Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Isolation and identification of *Bacillus thuringiensis* strains native of the Eastern Province of Saudi Arabia

Amina A. Hassan[1,2], Mohamed A. Youssef[2], M. M. A. Elashtokhy[2], I. M. Ismail[3], Munirah Aldayel[1] and Eman Afkar[4,5*]

Abstract

Background: *Bacillus thuringiensis* (*Bt*) produces a group of δ-endotoxin proteins designated as *cry* toxins. No doubt that *Bt* isolates are excellent potential candidates for biological control strategies.

Results: The present study showed that 12 *Bt* strains were isolated and characterized at morphological, biochemical, and molecular levels. All the tested 12 *Bt* strains were gram-positive, endospore-forming, and possessing typical *Bt* crystal structures under the scanning electron microscopy (SEM). Universal primers direct and reverse of five pairs were used to detect five *Cry*-type genes (*Cry1*, *Cry2*, *Cry3*, *Cry4*, *Cry7*, and *Cry8*) by the PCR sizes produced from the studied *Bt* strains. The 16S rRNA PCR technique, 16S gene primer, DNA template, dNTPs, and *Taq* polymerase produced unique and distinguishable restriction patterns used for the molecular characterization of the studied *Bt* strains. Based on the PCR products, the frequency of *Cry*-gene distribution among the tested strains w*as Cry1* 100%, *Cry4* 85%, and *Cry3* 62%, and *Cry2* and *Cry7* frequency was 54%. Based on the activity of insecticidal of the tested *Bt* strains, *Bt1*, *Bt9*, *Bt10*, and *Bt11* were extremely pathogenic; their pathogenicity ranged from 93 to 100% against dipteran and lepidopteran larvae, compared to the other *Bt* isolates. The nucleotide sequences of amplified 1500 bp conserved region of 16S rRNA genes of four strains blasted using NCBI database compared to NCBI database sequences, and they were reported as native strains of *Bt* showing high homology with the known *Bt* strains (99–100%). The nucleotide sequences of *Bt1*, *Bt9*, *Bt10*, and *Bt11* were placed in the GenBank database under accession numbers MN860017, MN843958, MN843959, and MN843960, respectively.

(Continued on next page)

* Correspondence: eafkar@kfu.edu.sa
[4]Preparatory Year Deanship, King Faisal University, P.O Box 31892, Al Hofuf, Al-Ahsa, Saudi Arabia
[5]Department of Botany and Microbiology, College of Science, Bani-Suef University, Beni Suef, Egypt
Full list of author information is available at the end of the article

(Continued from previous page)
Conclusion: The strategies of enhancing the sustainability of crops and vegetables that are targeted by a large number of pathogenic insects require a great effort of exploring novel species and strains of *Bt*. Herein, native strains of *Bt* were documented from the eastern province of Saudi Arabia that displayed bio-insecticidal action on larvae of Diptera and Lepidoptera.

Keywords: *Bacillus thuringiensis*, Isolation, *Cry* genes, PCR, Saudi Arabia

Background

Enhancing the resistance of crop and vegetable plants against pathogenic organism diseases is an excellent strategy to increase their productivity and achieve sustainable development. Several microorganisms induce toxins that can be used to control the pathogenicity of a wide range of plant pathogens. *Bt* is a soil organism that produces crystal inclusions during sporulation. The inclusions are toxic proteins encoded by *Cry* genes and shown to have toxic effects against different groups of insects, nematodes, and protozoa (Abo-Bakr et al. 2020). *Bt* toxic proteins neither affect human health nor non-target organisms. Several research articles proved that *Bt* synthesizes *Cry* toxins terminating the growth of pathogenic insect larvae.

Controlling crop and disease insects, *Bt* secretes parasporal crystal protein known as *Cry* toxins that have high toxicity towards specific orders of pathogenic insects. Taxonomic classification of these toxins placed themes in the delta category of insect toxins because of their intracellular position (Schnepf et al. 1998). Crystalline toxins or δ-endotoxins are considered the main factor conferring entomopathogenic properties to *Bt* (Bouslama et al. 2020). δ-endotoxins are composed of 74 types of toxins and holotypes of 295 that belong to the 3D protein family synthesized by *Bt* (Leopoldo et al. 2014). *Bt* delta toxins commercialized have been used as pesticides over decades as they have constitutive blocks of amino acids and different specificities against different orders of insects (Van Frankenhuyzen 2009). δ-endotoxins of *Bt* attack the insects' membrane pores and forming channels (Melo et al. 2016). The three-domain proteins have various complementary aspects of insect toxification. The first domain is responsible for the formation of pores; the second domain is specific in binding to the receptors in the epithelial cells of the insects' midgut; the third domain is functioning in stabilizing the bond between toxin and receptor that results in osmatic discrepancy and finally death of insect (Melo et al. 2016). Therefore, it is clear that the three domains of toxin protein have correlations and their activity can be described as complementary action. This complementary action does not affect the phylogeny or phyletic lineage among groups. It is important to mention that various *Bt* strains produce various toxins, each toxin specific for a certain category of insects. Recently, Cry genes have been expressed in transgenic crops and vegetables to resist pests. Using transgenic crops is a protocol for increasing the yields and minimizing the use of chemical insecticides (Yutao and Kongming 2019).

Bt Cry toxins are environmentally safe and effective pest control tools. The only negative point of using *Bt* to control pests is the development of new species of targeted insects that are able to resist these toxins, for example, *Plutella xylostella* (diamondback moth) has developed strains that resist *Bt* pesticides in different locations worldwide. It is documented that *B. thuringiensis* strain AB1 (Sri Lanka) has high toxicity against *P. xylostella* larvae that are resistant to the commercial *Bt* available in the market. Exploring new species of *Bt* will provide another way to cope up with the population of insects that are resistant to the known *Bt* biopesticides (Baragamaarachchi et al. 2019).

Polymerase chain reaction (PCR) is the most used method to characterize *Bt* genes. PCR protocol is precise and requires minor amounts of DNA and fast detection of the DNA sequences in a given organism. This protocol allows quick screening of large numbers of *Bt* species to identify novel *Cry*-type genes to determine their distribution (Yilmaz et al. 2017). The use of universal primers is a common method to detect the presence of *Cry* genes (Reinoso-Pozo et al. 2016).

In the current research, we aimed to isolate, identify, and screen for novel *Bt* that might be highly potent pathogenic from the eastern province of the Kingdom of Saudi Arabia.

Methods

Isolation and identification of native *B. thuringiensis* strains

Soil sampling was performed at six different regions at Al-Ahsa province in the eastern region of Saudi Arabia. Four samples are from cultivated soil (Al-Buhairia, Al-Batalieh, Ghubaiba Villages) and two from non-cultivated soil (El-Qaarh and El-Shoabah mountains). The top layer of soil was removed to avoid the destructive effect of atmospheric UV radiation on the viability of *Bt* spores. The samples were placed into zip-lock bags and stored in a refrigerator

at 4 °C until isolation (Palma 2015). Soil samples were suspended in saline 0.85% solution (1 g soil/saline solution); all samples were shacked at 250 rpm for 1 h. All soil samples were exposed to 75 °C for 20 min to kill most of the non-spore-forming cells. About 0.2 ml of each soil suspension was seeded onto nutrient agar (NA) medium, and incubation time was 24 h at 30 °C (Daniel et al. 2018). Bacterial colonies exhibiting *Bt* like phenotype flat, matte white color, dry, and uneven borders were picked up carefully and subcultured on nutrient agar medium for single-colony isolation (Palma 2015). The selected colonies were grown on T3 agar medium (per liter: 3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH 6.8], and 0.005 g MnCl (Travers et al. 1987)) and incubated at 30 °C for 72 h. The cells of *Bt* potential isolates grown on T3 agar medium were stained with Gram staining as well as spore-staining reagents. All *Bt* strains were examined for the presence of vegetative cells, spores, and parasporal inclusion (Padole et al. 2017). *Bt* isolates were further characterized by examining the enzymatic activities for catalase, urease, caseinase, gelatinase, amylase, and lecithinase according to Cinar et al. (2008). All *Bt* strains were given code names for recognition.

Preparation and capturing of insecticidal crystal proteins (ICPs)

The cells of *Bt* potential isolates were grown on T3 medium at 30 °C and 200 rpm for 7 days for spore induction. Spore suspension of *Bt* isolate was centrifuged at 15,000 rpm and 4 °C for 10 min to harvest spore-crystal mixtures. Pure spore crystals were fixed and sputter-coated with 10 nm Au/Pd using an SC7620 Mini-sputter coater; parasporal crystal protein were examined and captured using a LEO440 scanning electron microscope at 20 kV beam (Fig. 1) (Yilmaz et al. 2017).

Analysis of insecticidal crystal proteins (ICPs)

Proteins of parasporal crystal were solubilized, characterized, and accessed using 10% SDS-PAGE according to Laemmli (1970) with minor modification as follows: Aliquots of an overnight LB culture of *Bt* strains and *Bt*-HD1 reference strain were injected on to liquid T3 medium (Bozlagan et al. 2010) at 30 °C for more than 3 days. Ten milliliters from *Bt* broth was harvested by centrifugation at 7500 rpm for 20 min, 4 °C. The pelleted protein crystals were then suspended in 3 ml of sterilized distilled water (SDW) at 4 °C. The pelleted proteins were washed twice by the SDW at the same conditions; later, the pelleted crystal proteins were dissolved in 100 µl sterile distilled water and 100 µl of 2X breaking buffer composed of 125 mM Tris-HCl, pH 6.8, of sodium dodecyl sulfate (SDS) 4%, β-mercaptophenol 0.2%, glycerol 50%, bromophenol blue 0.02%. The *Bt* pellet was mixed well, boiled for 10 min, and allowed to cool down for 1 min, finally centrifuged for 15 min at 10,000 rpm, and loaded on to 12% SDS polyacrylamide gel page.

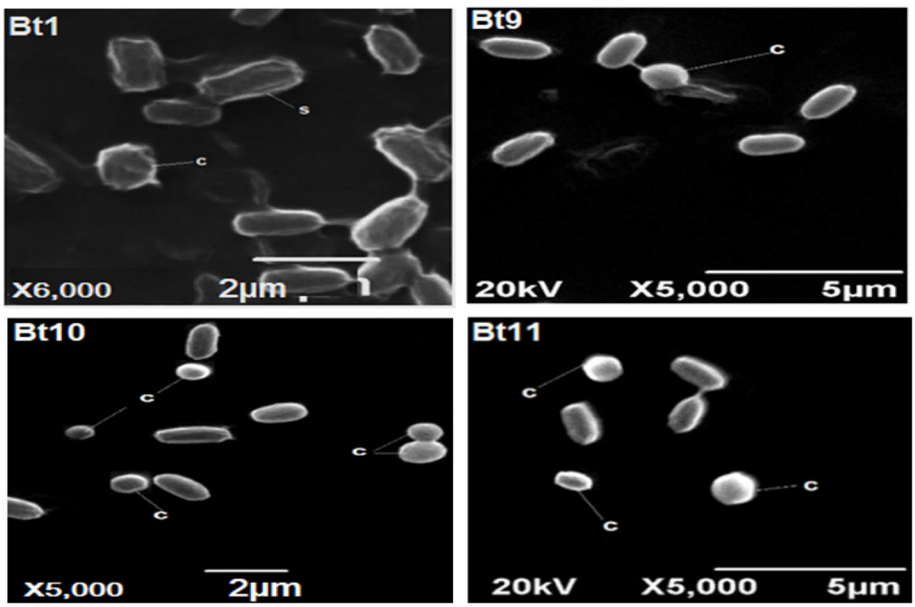

Fig. 1 Scanning electron microscopy (SEM) Bt strains Bt1, Bt9, Bt10, and Bt11 showing spore formation (S) and endo-crystals (C) are both typical characteristic features for Bt cells

Scanning of *B. thuringiensis* strains for *cry* genes DNA isolation

The tests were conducted as follows: 15 μl of *Bt* pure single colony was picked from nutrient agar culture and suspended in 150 μl SDW, the suspension was subjected to boiling for 5 min, followed by cooling at room temperature, and centrifuged at 10,000 rpm at 4 °C over 10 min. This supernatant comprising crude DNA was used in PCR amplification according to Carozzi et al. (1991).

Oligonucleotide PCR primers

Five pairs of universal primers (direct and reverse) were used to explore 5 *Cry*-encoding genes by sizes from PCR products. The sequences, gene recognized, and the expected Cry-gene sizes of PCR products are presented in Table 1.

Amplification reaction mixture

Two microliter DNA (about 20 ng) was isolated from each *Bt* strain, *Taq* DNA polymerase enzyme (1 unit), 10X buffer (2 μl), 2 μl $MgCl_2$ (2500 μM), 2 μl dNTPs (2500 μM), 2 μl primer (10 pmol), and 14.8 μl H2O (Miniatis 1989).

DNA amplification cycles

Perkin-Elmer GeneAmp PCR system (model 2400), used with temperature cycling program, was applied as described by Temnykh et al. (2000). The amplification steps were carried out as follows: a single cycle runs at 94 °C for 5 min, then 30 cycles composed of denaturation at 94 °C for 1 min, a single step of annealing at 55 °C for 1 min, next single step of synthesis at 72 °C for 2 min, followed by one step of extension at 72 °C for 7 min, and a final 4 °C infinitive. Each experiment was associated with negative and positive controls. The negative control was run in absence of a DNA template, and the positive control was run by a standard template. The PCR products were assessed by electrophoresis, using 1.4% agarose gels, after finishing the electrophoresis the gels stained with ethidium bromide, and UV light was used to capture the photographs. One hundred base pair DNA ladder purchased from Pharmacia was applied to access the PCR product band sizes.

Insects

Insects used in this study are the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), and the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). The required instars from both insects were kindly provided by Mr. Ibrahim Hamed and Ms. Marwa El-Saleh, Plant Protection Department, Faculty of Agriculture, Egypt.

Bioassay

Spore-crystal mixtures of *Bt* isolates were prepared according to Ammouneh et al. (2011). A single colony from preliminary identified *Bt* strains was cultured in 100 ml of T3 liquid medium and incubated on a shaker (150 rpm) at 30 °C for 5 days. Crystal proteins and *Bt* spores were collected and suspended in 1000 mM NaCl, centrifuged at 10,000 rpm at 4 °C for 10 min, washed with SDW two times, later suspended in SDW, and subjected to freeze-drying. Both *Bt* crystals and spore powders are used in further experimental work. Bio-insecticidal potentials of the native *Bt* strain studies in this work were tested against the cotton leafworm larvae (*S. littoralis*) and the fruit fly (*D. melanogaster*). Ten of 3rd to 4th instar tested larvae were put in 30-ml plastic cups in 3 replicates, with a total number of 30 larvae for each concentration which was maintained at 27 ± 2 °C, 60–65% RH, and 14:10 h (light:dark). Five milliliters (ug/ml^{-1}) of each native *Bt* strain lyophilized powders of the mixture of crystals and spores were introduced to the larvae and left at room temperature (Aramideh et al. 2010). Results of scoring mortality were recorded after 24 and 48 h for each treatment. Probit analysis program version 1.3 was used for calculating values of LC_{50} and slope. Bioassay experiments were

Table 1 Characteristics of universal primers for *cry*1, *cry*2, *cry*3, *cry*4, and *cry*7, 8 group genes

Primer pair	Sequence of primer	Gene recognized	Expected size (bp)
Un1(d) Un1(r)	5′CATGATTCATGCGGCAGATAAAC-3′ 5′-TTGTGACACTTCTGCTTCCCATT-3′	*cry1*	274 277
Un2(d) Un2(r)	5′-GTTATTCTTAATGCAGATGAATGGG-3′ 5′-CGGATAAAATAATCTGGGAAATAGT-3′	*cry2*	689 701
Un3(d) Un3(r),	5′CGTTATCGCAGAGAGATGACATTAAC3′ 5′-CATCTGTTGTTTCTGGAGGCAAT-3′	*cry3*	589 595 604
Un4(d) Un4(r)	5′-GCATATGATGTAGCGAAACAAGCC3′ 5′-GCGTGACATACCCATTTCCAGGTCC-3′	*cry4*	439
Un7,8(d) Un7,8(r)	5′AAGCAGTGAATGCCTTGTTTAC-3′ 5′-CTTCTAAACCTTGACTACTT-3′	*cry7* and *cry8*	420 423

performed using a spore-crystal mixture which was collected from each *Bt* isolate individually, and prepared serial concentrations ranged from 25 to 50 μg/ml^{-1} in sterile distilled water (dH_2O). The leaf-dipping technique was used for the tested cotton leafworm larvae. Fresh and clean leaves were dipped for 30 s in each original isolate concentration of *Bt* spore-crystal mixture separately and offered into instar larvae of *S. littoralis* larvae. For control, the same number of larvae was exposed to clean leaves dipped in dH_2O only. Also, 20 g from the fresh prepared artificial fruit fly feed was mixed well with each original isolate of *Bt* concentrations.

Molecular typing of native strains

Based on the insecticidal bioassay results, the strains of *Bt* that expressed the highest insecticidal activities against Diptera and Lepidoptera were selected for molecular typing 16S rRNA according to the methods of Rochelle et al. (1995). Universal primers for 16S rRNA, forward primer 8F 5′AGT TGA TCC TGG CTC AG 3′ and reverse primer 1492R 5′TAC CTT GTT ACG ACT T3′ were used to analyze the 16S rRNA. DNA templates were isolated from the 4 native (*Bt*) strains used in this experiment. The reaction was carried out as follows: 50 μl of the reaction mixture includes DNA template buffer, 16S gene primer, *Taq* polymerase, dNTPs, and $MgCl_2$. The program of PCR was performed in Bio-RAD i-cycler with these steps: (a) 94 °C for 40 s set for denaturation, (b) 55 °C for 50 s set for annealing, (c) 72 °C for 1.5 min set for extension; all running over 35 cycles, the initial temperature of denaturation was 94 °C for 3 min and 72 °C for 7 min for the final extension. A 1.5% agarose gel electrophoresis was used to analyze the PCR outcome fragments. The agarose gels were purified according to the catalog of purification kit purchased from (Promega Wizard SV Gel and PCR Clean Up-system Kit cat. #A9282). Purified PCR products were sent for sequencing at Macrogen (South Korea). The blast algorithm at NCBI database was used to set the comparison among the native *Bt* sequences, and the documented *Bt* sequences (*Bt1*, *Bt9*, *Bt10*, and *Bt11*) were stored in NCBI (Altschul et al. 1990).

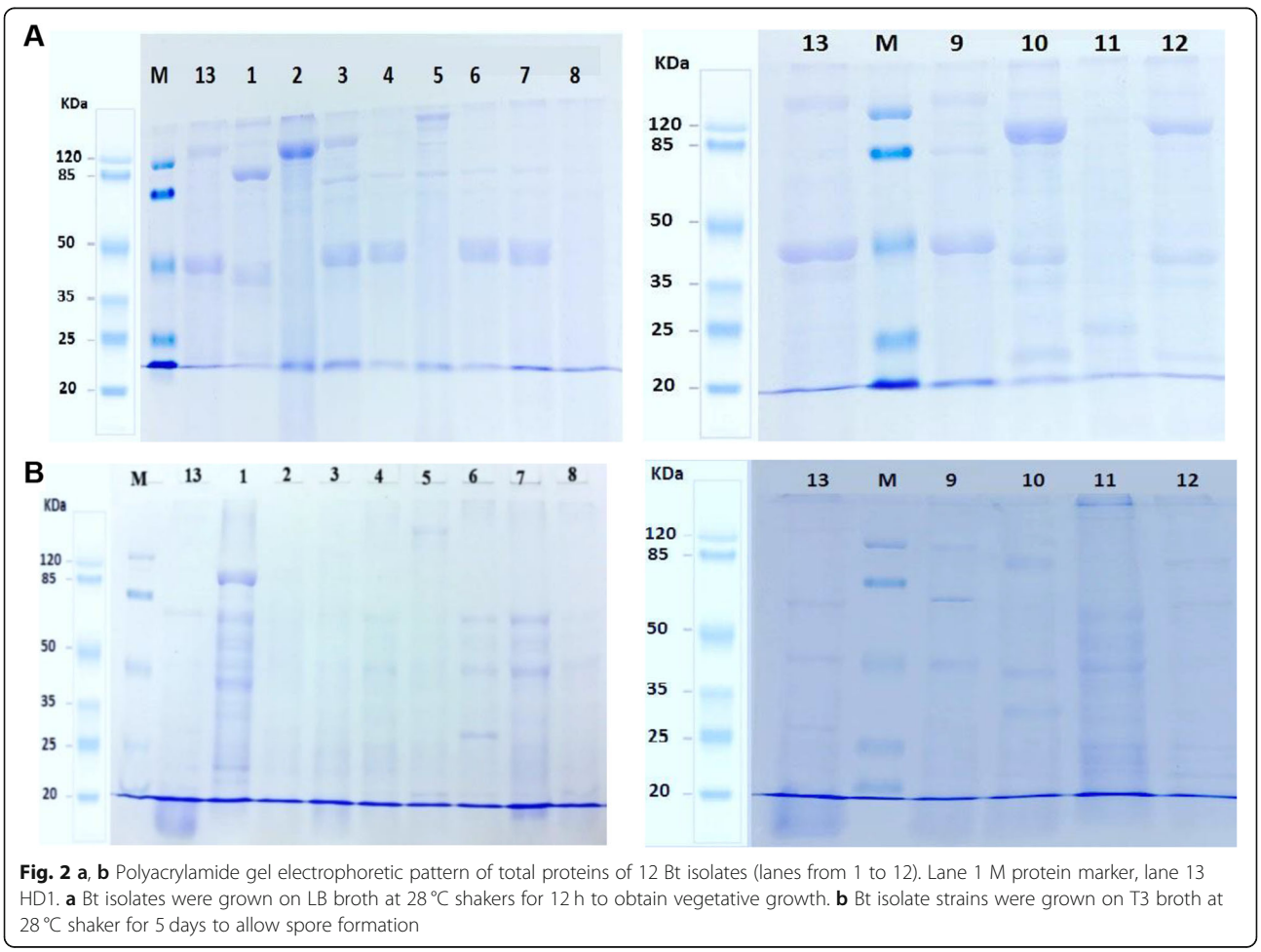

Fig. 2 **a**, **b** Polyacrylamide gel electrophoretic pattern of total proteins of 12 Bt isolates (lanes from 1 to 12). Lane 1 M protein marker, lane 13 HD1. **a** Bt isolates were grown on LB broth at 28 °C shakers for 12 h to obtain vegetative growth. **b** Bt isolate strains were grown on T3 broth at 28 °C shaker for 5 days to allow spore formation

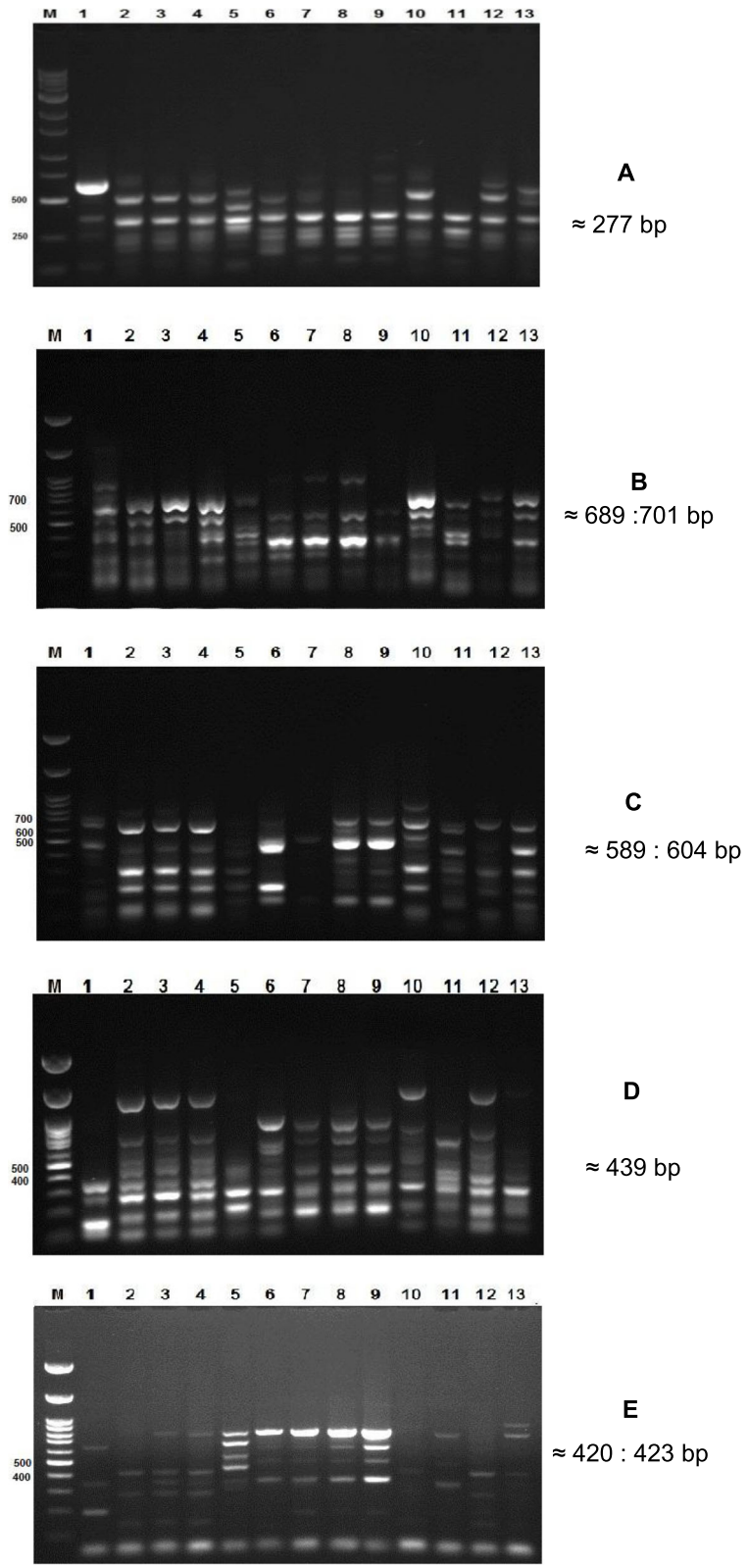

Fig. 3 Agarose gel electrophoresis of a PCR product of 16S rDNA region from Bt isolates with universal primers. Un1 (**a**), Un2 (**b**), Un3 (**c**), Un4 (**d**), and Un7,8 (**e**). DNA ladder (100 bp), 1 SA1, 2 SA2, 3 SA3, 4 SA4, 5 SA5, 6 SA6, 7 SA7, 8 SA8, 9 SA9, 10 SA10, 11 SA11, 12 SA12, 13 13: *B. thuringiensis subsp. kurstaki* HD-1 reference strain

Table 2 Frequency of *cry*-gene profile in *Bt* isolate strains identified by *cry*-gene universal primers

Cry-gene frequency (%)	*Bt1*	*Bt2*	*Bt3*	*Bt4*	*Bt5*	*Bt6*	*Bt7*	*Bt8*	*Bt9*	*Bt10*	*Bt11*	*Bt12*	*Bt13*
Cry1 (100%)	+	+	+	+	+	+	+	+	+	+	+	+	+
*Cry*2 (54%)	+	+	+	+	-	-	-	-	-	+	+	-	+
*Cry*3 (62%)	-	+	+	+	-	-	-	+	+	+	-	+	+
*Cry*4 (85%)	-	+	+	+	+	+	+	+	+	+	+	+	-
*Cry*7 andCry8 (54%)	-	+	+	+	+	-	-	-	-	+	-	+	+

Phylogeny of native strains *Bt*1, *Bt*9, *Bt*10, and *Bt*11

16S rRNA gene sequences were compared and submitted to the GeneBank database based on the nucleotide-nucleotide standard BLAST algorithm. The MEGA5 software was used to align the sequences, and the phyletic lineage was analyzed, and the tree was constructed. The neighbor-joining method inferred the history of evolution among the taxa. One thousand replicates were taken to represent the evolutionary history of the analyzed taxa, and the bootstrap consensus tree may be inferred (Saitou and Nei 1987).

Results

Isolation, identification, and characterizations of 12 *Bt* isolate from cultivated and non-cultivated soils, collected from 6 different regions of Al-Ahsa in the eastern province of Saudi Arabia, were reported. Four out of the 12 isolates were novel and highly toxic against *S. littoralis* and *D. melanogaster*. The isolated *Bt* strains were primarily identified based on their morphological characteristics (flat, matte white color, dry, and uneven borders) and their positivity towards gram-staining reagents. Moreover, the 12 *Bt* isolates showed positive results when examined for the activity of catalase, urease, caseinase, gelatinase, amylase, and lecithinase.

Spore-staining reagents declared the presence of parasporal crystal proteins (PCP), and pure spore suspensions of *Bt* isolates showed clear parasporal protein crystals (PCP) when examined under scanning electron microscopy (SEM) (Fig. 1).

Electrophoretic patterns of the 12 *Bt* strains along with a single type strain *Bt* subsp. *kurstaki* HD1 showed several bands of protein that had different molecular weights in a range of 130–20 kDa. Although each strain had its unique protein profile, there were common bands among different strains and the reference strain HD1 (Fig. 2a). Besides, the SDS-PAGE protein profile of the parasporal crystal protein was isolated after 72 h from sporulated *Bt* culture were also represented (Fig. 2b).

Direct and reverse universal primers consisted of 5 pairs that were used to detect PCR products by sizes of five *Cry*-type genes from the 12 isolates of *Bt*; the results revealed the presence of fragments for *Cry*1, *Cry*2, *Cry*3,

Table 3 Pathogenicity of 12 *Bt* isolates and a reference strain (HD1) against the 3rd instar larvae of *D. melanogaster* after 24 and 48 h post-treatment

Strain	*D. melanogaster* (Diptera)					
	24 h		Mortality %	48 h		Mortality %
	LC_{50} (μg/ml^{-1})	Slope ± SE		LC_{50} (μg/ml^{-1})	Slope ± SE	
1	25.18	0.8 ± 0.9	54	16.8	0.9 ± 0.9	60
2	56.7	1.7 ± 0.9	42	44.4	1.7 ± 1.0	48
3	22.1	0.4 ± 0.9	27	445.6	0.4 ± 0.9	30
4	31.4	1.8 ± 1.0	57	55.0	3.8 ± 1.3	61
5	66.6	5.2 ± 1.6	70	52.6	4.0 ± 1.8	75
6	62.5	4.8 ± 1.4	73	54.4	5.6 ± 1.6	80
7	125.4	0.4 ± 0.9	39	50.0	0.8 ± 0.9	50
8	59.4	3.7 ± 1.2	39	55.4	3.5 ± 2.5	46
9	48.1	4.2 ± 3.0	80	54.4	5.2 ± 1.6	93
10	55.0	4.4 ± 1.3	86	32.5	4.3 ± 1.7	86
11	19.9	0.4 ± 0.9	51	20.2	0.8 ± 0.9	63
12	37.8	3.0 ± 0.7	57	19.3	0.4 ± 0.9	70
HD1	36.6	1.3 ± 0.9	51	20.2	0.8 ± 0.9	63

Table 4 Pathogenicity of 12 *Bt* isolates and a reference strain (HD1) against the 3rd instar larvae of *S. littoralis* after 24 and 48 h post-treatment

Strain	*S. littoralis* (Lepidoptera)					
	24 h		Mortality %	48 h		Mortality %
	LC_{50} (μg/ml^{-1})	Slope ± SE		LC_{50} (μg/ml^{-1})	Slope ± SE	
1	41.1	5.3 ± 1.4	94	45.6	6.9 ± 1.2	100
2	43.4	4.7 ± 1.1	83	54.4	5.7 ± 1.0	96
3	26.1	0.7 ± 0.6	63	44.4	4.9 ± 0.7	93
4	45.4	3.4 ± 1.7	73	65.0	3.8 ± 1.4	80
5	68.5	0.7 ± 0.5	50	64.6	5.0 ± 1.6	93
6	57.5	0.8 ± 0.9	60	45.3	4.6 ± 1.5	86
7	23.4	0.7 ± 0.6	53	67.0	0.8 ± 0.9	63
8	61.4	4.9 ± 1.2	85	45.4	6.5 ± 1.5	100
9	56.1	5.1 ± 1.4	86	64.4	6.2 ± 1.5	100
10	34.0	0.6 ± 0.3	87	53.3	5.3 ± 1.7	94
11	57.9	5.3 ± 1.9	91	56.2	5.8 ± 1.8	100
12	39.7	3.2 ± 0.9	73	52.4	5.4 ± 1.6	93
HD1	51.5	5.3 ± 1.1	93	50.1	5.8 ± 1.3	100

*Cry*4, *Cry*7, and *Cry*8 genes (Table 1 and Fig. 3). *Cry*1 gene was frequently distributed among *Bt* isolate strains (100%), followed by *Cry*4 (85%) and *Cry*3 (62%), and *Cry*2, *Cry*7, and *Cry*8 are equally distributed (54%) among all *Bt* isolates (Table 2).

Bioassay of the 12 *Bt* isolates for larvicidal activity against 3rd instar larvae of the *D. melanogaster* as dipteran demonstrated that some strains were highly effective in killing the insect larvae than the reference strain (HD1). Depending on the values of LC_{50} and slope of the tested *Bt*, *Bt*5, *Bt*6, *Bt*9, and *Bt*10 isolates showed the highest mortality percentage for dipteran larvae after 24 h of exposure which ranged from 70 to 86% with LC_{50} values ranged from 48.1 to 66.6 μg/ml^{-1}, respectively. While the exposure after 48 h, the mortality percentage ranged from 75 to 93% with LC_{50} values ranged from 32.5 to 54.4 μg/ml^{-1}, respectively as shown in Table 3.

Regarding the larvicidal activity of *Bt* against *S. littoralis* as lipedopteran, the highest larva mortality percentage appeared with isolates *Bt*1, *Bt*8, *Bt*9, *Bt*10, and *Bt*11, which ranged between 87 and 94%, after 24 h of exposure with LC_{50} values between 34.0 and 61.4 μg/ml^{-1}, respectively, while after 48 h the mortality percentage increased to 100% with LC_{50} values ranged from 45.5 to 46.4 μg/ml^{-1} as shown in Table 4.

Referencing to these results, the highest effectiveness against both dipteran and lepidopteran insects (*Bt*1, *Bt*9, *Bt*10, and *Bt*11) were assigned to further molecular typing. The amplified sequences of these 4 strains were blasted against sequences from the NCBI database, using the blast algorithm (Altschul et al. 1990). The nucleotide sequencing of the amplified 1500 bp conserved region of 16SrRNA genes of all the *Bt* strains *Bt*1, *Bt*9, *Bt*10, and *Bt*11 were blasted using NCBI database showing high homology with *Bt* strains (99–100%).

The 4 native *Bt* (*Bt*1, *Bt*9, *Bt*10, and *Bt*11) were given the names SA1, SA9, SA10, and SA11 reflecting the site of isolation and reported in the GenBank under accession numbers MN860017, MN843958, MN843959, and MN843960, respectively (Table 5 and Fig. 4).

Table 5 Insecticidal *cry*-gene profiles of native *Bt* strains *Bt*1, *Bt*9, *Bt*10, and *Bt*11 along with their corresponding GenBank accession numbers and ICPs

Bt. strain ID	GenBank accession number(s)	ICPs	Insecticidal gene profiles
*Bt*1	MN860017	Present	*Cry*1, *Cry*2
*Bt*9	MN843958	Present	*Cry*1, *Cry*3, *Cry*4
*Bt*10	MN843959	Present	*Cry*1, *Cry*2, *Cry*3, *Cry*4, *Cry*7& *Cry*8
*Bt*11	MN843960	Present	*Cry*1, *Cry*2, *Cry*4

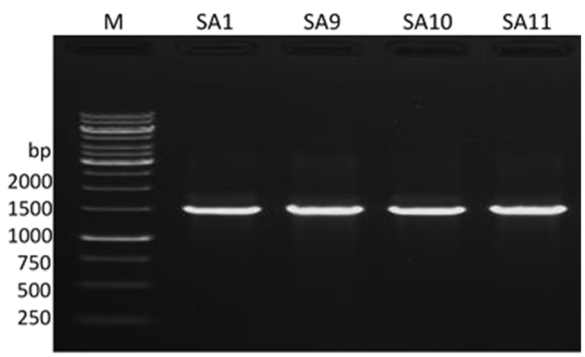

Fig. 4 Agarose gel electrophoresis of a PCR amplification of the 16S rRNA gene from Bt strains: SA1, SA9, SA10, and SA11. M, 1 kb DNA ladder GeneRuler™1 kb Fermentas, cat. # SM0311

The aligned sequences were challenged for neighbor-joining analysis to build the phylogenetic relationship. Nine clusters were produced with different percentages of similarities. *Bt*9 isolate showed a similarity of 15% with the two clusters of eight isolates with an internal similarity of 16%. The *Bt*10 isolate showed a similarity of 63% with the cluster containing four isolates with an internal similarity of 100% (Fig. 5).

Discussion

Bt toxins are highly selective and covering more than 50% of the market share (Lacey et al. 2015). The morphology of the isolated bacteria along with the results obtained from the biochemical tests was confirming that the 12 isolated bacterial strains belong to *Bt*. Similar results were obtained by Cinar et al. (2008); besides, the presence of parasporal crystal proteins (PCP) in the SDS-PAGE profile and the pure spore suspensions of the bacterial isolates is further confirming that the isolated bacteria belong to *Bt*. This result agreed with other studies on *Bt* protein crystal (Yilmaz et al. 2017).

Also, the diverse profiles of proteins on SDS-PAGE were consistent with the diverse insecticidal toxins due to the different chemical properties of insecticidal proteins. The presence of very highly crystal proteins soluble in high basic pH is aligned with similar findings by Bukhari and Shakoori (2010) while studying the molecular characterization of *Bt cry* genes and their mosquitocidal activity. The severe pathogenicity levels of *Bt* parasporal crystal proteins against larvae of *S. littoralis* and *D. melanogaster* were explained by the presence of various insecticidal parasporal crystal proteins and by the possibility of different binding sites on the larvae midguts, similar findings reported by Ammouneh et al. (2011) and Padole et al. (2017).

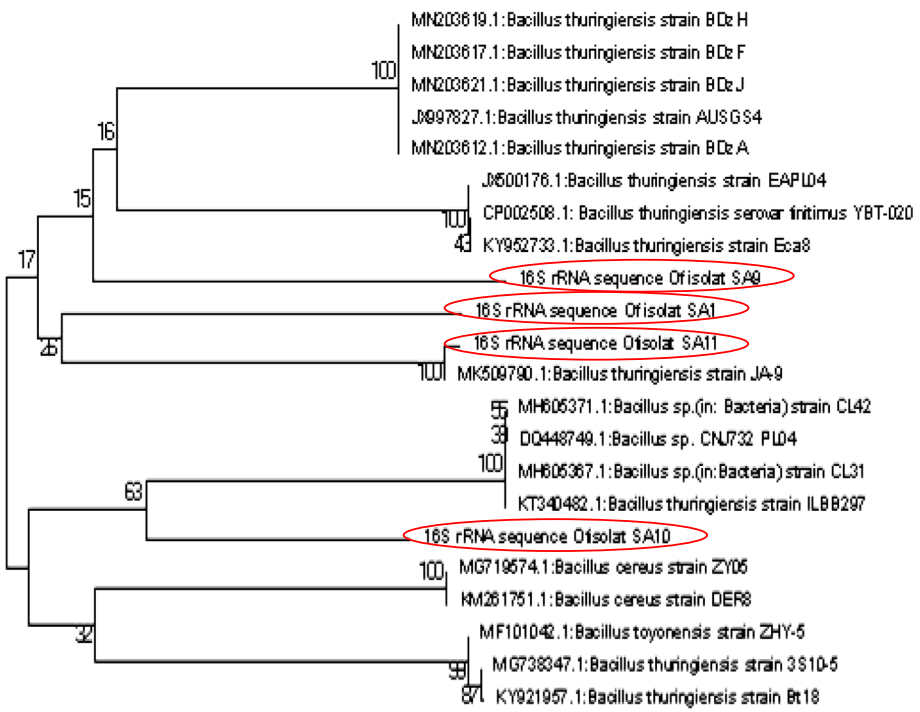

Fig. 5 Phylogenetic trees were constructed using MEGA 5 following the multiple alignments of the sequences by Cluster W. The trees were constructed using the neighbor-joining (NJ) method and the maximum parsimony option model, with 1000 bootstrap replicates to estimate the support for each branch

Cry-gene specific and competition-binding studies were carried out with lectins, and specific sugars confirmed that *Cry*1Ac and *Cry*1Fa share similar numbers of the midgut binding sites of both *Anticarsia gemmatalis* and *Chrysodeixis includes* (Bel et al. 2017). Also, 5 pairs of universal primers have been used to detect the highly conserved Cry-gene sequences and differentiate 34 out of about 60 genes known in the following groups: 20 *Cry*1, 3 *Cry*2, 4 *Cry*3, 2 *Cry*4, 2 *Cry*7, and 3 *Cry*8 genes (Ben-Dov et al. 1997); PCR screening using universal primers of two pairs to detect *Cry*1 and *Cry*2 gene families in their collection is performed by Lone et al. (2017). Therefore, Cry1 protein and other Cry proteins found in the 12 *Bt* isolates could have similar binding sites on the midguts of *S. littoralis* and *D. melanogaster*. Also, it was obvious that Cry1 genes were highly distributed in all *Bt* isolates (frequency of distribution was 100%). Recent proteomic data supported that Cry1C and Cry1D parasporal crystal proteins make up approximately 92% of the insecticidal crystal proteins in AB1 (Baragamaarachchi et al. 2019).

Although the 4 native *Bt* strains *Bt*1, *Bt*9, *Bt*10, and *Bt*11 exhibited high levels of pathogenicity against lepidopteran and dipteran, the percentage of mortality was 93% for lepidopteran, whereas it was 100% for dipteran. Besides, the *Bt*11 isolate showed a similarity of 100% with *Bt*9 isolate, while clustered with 25% of similarity with *Bt*1 isolate. These results strongly support the typing of *Bt*1, *Bt*9, *Bt*10, and *Bt*11 as *Bt* novel stains. It remains to match and compare the peptides in the parasporal crystal proteome of *Bt*1, *Bt*9, *Bt*10, and *Bt*11 to describe precisely their mode of action; this work is ongoing in our laboratories.

Conclusion

In this study, 12 strains of *Bt* were isolated, identified, and screened for novel *Bt* that might have highly potent pathogenic as the result of variable sequences that can be used as pest control. Here, we used universal primers, five pairs (direct and reverse) to explore five *Cry*-genes on different isolates of *Bt* from the eastern province of the Kingdom of Saudi Arabia. The 4 native strains of *Bt* types, *Bt*1, *Bt*9, *Bt*10, and *Bt*11, producing mostly *Cry1*, *Cry2*, *Cry3*, *Cry4*, *Cry7*, and *Cry8* genes that express insecticidal parasporal crystal proteins, were isolated and identified from the eastern province of Saudi Arabia. Continuous experimental work is needed to evaluate the possibility of culturing *Bt*: *Bt*1, *Bt*9, *Bt*10, and *Bt*11 for commercialization.

Abbreviations

B. thuringiensis strains: *Bacillus thuringiensis*; Cry: Crystal; SEM: Scanning electron microscopy; PCR: Polymerase chain reaction; ICP: Insecticidal crystal proteins; SDW: Sterilized distilled water; NA: Nutrient agar; rRNA: Ribosomal ribonucleic acid; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; dNTP: Deoxynucleotide triphosphate; NCBI: National Center for Biotechnology Information; LD_{50}: Lethal dose 50%; KD: Kilo Dalton

Acknowledgements

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia, for funding this research work through the research project grant number IFT20082. The authors also are grateful for Mr. Ibrahim Hamed and Ms. Marwa El-Saleh, from plant Protection Department, Faculty of Agriculture, Zagazig University, Egypt, for providing the instars of Diptera and Lepidoptera insects.

Authors' contributions

First through fourth author (A.H., M.A.Y., M.M.A.E., and I.M.I.) participated in setting the work idea, planning, designing, and executing the experimental work. The fifth author (M. A.) finished the scanning electron microscopy work. The sixth author (E.A.) participated in planning the experimental work, writing up the manuscript, editing, and sending the article for and publication. All authors have read and approved the manuscript

Funding

This research work is funded by the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia, research project grant number IFT20082. The role of the funder was support executing the experimental work and publication fees as well.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Biological Sciences, College of Science, King Faisal University, P.O Box 31892, Al Hofuf, Al-Ahsa, Saudi Arabia. [2]Genetics Department, College of Agriculture, Zagazig University, Zagazig, Egypt. [3]Department Of Microbial Molecular Biology, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza 12619, Egypt. [4]Preparatory Year Deanship, King Faisal University, P.O Box 31892, Al Hofuf, Al-Ahsa, Saudi Arabia. [5]Department of Botany and Microbiology, College of Science, Bani-Suef University, Beni Suef, Egypt.

Received: 31 August 2020 Accepted: 13 December 2020
Published online: 04 January 2021

References

Abo-Bakr A, Fahmy EM, Badawy F, Abd El-Latif AO, Moussa S (2020) Isolation and characterization of the local entomopathogenic bacterium, *Bacillus thuringiensis* isolates from different Egyptian soils. Egypt J Biol Pest Control 30:54–63

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Ammouneh H, Harba M, Idris E, Makee H (2011) Isolation and characterization of native *Bacillus thuringiensis isolates* from Syrian soil and testing of their insecticidal activities against some insect pests. Turk J Agric 35:421–431

Aramideh S, Saferalizadeh M, Pourmirza A, Bari M, Keshavarzi M, Mohseniazar M (2010) Characterization and pathogenic evaluation of *Bacillus thuringiensis* isolates from West Azerbaijan province-Iran. Afr J Microbiol Res 4:1224–1229

Baragamaarachchi RY, Samarasekera JKRR, Weerasena OVDSJ, Lamour K, Jurat-Fuentes JL (2019) Identification of a native *Bacillus thuringiensis* strain from Sri Lanka active against Dipel-resistant *Plutella xylostella*. Peer J **7**:e7535. https://doi.org/10.7717/peerj.7535

Bel Y, Sheets JJ, Tan SY, Narva KE, Escriche B (2017) Toxicity and binding studies of *Bacillus thuringiensis* Cry1Ac, Cry1F, Cry1C, and Cry2A proteins in the soybean pests *Anticarsia gemmatalis* and *Chrysodeixis (Pseudoplusia)* includens. Appl Environ Microbiol 83

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

Bouslama T, Chaieb I, Rhouma A, Laarif A (2020) Evaluation of a *Bacillus thuringiensis* isolate based formulation against the pod borer, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). Egypt J Biol Pest Control 30: 16–22

Bozlagan I, Abdurrahman A, Fatma O, Leyla A, Akbulut M, Yilmaz S (2010) Detection of the *cry1* gene in *Bacillus thuringiensis* isolates from agricultural fields and their bioactivity against two stored product moth larvae. Turk J Agric 34:145–154

Bukhari DA, Shakoori AR (2010) Isolation and molecular characterization of cry4 harboring *Bacillus thuringiensis* isolate from Pakistan and mosquitocidal activity of their spores and total proteins. Pak J Zool 42:1–15

Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Environ Microbiol 57:3057–3061

Cinar C, Apaydin 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

Daniel L, Ester R, Nelly L (2018) The characterization of *Bacillus thuringiensis* from soil habitat of Auky Island, Padaido District in Biak Numfor Regency and its toxicity against mosquito larva of *Anopheles sp*. Am J Infect Dis 14:77–81

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS (2015) Insect pathogens as biological control agents: back to the future. J Invertebr Pathol 132:1–41

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

Leopoldo P, Delia M, Colin B, Jesús M, Primitivo C (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins (Basel) 6:3296–3325

Lone SA, Malik A, Padaria JC (2017) Characterization of lepidopteran-specific *cry1* and *cry2* gene harboring native *Bacillus thuringiensis* isolates toxic against *Helicoverpa armigera*. Biotechnol Rep (Amst) 15:27–32

Melo AL, Soccol VT, Soccol CR (2016) *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. Crit Rev Biotechnol 36:317–326

Miniatis T (1989) In vitro amplification of DNA by a polymerase chain reaction. In: Sambrook J, Fritsch EF (eds) Molecular cloning-A laboratory manual, Book-2, vol 14. Laboratory Press USA, Cold Spring Harbour, pp 5–14

Padole DA, Moharil MP, Ingle KP, Munje S (2017) Isolation and characterization of native isolates of *Bacillus thuringiensis* from Vidarbha Region. Int J Curr Microbiol App Sci 6:798–806

Palma L (2015) Protocol for the fast isolation and identification of insecticidal *Bacillus thuringiensis* strains from the soil. Bt Res 6:1–3

Reinoso-Pozo Y, Del Rincón-Castro MC, Ibarra JE (2016) Characterization of a highly toxic strain of *Bacillus thuringiensis serovar kurstaki* very similarly to the HD-73 strain. FEMS Microbiol Lett 363:188

Rochelle PA, Will JAK, Fry JC, Jenkins GJS, Parkes RJ, Turley CM, Weightman AJ (1995) Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity. In: Trevors JT et al (eds) Nucleic Acids in the Environment © Springer-Verlag Berlin Heidelberg, pp 219–239

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

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 62:775–806

Temnykh S, Park WD, Ayres N, Cartinhour S, Hauck N, Lipovich L, Cho YG, Ishii T, McCouch SR (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100: 697–712

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

Van Frankenhuyzen K (2009) Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr Pathol 101:1–16

Yilmaz S, Ayvaz A, Azizoglu U (2017) Diversity and distribution of *cry* genes in native *Bacillus thuringiensis* strains isolated from wild ecological areas of East-Mediterranean region of Turkey. J Trop Ecol 58:605–610

Yutao X, Kongming W (2019) Recent progress on the interaction between insects and *Bacillus thuringiensis* crops. Philos Trans R Soc Lond Ser B Biol Sci 374: e20180316

Publisher's Note