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Detection of the *cry1* gene in *Bacillus thuringiensis* isolates from agricultural fields and their bioactivity against two stored product moth larvae

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Abstract: Sixty *Bacillus* spp. isolates were obtained from different agricultural fields in Kayseri province using selective media. Total DNA was isolated and analyzed by PCR using *cry1* general primers. Of the 60 tested isolates, 17 carried the *cry1* gene. *B. thuringiensis* subsp. *kurstaki* and all other isolates exhibited approximately 19-kb plasmid bands.

In addition to the 270-bp band, the standard strain and isolate I47 yielded 180-bp PCR products. The isolates and standard strain were toxic to *Ephestia kuehniella* Zeller and *Plodia interpunctella* Hubner larvae. Insecticidal activity of isolates I36, I37, and I40 against *Ephestia kuehniella* was higher than that of the standard strain when 10^6 spore-crystals mL^{-1} was used. LC_{99} values for isolates I36, I37, and I40 were 6.46, 6.28, and 6.24 for *E. kuehniella,* respectively, and these isolates were also effective against *Plodia interpunctella* larvae. LC_{99} values for isolates I36 and I40 were 6.69 and 5.07 for *P. interpunctella*, respectively. The results clearly show that isolates I36, I37, and I40 were effective at lower doses, as compared to the others.

Key words: *Bacillus thuringiensis*, *cry1* primer, *Ephestia kuehniella, Plodia interpunctella*

Tarım alanlarından izole edilen *Bacillus thuringiensis* izolatlarındaki *cry1* geninin belirlenmesi ve bu izolatların iki farklı depo zararlısı güve üzerindeki öldürücü etkilerinin incelenmesi

Özet: Bu çalışmada Kayseri'nin farklı bölgelerinden alınan toprak örneklerinden 60 farklı *Bacillus* spp. izolatı elde edilmiştir. Elde edilen izolatların total DNA'ları izole edildikten sonra *cry1* genel primerleri kullanılarak PCR analizleri yapılmıştır. Toplam 60 izolattan 17'sinin *cry1* geni taşıdığı tespit edilmiştir. *B. thuringiensis* subsp. *kurstaki* ve diğer izolatlardan yaklaşık 19 kb büyüklüğünde plasmid bandları elde edilmiştir.

Standart suş ve I47 izolatının, yapılan PCR analizi sonucunda 180 bç büyüklüğündeki banda ilave olarak 270 bç büyüklüğünde bir band daha oluşturduğu görülmüştür. Standart suş ve diğer izolatların *Ephestia kuehniella* ve *Plodia interpunctella* larvaları üzerinde öldürücü etki gösterdikleri tespit edilmiştir. *Ephestia kuehniella* larvalarına karşı I36,

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I37 ve I40 izolatlarının 10^6 spor-crystal mL^{-1} konsantrasyonları uygulandığında toksik etkilerinin standart suşa göre daha yüksek olduğu belirlenmiş ve aynı izolatların *Plodia interpunctella* larvalarına karşı da etkili oldukları gözlenmiştir. I36, I37 and I40 izolatlarının LC_{99} değerleri *E. kuehniella* larvaları için sırasıyla 6.46, 6.28 ve 6.24 olarak hesaplanmıştır. I36 ve I40 izolatlarının LC_{99} değerleri ise *P. interpunctella* larvaları için sırasıyla 6.69 ve 5.07 olarak hesaplanmıştır. I36, I37 ve I40 izolatlarının düşük dozlarda diğer izolatlarla kıyaslandığına daha etkili olduğu görülmüştür.

Anahtar sözcükler: *Bacillus thuringiensis*, *cry1* primeri, *Ephestia kuehniella, Plodia interpunctella*

Introduction

Bacillus thuringiensis Berliner *(Bt)* is a gram-positive, rod-shaped sporulating bacterium (Konecka et al. 2007). During sporulation it produces parasporal crystals known as δ-endotoxins, which are not produced by other species it resembles, namely *B. cereus*, *B. mycoides*, and *B. anthracis* (Koneman 1997; De Respinis et al. 2006; Soberon et al. 2007; Bizzarri and Bishop 2008). δ-Endotoxins consist of 2 multigenic classes, Cry and Cyt (Dronina et al. 2006; Soberon et al. 2007). Cry proteins interfere with insect development and are toxic to certain insects of Lepidoptera, Coleoptera, Hymenoptera, Diptera, Homoptera, Mallophaga, Acari, and Nematodes (Konecka et al. 2007; Soberon et al. 2007; Cinar et al. 2008). Due to the action of its toxic substance, *Bt* is used worldwide as a biological insecticide against insect pests on a commercial scale (Konecka et al. 2007; Bizzarri et al. 2008). Following the ingestion of sporulated cells, the inactive crystal protoxins are solubilized and activated by trypsin-like proteases in the alkaline midgut of susceptible insect larvae (Sauka et al. 2007; Bondzio et al. 2008). This condition eventually results in insect death by changing its apical potential, which is necessary as a driving force for nutrient uptake and pH regulation (Bondzio et al. 2008). Interest in biological control agents, such as the entomopathogenic *Bacillus* species, is enormous because of the drawbacks to the use of chemical insecticides on human food crops (Brock and Madigan 1991). The advantage of using entomopathogens against pests is that they are insect specific and do not affect many beneficial organisms, including humans, and they do not present resistance problems or pollute the environment (Oliveira et al. 2006).

Ephestia kuehniella and *Plodia interpunctella* are considered among the most destructive pests in stored products. By feeding in the larval stage they cause serious economic loss (Simmons and Nelson 1975). The aims of the present study were to isolate effective *Bacillus thuringiensis* strains specific to lepidopteran pests, characterize their *cry* genes using *cry1* general primers in PCR, and to investigate the effect of the sporulated cells of those isolates against *Ephestia kuehniella* and *Plodia interpunctella.*

Materials and methods

Soil sampling and isolation of *B. thuringiensis* strains

Soil samples from 4 different regions of Kayseri (Turkey) province were used to isolate *B. thuringiensis* strains (Table 1). The soil surface was scraped with a sterile spatula and the samples were taken from depths of 2-5 cm. Ten grams of soil sample was placed in cups and kept at 4 °C until isolation of *B. thuringiensis.*

B. thuringiensis was isolated from the soil samples according to the method described by Travers et al. (1987). One gram of each soil sample was suspended in 20 mL of LB medium buffered with 0.25 M sodium acetate. The mixture was incubated in a rotary shaker at 250 rpm for 4 h at 30 °C. At the end of the incubation period 1.5 mL of the mixture was sampled and placed into microfuge tubes and subjected to 80 °C for 5 min to eliminate the vegetative forms. Then the samples were plated on LB agar medium and incubated overnight at 30 °C. The next day different colonies were randomly selected and cultured on T3 agar medium (3 g of tryptone L^{-1}, 2 g of tryptose L^{-1}, 1.5 of 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}, and 15 g of agar L^{-1}) overnight at 30 °C. At the end of the incubation period Gram staining was carried out and colony morphology was evaluated. Native *Bt* isolates were kept at 4 °C for further analysis.

Table 1. Locations of *B. thuringiensis* isolated soil samples.

Locations	GPS Position	Altitude (m)
Erciyes University campus	38°42′24.94″N; 35°32′01.35″E,	1096
Tomarza (Center)	38°26′44.45″N; 35°48′01.13″E,	1397
Tomarza (Alakuşak)	38°28′58.89″N; 35°40′85.35″E,	1570
Tomarza (Süvegen)	38°23′39.31″N; 35°56′30.59″E,	1350

Total DNA isolation

Total DNA of the native *B. thuringiensis* was isolated according to the method described by Bravo et al. (1998). *Bacillus thuringiensis* subsp. *kurstaki* HD1 was used as the standard strain. The isolates were grown on nutrient agar for 12 h at 30 °C. A small amount of each sample was taken from growing colonies and placed into 100 µL of sterile distilled water in a microfuge tube and vortexed. The mixture was kept at –70 °C for 20 min, and then the cells were transferred into boiling water and incubated for 10 min. At the end of the incubation period the mixture was centrifuged at 10,000 × g for 20 s and the upper phase was transferred to sterile tubes and kept at –20 °C until used for PCR.

Identification of the *cry1* gene

To identify the *B. thuringiensis* strains among the isolated *Bacillus* species and to prove the existence of *cry1* genes, PCR analysis was carried out using *cry1* general primers (Bravo et al. 1998). *B. thuringiensis* subsp. *kurstaki* HD1 was used as the standard strain. PCR mixtures were prepared using 0.2-0.4 µM CJI-1 (5' TGTAGAAGAGGAAGTC TATCCA 3') and CJI-2 (5' TATCGGTTTCTGGGAA GTA 3') primers, 2 U of Taq DNA polymerase, 200 µM dNTP, 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, and 15 µL of DNA solution in a volume of 50 µL. PCR conditions were as follows: a single denaturation step for 2 min at 95 °C, followed by 30 amplification cycles—including denaturation—at 95 °C for 1 min, annealing at 48 °C for 1 min, elongation at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplifications were carried out in a Biometra thermal cycler. After electrophoresis of 10 µL of each PCR product on 3% agarose-EtBr gel, DNA bands were visualized in a gel documentation system (Bio-Doc analyzer).

Plasmid DNA isolation from native *Bacillus thuringiensis* isolates

Plasmid DNA of the isolates and reference strain were obtained according to the method described by Anderson and McKay (1983). The *B. thuringiensis* subsp. *kurstaki* HD1 strain was used as a positive control. Isolated plasmids (20 µL of each) were electrophoresed in 0.8% agarose-EtBr gel and visualized in a gel documentation system (Bio-Doc analyzer).

Bioassays

The effect of PCR-positive *B. thuringiensis* isolates on insect larvae was determined according to the method described by Obeidat et al. (2004). Of the 17 *cry1*-positive isolates, 11 isolates and *Bacillus thuringiensis* subsp. *kurstaki* HD1 were cultured in 100 mL of T3 liquid medium and incubated for 7 days at 30 °C with continuous shaking at 250 rpm. Samples were centrifuged at 5000 rpm for 15 min. Pellets (spores and parasporal crystals) were washed twice in 20 mL of sterile distilled water and centrifuged at 5000 rpm for 5 min. The pellets were re-suspended in 20 mL of sterile distilled water and stored at 4 °C for further use in toxicity experiments.

Spore-crystal suspensions of the *Bt* isolates and standard strain (*B. thuringiensis* subsp. *kurstaki* HD1) were examined to determine their toxicity to third instar larvae of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae). Larval food was prepared by soaking 1 g of peanut pieces in 10 mL of each bacterial suspension for 5 min using 3-fold serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}). The food was then dried and placed in a petri dish in which 10 larvae were placed. The toxicity of each strain was assayed in triplicate, both for the original spore-crystal

suspension and the dilutions. The petri dishes were incubated at 25 ± 2 °C and 70 ± 10% RH, with a photoperiod of 16:8 (L:D) for 7 days. Mortality was scored in comparison with a parallel control in which peanut pieces soaked in sterile distilled water instead of bacterial suspension was used to correct test mortality using Abbot's formula (Daffonchio et al. 1998).

Analysis of variance and the LSD test were used to detect differences in mean mortality between the isolates using SPSS for Windows (SPSS Version 10.0, 2001, SPSS, Chicago, IL, USA). LC_{50} and LC_{99} values were determined by probit analysis.

Results

In all, 60 *Bacillus* spp. isolates were obtained from the soil samples from different regions of Kayseri using selective media. Total DNA was isolated and analyzed by PCR using *cry1* general primers. Of the 60 isolates, 17 carried the *cry1* gene. Plasmids of those strains were isolated and their insecticidal activity was tested against *Ephestia kuehniella* and *Plodia interpunctella* larvae.

Plasmid DNA isolation was carried out according to the method described by Anderson and McKay (1983). The plasmid profile of the standard strain *(B. thuringiensis* subsp. *kurstaki*) and that of 17 isolates was compared. Determination of the precise number and size of the *B. thuringiensis* plasmids is difficult, as the number and size of *B. thuringiensis* subsp. *kurstaki* plasmids were reported to vary when different isolation procedures were used. *B. thuringiensis* subsp. *kurstaki* and all other isolates exhibited an approximately 19-kb plasmid band (Figure 1).

PCR analysis of the *cry1* gene

Many types of PCR methodologies have been widely used to detect *cry* genes by many researchers and many novel genes were recently reported worldwide (Porcar and Perez 2003). To detect those genes new primer sequences were designed. In the present study *cry* genes were amplified using *cry1* general primers. Agarose gel analysis of 17 isolates and the standard strain was carried out, and the results were compared.

Among the tested isolates, I33, I41, I47, I48, I49, and I56 produced 270-bp PCR products using *cry1* primers. In addition to this 270-bp product, isolates I47 and I48 yielded additional 180- and 360-bp PCR products, respectively. Isolates I34, I35, I36, I37, I38, I40, I46, I51, and I54 yielded 2 bands approximately 400 and 450 bp in size with *cry1* primers (Table 2, Figure 2).

The effects of *Bacillus thuringiensis* isolates against *E. kuehniella* and *P. interpunctella* larvae

The toxicity of the spore-crystal mixture of the native isolates and reference strains that gave positive

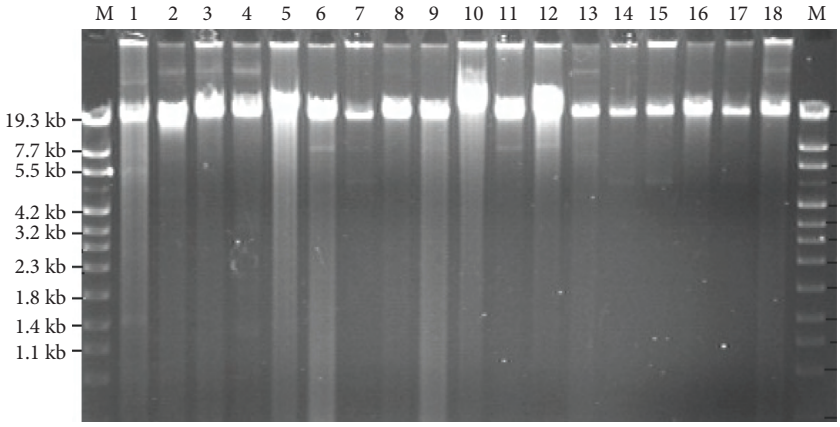

Figure 1. Agarose gel electrophoresis of the plasmid profiles of reference strain and other Bt isolates. M-Marker λ DNA Pub-Mix, 1) *B. thuringiensis* subsp. *kurstaki* HD1, 2) I35, 3) I36, 4) I37, 5) I40, 6) I 46, 7) I48, 8) I51, 9) I54, 10) I56, 11) I41, 12) I33, 13) I38, 14) I47, 15) I49, 16) I57, 17) I50, 18) I34.

Table 2. The size of PCR products amplified by using *cry1* general primer from plasmids of reference strain and different *Bt* isolates.

Isolates	Product size (bp)
B. thuringiensis subsp. *kurstaki* HD1, I47	270, 180
I33, I41, I49, I56	270
I34, I35, I36, I37, I38, I40, I 46, I51	460-470, 400-410
I48	350-360, 270
I54	460-470, 400-410, 350-360
I50, I57	120

PCR results was assayed on the larvae of the Mediterranean flour moth *Ephestia kuehniella* and the Indian meal moth *Plodia interpunctella*. Some of the isolates and the standard strain were highly toxic to the larvae tested (Figure 3). After 7-day exposure the 50% (LC_{50}) and 99% lethal concentration (LC_{99}) of the spore- crystal mixture obtained from the isolates and reference strains were calculated (Tables 3 and 4). For *E. kuehniella* larvae the mortality rate obtained with isolates I36, I37, and I40 was higher than that with the reference strain when 10^6 spore-crystals mL^{-1} was used, and this difference was statistically significant ($F = 590.134$, $SD = 12$, $P < 0.0001$). LC_{99} values obtained with isolates I36, I37, and I40 were 6.46, 6.28, and 6.24 for *E. kuehniella,* respectively. Similar mortally rates were obtained with isolate I33 and the reference strain (HD1) at the dose of 10^6 spore-crystals mL^{-1} for 7 days. The LC_{99} value obtained with isolate I33 was 6.69 for *E. kuehniella.* The insecticidal activity of isolates I33, I36, and I40, and the standard strain on the larvae of *Plodia interpunctella* is shown in Table 4. Isolate I40 caused the highest mortality rate (100%) and there were significant differences between the tested isolates ($F = 1122.463$, $df = 4$, $P < 0.0001$). Isolates I36 and I40 caused similar mortality in both *P. interpunctella* and *E. kuehniella* at the end of the 7^{th} day. LC_{99} values obtained with isolates I36 and I40 were 6.69 and 5.07 for *P. interpunctella*, respectively. These results clearly show that isolates I36, I37, and I40 were more effective at lower doses than the other isolates and standard strain.

Discussion

In the present study 60 different putative *Bacillus* spp. isolates were obtained from soil in different regions of Kayseri, and the presence of *B. thuringiensis* strains highly toxic to *E. kuehniella* and *P. interpunctella* larvae was confirmed.

Morphological characterization and Gram staining of the isolates were carried out. Although colony morphology of the isolates was similar to that of the reference strain (HD1), morphological characterization only is not reliable for identification

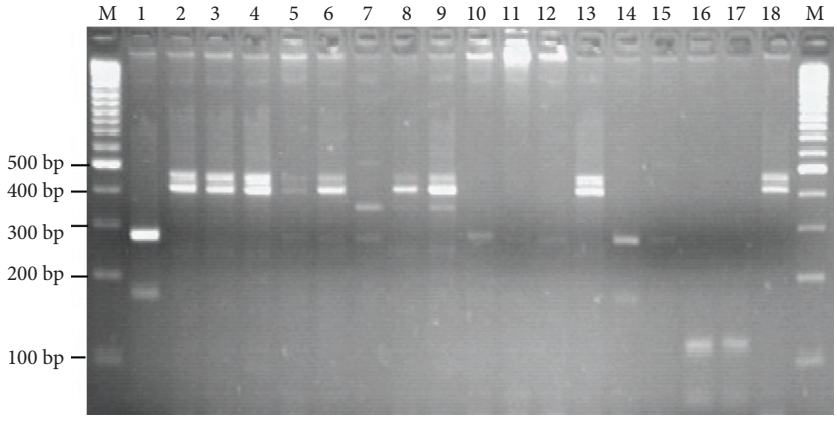

Figure 2. Agarose gel (3%) electrophoresis of the PCR product amplified by using *cry1* primer from reference strain and other isolates. M) DNA Ladder 100 bp, 1) *B. thuringiensis* subsp. *kurstaki* HD1, 2) I35, 3) I36, 4) I37, 5) I40, 6) I46, 7) I48, 8) I51, 9) I54, 10) I56, 11) I41, 12) I33, 13) I38, 14) I47, 15) I49, 16) I57, 17) I50, 18) I34.

Figure 3. Insecticidal activity of standard strain (HD1) and I36, I40 isolates on *Ephestia kuehniella* (left column) and *Plodia interpunctella* (right column) larvae. A and B, HD 1; C and D, I36; E and F, I40.

of isolates. As different *Bt* isolates might have a similar colony morphology, molecular characterization of the isolates is better than either serological or morphological characterization (Iriarte et al. 2000). Cry proteins are encoded by *cry* genes that are frequently carried on plasmids; to date, nearly 300 *cry* genes have been identified and classified into 51 groups and subgroups on the basis of amino acid sequence similarity (Crickmore et al. 2007).

Following PCR analysis, some of the PCR-positive isolates were selected for insect toxicity assay. The selected isolates were toxic to *Ephestia kuehniella* and *Plodia interpunctella* larvae. *B. thuringiensis* δ-endotoxins are well known for their ability to control a variety of insect pests, including members of the Lepidoptera, Coleoptera, and Diptera (Tanada and Kaya 1993).

As the genes that encode insecticidal crystal proteins are commonly located on *B. thuringiensis* plasmids, we performed relevant studies on the *B. thuringiensis* isolates. All the isolates tested in this study, including *B. thuringiensis* subsp. *kurstaki*, yielded 1 plasmid of approximately 19 kb. Nucleotide sequencing of the *cry1* gene, serological tests, and

Table 3. Toxicity of reference strain and some *Bt* isolates against larvae of *E. kuehniella*.

Isolates	C (Spore-crystals mL^{-1})	% Mortality after 7 days	*LC values	
			LC_{50}	LC_{99}
Control	Distilled water	0.00 H¥	-	-
HD1	10^6	48.18 E	6.01	5.9
	10^5	29.20		
	10^4	14.60		
I33	10^6	51.00 E	5.97	6.69
	10^5	33.99		
	10^4	16.98		
I35	10^6	29.20 G	6.25	6.74
	10^5	29.20		
	10^4	9.72		
I36	10^6	86.67 A	5.93	6.46
	10^5	80.00		
	10^4	63.33		
I37	10^6	85.39 A	5.29	6.28
	10^5	48.79		
	10^4	36.60		
I40	10^6	90.40 A	5.00	6.24
	10^5	60.25		
	10^4	45.20		
I41	10^6	70.69 C	5.77	6.75
	10^5	80.78		
	10^4	40.39		
I46	10^6	81.33 B	5.55	6.49
	10^5	65.05		
	10^4	56.93		
I48	10^6	59.72 D	5.88	6.46
	10^5	29.86		
	10^4	14.92		
I51	10^6	43.46 E	6.10	6.73
	10^5	27.16		
	10^4	21.73		
I54	10^6	57.00 D	5.89	6.57
	10^5	28.50		
	10^4	28.50		
I56	10^6	36.60 F	6.13	6.62
	10^5	30.49		
	10^4	00.00		

*LC50 and LC99 = log (spore-crystal concentration mL^{-1}). C: Concentration.
¥Means followed by the same upper case letter are not significantly different at $P < 0.05$

Table 4. Effect of various concentrations of spore-crystal mixture of reference strain and other *Bt* isolates against larvae of *P. interpunctella*.

Isolates	C (Spore-crystals mL^{-1})	% Mortality after 7 Days	*LC values	
			LC_{50}	LC_{99}
Control	Distilled water	0.00 H[¥]	-	-
HD1	10^6	19.46 D	6.28	6.65
	10^5	9.72		
	10^4	00.00		
I33	10^6	25.50 C	6.27	6.69
	10^5	8.490		
	10^4	8.490		
I36	10^6	80.00 B	5.34	6.34
	10^5	63.33		
	10^4	23.33		
I40	10^6	100.00A	4.55	5.07
	10^5	97.86		
	10^4	75.33		

[¥]Means followed by the same upper case letter are not significantly different at $P < 0.05$
*LC50 and LC99 = log (spore-crystal concentration mL^{-1}). C: Concentration.

insecticidal activity testing of the isolates could provide better clues about their genomes.

All *Bt* isolates used in the present study exhibited a similar plasmid profile. Native *Bt* isolates were also characterized utilizing *cry1* primers. *B. thuringiensis* subsp. *kurstaki* was used as the reference strain. PCR analysis of total DNA of the isolates produced the same PCR product as the *cry1* primers did.

PCR products of isolates I33, I41, I47, I48, I49, and I56 were in agreement with the standard strain *B. thuringiensis* subsp. *kurstaki*. Eight of the putative *Bt* isolates (I34, I35, I36, I37, I38, I40, I46, and I51) yielded larger PCR products than the standard strain. Such variation might occur among the strains of bacteria; however, sequence analysis must be carried out to characterize the *cry* genes of those isolates and for comparison with the sequences of reference strains.

Öztürk et al. (2008) reported that their isolate produced 272-bp products using CJI and CJII general primers. They also reported that the strain (F21) was more effective against *E. kuehniella* larvae than *B. thuringiensis* subsp. *kurstaki*, and caused 83% mortality. Apaydın et al. (2005) examined the effects of different *B. thuringiensis* strains on *E. kuehniella*. They observed that 1 strain (85PPb), identified as serovar *morrisoni*, caused a high level of mortality (84%) and was positive for *cry1* and *cry2* genes. Similarly, a novel *B. thuringiensis* strain (serovar *kurstaki*) isolated from Tunisian soil was reported to be toxic to lepidopteran insects, including *E. kuehniella*, due to Cry1Aa, Cry1Ac, and Cry2Aa proteins (Tounsi et al. 1999; Tounsi and Jaoua 2002, Tounsi and Jaoua 2003; Tounsi et al. 2003). Some other isolates effective against lepidopteran larvae were reported by Bobrowski et al. (2001). Although producing PCR products that differ in size, some of the isolates (I36, I37, I40, and I46) were more toxic to lepidopteran larvae when compared with the standard strain. Those isolates might be different strains bearing different *cry* genes. Nucleotide sequence analysis of the *cry* genes of those isolates might be more helpful in explaining the nature of the observed insecticidal activity.

Lepidoptera contain some of the most destructive insect pests of economic importance to crops; therefore, it is important to discover new and/or more potent insecticidal crystal proteins from *B. thuringiensis* strains that can be effectively used in the biological control of insect pests. Toxicity test results obtained in the present study show that some of the isolates were more toxic than the reference strain (*B. thuringiensis* subsp. *kurstaki*). To the best of our knowledge this is the first study on *B. thuringiensis* isolates from Kayseri.

In conclusion, the effective isolates reported in the present study will contribute to the control of 2 economically important stored product pests, although further research is needed to identify the *B. thuringiensis* strains that are active against these and other stored product pests. Additional research on improving protoxin solubility, proteolytic activation, and binding to midgut receptors is planned with these potentially useful *B. thuringiensis* isolates. Further research on the identification of the isolates and their genetic relatedness using DNA markers, their molecular characterization, and bioformulation as a commercial biopesticide are among our future goals.

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