY; lane 5, 23T; lane 6, 106T; lane 7, 69T; lane 8, 71T; lane 9, 153T; lane 10, 158T; lane 11, 145T; lane 12, Bt subsp. *kurstaki*.

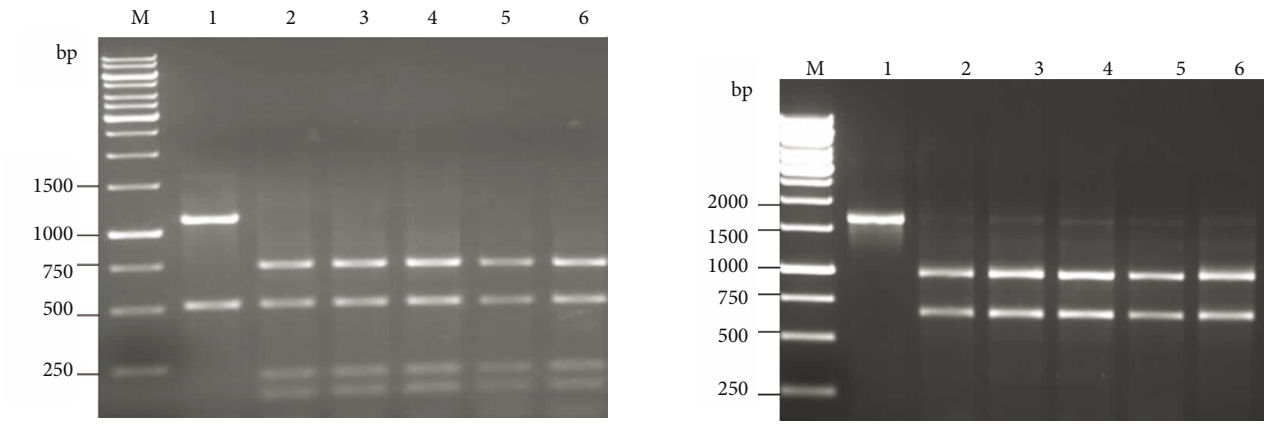

Figure 3. PCR-RFLP analysis for PCR products of *cry1* gene. **A)** *Xba*I restriction; **B)** *Pst*I restriction. M: marker; lane 1: Bt *kurstaki*; lane 2: 13 MY; lane 3: 42 MY; lane 4: 45MY; lane 5: 23T; lane 6: 106T.

strain and the toxic isolates were found to be different from each other. The reference strain produced 1500-bp DNA fragments, whereas the toxic isolates produced 2 different DNA fragments of 900 bp and 600 bp (Figure 3B). Based on PCR-RFLP analysis, the results indicate that the toxic isolates differ from the reference strain with respect to the *cry1* type gene. Cloning of whole sequence of these *cry1* genes for more detailed investigation will be carried out in the near future.

4. Discussion

In this work, Bt occurrence was examined in fig-related areas of Aydın where no commercially available Bt strains have been applied before to control insect pests. Fig is susceptible to more than 5 species of insect pests (Akşit et al., 2003); *C. cautella* and *C. hemipterus* are among the most destructive. In order to protect fig fruits from agricultural pests, it is important to discover Bt-based biopesticides compatible with human and environmental health.

Samples of soil, fig leaves, and fig fruit for Bt isolation were collected from 8 different locations of fig groves in Aydın Province (Table 1). It was found that 83% of 380 total samples yielded Bt. Although no bacterial growth was observed in some of the leaf and fruit samples, all soil samples contained Bt strains. This finding is in agreement with several previous reports (Martin and Travers, 1989; Bel et al., 1997; Uribe et al., 2003; Apaydin et al., 2005; Cinar et al., 2008; Gao et al., 2008) that demonstrated that the soil is the richest source of Bt isolates.

This study was the first to describe the distribution of Bt in the fig groves of Aydın Province. The Bt index serves as an indicator of success in Bt isolation. The highest index of

0.60 and the lowest index of 0.38 were detected in Kuyucak and central Aydın, respectively (Table 1). In addition, the Bt index of sample types showed variations according to the locations. For example, the highest Bt index of 0.70 for the leaf samples was observed in the Germencik area, whereas the lowest Bt index of 0.29 was detected in the Köşk region. Among the fruit samples, the highest Bt index of 0.58 was detected in the Bozdoğan area, while the lowest index of 0.28 was recorded in the central Aydın region. The highest Bt index of 0.72 and the lowest Bt index of 0.40 were recorded in soil samples obtained from the Kuyucak and central Aydın regions, respectively. Taken together, the Bt index shows variations according to sample types and locations. The soil sample of the Kuyucak region was the best source for Bt isolation based on the highest Bt index of 0.72. Finally, the central Aydın region was not a better source for the Bt isolation from the soil and the fruit samples. Although all these locations have the same geographic and mild Mediterranean climate, observed differences in Bt index could be due to the pH level and nutritional value of the soil as well as the accumulation of environmental toxins. Further detailed investigations of ecological parameters will explain the possible reason for the differences in the Bt index observed among these locations.

Because of an association between target insect spectra and the type of *cry* gene (Chambers et al., 1991), the type and the subgroups of *cry* genes were identified in this study. A total of 288 crystal-forming Bt isolates were screened with primers for 5 different *cry* genes and it was found that 222 isolates contained at least 1 type of *cry* gene distributed through 11 different *cry* gene profiles. The percentage of the isolates containing only the *cry1* gene was the highest (36%) compared to the isolates harboring *cry2* (3%), *cry3* (1%), *cry4* (2%), and *cry9* (2%) genes. This is consistent with previous investigations (Chak et al., 1994; Ben-Dov et al., 1997; Bravo et al., 1998; Martinez et al., 2005; Jara et al., 2006; Gao et al., 2008) that reported the *cry1* gene as the most abundant.

As indicated previously (Bravo et al., 1998; Thammasittirong and Attathom, 2008; Nazarian et al., 2009; Vidal-Quest et al., 2009), we also observed that the *cry2* gene was present in combination with the *cry1* gene and this combination was the most frequent (40%) out of all of the *cry* gene combinations (Figure 1). Moreover, some of the Bt strains were found to contain other combinations of the *cry* genes, such as the lepidopteran-active *cry1* gene and the coleopteran-active *cry3* gene. In fact, observation of 16 different genetic profiles of the *cry* genes suggests that Bt isolates have some degree of genetic information exchange.

In order to evaluate the toxic potential of the isolates, bioactivity assays were carried out. A total of 102 Bt isolates containing lepidopteran-specific *cry1* and/or *cry2* genes were selected to test their bioactivity against *C. cautella*, which causes a major loss in dry fig quality. The isolates 13MY and 42MY exhibited a larval toxicity around 97%. Similarly, Hubert et al. (2008) fed *C. cautella* larvae with a diet of transgenic maize containing Cry1Ab toxin and observed a mortality rate around 100%.

In addition, 28 Bt isolates harboring *cry3* and/or *cry9* genes were examined to determine their toxicity against *C. hemipterus* larvae. Three of them (69T, 71T, and 153T) showed the highest toxicity. On the other hand, the reference strain Bt *aizawai*, which contains the *cry9* gene with toxic activity against various insect species, displayed only 12% toxicity on *C. hemipterus* larvae. According to the results of Yu et al. (2006) and Nazarian et al. (2009), Bt isolates containing *cry7* and *cry8* were found to be more effective against coleopteran-specific pests. In addition, Brizzard and Whitley (1988) reported that Cry1B and Cry3 proteins were effective against coleopteran larvae. However, the isolates of 13MY and 42MY harboring the *cry1B* and the *cry3* genes were not as effective as the other toxic isolates against *C. hemipterus*, indicating that these genes could be poorly expressed due to a weak promoter of the isolates 13MY and 42MY. On the other hand, it might be possible that a novel *cry3*-type gene as well as *cry7/8* gene may have been expressed in the isolates exhibiting high levels of toxicity against *C. hemipterus* larvae. Therefore, the cloning and the sequencing of *cry3* genes of effective Bt strains 69T, 71T, and 153T will clarify whether these strains contain novel *cry3*-type genes.

Substantial evidence indicates that continuous exposure of the pests and their environment to the same Bt strain causes an insect resistance. Several approaches have been applied by pest resistance management programs to delay the resistance. For example, application of an alternative Bt strain, minimal exposure to a toxin, combinations of Bt toxins, and high-dose toxin strategies have been used (Chilcutt and Tabashnik, 1997; Hoy, 1998). Therefore, Bt strains of this study at various toxicity levels can be strong candidates to serve as alternative biopesticides for *C. cautella* in the case of development of a pest resistance to Bt *kurstaki*-based products.

In conclusion, Bt occurrence in fig-related areas was investigated in this study. The highest Bt index was obtained in the Kuyucak region. Among the *cry* genes, *cry1* and the combination of *cry1* and *cry2* genes were found to be the most frequent, with occurrences of 36% and 40%, respectively. According to LC_{50} values and fiducial limits, the isolates 13MY, 42MY, and 45MY showed the highest toxicity levels to *C. cautella* larvae. Furthermore, 3 other strains caused a toxicity determined as 50% to 65% mortality against *C. hemipterus* larvae. As a result, these strains may serve as important candidates for the development of commercialized biocontrol agents as well as alternative biopesticides in the face of resistance

to repeatedly used pesticides. Future work will continue for the sequencing and the cloning of the *cry* genes for the identification of novel *cry* genes and the recombinant Cry protein production.

Acknowledgment
This work was supported by grant 108T178 from the Scientific and Technological Research Council of Turkey (TÜBİTAK).

References

Abbott WS (1925). A method of computing the effectiveness of an insecticide. J Econ Entomol 18: 265–267.

Akşit T, *Özsemerci* F, *Çakmak* İ (2003). Aydın ilinde incir ağaçlarında saptanan zararlı türler. Türk Entomol Derg 27: 181–189 (in Turkish).

Apaydin O, Yenidünya AF, Harsa S, Günes H (2005). Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey. World J Microbiol Biotechnol 21: 285–292.

Ausebel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994). Current Protocols in Molecular Biology 3. New York, NY, USA: John Wiley & Sons.

Bel Y, Granero F, Alberola TM, Martinez-Sebastian MJ, Ferre J (1997). Distribution, frequency, and diversity of *Bacillus thuringiensis* in olive tree environments in Spain. Syst Appl Microbiol 20: 652–658.

Ben-Dov E, Wang Q, Zaritsky A, Manasherob R, Barak Z, Schneider B, Khamraev A, Baizhanov M, Glupov V, Margalith Y (1999). Multiplex PCR screening to detect *cry9* genes in *Bacillus thuringiensis* strains. Appl Environ Microbiol 65: 3714–3716.

Ben-Dov E, Zaritsky A, Dahan E, Barak Z, Sinal R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N et al. (1997). Extend screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. Appl Environ Microbiol 63: 4883–4890.

Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Peña G et al. (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 64: 4965–4972.

Bravo A, Likitvivatanavong S, Gill SS, Soberón M (2011). *Bacillus thuringiensis*: a story of a successful bioinsecticide. Insect Biochem Mol Biol 41: 423–431.

Brizzard BL, Whiteley HR (1988). Nucleotide sequence of an additional crystal protein gene cloned from *Bacillus thuringiensis* subsp. *thuringiensis*. Nucleic Acids Res 16: 2723–2724.

Ceron J, Covarrubias L, Quintero R, Ortiz A, Ortiz M, Aranda E, Lina L, Bravo A (1994). PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 60: 353–356.

Ceron J, Ortiz A, Quintero R, Güereca L, Bravo A (1995). Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 61: 3826–3831.

Chak KF, Chao DC, Tseng MY, Kao SS, Tuan SJ, Feng TY (1994). Determination and distribution of *cry* type genes of *Bacillus thuringiensis* isolates from Taiwan. Appl Environ Microbiol 60: 2415–2420.

Chambers JA, Jelen A, Gilbert MP, Jany CS, Johnson TB, Grawn-Burke C (1991). Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J Bacteriol 173: 3966–3976.

Chilcutt CF, Tabashnik BE (1997). Independent and combined effects of *Bacillus thuringiensis* and parasitoid *Cotesia plutella* (Hymenoptera: Broconidae) on susceptible and resistant diamondback month (Lepidoptera: Plutellidae). J Econ Entomol 90: 397–403.

Cinar C, Apaydın O, Yenidunya AF, Harsa S, Gunes H (2008). Isolation and characterization of *Bacillus thuringiensis* strains from olive-related habitats in Turkey. J Appl Microbiol 104: 515–525.

Feitelson JS, Payne J, Kim L (1992). *Bacillus thuringiensis*: insects and beyond. Bio/Technology 10: 271–275.

Gao M, Li R, Dai S, Wu Y, Yi D (2008). Diversity of *Bacillus thuringiensis* strains from soil in China and their pesticidal activities. Biol Control 44: 380–388.

González JM, Dulmage HT, Carlton BC (1981). Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*. Plasmid 5: 351–365.

Höfte H, Whiteley HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53: 242–255.

Hoy MA (1998). Myths, models and mitigation of resistance to pesticides. Phil Trans R Soc Lond B 353: 1787–1795.

Hubert J, Kudlíková-Křížková I, Stejskal V (2008). Effect of MON 810 *Bt* transgenic maize diet on stored-product moths (Lepidoptera: Pyralidae). Crop Prot 27: 489–496.

Jara S, Madvell P, Orduz S (2006). Diversity of Bt strains in the maize and bean phylloplane and their respective soils in Colombia. J Appl Microbiol 101: 117–124.

Kuo WS, Chak KF (1996). Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. Appl Environ Microbiol 62: 1369–1377.

Laemmli UK (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

Magana C, Hernandez-Crespo P, Ortego F, Castanera P (2007). Resistance to malathion in field populations of *Ceratitis capitata*. J Econ Entomol 100: 1836–1843.

Martin PAW, Travers RS (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl Environ Microbiol 55: 2437–2442.

Martínez C, Ibarra JE, Caballero P (2005). Association analysis between serotype, *cry* gene content, and toxicity to *Helicoverpa armigera* larvae among *Bacillus thuringiensis* isolates native to Spain. J Invertebr Pathol 90: 91–97.

Nazarian A, Jahangiri R, Jouzai GH, Seifinejad A, Soheilivand S, Bagheri O, Keshavarzi M, Alamisaeid K (2009). Coleopteran-specific and putative novel *cry* genes in Iranian native *Bacillus thuringiensis* collection. J Invertebr Pathol 102: 101–109.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 775–806.

Seifinejad A, Salehi Jouzani GR, Hosseinzadeh A, Abdmishani C (2008). Characterization of Lepidoptera-active *cry* and *vip* genes in Iranian *Bacillus thuringiensis* strain collection. Biol Control 44: 216–226.

Smith R, Couche G (1991). The phylloplane as a source of *Bacillus thuringiensis* variants. Appl Environ Microbiol 57: 311–315.

Thammasittirong A, Attathom T (2008). PCR-based method for the detection of *cry* genes in local isolates of *Bacillus thuringiensis* from Thailand. J Invertebr Pathol 98: 121–126.

Travers RS, Martin PAW, Reichelderfer CF (1987). Selective process for efficient isolation of soil *Bacillus* spp. Appl Environ Microbiol 53: 1263–1266.

Uribe D, Martinez W, Ceron, J (2003). Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. J Invertebr Pathol 82: 119–127.

Vidal-Quist JC, Castañera P, González-Cabrera J (2009). Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in Spain and evaluation of their insecticidal activity against *Ceratitis capitata*. J Microbiol Biotechnol 19: 749–759.

Yu H, Zhang J, Huang D, Gao J, Song F (2006). Characterization of *Bacillus thuringiensis* strain Bt185 toxic to the Asian cockchafer: *Holothrichia parallela*. Curr Microbiol 53: 13–17.