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Bio-Insecticidal Efficacy and Molecular Characterization of *Cry* Genes in *Bacillus thuringiensis* Isolated from South Punjab Districts, Pakistan, against *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

(Keberkesanan Bio-Insektisid dan Pencirian Molekul Gen *Cry* dalam *Bacillus thuringiensis* yang Dipencilkan dari Daerah Punjab Selatan, Pakistan terhadap *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

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ABSTRACT

Lepidopteran insects can severely reduce crop productivity through extensive defoliation during outbreaks. Increasing resistance to chemical insecticides has emphasized the need for environmentally safer alternatives such as *Bacillus thuringiensis* (*Bt*)-based biopesticides. Since the insecticidal activity and *Cry* gene diversity of *Bt* strains vary geographically, this study aimed to isolate and characterise indigenous *Bt* strains from South Punjab, Pakistan, active against *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). *Bt* isolates were recovered from soil samples using the acetate enrichment method and characterised through morphological, proteomic, and molecular analyses. Scanning electron microscopy showed five crystal morphologies, predominantly bipyramidal crystals. SDS-PAGE analysis showed major protein bands at approximately 130, 60, and 40 kDa, indicating diversity in crystal toxin composition. Among 93 isolates, 20 (21.5%) exhibited toxicity against second instar larvae of *S. litura* in preliminary diet bioassays. At 400 µg/g crystal protein concentration, 14 isolates caused >50% larval mortality. Isolate JTO-9 from Muzaffargarh showed the highest pathogenicity, causing 100% mortality with an LC_{50} value of 91.62 µg/g diet after 96 h exceeding the toxicity of reference strains HD-1 and HD-73. PCR analysis showed that 65% of active isolates carried *Cry1* genes, while 70% possessed *Cry2* genes. Detection of *Cry1Aa*, *Cry1Ab*, *Cry1Ac*, *Cry2Aa*, *Cry2Ab*, and *Cry2Ac* genes identified eight distinct *Cry* gene profiles among nine isolates. This study provides the first comprehensive characterization of indigenous *Bt* isolates from South Punjab, Pakistan, and identifies JTO-9 as a promising candidate for the development of effective biopesticides against *S. litura*. These isolates may support pest management in agricultural ecosystems.

Keywords: *Bacillus thuringiensis*; *biopesticides; Cry* genes; indigenous isolates; *Spodoptera litura*

ABSTRAK

Serangga lepidoptera boleh mengurangkan produktiviti tanaman dengan ketara melalui pengguguran daun yang meluas semasa wabak. Peningkatan rintangan terhadap racun serangga kimia telah menekankan keperluan untuk alternatif yang lebih selamat dari segi alam sekitar seperti biopestisid berasaskan *Bacillus thuringiensis* (*Bt*). Memandangkan aktiviti insektisida dan kepelbagaian gen *Cry* bagi strain *Bt* berbeza-beza secara geografi, kajian ini bertujuan untuk memencilkan dan mencirikan strain *Bt* asli dari Punjab Selatan, Pakistan, yang aktif terhadap *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). Pencilan *Bt* diperoleh daripada sampel tanah menggunakan kaedah pengayaan asetat dan dicirikan melalui analisis morfologi, proteomik dan molekul. Mikroskopi elektron imbasan mendedahkan lima morfologi kristal, terutamanya kristal bipiramida. Analisis SDS-PAGE menunjukkan jalur protein utama pada kira-kira 130, 60 dan 40 kDa, menunjukkan kepelbagaian dalam komposisi toksin kristal. Antara 93 pencilan, 20 (21.5%) menunjukkan ketoksikan terhadap larva instar kedua *S. litura* dalam bioasai diet awal. Pada kepekatan protein kristal 400 µg/g, 14 pencilan menyebabkan kematian larva >50%. Pengasingan JTO-9 dari Muzaffargarh menunjukkan kepatogenan tertinggi, menyebabkan kematian 100% dengan nilai LC_{50} 91.62 µg/g diet selepas 96 jam melebihi ketoksikan strain rujukan HD-1 dan HD-73. Analisis PCR mendedahkan bahawa 65% pencilan aktif membawa gen *Cry1*, manakala 70% mempunyai gen *Cry2*. Pengesanan gen *Cry1Aa*, *Cry1Ab*, *Cry1Ac*, *Cry2Aa*, *Cry2Ab* dan *Cry2Ac* mengenal pasti lapan profil gen *Cry* yang berbeza antara sembilan pencilan. Kajian ini menyediakan pencirian komprehensif pertama pencilan *Bt* asli dari Punjab Selatan, Pakistan dan mengenal pasti JTO-9 sebagai calon yang berpotensi untuk pembangunan biopestisid yang berkesan terhadap *S. litura*. Pencilan ini mungkin boleh menyokong pengurusan perosak dalam ekosistem pertanian.

Kata kunci: *Bacillus thuringiensis*; *biopestisid;* gen *Cry*; pencilan indigenus; *Spodoptera litura*

INTRODUCTION

The cotton cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is a sporadic and polyphagous insect pest that significantly affects the productivity of numerous crops worldwide (Arumugam et al. 2015). This pest is reported to infest more than 120 plant species, including economically important crops such as cotton, soybean, tobacco, maize, vegetables, and ornamental plants, and can cause severe yield losses of up to 50-80% during outbreak conditions (EPPO 2008a; CABI 2023). In several countries, including Pakistan, the heavy use of chemical insecticides to control insect pests has caused major problems, including the development of insect resistance (Ahmad & Gull 2017). Chemical insecticides can be replaced with microbial insecticides, which are safer for humans and other organisms and are better for the environment (Bravo & Soberon 2023).

Among the most effective microbial pesticides, *Bt* is a Gram-positive spore-forming bacterium. During its stationary phase of growth, it produces Crystalline proteins known as delta endotoxins (Dominguez-Arrizabalaga et al. 2020). *Bt* is a promising microbial insecticide due to its specificity, environmental safety, and production of *Cry* toxins, with its selective mode of action and compatibility with integrated pest management enhancing its effectiveness as an alternative to chemical pesticides (Schunemann, Knaak & Fiuza 2014). Its primary habitat has been described as soil, although it has also been isolated from vegetation, stored grains, dead insects, sediments, and aquatic environments (Baig & Mehnaz 2010b). The major function of *Cry* proteins is to lyse midgut epithelial cells of insects by inserting into the membrane and triggering the development of pores. The Crystals are then dissolved in the alkaline environment of the midgut lumen and activated by the host protease (Bravo et al. 2013). Based on the incidence, magnitude, and spatial distribution of field-evolved resistance, its impact can range from negligible to severe (Tabashnik et al. 2014).

Each ecosystem potentially harbours unique *Bt* strains that remain undiscovered but have the ability to be toxic to specific groups of insects. Hence, discovering new *Bt* strains and characterising their *Cry* genes is important to differentiate the activity of *Cry* proteins (Seifinejad, Jouzani & Hosseinzadeh 2008). Multiple studies from countries like Saudi Arabia (El-Ghiet et al. 2023), Thailand (Hemthanon, Promdonkoy & Boonserm 2023), Mexico (Bel, Andres-Anton & Escriche 2023), Iran (Rashki et al. 2021), Brazil (Pinheiro & Valicente 2021), Egypt (Abo-Bakr et al. 2020), China (Liu et al. 2020), and India (Rabha et al. 2017) have documented the diversity of *Bt* in the soil. However, no comprehensive investigation has been conducted on the *Cry* genes in the south region of Punjab, Pakistan, against *S. litura*. Therefore, it seems important to search for new *Bt* strains to assess their effectiveness in pest management and to ascertain the distribution characteristics of *Cry* genes.

In previous research conducted using soil samples from across Pakistan, the *Bt* isolate MS-SBSBt1 demonstrated greater toxicity to *S. litura* compared to reference strains (Ahmad & Shakoori 2013). In another study, genome analysis of the indigenous *Bt* strain (T407) showed the presence of multiple *Cry* and *vip* toxin genes, indicating a strong potential for development as a broad-spectrum biocontrol agent against several lepidopteran pests (Gothandaraman et al. 2025). The varied ecosystems of Punjab, Pakistan, provide ideal conditions for the growth of microorganisms and impart ample opportunities for the isolation of novel bacteria. *Bt* isolates from soil samples may help uncover new insect pathogens with various *Cry* gene combinations. Therefore, *this study aimed to isolate and characterise native Bt strains from the soils of Punjab, Pakistan, and to determine their Cry gene composition and insecticidal activity against S. litura.*

MATERIALS AND METHODS

SAMPLE COLLECTION

The southern region of the Punjab province of Pakistan extends over 1200 km along the Indus River and is denoted as the cotton belt or cotton zone. Representative soil samples were collected up to a depth of 8-10 cm across six districts of South Punjab, namely Rahim Yar Khan, Multan, Rajanpur, Vehari, Muzaffargarh, and Bahawalpur (Figure 1). A geographic map of the sampling districts was generated using QGIS software (version 3.24) based on recorded coordinates to illustrate the distribution of sampling sites and inter-district distances.

Using a sterile spatula, surface material was scraped off in order to gather samples (10 g soil per sample). A total of 150 soil samples were selected from areas with no previous history of using *Bt*-based product applications. They were kept in sterile HDPE bags and stored at 4 ºC till further processing.

ISOLATION AND CHARACTERISATION OF *Bt* STRAINS

The acetate-heat selection method by Travers, Martin and Reichelderfer (1987) was followed for the isolation of *Bt*. Briefly, 1 g of each soil sample was added in 10 mL sterile dH_2O and heated for 30 min at 80 ºC to eliminate unwanted non-spore-forming and most of spore-forming bacteria. One mL of suspension was used to inoculate 10 mL LB broth (Miller) buffered with 0.25 M sodium acetate (pH 6.8). Afterward, the suspensions were incubated for 4 h at 30 ºC, and heat treatment was applied at 30 ºC for 5 min. All suspensions were diluted (10^{-1}), and 100 µL of each sample was plated on T3 agar plates containing phosphate buffer (tryptone 3 g/L, tryptose 2 g/L, yeast extract 1.5 g/L, $MnCl_2$ 0.005 g/L, disodium phosphate 8.9 g/L, monosodium phosphate 6.9 g/L, and agar 15 g/L; pH 6.8-7.0) and incubated for 72 h until sporulation occurred. *Bt*-like colonies (cream coloured) were labelled and subcultured

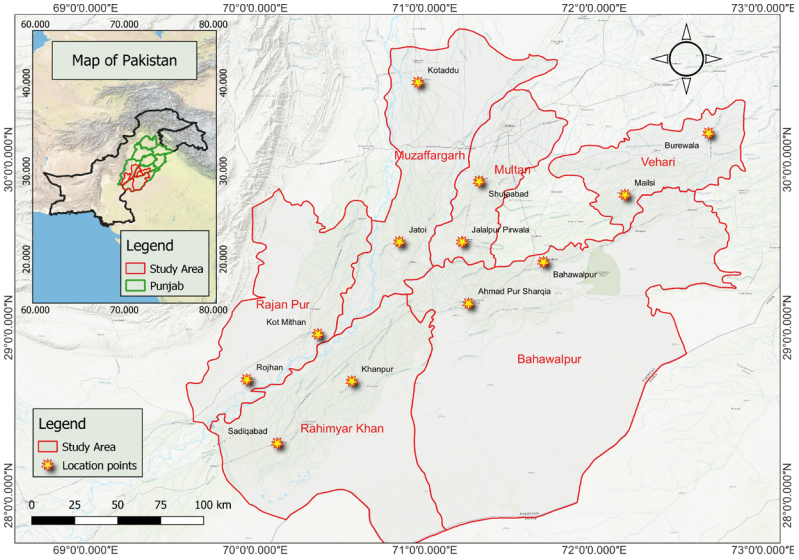

FIGURE 1. Map showing the sampling sites of *Bacillus thuringiensis* isolates across different districts of South Punjab, Pakistan

three times to obtain a pure culture. Plates were incubated at 30 ± 2 ºC for 48 h and inspected for colony shape and parasporal Crystals using microscopy. Stock samples were prepared in 80% glycerol and kept at -80 ºC for further investigation. *Bt* subsp. *kurstaki* HD-73 (4D4) and HD-1 (4D6) were used as references and procured from BGSC (*Bacillus* Genetic Stock Centre, Ohio State). The *Bt* index was determined by the following formula: Total number of *Bt* isolates divided by the overall sum of isolates with *Bacillus* morphology.

SCANNING ELECTRON MICROSCOPY

Bt isolates were cultured on nutrient agar plates using a single colony as inoculum and incubated at room temperature for 5 days to allow complete sporulation. The spore-Crystal mixture was suspended in ice-cold 1 M NaCl for 5 min, washed 3 times with ice-cold dH_2O and then pellets were suspended in dH_2O. The Crystals were analysed under a scanning electron microscope using the ZEISS EVO LS10 instrument. The SmartSEM software was used to observe differences between crystal shapes of isolates at 24,000× magnifications at a voltage of 1500 kV (Kati et al. 2007).

ISOLATION OF SPORE-CRYSTAL MIXTURE

One colony of each *Bt* isolate was transferred into 5 mL T3 broth and incubated at 200 rpm for 48-60 h until more than 90% of cells had lysed. The broth was centrifuged at 10,000 rpm for 10 min at 4 ºC. The pellet was washed once with ice-cold Tris-EDTA buffer containing 1 mM phenyl methyl sulphonyl fluoride (PMSF), once with ice-cold 0.5 M NaCl, followed by twice with Tris-EDTA buffer with 0.5 mM PMSF. The spore-crystal mixture was kept at -20 ºC in 100 µL sterile dH_2O with 1 mM PMSF.

CHARACTERISATION OF SPORE-CRYSTAL COMPOSITION

Protein aliquots (10 µL) of each *Bt* isolate were analysed by SDS-PAGE at 37 ºC for 3 h on 4% stacking and 10% resolving gel in the Bio-Rad Mini Protean 3 Cell System (Laemmli 1970). Electrophoresis was carried out at 15 V for 15 min, followed by 100V for 2 h. Gels were stained with Coomassie brilliant blue stain and a high molecular weight standard marker was used to estimate the molecular masses of the protein samples.

INSECT SAMPLING

The S. litura larvae were collected from agricultural fields in Punjab and reared under controlled laboratory conditions (27 ± 2 °C, 65 ± 10% relative humidity, 14:10 h (light:dark) photoperiod) on an artificial diet (Gupta et al. 2005). The diet comprised chickpea flour (100 g), yeast powder (20 g), agar (15 g), ascorbic acid (3 g), methyl paraben (2 g), and sorbic acid (1 g) dissolved in 1000 mL dH_2O and supplemented with a multivitamin solution. Agar was first dissolved in boiling distilled water, after which the remaining ingredients were added gradually with continuous stirring to ensure uniform mixing, and the diet was allowed to solidify before use. Neonates were reared in groups, while later instars were maintained individually to prevent cannibalism. Larvae were identified based on morphological characteristics, including body colouration, longitudinal stripes, and head capsule features. The second instar larvae were used for bioassays.

TOXICITY EXPERIMENTS

Initially, three-day-old, ready-to-hatch egg masses with yellowish brown hairs were placed in a container. The neonate larvae hatched from these egg masses were reared

on an artificial diet. Second-instar larvae of *S. litura* were identified based on body length (4-6 mm), pale green to light brown colouration, and a head capsule width of approximately 0.5 mm (EPPO 2008b). The *in-vitro* bioassays against second instar larvae of *S. litura* were performed by the diet overlay method. The preliminary bioassay with a single dose of 50 µg/g of diet was used to identify pathogenic strains, whereas the dose-mortality bioassay was performed on identified pathogenic strains to determine the LC_{50} needed to kill 50% of the target insect using different diet concentrations (10 µg/g, 50 µg/g, 100 µg/g, 200 µg/g, 300 µg/g, and 400 µg/g). Three replicates with 30 larvae were used for each concentration. The treated diet was allowed to dry at room temperature, and three second-instar larvae were placed in each container (4 × 6 cm). The reference *Bt* strains were utilised as the positive control, while autoclaved deionised water was used as the negative control. The larval mortality was recorded after 4 days. The total mortality of the insects after treatment was converted into percentage values. The data underwent an arcsine transformation and were analysed statistically. The LC_{50} values of *Bt* isolates were evaluated using Probit analysis by SPSS (version 25).

PCR ANALYSIS OF ISOLATES AND *CRY* GENE IDENTIFICATION

PCR analysis was performed to identify *Cry* genes. All isolates producing Crystal proteins were screened for *Cry1* and *Cry2* genes by two pairs of universal primers as previously described by Ben-Dov et al. (1997). Further identification of *Cry1Aa*, *Cry1Ab*, *Cry1Ac* (Ben-Dov et al. 1997), *Cry2Aa*, *Cry2Ab*, and *Cry2Ac* (Ceron et al. 1994) was conducted using six pairs of specific primers. The genomic DNA was obtained by the method described by Bravo et al. (1998). *Bt* strains were grown on nutrient agar plates at 37 °C. A loopful of cells was transferred to 0.2 mL H_2O, frozen at -80 °C for 20 min, and then transferred to boiling water for 10 min. The cell lysate was centrifuged at 10,000 × *g* for 10 s, and the supernatant was utilised as a DNA sample in the PCR.

RESULTS

ISOLATION OF *Bacillus thuringiensis*

Out of 150 samples, 266 *Bt*-like colonies were selected for the examination of spore and Crystal formation. The ratio of *Bt* colonies to total colonies separated (*Bt* index) ranged from 0.20 to 0.41, with an average value of 0.30. Geographically, the highest *Bt* isolates (5.3%) were obtained from Multan (*Bt* index 0.41), which contained 110 *Bt*-like colonies, of which 46 were identified as *Bt*. The next richest sample was Muzaffargarh (*Bt* index 0.38), followed by Rajanpur with a 0.32 index corresponding to *Bt* (Table 1).

CHARACTERIZATION OF *Bt* ISOLATES

Among 266 isolates, five different Crystal shapes were observed in 35% (93) isolates. The strains exhibited a single type of Crystal morphology in 51.61% (48) of the isolates, while the remaining 38.71% (45) showed multiple types of Crystal morphology. The percentages of Crystal shapes were 34.41% (32) bipyramidal & cuboidal, 24.73% (23) spherical, 15.05% (14) cuboidal, 8.60% (08) irregular & spherical, 8.60% (8) bipyramidal, 5.38% (5) rod & irregular, and 3.23% (3) irregular pointed. Most of the isolates, 40 (43.01%), displayed bipyramidal Crystals (Figure 2).

SDS-PAGE ANALYSIS

SDS-PAGE was used to analyse the Crystal protein profiles of 93 strains. The *Bt* strains had different molecular weights of proteins, specifically 135, 130, 120, 100, 95, 75, 70, 65, 60, 45, 35, 28, and 25 kDa (Figure 3). The presence of several

TABLE 1. Distribution of *Bacillus thuringiensis* soil isolates found in soil samples collected from different sites of South Punjab

District[a]	Samples analysed	No. of *Bt* positive samples obtained	No. of samples yielding *Bt* (%)	No. of *Bt*-like colonies examined	No. of *Bt* positive colonies	Mean *Bt* index[b] (*Bt* isolates)
Rahim Yar Khan[1]	24	02	1.3%	31	08	0.25
Multan[2]	33	08	5.3%	110	46	0.41
Rajanpur[3]	23	03	02%	46	15	0.32
Vehari[4]	21	02	1.3%	21	05	0.23
Muzaffargarh[5]	26	04	2.6%	39	15	0.38
Bahawalpur[6]	23	01	0.6%	20	04	0.20
Total	150	20	13.33%	266	93(34.96)	0.30

[a]Sites; [1], Sadiqabad (SDK), Khanpur (KPR); [2], Jalalpur Pirwala (JLP), Shujaabad (SHJ); [3], Rojhan (Roj), Kot Mithan (KOM); [4], Mailsi (MA), Burewala (BRW); [5], Jatoi (JTO), Kotaddu (ADK); [6], Bahawalpur-1 (BHV-1), Ahmad Pur Sharqia (ADP); [b]Mean *B. thuringiensis* index (*Bti*)= The proportion of *Bt* Crystal-producing isolates to non-*Bt* spore-forming *Bacilli*

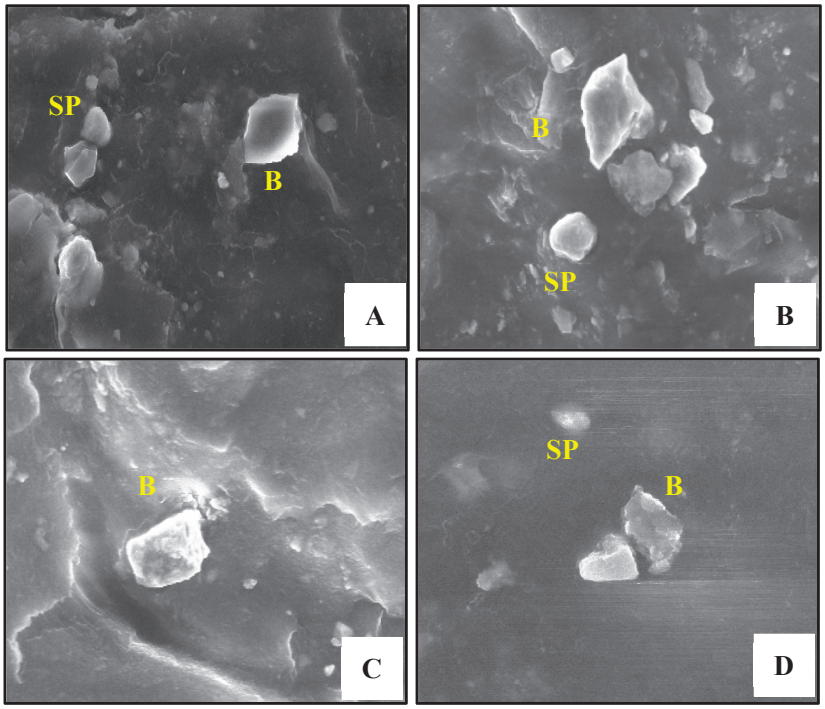

FIGURE 2. Electron micrograph of *Bacillus thuringiensis* isolates. A-D: Bipyramidal spore-Crystal morphology of *Bt* isolates (SHJ-20, JTO-6, BRW-19, ADK-23). B: bipyramidal; SP: spore)

proteins suggests that *Bt* strains may possess a range of *Cry* genes and insecticidal properties. Out of the 93 *Bt* strains, 19 strains (20.43%) were found to produce only one band, while 74 *Bt* strains (79.56%) exhibited multiple bands with varying molecular weights. The majority of the *Bt* strains (63, 67.74%) synthesised proteins with molecular weights ranging from 130, 60, and 40 kDa. The proteins with a molecular weight of 120 - 135 kDa were the most abundant proteins found in 38 (41.93%) *Bt* strains. Out of the total strains, 18 (19.35%) were discovered to contain proteins within the range of 35-100 kDa, and 9 strains (9.68%) were found to synthesise proteins with a molecular weight of 25 kDa. Proteins with molecular weights of around 130 and 60 kDa indicated the existence of genes associated to *Cry1* and *Cry2*, respectively.

M: High-range protein marker, Lane 1: HD-1, Lane 2: HD-73, Lane 3: JLP-10, Lane 4: BRW-19, Lane 5: KOM-16, Lane 6: JTO-9, Lane 7: SHJ-14. M: Mid-range protein marker, Lane 8: SHJ-31, Lane 9: SDK-1, Lane 10: MA-11, Lane 11: ADK-23, Lane 12: ROJ-12

BIO-INSECTICIDAL ACTIVITY

The preliminary bioassay was performed using a single dose of 50 µg of proteins per gram of diet to identify pathogenic strains. Out of the 93 local *Bt* strains, 20 (21.51%) showed insecticidal activity (>20% mortality) and were screened to perform further analysis. During the dose-mortality bioassay, mortality rates ranged from 40 to 100% for isolates and reference strains (Table 2). The most optimal outcomes were obtained on the fourth day across all treatments. After being inoculated, *S. litura* larvae treated with native *Bt* strains noticeably slow down the rate of feeding, turn black and eventually die owing to septicaemia and hunger. The highest mortality rate of 77 to 100% was recorded for reference strains HD-1, HD-73, and isolate JTO-9. Out of the 20 isolates, one isolate (JTO-9) showed 100% mortality, six isolates (KPR-6, JLP-10, SHJ-14, SHJ-20, BRW-19, and ADK-23) resulted in mortality rates ranging from 61 to 76%, seven isolates (SDK-1, JLP-15, SHJ-17, SHJ-31, ROJ-12, KOM-16, and MA-11) resulted in mortality rates ranging from 51 to 60%, and six isolates (JLP-3, SHJ-24, KOM-21, JTO-6, ADK-25, and ADP-4) resulted in mortality rates ranging from 40 to 50% against second instar larvae of *S. litura*.

Out of ninety-three *Bt* isolates, the LC_{50} value of the twenty *Bt* strains ranged from 91.62 µg/g to 532.81 µg/g for *S. litura*. The *Bt* isolate JTO-9 from Muzaffargarh demonstrated the highest level of pathogenicity against *S. litura*, with an LC_{50} value of 91.62 µg/g after 96 h, followed by reference strains with LC_{50} values of 150.63 µg/g and 193.87 µg/g after 96 h (Figure 4). The ADP-4 strain from Bahawalpur exhibited the lowest toxicity, as shown by the LC_{50} value of 532.81 µg/g after 96 h. The bioassay results indicated that the *Bt* isolates, especially JTO-9, SHJ-14, and

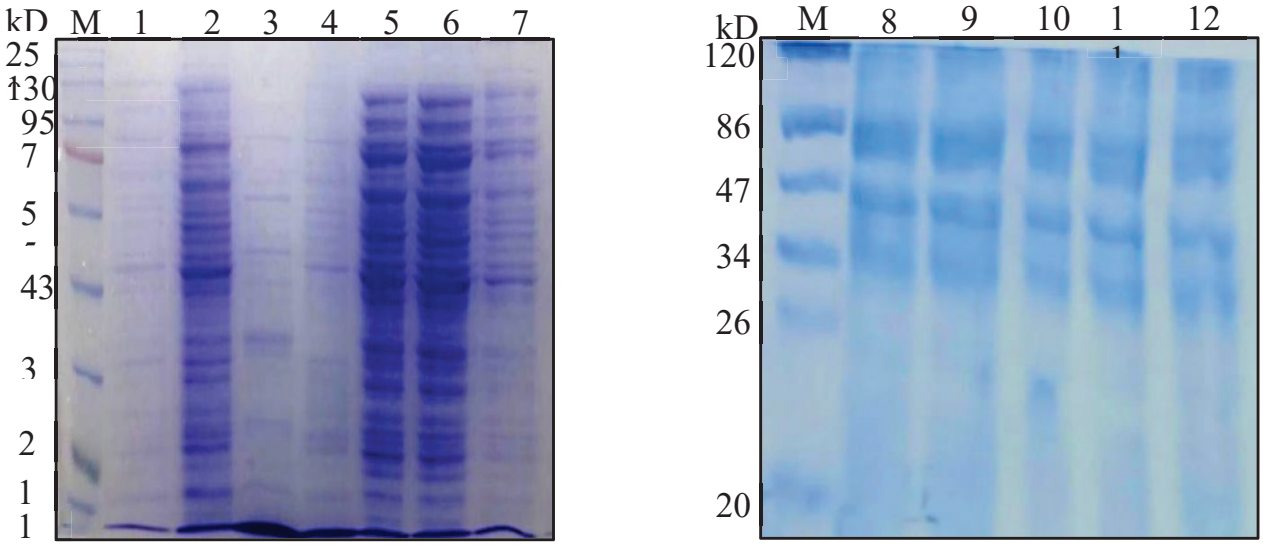

FIGURE 3. The SDS-PAGE gels exhibit the distinct protein patterns identified in the collection of *Bacillus thuringiensis* strains

TABLE 2. The LC_{50} values of Bacillus thuringiensis isolates against Spodoptera litura following 96-h post-treatment

Sr No.	Isolates	Mortality percentage	LC_{50} µg/g[a]	UL-LL[b]	Slope ± SE[c]	X^2
1	HD-1	83.33%	150.63	111.62-205.95	1.63 ± 0.25	3.82
2	HD-73	77.00%	193.87	147.80-264.56	1.81 ± 0.28	3.08
3	SDK-1	57.00%	309.70	225.17-514.13	1.61 ± 0.31	0.96
4	KPR-6	62.00%	311.38	226.49-644.26	1.80 ± 0.34	0.30
5	JLP-3	46.66%	480.56	313.44-1109.93	1.49 ± 0.35	0.59
6	JLP-10	67.00%	290.69	199.30-446.21	1.14 ± 0.23	2.22
7	JLP-15	56.66%	353.01	239.35-690.33	1.58 ± 0.33	0.95
8	SHJ-14	70.00%	242.80	196.03-314.65	2.432 ± 0.41	0.37
9	SHJ-17	53.33%	390.73	271.88-765.37	1.55 ± 0.33	0.68
10	SHJ-20	66.00%	275.31	202.28-469.23	1.39 ± 0.26	0.88
11	SHJ-24	50.00%	476.09	295.41-1261.41	1.21 ± 0.26	0.56
12	SHJ-31	53.33%	385.44	270.27-739.02	1.55 ± 0.33	0.90
13	ROJ-12	60.00%	324.75	231.65-557.57	1.33 ± 0.26	0.30
14	KOM-16	60.00%	321.96	226.49-644.26	1.54 ± 0.29	0.64
15	KOM-21	44.00%	485.39	323.36-1224.52	1.39 ± 0.32	0.39
16	MA-11	53.33%	442.91	282.43-1068.61	1.25 ± 0.27	0.95
17	BRW-19	64.00%	301.84	205.13-496.61	1.42 ± 0.27	1.82
18	JTO-6	47.00%	466.81	304.60-1130.62	1.59 ± 0.36	0.76
19	JTO-9	100.00%	91.62	29.04-207.91	1.61 ± 0.23	4.39
20	ADK-23	63.33%	303.42	211.87-535.90	1.52 ± 0.29	1.70
21	ADK-25	50.00%	528.82	318.40-1411.20	1.17 ± 0.27	0.30
22	ADP-4	40.00%	532.81	340.80-1622.16	1.40 ± 0.31	0.96

[a] µg of toxin concentration of *B. thuringiensis* isolates per gram of diet killing 50%; [b] 95% fiducial limits of upper and lower concentration; [c] slope of the concentration and its standard error

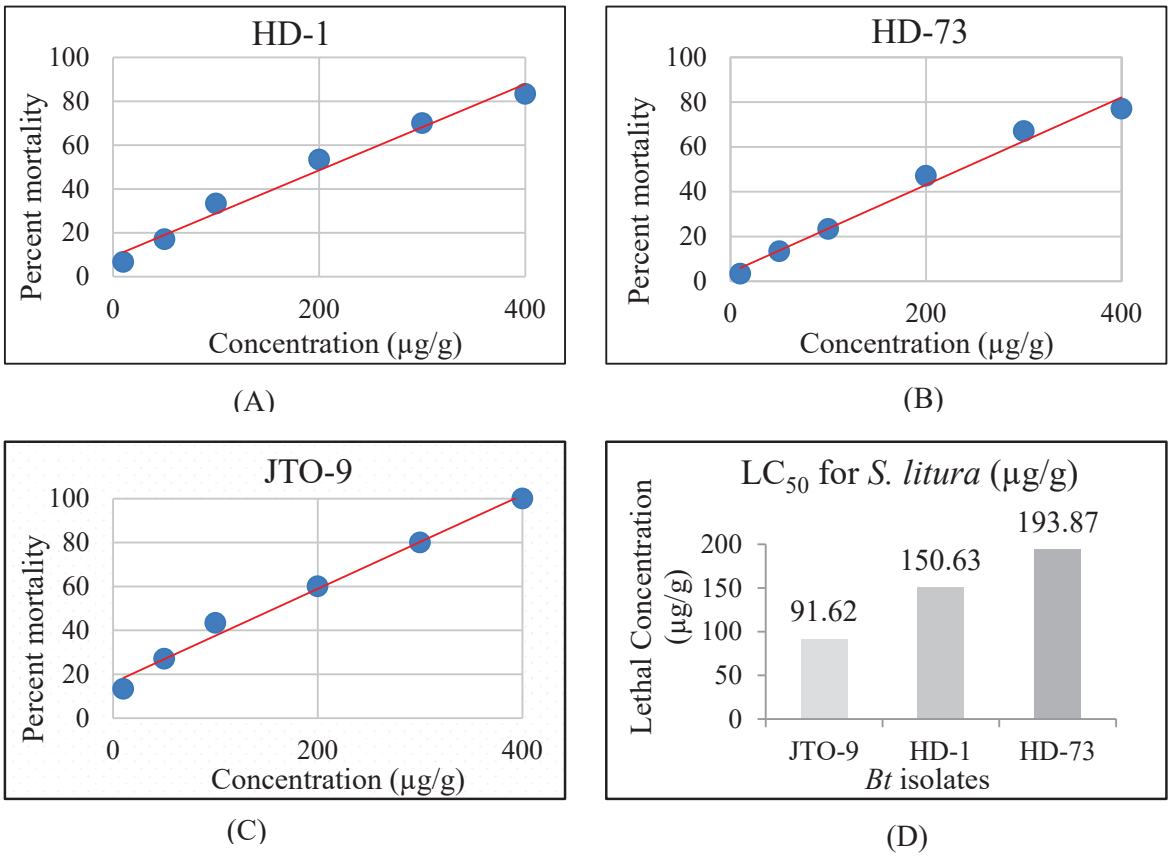

FIGURE 4. Mortality percentages of *Spodoptera litura* second instar larvae treated with six different concentrations of *B. thuringiensis* isolates. A: *Bt kurstaki* HD-1, B: HD-73, C: JTO-9 isolate, D: The LC_{50} values of most effective *Bt* strains against second instar larvae of *S. litura*

JLP-10, can be analysed further at the molecular level for their characteristics, as it is essential to identify novel *Bt* strains for the effective biological management of insect pests.

MOLECULAR CHARACTERIZATION

SCREENING WITH *CRY*1 AND *CRY*2 PRIMERS

The *Bt* isolates were subjected to screening by PCR to identify native strains active against lepidopteran insects (Table 3). PCR analysis using universal primers for *Cry1* and *Cry2* genes resulted in the amplification products of approximately 277 bp and 701 bp, respectively. Out of 20 native strains, the *Cry1* gene was found in 13 isolates; however, the *Cry2* gene was detected in 14 isolates (Figure 5). The PCR products of native *Bt* strains were electrophoresed to check for the target sequences of the *Cry1* and *Cry2* genes in 1% agarose gel. Nearly all of the isolates, 65% (13), except one isolate (JTO-6) containing the *Cry1* gene, also had the *Cry2* gene, while 60% (12) of the isolates containing the *Cry*2 gene also contained the *Cry1* gene. Collectively, these genes were present in 30% of *Bt* isolates collected from Multan, 20% from Muzaffargarh, 10% from Vehari and Rajanpur, and 5% from Rahimyar Khan and Bahawalpur.

(A): *Cry*1 (701 bp) universal primer product. M: 1 kb DNA marker, Lane 1: HD-73, Lane 2: SDK-1, Lane 3: JLP-10, Lane 4: JLP-15, Lane 5: SHJ-14, Lane 6: SHJ-17, Lane 7: SHJ-20, Lane 8: ROJ-12, Lane 9: KOM-16, Lane 10: MA-11, Lane 11: BRW-19, Lane 12: JTO-6, Lane 13: JTO-9, Lane 14: ADK-23. (B) *Cry*2 (277 bp) universal primer product. M: 1 kb DNA marker, Lane 1: HD-1, Lane 2: SDK-1, Lane 3: JLP-10, Lane 4: JLP-15, Lane 5: SHJ-14, Lane 6: SHJ-17, Lane 7: SHJ-20, Lane 8: SHJ-31, Lane 9: ROJ-12, Lane 10: KOM-16, Lane 11: MA-11, Lane 12: BRW-19, Lane 13: JTO-9, Lane 14: ADK-23, Lane 15: ADK-25

IDENTIFICATION OF SPECIFIC *CRY1* AND *CRY2* GENES

The *Bt* strains exhibited amplification with general primers for *Cry* genes were further characterised specifically. The PCR product of *Cry1Aa*, *Cry1Ab*, and *Cry1Ac* showed bands of approximately 246 bp, 216 bp, and 180 bp, while *Cry2Aa*, *Cry2Ab*, and *Cry2Ac* produced amplicons of 498 bp, 546 bp, and 725 bp (Figure 6). Out of all the strains

TABLE 3. Primer sequences used for the PCR screening of *Cry* genes

Target gene	Sequence (5′- to 3′-)	Expected size (bp)	Reference
Cry1-F[a]	CATGATTCATGCGGCAGATAAAC	277 bp	Ben-Dov et al. (1997)
Cry1-R[b]	TTGTGACA CTTCTGCTTCCCATT		
Cry2-F	GTTATTCTTAATGCAGATGAATGGG	701 bp	
Cry2-R	CGGATAAAATAATCTGGGAAATAGT		
Cry1Aa-F	TTATACTTGGTTCAGGCCC	246 bp	Ceron et al. (1994)
Cry1Aa-R	TTGGAGCTCTCAAGGTGTAA		
Cry1Ab-F	AACAACTATCTGTTCTTGAC	216 bp	
Cry1Ab-R	CTCTTATTATACTTACACTAC		
Cry1Ac-F	GTTAGATTAAATAGTAGTGG	180 bp	
Cry1Ac-R	TGTAGCTGGTACTGTATTG		
Cry2Aa-F	GTTATTCTTAATGCAGATGAATGGG	498 bp	Ben-Dov et al. (1997)
Cry2Aa-R	GAGATTAGTCGCCCCTATGAG		
Cry2Ab-F	GTTATTCTTAATGCAGATGAATGGG	546 bp	
Cry2Ab-R	TGGCGTTAACAATGGGGGGAGAAAT		
Cry2Ac-F	GTTATTCTTAATGCAGATGAATGGG	725 bp	
Cry2Ac-R	GCGTTGCTAATAGTCCCAACAACA		

F[a] and R[b]: forward and reverse primers, respectively

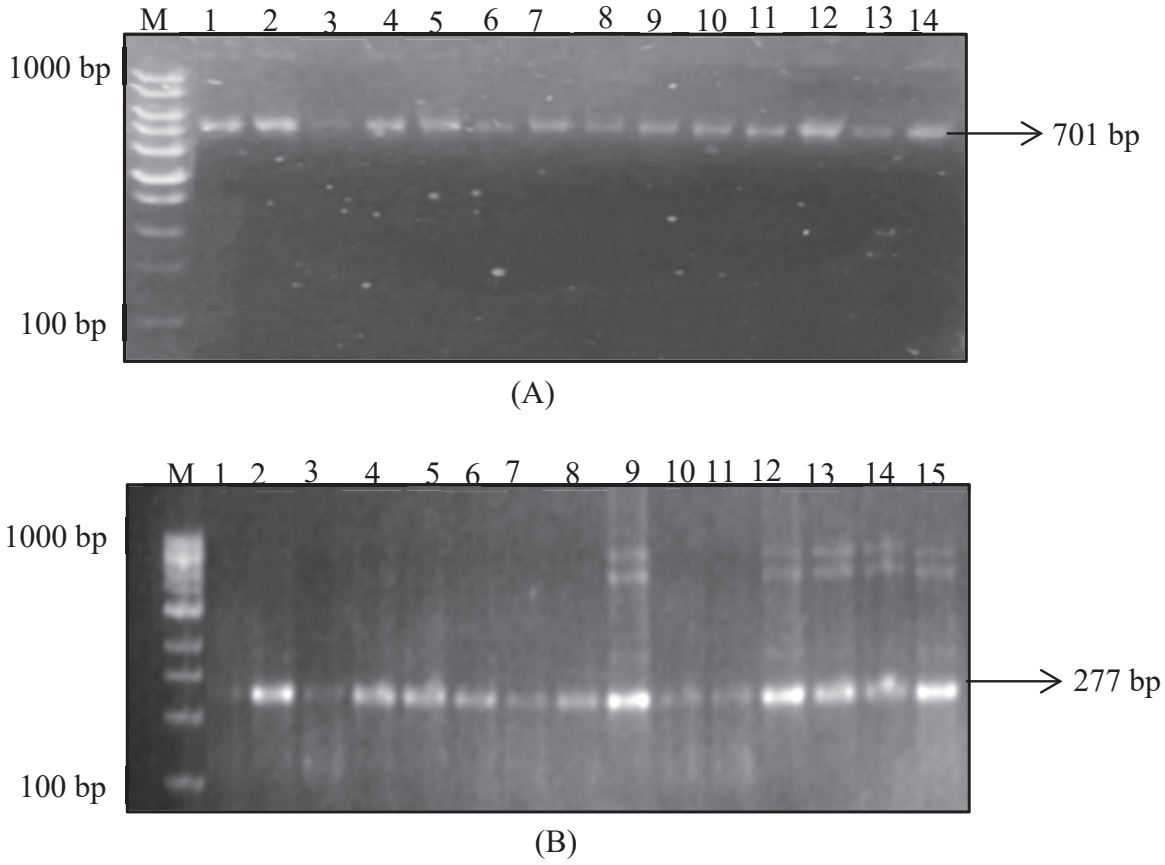

FIGURE 5. PCR analysis to identify general *Cry* protein genes from local *Bacillus thuringiensis* isolates

tested, 30.76% (4) *Cry*1-positive and 35.71% (5) *Cry*2-positive strains did not show a reaction with any specific primers. These strains may contain novel *Cry* genes; other than those specific primers were used in this work. *Cry1Aa* and *Cry1Ab* genes were detected in 15.38%, *Cry1Ac* 46.15%, *Cry2Aa* 14.28%, and *Cry2Ab* and *Cry2Ac* were found in 35.71% of the isolates, respectively. *Bt* isolates showed 8 *Cry* gene profiles, with profile No. 7 (*Cry1Ac, Cry2Ac*) being the most prevalent (10%) and profile No. 5 (*Cry1Ac*, *Cry2Aa, Cry2Ab*) being the most effective (Table 4). None of the *Cry*-specific genes were amplified from the *Bt* isolate of Bahawalpur.

(A) M: 1 kb DNA marker, Lane 1-2: *Cry1Aa* (246 bp) gene (SHJ-20, JLP-10), Lane 3-4: *Cry1Ab* (216 bp) gene (SHJ-14, ADK-23) (B) Lane 1-6: *Cry1Ac* (180 bp) gene (SHJ-14, ADK-23, JTO-9, SDK-1, KOM-16, ROJ-12) (C) Lane 1-2: *Cry2Aa* (498 bp) gene (JLP-10, JTO-9) (D) Lane 1-5: *Cry2Ac* (725 bp) gene (SHJ-20, SDK-1, KOM-16, ROJ-12, MA-11), Lane 6-10: *Cry2Ab* (546 bp) gene (SHJ-20, JLP-10, SHJ-14, JTO-9, SDK-1)

DISCUSSION

The south region of Punjab comprises a total of 16 districts. This region has been significantly important due to its geographical features and fertile soil (Hussain et al. 2020). Analysing native *Bt* isolates from six districts of South Punjab demonstrated that soil is an important source of *Bt* in terms of both variability and richness. A total of

TABLE 4. *Bacillus thuringiensis Cry* gene profiles of isolates collected from South Punjab

Profile No.	Gene profile	Isolates	Frequency (%)
1	*Cry1Aa*, *Cry2Ab, Cry2Ac*	SHJ-20	5%
2	*Cry1Aa*, *Cry2Aa, Cry2Ab*	JLP-10	5%
3	*Cry1Ab, Cry1Ac, Cry2Ab*	SHJ-14	5%
4	*Cry1Ab, Cry1Ac*	ADK-23	5%
5	*Cry1Ac*, *Cry2Aa, Cry2Ab*	JTO-9	5%
6	*Cry1Ac, Cry2Ab, Cry2Ac*	SDK-1	5%
7	*Cry1Ac, Cry2Ac*	KOM-16, ROJ-12	10%
8	*Cry2Ac*	MA-11	5%

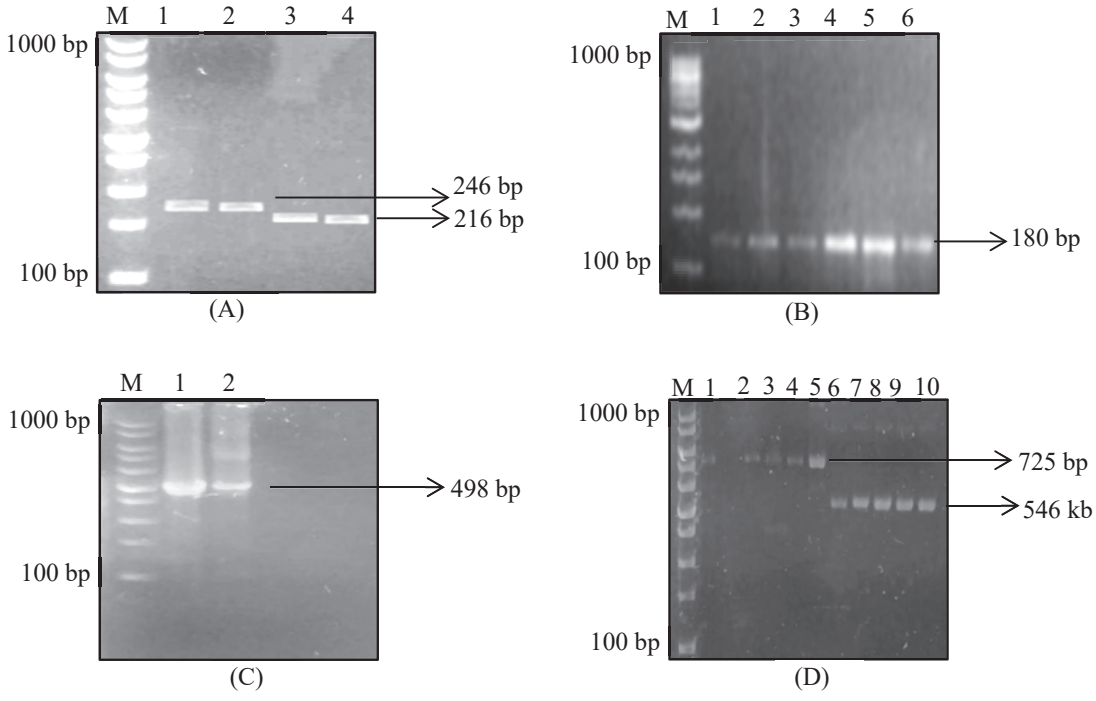

FIGURE 6. PCR analysis to identify specific *Cry* protein genes from local *Bacillus thuringiensis* isolates

266 colonies resembling *Bt* were obtained from 150 soil samples, and 34.96% (93) isolates were recognised as *Bt* with an average *Bt* index of 0.30. Previous studies have reported different values of *Bt* isolation and index in various soil samples (Aynalem et al. 2021). Kati et al. (2007) showed that among 11,000 soil samples collected from 30 different countries, *Bt* was found to be present in around 50 to 70% of the total samples. Specifically, it was detected in Asian and agricultural soils as 94.3% and 87.1%, respectively. Unalmis et al. (2015) identified 28 (47%) *Bt* isolates out of 60 bacterial colonies due to the presence of crystalline inclusions. The precise cause for the disparity in the *Bt* index is difficult to determine; however, Vilas-Boas and Lemos (2004) proposed that the *Bt* index could be influenced by various biotic factors such as vegetation cover, presence of specific microorganisms in the soil, prevalence of certain insect species, and abiotic factors like soil texture, nutrient availability, humidity, pH, and temperature.

In addition to environmental and ecological influences on *Bt* distribution, the morphological and protein characteristics of the isolates provide further insight into their diversity and functional potential. In this study, the majority of *Bt* isolates were screened by the shape of crystals and protein profiles. The isolates producing bipyramidal and cuboidal crystals (formed by *Cry1* and *Cry2* proteins) were identified more readily than sphere, irregular pointed, or rod-shaped crystals. Therefore, the high proportion of *Cry1* and *Cry2* genes is found in active *Bt* strain collections. During sporulation, the synthesis and storage of *Cry* proteins in crystal form serve to protect proteins against degradation caused by proteases, mainly produced during the stationary phase (Deng et al. 2014). The morphology and composition of the *Bt* proteins are intricately linked to their insecticidal potential and selectivity (Huang et al. 2006). In SDS-PAGE analysis, the predominant pattern of proteins was observed at molecular weights of approximately 130, 60, and 40 kDa of molecular weights. As a result, it was considered that the tested *Bt* isolates harboured a significant range of *Cry* proteins.

While morphological and protein profiles indicate phenotypic diversity, molecular characterisation of *Cry* genes offers a more precise understanding of the genetic basis of insecticidal potential. The PCR results showed that the *Cry2* gene had the highest frequency among the isolates (70%). In addition, 60% (12) of the isolates that tested positively for the *Cry1* gene also carried the *Cry2* gene. The proximity of both *Cry1* and *Cry2* genes on the genome and their coevolution (Ruan et al. 2015) may account for their coexistence. Multiple studies have found that *Cry1* and *Cry2* genes frequently co-occur (Zhang, Yu & Deng 2000). According to Wang et al. (2003), 90.7% of the strains harbouring the *Cry1* gene also had the *Cry2* gene. However, in contrast to our findings, Salama, Abd El-Ghany and Saker (2015) found that the *Cry1* gene was the most prevalent, with a frequency of 83.33% in the isolates. The *Cry* gene profiles of isolates from different localities exhibited variations, and a total of 8 *Cry* gene profiles were identified, which is lower than the 10 profiles detected in Mali (Fane et al. 2016) and the 19 profiles in Thailand (Boonmee, Thammasittirong & Thammasittirong 2019). However, Wang et al. (2003) observed the greatest number of *Bt Cry* gene combinations (43 profiles) from China.

Importantly, linking *Cry* gene composition with biological activity enables a clearer understanding of how genetic diversity translates into larval toxicity. The highest insecticidal activity (100%) was observed for the JTO-9 isolate in dose-mortality bioassays with six doses of *Bt Cry* proteins against *S. litura* larvae is possibly attributed to the presence of *Cry1* toxin, which targets lepidopteran insects, and *Cry2* toxin, which targets both lepidopteran and dipteran insects (Soberon, Monnerat & Bravo 2018). The toxicity levels of *Bt* strains against target insects are impacted by various parameters such as gene profiles, synergism, and expression levels (Hongyu, Ziniu & Wangxi 2000). Districts with intensive cropping systems, such as Multan and Muzaffargarh, yielded isolates harbouring multiple *Cry1* and *Cry2* genes and exhibiting higher mortality against *S. litura*. In contrast, isolates from districts with distinct environmental and cropping patterns showed fewer toxin genes and comparatively lower bio-efficacy. This suggests that local agroecological factors shape *Bt* population structure and toxin diversity. Similar geographic structuring of *Bt* toxin diversity has been reported in other regions (Baig & Mehnaz 2010a; Seifinejad et al. 2008). The gene profile of the most effective isolate, JTO-9 from Muzaffargarh showed a combination of *Cry1Ac*, *Cry2Aa,* and *Cry2Ab* genes. Interestingly, other subsequent toxic isolates i.e., SHJ-14, JLP-10, and SHJ-20 from Multan harboured partial gene profiles of the most toxic isolate JTO-9 with *Cry2Ab* gene observed as common among these isolates. Profile 5 (*Cry1Ac*, *Cry2Aa,* and *Cry2Ab*) exhibited the highest toxicity against *S. litura*, likely due to additive effects of multiple *Cry* toxins with possible synergistic enhancement of midgut receptor targeting, whereas *Cry2* toxins alone showed lower activity but increased toxicity when combined with *Cry1* toxins.

The enhanced toxicity observed in isolates harbouring multiple *Cry* genes can be further explained by the mode of action and receptor-binding behaviour of *Cry* proteins. Lu et al. (2013) demonstrated that *Cry2Ab* protein bound with high affinity to the receptors on the midgut epithelial cell membrane (BBMV) of fifth instar larvae of *S. litura*. Presumably, the *Cry2Aa* protein binding affinities contributed to its attachment to the membrane receptor and integration into the membrane, resulting in the formation of a pore and septicaemia. Furthermore, Ibargutxi et al. (2008) showed that combining *Cry1Ac* and *Cry2Ab* proteins had an additive effect on spiny bollworm, *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae) as well as a synergistic effect on *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Moreover, Yunus, Makhdoom and Raza (2011) observed that a combination of *Cry1Ac*

and *Cry2Aa* proteins had a synergistic effect against *Earias vitella*. In this study, *Cry1Ac* is considered the primary contributor to larval mortality against *S. litura*, whereas *Cry2Ab* generally acts as a secondary toxin, contributing mainly through additive or synergistic effects (Hernandez-Rodriguez et al. 2008).

These differences in toxin interaction at the midgut level are closely associated with their receptor-binding mechanisms, which have important implications for resistance management. Differences in midgut receptor recognition between *Cry1* and *Cry2* proteins have important implications for resistance management in *S. litura*. *Cry1* toxins mainly rely on cadherin, aminopeptidase N and alkaline phosphatase receptors for toxicity, whereas *Cry2* toxins interact with alternative or partially overlapping binding sites. This receptor divergence provides a mechanistic basis for the effectiveness of *Cry1–Cry2* toxin combinations, as alterations in *Cry1*-binding receptors that confer resistance may not compromise *Cry2* activity. Consequently, the use of *Bt* strains or formulations expressing multiple Cry toxins can help delay resistance development in *S. litura* populations (Bravo et al. 2011).

Despite the strong association between *Cry* gene profiles and toxicity, certain limitations should be considered when interpreting these findings. Prior research showed that *Cry* gene patterns could indicate the insecticidal activity of *Bt* strains, but not their specific toxicity levels. There may be other genes in these strains that were not examined in this study, which could also contribute to their overall toxicity. To accurately identify all *Cry* genes associated with the toxicity of the strains, additional studies at the genomic and transcriptomic levels will be necessary. Additionally, conducting bioassays with varying doses of Cry protein combinations on a larger number of different insects may help to identify potentially harmful isolates that can be used to control insects.

Notwithstanding these limitations, the findings of this study provide important insights into the application of indigenous *Bt* isolates for sustainable pest management. Overall, this study supports the targeted and specific design of *Bt*-based biopesticides for *S. litura* by identifying highly effective local isolates and linking their bio-efficacy to distinct *Cry1* and *Cry2* gene combinations and expressed toxin profiles. The integration of molecular, phenotypic, and geographic evidence provides a rational framework for selecting and formulating Cry toxin combinations that enhance efficacy while maintaining host specificity and supporting resistance management.

CONCLUSION

Pakistan's diverse agro-ecological zones offer a unique reservoir of microbial biodiversity. The research aimed to uncover novel toxins and genetic determinants within local *Bt* strains, potentially advantageous for pest control tailored to the specific challenges faced by Pakistani agriculture. The study highlights the global significance of *Bt* diversity and its impact on pest management, emphasising the need for region-specific insights. Furthermore, the research underscores the pivotal role of *Bt* in sustainable agriculture, aiming to reduce dependence on chemical pesticides. The present research identified the variety of *Cry* genes in local *Bt* strains obtained from unexplored agricultural areas in South Punjab. The bioassay investigations conducted on *S. litura* demonstrated the efficacy of native strains in pest management. The used collection contained new strains that possess distinct *Cry* gene combinations, expressing a variety of crystal proteins. These strains can be further evaluated for their efficacy against other lepidopteran insects and have the potential to be developed into biopesticides. This study concludes that *Bt* is a potential microbial agent for managing cotton cutworm (*S. litura*) and can be utilised in the preparation of biopesticides for pest management purposes. Using *Bt* as a biopesticide can help eradicate agricultural pests and reduce the environmental risks associated with chemical pesticides.

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