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Potential of Syrian *Bacillus thuringiensis kurstaki* Strains for Bioinsecticide Production

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Abstract- Six strains of *Bacillus thuringiensis kurstaki* (designated as SSy111-c, SSy125-c, SSy141-c, SyE3s, SyG41, and SyG46), previously isolated from Syrian soil samples and deceased larvae, were chosen due to their superior toxicity to lepidopteran larvae when compared to hundreds of local *Bt* isolates and *Bt kurstaki* HD-1, the reference strain. Scanning electron microscopy analysis revealed that these strains produce bipyramidal and cubical crystal proteins akin to those of the HD-1 strain. Plasmid pattern analysis demonstrated that these strains exhibit a plasmid profile like to that of HD-1. The digestion of 352 bp *gyrB* PCR fragments corresponding to the *gyrB* genes of the six strains with *Sau3*AI or *Eco*RI produced a single pattern type, mirroring the standard strain HD-1. PCR screening confirmed that all six strains contain *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1I*, *cry2Aa*, *cry2Ab*, and *vip3A* genes, which are similar to the HD-1. Analysis of protein profiles in the crystal protein extracts of the tested strains displayed two bands, approximately 130 and 65 KDa, matching the size of proteins produced by the HD-1 strain. However, comparative assessment of proteins production between the local strains and HD-1 indicated that two of them, namely SyG41 and SyG46, exhibited the highest δ-endotoxin/spore ratio almost 40 % more than the HD-1 strain. Consequently, these two local strains hold promise for future applications in the production of cost-effective local bioinsecticides, not only due to their potent toxicity against lepidopteran insect pests but also their potential for large-scale bioinsecticide production.

Keywords- Bioinsecticide, *Bacillus thuringiensis*, *kurstaki*, *δ*-endotoxin.

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1. Introduction

Bacillus thuringiensis (*Bt*), bacterial pathogen that synthesizes crystalline δ-endotoxin proteins (Cry) during sporulation, is a spore-forming and a Gram-positive bacterium. These proteins exhibit toxicity against a broad spectrum of insects, nematodes and human-cancer cells (da Silva et al., 2022). Additionally, certain *Bt* strains secrete vegetative insecticidal proteins (Vips) during vegetative growth mimicking the effects of Cry proteins intoxication (Chakroun et al., 2016).

Bt strains which are toxic to Lepidopteran insects comprise a diverse group of organisms. These strains are isolated from different natural environments and these are further developed by cloning many δ -endotoxin genes (*cry*) (Pinheiro and Valicente 2021). The *cry1*, *cry2* and *cry9* classes have been specified as Cry proteins toxic to lepidopteran larvae, making these genes popular choices for development of bioinsecticide and transgenic plants (Shelton et al., 2002; Roh et al., 2007; Crickmore et al., 2021). In general, *Bt* strains toxic to lepidopteran larvae produce big bipyramidal Cry proteins (130-140 kDa) and smaller cuboidal Cry proteins (65 kDa) which extended their toxicity spectrum, including mildly toxicity to mosquito larvae (Sauka and Benintende 2008). *Bt kurstaki* HD-1strain (HD-1) stand out as a highly effective strain in the commercial bioinsecticides formulations containing Cry1 and Cry2 proteins which show high levels of toxicity to more than one hundred species of Lepidoptera (Li et al., 2002; Federici et al., 2006; Glare and O'Callaghan, 2000).

The isolation and characterization of *Bt* strains and their use in the production of bioinsecticides have been established as worldwide practices for several decades (Kumar et al., 2021; Sánchez–Yáñez et at., 2022; Sanahuja et al., 2011). These practices will help in developing the production of local microbial biopesticides and promoting sustainable agricultural practices, reducing the negative environmental impacts of chemical pesticides, promoting food security, and improving human health especially in the developing countries (Fenibo et al., 2021; Sethi et al., 2022,).

Six locally interesting *Btk* strains are described in this study that were isolated previously from Syrian soil and dead larvae. These strains showed high insecticidal activity against Lepidoptera compared to the reference strain HD-1 and hundreds of *Bt* local isolates. The analysis included electron microscopy observation of the spore–crystal mixture, plasmid patterns, *gyrB*-RFLP analysis, *cry* and *vip* gene content and protein profiles. Further, δ-endotoxins production per spore between these strains and HD-1 was compared.

2. Materials and Methods

A. Bacterial strains

The six local *Bt* strains named SSy111-c, SSy125-c, SSy141-c, SyE3s, SyG41 and SyG46 that were isolated previously from Syrian soil (SSy) and dead larvae of *Ephestia kuehniella* (SyE) and *Galleria mellonella* (SyG) (Ammouneh at al., 2011; 2013) were used in this study. *Bt aizawai* B401, *Bt israelensis* T14 and *Bt kurstaki* HD-1 were obtained from Wuhan Institute of Virology (Lab of Biological Control of Arbovirus Vectors) and used as reference strains. All the bacterial strains were plated on nutrient agar (NA) and incubated at 30 ºC until bacterial colonies developed. The plates were stored in the fridge at 4ºC and sub-cultured every two weeks. For long-term storage, all the strains were stored in 20% glycerol and kept in a deep freezer at -80 ºC. T3 culture media (Travers et al., 1987) was used to monitor the sporulation

and parasporal crystal formation during growth. To grow the bacterial strains to extract genomic or plasmid DNA, Luria-Bertani (LB) culture media (Sambrook et al., 1989) was used.

B. Electron microscopy

Scanning electron microscope (SEM) was used to observe spore and crystal morphologies of the *Bt* strains. To prepare the samples, *Bt* culture was grown in a loop in 5-mL of T3 medium at 30 °C. An orbital shaker at 200 rpm was used in this process for 72 hours. After incubation, the culture was centrifuged for 10 minutes at 9700 g. Cell residues were removed by resuspending the resultant pellets in 0.5 M NaCl and incubating at 37 ºC for 30 minutes. After another centrifugation step for 10 minutes at 9700 g, sterilized distilled water (SDM) was used to wash the pellet three times. Subsequently, a 10 µL aliquot of the spore–crystal mixture, dissolved in 1 mL of sterile distilled water (SDW), was placed on a microscope slide. The samples, after drying, were examined under the SEM (TESACN at 30 kV).

C. Plasmid isolation

QIGEN plasmid Midi Kit (QIAGEN, cat. No.12145) was used for Plasmid DNA extraction according to the manufacturer's recommendations with one additional step which involved lysozyme treatment. The plasmids were analyzed by electrophoresis for 5 to 7 hours at 64V and at 4 ºC in a 0.5% agarose gel. The gels were photographed under UV after staining them in ethidium bromide.

D. PCR Amplification

Using PCR and specific primer directed toward the identification of subgroups of *cry1*, *cry2, and vip* genes, *cry* and *vip* genes were screened (Table 1). Primers employed for amplifying *gyrB* were chosen based on the method outlined by Manzano et al. (2003), targeting the conserved region of *gyrB*. Genomic DNA extraction kit (BIOTOOLS) was used for extracting of the *Bt* genomic DNA. Each PCR reaction contained 20 ng genomic DNA, 1 U of *Taq* DNA polymerase; 0.4 µM of each primer; 0.2 mM each of Deoxynucleotide triphosphate (dNTP) dATP, dCTP, dGTP, and dTTP (Promega); 3% dimethyl sulfoxide (DMSO) and 2 mM $MgSO_4$. Under the following conditions, Bio-Rad T gradient thermocycler was used: one denaturation step (5 minutes step at 95 °C), then 30 amplification cycles (1 minute at 95 °C, 1 minute at 48-58 °C and 1 minute at 72 °C) and finally, an extra extension one step of 10 minutes at 72 ºC. 1-2% agarose gels were used to separate the PCR products (according to the size of the fragments to be separated) and photographed under UV after ethidium bromide was added.

Table 1

Primers used in PCR screening

Primer pair	Gene (s) recognized	Product size (bp)	Annealing temperature (ºC)	Sequence	Reference
UN1	*cry1*	277	52	5`CATGATTCATGCGGCAGATAAAC (d) 5`TTGTGACACTTCTGCTTCCCATT (r)	Ben-Dov et al.,1997
UN2	*cry2*	700	54	5`GTTATTCTTAATGCAGATGAATGGG (d)	

				5`CGGATAAAATAATCTGGGAAA TAGT (r)	
Cry1I	*cry1I*	1137	48	5`ATGAAACTAAAGAATCCAGA(d ) 5`AGGATCCTTGTGTTGAGATA (r)	Masson et al., 1998
EE-2Aa	*cry2Aa*	498	60 (with UN2d)	5`GAGATTAGTCGCCCCTATGAG (r)	Ben-Dov et al.,1997
EE-2Ab	*cry2Ab*	546	60 (with UN2d)	5`TGGCGTTAACAATGGGGGGAG AAAT (r)	
EE-2Ac	*cry2Ac*	725	60 (with UN2d)	5`GCGTTGCTAATAGTCCCAACAA CA (r)	
CJ1	*cry1Aa*	246	52	5`TTATACTTGGTTCAGGCCC (d)	Ceron et al., 1994
CJ2				5`TTGGAGCTCTCAAGGTGTAA (r)	
CJ6	*cry1Ac*	180	50	5`GTTAGATTAAATAGTAGTGG (d)	
CJ7				5`TGTAGCTGGTACTGTATTG (r)	
SB-2	*cry1Ab*	858	42	5`TCGGAAAATGTGCCCAT (d)	Bourque *et al.*, 1993
U3-18c				5`AATTGCTTTCATAGGCT (r)	
Vip1s	*vip1*	484	54	5`GTGGAAAYTAACYGYTACTGA A (d)*	Yu et al., 2011
Vip1a				5`CCRCACCATCTATAMACAGTA AT (r)	
Vip2s	*vip2*	804	60	5`TTATTTTAATGGCATTTATGGA TTTGCC (d)	
Vip2a				5`GCAGGTGTAATTTCAGTAAGTG TAGAG (r)	
V1F:	*vip3*	444	60	T5`TATTTTAATGGCATTTATGGA TTTGCC(d)	Fang et al., 2007
V1R				5`GCAGGTGTAATTTCAGTAAGTG TAGAG (r)	
V2F	*vip3*	364	60	5`CTTCTGAAAAGTTATTAAGTCC AGAAT (d)	
V2R				5`TTACTTAATAGAGACATCGTAA AAA (r)	
Vip3Aab-F	*vip3Aa* &	1621	45	5`TGCCACTGGTATCAARGA (d)	Beard et al, 2008
vip3Aab-R	*vip3Ab*			5`TCCTCCTGTATGATCTACATAT GCATTYTT (r)	

* (M=A or C; R =A or G; Y= C or T)

E. Restriction enzyme digestion

The PCR products of *gyrB* region were digested using tow restriction enzymes *Eco*RI and *Sau3*AI (Pharmacia). To analyze the digests; samples were loaded into 2 % agarose gels and separated by electrophoresis. Agarose gels were stained in ethidium bromide solution. Visualization of the fragments was done on a UV transilluminator, and the agarose gel was photographed.

F. Analysis of crystal proteins

The purified crystal proteins of each isolate were analyzed according to the procedure described by Ammouneh et al., (2011). Proteins were separated on a 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie blue dye as described by Laemmli (1970).

G. Estimation of colony forming units for sporulated Bt cultures

As described by Zouari et al. (1998), *Bt* strains were grown in 250 ml flasks containing 50 ml liquid medium. One-liter of the medium consist 15g glucose, 5.4g ammonium sulphate, 5g yeast extract, 5g glycerol, 1g KH_2PO_4, 1g K_2HPO_4, 0.3g MgSO4, 0.01g $MnSO_4$ and 0.01g $FeSO_4$. Before sterilizing the medium, the pH was adjusted to 7 and then 1g of $CaCO_3$ was added in each flask for pH stabilization. After inoculation of the bacteria, the flasks were incubated at 30°C for 72 hours and 200 rpm in a rotary shaker to get more than 90 % of spores-crystals mixture which can be observed under the microscope. By transferring one ml of the *Bt* culture into Eppendorf tube which is heated for 10 minutes at 80°C, the number of spores was estimated. Appropriate dilutions of this culture were spread on LB plates in triplicate. Incubation of the plates were done then at 30 °C overnight. For each dilution, the colony-forming units per ml (CFU/ml) were calculated from each replicate.

H. δ-endotoxin yield estimation

Spore-crystal mixture was obtained by centrifuging one ml from the sporulated culture. To solubilize the crystals, the spore-crystal pellet was washed twice in 1M NaCl and twice in SDW and then was resuspended in sterile 50mM NaOH solution at 30°C for 3 hours. By centrifugation, the solubilized proteins were separated from the spores. δ-endotoxin concentration of each *Bt* strain per ml was calculated employing the Bradford's method with different concentrations of Bovine Serum Albumin (BSA) for standard graph (Bradford, 1976). The average of the three replicates were the data related to the determination of δ-endotoxin. It was characterized by their Standard Deviation (SD). The yield of δ-endotoxin per cell for each strain was calculated by dividing the mean of δ-endotoxin values (mg/l) by the mean of CFU values (spores/l)

3. Results

A. The ultrastructure of the crystal proteins

The analysis of crystal proteins from the six local strains was obtained by SEM. As shown in Figure 1, the crystal morphology showed two types bipyramidal and cuboidal crystal proteins with a perfectly regular shape for both, like the strain HD-1. Spore-crystal protein mixtures of the tested strains showed large bipyramidal crystals (ranging between ~ 800 and 1600 nm), small cuboidal crystals ranging between ~500 and 800 nm) and ovoid spores of ~1400 nm long.

B. Plasmid pattern and gyrB PCR-RFLP

Plasmid profiling of the six local strains showed two large plasmid bands (around 50 and 75 kb), three medium plasmid bands (6 to 10 Kb) and three small plasmid bands (from approximately 0.5 to 2 kb). These bands are similar in size to the *Bt kurstaki* HD-1 standard strain, whereas the plasmid profiles of *Bt israelensis* T14 is totally different (Table 2).

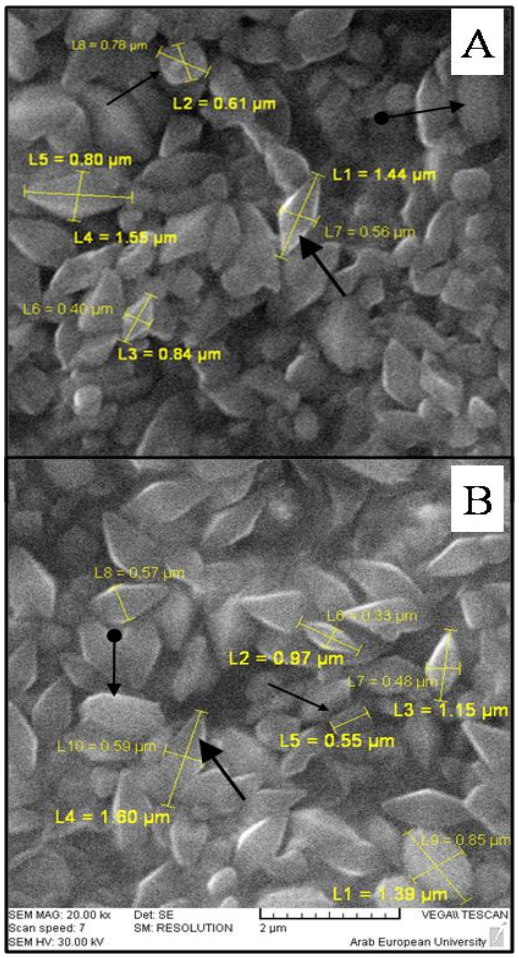

Figure 1. *Scanning electron micrographs of sporulated cultures of a local isolate (A-SyG46) and reference strain (B-HD-1) of Bacillus thuringeinsis kurstaki, showing typical parasporal inclusion. The big arrow: Bipyramidal, the small arrow: cuboidal, the arrow with dot: spores.*

The Sau3AI digestion of the 352 bp gyrB PCR fragments from the gyrB genes showed one pattern in all the tested strains. This pattern also yielded three fragments of 30, 150 and 170 bp same as the HD-1 pattern (Figure 2-A). Whereas, only two fragments of 170 and 182 bp with *Bt israelensis* T14 strain yielded. Additionally, the EcoRI digestion of gyrB PCR fragment gave one pattern for all the tested strains which also were similar to HD-1'pattern (Figure 2-B). Accordingly, two fragments of 37 and 315 bp were obtained

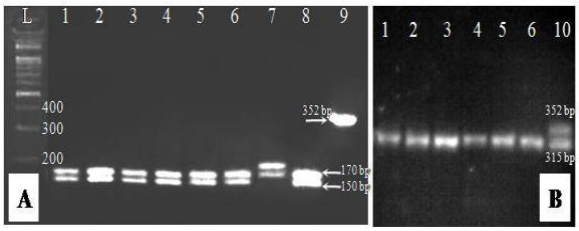

Figure 2. *Sau3AI (A) and EcoRI (B) digestion of gyrB PCR products from Bacillus thuringiensis strains. Lane L: DNA 100 bp ladder, lane 1: SSy111-c, lane 2: SSy125-c, lane 3: SSy141-c, lane 4: SyE3s, lane 5: SyG41, lane 6: SyG46, lane 7: Bt israelensis T14 (182 and 170 bp), lane 8: Bt*

kurstaki HD-1 (170 and 150 bp), lane 9: uncut gyrB PCR product of 352 bp and lane 10: uncut (352 bp) and digested (315 bp) gyrB PCR product of Bt kurstaki HD-1.

C. cry gene contents and protein profile

Six different cry genes and one vip gene were identified by PCR in all the tested strains (Figure 3). Amplifications corresponding to *cry1Aa, cry1Ab, cry1Ac, cry2Aa, cry2Ab, cry1I* and *vip3Aa* genes were achieved using specific primers. All the strains produced amplification fragments like the sizes of fragments obtained by the HD-1 strain (Table 2).

Table 2

Molecular characterization of the six local Bacillus thuringiensis kurstaki strains

strains	Crystal protein	No.of plasmids	*Cry* and *vip* gene profile	Major protein profile bands (kDa)	Toxin yield [mg toxin (10^{10}spores)$^{-1}$]
HD-1[†]	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	1.58 ± 0.3
SSy111-c[‡]	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	1.92 ± 0.2
SSy125-c	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	1.77± 0.5
SSy141-c	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	1.99 ± 0.1
SyE3s	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	1.77 ± 0.4
SyG41	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	2.2 ± 0.6
SyG46	BP, C	8	*1Aa, 1Ab, 1Ac, 2Aa, 2Ab, 1I, vip3A*	65/130	2.17 ± 0.2

HD-1: *Bacillus thuringiensis kurstaki* HD-1, [‡] Sy: The local strains recovered from soil (SSy) and dead larvae, *Galleria mellonella*, (SyG). BP: bipyramidal and C: cubical crystal proteins

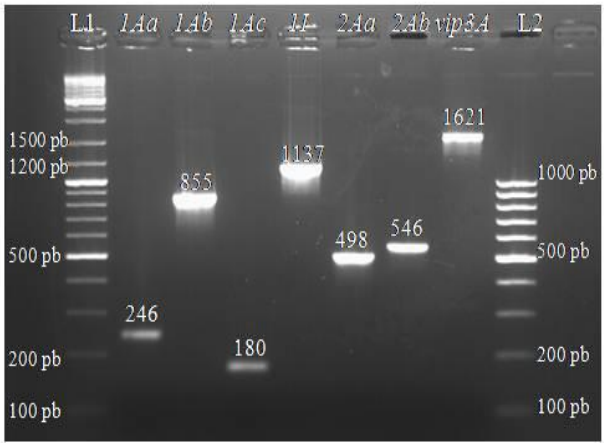

Figure 3. *Agarose gel (2 %) electrophoresis of PCR products amplified from the native Bt strain SyG46 using primers to detect cry1Aa (CJ1, CJ2), cry1Ab (SB-2, U3-18c), cry1Ac (CJ6, CJ7), cry1I (cry1Id, cry1Ir), cry2Aa (EE-2Aa, UN2d), cry2Ab (EE-2Ab, UN2d) and vip3Aa/b (Vip3Aab-F, Vip3Aab-R) genes. Lane L1: DNA 1 Kb ladder, lane L2:100 pb ladder.*

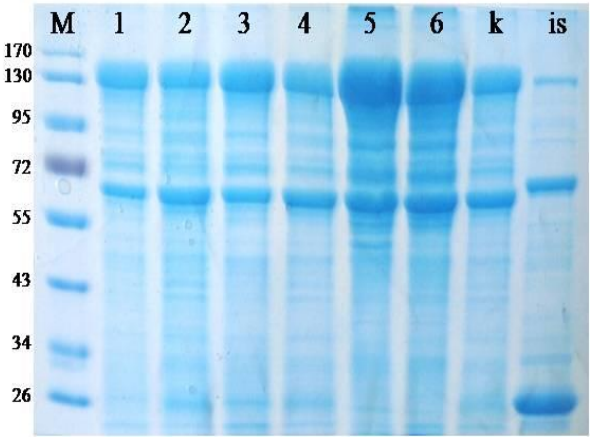

Figure 4. *SDS-PAGE of spore-crystal mixture from six local Bacillus thuringiensis kurstaki strains. Lane M: Molecular Marker given in kDa (Fermentas); lane 1: SSy111-c, lane 2: SSy125-c, lane 3: SSy141-c, lane 4: SyE3s, lane 5: SyG4 1, lane 6: SyG46, lane K: Bt kurstaki HD-1 and lane is: Bt israelensis T14.*

D. Study of spore and δ-endotoxin production

The comparison of δ-endotoxin production between the local strains and HD-1 are summarized in Table (2) and revealed that SyG41 and SyG46 exhibited the highest production levels at 2.2 and 2.17 [mg toxin (10^{10}spores)$^{-1}$], respectively. This production is almost 40% greater than that of HD-1, which produced 1.58 [mg toxin (10^{10}spores)$^{-1}$]. Additionally, SSy141-c, SSy111-c, SyE3s, and SSy125-c produced toxin at rates of 1.99, 1.92, 1.77, and 1.77 [mg toxin (10^{10}spores)$^{-1}$], respectively.

4. Discussion

Syria has seen limited screening programs for *Bt* strains, with only a few reports providing insights into the isolation, toxicity, insecticidal gene content, and polypeptide patterns of Syrian *Bt* isolates. Ammouneh et al., (2011, 2013) previously reported on 65 Syrian *Bt* isolates, focusing on their bioactivity against *E. kuehniella* larvae. Among these isolates, several exhibited higher toxicities than the standard strain HD-1, with six of them (SSy111-c, SSy125-c, SSy141-c, SyE3s, SyG41, and SyG46) belonging to serovar *kurstaki* and showing remarkable activity against *E. kuehniella* larvae. These strains exhibited Lethal dose 50% (LC_{50}) values substantially lower than HD-1, with some strains like SyG41 and SyG46 demonstrating almost six times higher toxicity (Ammouneh et al., 2013).

Furthermore, these local strains displayed enhanced toxicity against other lepidopteran larvae, such as *Spodoptera exigua*, *Helicoverpa armigera* and *Galleria mellonella*, when compared to HD-1 (Table 3, unpublished data). The remarkable high toxicity of these local *Bt* strains underscore the importance of regional screening in the quest for isolation of novel *Bt* strains which could be used as biocontrol agents for controlling local insect pests. Moreover, the use of indigenous strains offers economic benefits by reducing reliance on imported pesticides and fostering local biotechnological innovation. Therefore, we consider these new strains exhibited a significantly greater potency than the standard strain HD-1 against lepidopteran pest insects and those were applied for further analyses in order to assess their potential as an active ingredient in local biopesticides.

Crystal protein morphology is often indicative of the target insect range of *Bt* strains. Bipyramidal crystals are typically targeting Lepidoptera, while cuboidal crystals may affect both Lepidoptera and Diptera (Federici et al., 2006). The observed bipyramidal and cuboidal crystal protein patterns in the local strains align with their enhanced toxicity against lepidopteran pests. The bipyramidal crystal shape is mainly associated with Cry1 toxins whereas the cubic square crystal contains mostly Cry2 toxin (Silva et al., 2004; Bravo et al., 2007).

Table 3

The insecticidal activity of six local Bacillus thuringiensis kurstaki strains against the larvae of some insect pests

Insect Strains	*Ephestia kuehniella*	*Spodoptera exigua*	*Helicoverpa armigera*	*Galleria mellonella*	*Culex quinquefasciatus*	types of *cry* genes
HD-1[†]	+++	+++	+++	++	+	*cry1, cry2*
T14[†]	-	-	-	-	++++	*cry4, cry11*
B401[†]	++	++	++	++++	-	*cry1, cry9*
SSy111-c[‡]	+++	+++	+++	+++	+	*cry1, cry2*
SSy125-c	++++	+++	+++	+++	+	*cry1, cry2*
SSy141-c	++++	+++	+++	+++	+	*cry1, cry2*
SyE3s	++++	+++	+++	+++	+	*cry1, cry2*
SyG41	++++	++++	++++	+++	+	*cry1, cry2*
SyG46	++++	++++	++++	+++	+	*cry1, cry2*

++++: very high toxicity, +++: high toxicity, ++: toxic, +: low toxicity, -: no toxicity.
[†] Reference strains used as controls, HD-1: *Bt kurstaki*, T14: *Bt israelensis*, B401: *Bt aizawai.*
[‡] Sy: The local strains recovered from soil (SSy) and dead larvae, *Galleria mellonella* (SyG) or *Ephestia kuehniella*

PCR has been used for long time to Identify the *cry* genes which will help in the prediction of the insecticidal activities of *Bt* strains (Porcar and Juarez-Perez 2003). Additionally, the PCR used to determine the distribution of *cry* genes within a collection of *Bt* strains (Crickmore et al., 2021). However, toxic potency of *Bt* strains depends also on the type and subgroups of *cry* genes and their expression (Saadaoui et al., 2009; da Silva et al., 2022). While Plasmid and *gyrB* PCR-RFLP patterns, *cry* and *vip* gene content, and protein profiles of these local *kurstaki* strains resemble those of the standard strain HD-1, their significantly lower LC_{50} values suggest a complex interplay of factors, potentially involving gene expression at the protein level and synergistic interactions among *cry* genes. Therefore, comprehensive characterization of *Bt* strains should encompass more than PCR screening and protein profile analysis, including a focus on δ-endotoxin / spore ration for each strain.

5. Conclusion

It can be concluded from this study that these local *Bt* strains, particularly SyG41 and SyG46, have great potential to control the lepidopteran pests and hold promise for future applications in

the production of local bioinsecticides. This recommendation is not only based on their high toxicity towards lepidopteran insect pests but also on their potential to produce large quantities of bioinsecticides at a reduced cost. This opens avenues for harnessing these local strains for development of effective, environmentally friendly, and sustainable bioinsecticides serving as alternatives to conventional chemical insecticides.

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