Research Article

Toxicity Analysis of Local Isolates of *Bacillus thuringiensis* and CRY1F Protein against Selected Lepidoptera Pests

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Abstract | *Bacillus thuringiensis* has the potential to control a variety of insect pests, particularly the species of Lepidoptera. The goal of the current investigation was to evaluate the bio larvicidal effects of endotoxins produced by *B. thuringiensis* from the CRY1F gene against widely encountered lepidopteran pests (*Spodoptera litura* and *Helicoverpa armigera*). Insect-proof netting was used to contain the vegetable plants in a small field. *B. thuringiensis* with active CRY1F was cultivated in L*uria-Bertani* (LB) media under controlled laboratory conditions. The goal was to maximize colony growth for the preparation of spore/crystal mixtures at various concentrations. The larvae of *S. litura* and *H. armigera* were reared in a laboratory under control. Following tests, the LC_{50} (96-hours) of the CRY1F protein-crystal mixture against lepidoptera pests was determined to be 158.37 µg/ml for *S. litura* and 170.73 µg/ml for *H. armigera*. The endotoxin mixes of CRY1F exhibit considerable potency, causing 100% mortality in *S. litura* with 500 µg/ml and *H. armigera* with 600 µg/ml.. The overall findings showed that the *Bt* local isolates and CRY1F protein endotoxins were effective against both larvae. The bio larvicidal action of *Bt* local isolates and CRY1F endotoxins against various lepidopteron pests indicate promising results for pest control.

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Introduction

Insects, comprising 80% of all animals and stand as the most successful group on earth (Lawton *et al*., 2022). The harm caused by insect pest are both directly by pest infestations and indirectly by disseminating plant pathogen (Rosenzweig *et al*., 2014; Lehmann *et al*., 2020). Annual yield losses result in an estimated 18–20% of the world's crops being lost to insect pests, valued at around USD 470 billion. Of this, 30–35% is attributed to Indian agriculture, accounting for approximately USD 36 billion in losses (Yadav and Kumar, 2021). These losses decreased to 17.5 percent in the twenty-first century from 23.3 percent in the early 2000s, a good development that is largely attributable due to the increased use of transgenic crops (Ceccarelli and Pietropaoli, 2022).

Lepidoptera insects are the most insect pests, causing yield losses in both quantity and quality all over the world (Rasib *et al*., 2020). Among the lepidopteron pest, *Spodoptera litura* (Lepidoptera: Noctuidae) commonly known as cutworm or tobacco cutworm is a destructive insect causes significant yield losses (Jafir *et al*., 2021). It is a significant crop pest in many Asian countries, and because of its army-like march, it is also well-known in Pakistan and the Indo-Pak region. (Abbas *et al*., 2012). Likewise, *Helicoverpa armigera* (Lepidoptera: Noctuidae) is also a significant economic and agricultural pest in many part of the globe. Females can produce up to 2,500 eggs in their lifetime, which they deposit in clusters on cotton leaves and maize silks. Larvae develop a cannibalistic nature as they mature, and one larva (sometimes two) usually settles on each corn ear. When larvae are fully developed, they relocate to the soil where they pupate for about two weeks before emerging as moths. Adults are nocturnal, have a lifespan of two to three weeks, and eat nectar (Butter, 2021).

Insect pests can be controlled by various methods i-e., the use of chemical insecticides, resistant varieties, bio-pesticides, cultural control, bio-agents, etc., when the insect's population crosses the threshold the use of chemicals is the only option for the suppression of pest (Vinha *et al*., 2020). Alternative approaches to reducing reliance on chemical pesticides are needed to minimize these impacts (Riaz *et al*., 2021).

Various biological methods have been introduced which reduces-pests and are also environmentally friendly (Hafeez *et al*., 2021). To control insect pests, bio-agents such as insect pathogens, predators, and parasitoids (fungi, bacteria, viruses, nematodes *etc*) are used (Ahmad, 2021). The use of bio-pesticides reduces chemical residues as well as pest and pathogen resistance, resulting in an increase in yield of up to 10% to 20% (Buragohain *et al*., 2021). Various strains of *B. thuringiensis* are entomo-pathogens that are mostly used all over the world to control pests and are commercially available (Wong and Teh, 2021). Biopesticides account for over 2% of the worldwide crop protection market. Out of which, (*Bt*) products based on *B. thuringiensis* make up around 90% of all biopesticides. (De-Bock *et al*., 2022). The basic reasons for the success of (*Bt*) *B. thuringiensis* based products are: sustained and rapid larvicidal activity, easy utilization of standard equipment during the process, and negligible harmful effects on beneficial and friendly insects (Kamatham *et al*., 2021).

Keeping in view the economic importance and eco-friendly effect of *B. thuringiensis*, a study was conducted to find the efficacy of CRY1F *Spodoptera litura* and *Helicoverpa armigera*. The present studies comprise the isolation of *B. thuringiensis* from local soil samples of district Swabi, the efficacy of different concentrations of local isolates of *B. thuringiensis* harboring CRY genes against selected lepidopteran pests and to compare the toxicity of local isolates and CRY1F harboring strain against *Spodoptera litura* and *Helicoverpa armigera* larvae.

Materials and Methods

Sample collection

Soil samples were collected from one inch beneath the soil-containing surface area and sand from different localities of Swabi. The selected samples of soil were having high organic content. The sand and soil samples were kept in a zipped plastic bag, labeled and were brought to the laboratory (Nguyen *et al*., 2018).

Screening of Bt strains

For each 1.0 g of soil, 20 ml of LB medium (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) was mixed thoroughly, and sodium acetate was added to the aforementioned mixture to a final concentration of 0.3 M and incubated for 5 hours at 37°C and 250 rpm in incubating shaker. The sample was cooked for 20 minutes at 80 °C. Serial dilutions of the treated sample was made, and 250 ml of the dilute sample

was disseminated on nutrient rich LB agar plates (3g/ LTryptone, 1.5 g/L yeast extract, 2 g/L tryptose, 0.005 g/L MnCl2, 6.9 g/L NaH2PO4, 8.9 g/L Na2HPO4, and 15 g/L Agar and incubated for 24 hours at 37°C. Colonies exhibiting morphology similar to *Bt* (off-white colour, complete margin dry, and rich colony growth) was chosen randomly and purified using agar LB petri Plates (Liang *et al*., 2022).

Characterization of isolated Bt species

Screening of isolated colonies were carried out to identify the *Bt* species. Various identification methods were applied for the identification and purification of *Bt.* Strains.

Gram staining

For making smear the drop of water was added and 24 hrs old bacterial colonies on a clean glass slide. The slide was air dried for 1-2 minutes fixed by exposure to heat on flame and stain with crystal violet (mixture of solution A and B were rinsed with distilled water after 30-60 seconds (Solution A had 95ml of ethanol and 2g of crystal violet in it; Solution B had 1L of distilled water and 9g of ammonium oxalate). Lugols Iodine Solution (potassium iodide 20g, iodine 10g, distilled water 100 ml) was added to the smear and kept at room temperature for 40-60 seconds. Slides were cleaned with distilled water, immersed in 500 ml of acetone, 475 ml of pure ethanol, and 25 ml of distilled water were used to decolorize the acetone alcohol. After a brief period of rinsing with water, the mixture was covered with a safranine solution (safranine 0.25 g, ethanol 10 ml, and distilled water 100 ml).) for 3min and were observed for Gram-positive rods under the microscope (Petersen and McLaughlin, 2016).

Endospore staining

Plates containing T3 medium (Yeast extract 1.5 g/L, Agar 15g/L, Tryptose 2g/L, Tryptone 3g/L, NaH2PO4 6.9g/L, Mncl2 0.005g/L and Na2HPO4 8.9g/L) were used to grow Gram-positive rods by incubation at 37°C for 98 hrs. Sporulated colonies was stained by a modified procedure based on Schaeffer and Fulton (1933) to verify the spores. Sporulated colonies were used for the preparation of the smear which was covered with 5% aqueous malachite green. The slide was kept on boiling water bath for 6-8 min, rinsed with excess of distilled water. The smear was stained with 0.1 % carbol fuchsin and rinsed with distilled water after one minute again, then air dried and examined under the microscope. The bacterial rods with green colored oval or spherical bodies within pinkish sporangia were chosen for further analysis (Bukhari and Shakoori, 2010).

Voges-proskauer (V-P) test

Five ml of glucose phosphate peptone medium (1g peptone and 1g di-potassium hydrogen phosphate in 200 ml distilled water). pH were adjusted to 7.6 and then 1g of glucose was added. Then these were put in test tubes and were autoclaved for 15 minutes at 15lbs pressure at 121°C. In these test tubes, the isolates were injected and 48 hours of incubation at 37°C and one ml of 40% potassium hydroxide solution and 3 ml of ά-naphthol were added in these test tubes after 48 hours. After 2-5 minutes the appearance of a pink shade shows the presence of *Bt*.sp (Baker-Moffatt, 2017).

Catalase test

Well isolated colony of the local isolates were added in a test tube containing 2 to 3 ml solution of 3% hydrogen peroxide by means of a sterile wire loop. The appearance of bubbles within a few seconds shows catalase activity (Reiner, 2010).

Motility test

Local isolate colony was inoculated by using a straight sterilized wire in a single pass to the center of the test tube. The test tube already had the autoclaved media (5ml) which was prepared by adding 0.75g agar in 200ml of peptone water (peptone 2mg, NaCl 1g, distilled water 200ml). Then these test tubes were over nightly incubated at 37°C. Motile bacteria exhibits diffuse proliferation in all semisolid agar media (Gude *et al*., 2020).

Bacteria culture

B. thuringiensis CRY1F obtained from University of Tennessee's, Department of Entomology and Plant Pathology in Knoville, Tennessee. *Bt* local isolates were grown in the lab as described in the previous section. These spores were collected and added to/increased by employing the techniques of Sambrook *et al*. (2001) in a media containing tryptone, yeast extract, sodium chloride, and agar for jelling. Ten grams (10 g) of Ten grams of NaCl, ten grams of tryptone, and ten grams of 950 ml of pure water were used to weigh and dissolve yeast extract inside a clean conical flask. To dissolve the mixture we heated it on a hot plate. Adding up to 1000 ml and 15 grams of agar was

the total volume. Wool cotton stoppers were used to plug the conical flask, and aluminum foil was used to cover it. After being autoclaved for15 minutes at 121 °C to sanitize it, after sterilization, the medium was cooled to 45–50°C, and then it was put into sanitized petri dishes (approximately 20 ml per petri plate) in an environment free of disease. After allowing the plates to solidify, they were kept at 37°C for 24 hours, and the sterility of the media was assessed (De-Thier, 2020).

Colony Forming Unit (CFU)

Ten mg spores were taken in three tubes (1.5 ml). The spores were shifted to the desiccator in which the temperature was maintained at 65°C for three hours. The dried spores were weight and re-suspended in 1ml of sterile water. Then, the serial dilution of 100 μg /ml were made. It was then heated for 10 min at 90°C to demolish the vegetative cells in a water bath 100μl was smeared on nutritional agar and were incubated at 37°C, then after 72 hrs. They counted the colonies in order to ascertain the quantity of spores.

$$\text{CFU/ml} = \frac{\text{(Average Count}}{\text{(Dilution plated)(ml plated)}}$$

OR

$$\text{CFU/dose} = \frac{\text{(Average Count)} \times \text{(mL used to rehydrate)}}{\text{(Dilution used)} \times \text{(mL plated)} \times \text{(Number of dose)}}$$

Formulation of spray

Spore formulation was enhanced by spraying-dried wet-table powder, Milk powder was employed as a feeding stimulant, Tween 20 (wetting agent), silica smoke (as a free-ßowing agent) UV protection (in the form of polyvinyl alcohol) and antifoam solution (F-I formulations),. *B. thuringiensis* was found in every formulation at a 10% (wt: wt dry basis) concentration and a 30% (wt: vol) total solids content. The mixers (1 liter) were spray dried at ambient temperature with input and exit air temperatures of 120^{0}C and 70^{0}C, respectively. An agitator was employed to combine the feed solutions prior to spray drying. Physical characteristics (particle size, moisture content, wettability, and suspension) and biological experiments (activity assessment and viable spore count) were carried out for each formulation (Teera-Arunsiri, 2003).

Host plant culture

In order to grow the vegetables, the field was selected in Swabi and was prepared by adding the recommended fertilizers. This plot was further divided into 5 sub plots (4*4) feets having a buffer zone of 2 feet. Tomato, brinjal, chili and okra crop were grown in each sub plot by keeping their recommended distance between plants and rows. All other agronomic practices were kept constant throughout the experimental time (Whiting *et al*., 2003). No chemical treatments were applied. The insect arrival were monitored daily (Table 1 and 2).

Table 1: *Plots for Vegetable Growth (Layout).*

Sr. No	Name of Vegetable	Plot Area	Spacing between Vegetables(cm)
1	Tomato	4×4	80 × 30
2	Brinjal	4×4	45×30
3	Chili and Capsicum	4×4	60 × 45/60 × 30
5	Okra	4×4	45 × 15/80 × 45

Table 2: *Lepidoptera Pest on Vegetables.*

Sr. No	Vegetable Name	Insect Pest Name	Scientific name
1	Tomato	Army worm	Spodoptera litura
		Fruit borer or cotton bollworm	Helicoverpa armigera
2	Brinjal	Army worm	Spodoptera Litura
		Fruit Borer or cotton bollworm	Helicoverpa armigera
3	Chili and Capsicum	Army worm	Spodoptera litura
		Fruit Borer or cotton bollworm	Helicoverpa armigera
4	Okra	Fruit Borer or cotton bollworm	Helicoverpa armigera

Insect culture

Eggs batches of *Spodoptera Litura* and *Helicoverpa armigera* were collected by cutting the leaf portion along with egg batches (without disturbing the eggs) of the mentioned insects from different vegetable plots at Insect Bio-control Laboratory (Insectary), NARC, Islamabad. The cut portion of the leaf along with the eggs were shifted to the laboratory where it was kept in separate plastic boxes. The boxes were covered with muslin/ ventilated cloth and were tightened with rubber bands. These boxes were kept at constant temperature of 28 ± 3 °C, 75±5 RH and with photoperiod (16:8) light and dark, and were monitored daily for egg hatching. Upon hatching fresh food obtained from host plants was provided daily till the larvae reaches to 2nd instar (Arif *et al*., 2018).

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Table 3: *Efficacy of locally isolated Bt harboring cry gene.*

Strain name *Bt* CRY 1F gene	Different concentrations of locally isolated harboring gene µg/ml										
	00	100	200	300	400	500	600	700	800	900	1000
Spodopter a litura	00 %	19 %	38 %	55 %	74 %	94 %	100 %	100 %	100 %	100 %	100 %
Helicoverp a ameriga	00 %	14 %	28 %	42 %	56 %	70 %	85 %	100 %	100 %	100 %	100 %

Table 4: *Efficacy of different concentrations of spores/ crystal of Bt CRY1F on mortality % of Spodoptera litura and Helicoverpa armigera after 96 hrs.*

Strain name *BT* CRY 1F gene	Different concentrations of spores /crystal mixture µg/ml										
	00	100	200	300	400	500	600	700	800	900	1000
Spodopter a litura	00 %	30 %	55 %	80 %	95 %	100 %	100 %	100 %	100 %	100 %	100 %
Helicoverp a ameriga	00 %	35 %	50 %	65 %	85 %	94 %	100 %	100 %	100 %	100 %	100 %

Bioassays

For bioassay, 100 larvae of 2nd instar of different lepidopteran larvae were collected from the culture. 10 larvae of each were kept in a petri dish separately. Ten concentrations (100µg/ml, 200µg/ml, 300µg/ml…..1000µg/ml) of CRY1F and local *Bt* isolates were prepared. Some parts of the host vegetables (tomato, brinjal) were cut into equal sizes of 2cm. These cut stem pieces were treated with different concentrations and were kept in a petri dish as food for larvae. The larvae were observed daily for 96hrs. After 96hrs the total dead and live larvae were counted and recorded (Galdino and Visintin, 2011).

Statistical analysis

The Abbot's formula was used for the mortality rate in treated and untreated (control) diets for Lepidoptera larvae.

$$\frac{X-Y}{X} \times 100 = \text{Percent Control}$$

Where;

X: Percentage of living larvae in a safe environment;

Y: Proportion or percentage of larvae who live on treated petri dish.

X-Y: Percentage of larvae that died as a result of the exposure to treatments and the control or expressed value is equal to the percent killed by the procedure (X-Y) divided by the percent live in the check (X) (Abbot, 1987).

Data were analyzed by finding the fatal concentration required to wipe out 50% of a population using Log Dose Probit (LC_{50}) with a 95% confidence interval by using the statistics software SPSS.

Results and Discussion

Efficacy of local Bt isolates on mortality percentages of Spodoptera litura and Helicoverpa armigera after 96 hrs

The results present in Table 3 shows efficacy of different concentrations of local *Bt* isolates on mortality percentages of *S. litura* and *H. armigera* . The higher concentration of CRY gene (1000 µg/ml) caused maximum mortality of 100% of the both larvae species. While the lower concentration of 100 µg/ml the mortality of *S. litura and H. armigera* caused 19 and 14% mortality, respectively. With a concentration of 200µg/ml the mortality of *S. litura was* (38%) and *H. armigera* (28%). Similarly with 300 µg/ml the mortality of *S. litura and H. armigera* (55 and 42%) respectively. When the concentration was 400 µg/ml the mortality was 74% and 56% for *S. litura and H. armigera* respectively. When the concentration was 500 µg/ml the mortality of *S. litura and H. armigera* 94% and 70% respectively. The mortality was 100% for *S. litura* with 600 µg/ml concentration while for *H.armigera* 85% respectively. On ward 600 µg/ml the mortality was 100% for *S. litura and H. armigera*. Furthermore, the mortality was 100% for *S. litura and H. armigera* when the concentration was 700, 800, 900 and 1000 µg/ml respectively.

Efficacy of different concentrations of spores/ crystal against Spodoptera litura and Helicoverpa armigera after 96 hrs

The efficacy of different concentrations of spore/ crystal of *Bt CRY*1F on % mortality of *S. litura* and *H. armigera* after 96 hrs (Table 4) showed that higher concentrations of CRY1 gene 1000 µg/ml caused maximum mortality (100 %) of the tested both larvae species. With a lower concentration of 100 µg/ml the

mortality of *S. litura and H. armigera* was 30 and 35%, respectively. With the concentration of 200µg/ml the mortality of *S. litura was* (55%) and *H. armigera* (50%). Similarly with 300 µg/ml the mortality of *S. litura and H. armigera* (80 and 65%) respectively. When the concentration was 400 µg/ml the mortality was 95 and 85% for *S. litura and H. armigera* respectively. When the concentration was 500 µg/ml the mortality of *S. litura and H. armigera* 100 and 94% respectively. The mortality was 100% for *S. litura and H. armigera* when 600 µg/ml were used respectively. Onward 600 µg/ml the mortality was 100% for *S. litura and H. armigera*. Furthermore, the mortality was 100% for *S. litura and H. armigera* when the concentration was 700, 800, 900, and 1000µg/ml respectively.

Toxicity of B. thuringiensis local isolates harboring CRY gene against Spodoptera litura and Hpodoptera armigera after 96hrs

The outcome of demonstrated *B. thuringiensis* CRY 1F gene against *S. litura* and *H. armigera,* after 96 hrs is present in (Table 5). The LC_{50} or fatal concentration required to kill half of a population was discovered using Log Dose Probit, and a 95% confidence interval was established. The LC_{50} of *Spodoptera litura* 229.99 with a lower and upper bounds of 167.64µg/ml and 285.85µg/ml respectively. For *Helicoverpa armigera* the LC_{50} was 210.92µg/ml with 148.68 µg/ml and 267.02µg/ml lower and upper bound respectively.

Table 5: *Toxicity of B. thuringiensis local isolates harboring CRY gene against Spodoptera litura and Helicoverpa armigera after 96hrs.*

Specie Name	Treated period (hrs)	Effective dose LC50 µg/ml	Lower Bound	Upper bound
Spodoptera litura	96	229.33	167.64	285.85
Helicoverpa armigera	96	210.921	148.68	267.02

Toxicity of CRY 1F

Toxicity of B. thuringiensis CRY 1F gene against Spodoptera litura and Helicoverpa armigera after 96hrs

The results showed that after 96 hours, *S. litura* and *H. armigera* were toxic to the *B. thuringiensis* CRY1F gene (Table 6). To determine the lethal dosage required to kill half of a population, the LC50 were calculated using Log Dose Probit, which created a confidence interval of 95%. The LC LC_{50} of *S. litura* was 158.37µg/ml with a lower and upper bounds of 105.16µg/ml and 204.33µg/ml respectively. For *H. armigera* the LC_{50} was 170.73µg/ml with 108.49µg/ml and 224.34µg/ml lower and upper bound respectively.

Table 6: *Toxicity of B. thuringiensis CRY1F gene against Spodoptera litura and Helicoverpa armigera after 96hrs***.**

Specie Name	Treated period (hrs)	Effective dose LC50 µg/ml	Lower bound	Upper bound
Spodoptera litura	96	158.37	105.16	204.33
Helicoverpa armeriga	96	170.73	108.49	224.34

The use of micro bio-agent against insect pest is essential to decrease the harmful effect of chemical pesticides (Lone *et al*., 2017). Among benficial micro-organism, *Baccillus threungenus* isolates were commonly used against many insect pest and having significant effect on the harmful insect. (Miller and Allesina, 2021). In the same content during the present study 63Bt isolate were recovered from soil samples. The results demonstrated that the various isolates varied from one another in terms of colony color, margin, surface, and height. Bt's distinct colony morphology was described as having an uneven colony edge, flat to slightly raised colony height, and white to off-white colony color (Chai *et al*., 2016). In order to identify *Bt* isolates culturally described isolates were next subjected to morphological examinations using Gram staining, V-P test, mortality test and Catalase test as the appropriate parameters. All of the isolates were spore-forming, rod-shaped, and Gram-positive bacteria. According to a similar finding, Gram-positive, rod-shaped, and spore-forming soil-dwelling bacteria made up the *Bt* isolates. (Adedayo and Uthman, 2021). The study of the gram staining revealed that only 31 of the total 40 isolates had paranormal inclusion and were categorized as *Bt* whereas the remaining 9 isolates lacked inclusion of paranormal. According to studies, *Bt*'s distinctive crystal protein inclusions set it apart from other *Bt* species (Gebremariam *et al*., 2021).

The distribution of crystal proteins among isolates may vary according to the environmental conditions, which may have an impact on genetic variation. *S. litura* larvae were used for the initial pathogenicity screening of entomopathogenic bacterial isolates. Twenty (64.5%) of the 31 examined isolates were shown to be virulent against *S. litura*, with percentage larval mortality ranging from 2.50 to 62.50, 10

to 75, and 20 to 95 within 48, 96, and 144 hours after application, respectively. P 0.01 indicated that the examined isolates varied in their toxicity toward *S. litura*. Within 48 hours of infection, *Bt*'s bio-insecticidal evaluation against *S. litura* larvae showed a considerable reduction (Mukherjee *et al*., 2019). Research findings have identified the *Spodoptera litura* larva as the most effective model host for studying the interactions between *Bacillus thuringiensis* (Bt) and insect pests. (Baranek *et al*., 2021). Furthermore, in laboratory and field conditions, the evaluation of the insecticidal properties of *Bt* isolates led to a considerable death rate against *S. litura* (Dutta *et al*., 2022). Due to the microbiological comparison to morphological, cultural, and physiological classification techniques, molecular characterization of *Bt* isolates and other *B. cereus* groups is a more successful methodology. Twenty isolates were ultimately chosen for the molecular characterization investigation after being screened from the preliminary bioassay evaluation. This outcome is consistent with the study, which found that after 48 hours of treatment with several *B. tabaci* isolates, nymphal mortality ranged from 34.84 to 51.21 percent. After being treated with culture broth containing spore crystal complex, (Salazar-Magallon *et al*., 2015) observed that more than 92 percent of 3rd to 4th nymphal instar whitefly mortality had occurred. 39 *Bt* isolates (30%) out of a total of 130 *Bt* isolates in Ethiopia showed insecticidal efficacy against Aedes africanus. (Azizoglu *et al*., 2020). The biocontrol agent *Bascillus thurengensis* was utilized in this study to combat a variety of lepidoptera pests, including vegetables pest. *Spodoptera litura, Helicoverpa amerigara, Empoasca devastans, Bemisia tabeci, Gnorimoschema opercullela, Scirpophaga novella, and Emmalocera depressella* are major pests used in this study. Pest insects seriously reduce crop productivity by up to 90%.

Chemical pesticides used as a control measure cause insect pests to develop resistance (Kariyanna *et al*., 2020). Therefore, in this work, we avoided using chemical pesticides and evaluated CRY 1F's effectiveness against the Lepidoptera pest. Their biology is influenced by the relationship between insects and microorganisms. The community of bacteria that affect the history of life of insects, especially pest species, has received attention. Examples of microbes that exhibit pest insect behavior include bacteria, fungi, and protozoa (Gurung *et al*., 2019). Therefore, we employed *Bascillus thurngensis*, which can harm pests at an early stage. As a pest grows, it becomes more difficult to control. Later stages also saw a rise in the quantity of insecticide utilized. As it was early in the investigation, we concentrated on 2nd instar larvae.

Insect pests of economically significant crops including cotton, maize, and soybean can be effectively controlled with *Bt* CRY toxins. The regulated laboratory rearing of *S. litura* culture resulted in the satisfactory development of mulberry leaves and semi-synthetic feeds. After numerous attempts, we were unable to produce the second generation of *S. litura* larvae fed on spinach leaves because the population of larvae raised on spinach leaves was lost between the fourth and fifth instar. This implies that leaf worms have a higher survival rate on mulberry leaves than they do on spinach leaves, which highlights spinach's status as a rare host plant according to (Pathma *et al*., 2021). To ensure a semi-synthetic diet was used for bioassays, with the final concentration of the test protein solution. According to bioassay data, the insect pest *S. litura* is toxic to the CRY1F protein. After 48 hours, reduced growth was apparent in larvae fed on CRY1F-contaminated protein. Larvae started dying 72 hours after being exposed to the CRY1F protein. The proteins we employed were protoxins since trypsin digestion or stomach fluids did not activate them before usage. Similar to other CRY protoxins (Banyuls, 2017), these protoxins are activated by larval midgut fluids to become active poisons in the insect gut. According to table 5, the LC50 for the CRY1F protoxin is 500 μg/ml. At the highest tested concentration, there was 100% fatality. For the control of *S.litura* and *H. armigera*, CRY1F can be an effective option as a biopesticide. This and other susceptible insects could be fought off by transforming crop plants with the CRY1F gene and other *Bt* insecticidal genes. Based only on Bernardi *et al*. (2012)'s findings, it may be concluded from the LC50 that the CRY1F protein is more harmful to *S.litura* than to *H.armigera*. CRY1F has a four times lower LC50 for *H. armigera* in than the earlier reported LC50 for *S. litura* by (Visser, 2020). The armyworm is four times more vulnerable to CRY1F than the cotton bollworm. CRY1F has an almost higher LC50 for *S. litura* (158.37 μg/ml) than for *H. armigera* (108.73 μg/ml), and it is 5:7 times more toxic to *H. armigera*, which is consistent with earlier findings. However, the variance in these insects' susceptibility to CRY1F is greater than 7-fold (Bel *et al*., 2017), which may be caused by the employment of various microorganisms and systems for producing toxins. The similar pattern of relative toxicity was yet

seen, with CRY1F the poison that both insects have most susceptible to, as previously described (Boaventura, *et al*., 2020). Given Since the CRY1F poisons found in lepidopteran pests do not share binding sites with these toxins, these data support local isolates for pyramiding with CRY1F in fruit borers to manage *S. litura* and *H. armigera* more effectively and prevent the emergence of resistance (Gupta *et al*., 2021).

By creating a variety of insecticidal parasporal crystal proteins, the various *Bt* isolates have demonstrated their ability to effectively control insect pests (Jurat-Fuentes and Crickmore, 2017). The most promising isolates of *Bt* were evaluated in response to different concentrations (1 105 - 1 109 spores/ml) of *B. tabaci* nymphs in a lab setting for 144 hours. The range of isolates' LC50 values was found to range from 9.67 106 to 3.32 107 spores per milliliter (ml). *Bt* AAUDS-16 isolates had the highest level of virulence, and isolates AAUES-69D came in second with respective LC50 values of 9.67 106 and 1.16 107 spores/ml. The concentrations of toxin-spore suspension of particular *B. tabaci* isolates were shown to rise together with an increase in the *B. tabaci* mortality rate percentage. In line with this, (Syed *et al*., 2020) also discussed the rise in *Bt* isolate toxin-spore concentration and the percent death of whitefly pests. Significant variations in the percentage mortality of the tested *B. tabaci* under various actions were found. Similarly, differences in the efficacy of various *Bt* isolate concentrations were described (Patel *et al*., 2018). The virulent isolates all had a maximum growth rate at about 30 °C when their capacity to proliferate at various temperature ranges between 15 and 40 °C was assessed. AAUDS-16 and AAUES-69D were the isolates that grew the fastest, at 2.07 generation per hour (1.86 generation per hour). Woldetenssay and Ashenafi (2009) discovered that the effects of temperature on the growth rate of isolates from different agro ecological zones of Ethiopia were greatest at 30 °C, despite the fact that all isolates developed well in temperatures ranging from 12 to 39 °C. Additionally, *Bt* isolates were shown to grow across a range of temperatures (from 25 to 50 °C), with the temperature range between 30 and 35 °C showing the highest rates of isolate growth (Abo-Bakr *et al*., 2020). The isolates of AAUES-69D and AAUDS-16 have generally demonstrated significant insecticidal potential against nymphs of *B. tabaci*, with minimal LC50 values of 1.16 107 and 9.67 106 spores/ml, respectively, and maximum growth rates of 2.07 and 1.86 generation/h in response to a wide range of temperature. CRY1F is a good option for usage against these insect pests due to its high toxicity to *S. litura* and *H. armigera*. The parent CRY toxins are both active against *S. litura* and *H. armigera* larvae in bioassay assays, activating insecticidal action against *S. litura*. The hybrid construct has undergone proper processing and has been validated as a stable active toxin. Based on the activity against *H. armigera.* Even at greater concentrations, CRY1F was unable to kill or prevent the growth of *S. litura* larvae, according to bioassays with this species, which demonstrate that domain substitution removes CRY1F from its harmful effects. The outcomes of our competition binding experiments with CRY1F local isolates of proteins have separate binding sites from CRY1F, it was shown that CRY1F does not share binding sites with local isolates for *H. armigera,* which is consistent with behaviour predicted for Helicoverpa species (Hernández-Rodrguez *et al*., 2008). CRY1F have a single population of binding sites in *H. armigera*, as was just recently found. Because it has been projected that CRY1F can attach with various modalities binding to several binding site in a variety of lepidopteran insects, our ligand blots confirm this theory and show that each of the relevant proteins has many binding sites (Jakka *et al*., 2015; Herrero *et al*., 2016). (A comparison of the bioassay of CRY1F protein and local isolates for *S. litura* and *H. armigera*. These results also imply that depending on the insect, the same poison may act in a different way. The aforementioned notion should, however, be further confirmed by carrying out research using various techniques and by taking into account other significant models for the function of Cry proteins. These exceptional isolation characteristics are crucial to the screen since they demonstrate their competitiveness for upcoming biopesticide applications for sustainable crop development in fields with a wide temperature range.

Conclusions and Recommendations

The CRY1F protein and local isolates provide insight into the results of *S. litura* and *H. armigera* bioassays. This knowledge would be useful for understanding the binding mechanisms of the CRY proteins and for creating novel toxins to manage these insect pests. The present study showed the toxicity isolates *Bt* from soil samples. *S. litura* and *H. armigera* were used to test *Bt*'s insecticidal abilities. The percentage mortality effects of isolates against *S. litura* and *H. armigera* were

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significantly different. Therefore, these isolates should be significant candidates for various insecticidal toxin research and usage as gene sources for the development of transgenic tomato plants. They also have the potential to biologically control *S. litura* and *H. armigera*.

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Novelty Statement

The novelty of the study is testing the toxicity of Bacillus thuregensis and Cry1f genes against lepidopteran insect pest under laboratory conditions for the first time in NARC, Islamabad.

Author's Contribution

Yusra Karim: Conducted the experiment.
Munawar Saleem Ahmad: Designed the experiment
Javed Khan: Co-supervised the research work.
Imtiaz Khan and Said Hussain Shah: Drafting and statistical analysis.
Syeda Anika Shamsher and Imran Qazi: Helped in experiment and data collection.
Habib-Ur-Rahman Kakar and Wajih Ullah: Helped in statistical analysis and editing.

Conflict of interest
The authors have declared no conflict of interest.

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