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Impact of midgut microbiota with Bt toxin to enhance the insecticidal activity against Spodoptera litura (Fab.) (Lepidoptera: Noctuidae)

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ABSTRACT

Biological pest control and management using the gutbiota species is recently considered to be a safe and cost-effective strategy. This study investigates the effect of gut biota in combination with *Bt*-toxin in inducing mortality among the pest host Tobacco caterpillar (*Spodoptera litura*). Egg mass of *S. litura* were collected and larvae were maintained in lab conditions. *Bacillus thuringiensis* (*Bt*) was cultured from soil sample and the *Bt*-toxin Cry1, Cry2 were confirmed by PCR and extracted by centrifugal method. DNA was extracted from the larval midgut microbiota, and Sanger sequencing was performed for candidate 16SrRNA regions. Mortality of *S. litura* larvae treated with formulations of *Bt*-Toxin combined with *E.mundtii*, *E. gallinarum*, *Acinetobacter* sp, *Bacillus* sp, *E. cloacae*, *E. coli*, and *P. aeruginosa* were analyzed. Highest mortality of 72% and 60% were observed with *Bt*-Toxin + *E. cloacae* and *Bt*-Toxin +*Bacillus* sp combinations, respectively. In addition, One-way ANOVA analysis indicated significant ($p \leq 0.001$) difference among the mortality across the combination groups ($F [7,392] = 16.539$). Hence this present study contemplates the possibility of utilizing *E.cloacae* as a preferable biocontrol agent for effective downstream pest control and management.

INTRODUCTION

Agriculture is the backbone of the majority of population of the Indian subcontinent. Both agriculturally important staple and cash crops imply the livelihood and welfare of both the farmer communities and general populace. Yet the crop varieties are under the constant attack by various biotic and abiotic stress factors. Insects represent a major group of biotic stressors. Insecticide spray, albeit being considered as one of the common intervention strategies, has the downside of toxicity accumulation. Hence alternative insect control methods are being contemplated. Although *Bacillus thuringiensis* (*Bt*) -based transgenic plants were used as an alternative to insecticides, increased resistance against *Bt*-toxin (Kain *et al.*, 2022; Benowitz *et al.*, 2022) gave way to *Bt*-Gut biota combination based insecticidal approaches.

The Tobacco caterpillar (*Spodoptera litura* (Fab.) is a pest insect which poses serious threat to agriculturally important crops. One of the novel and eco-friendly control methods against *S. litura* is the exploitation of the *Bt*-toxin combined with parasite gut microbes, which could negatively modulate the development of their insect host. Gut microbes are reported (Xia *et al.*, 2020) to mediate crucial cellular and physiological pathways of *S. litura*. Gut pathogenic microbes such as *Serratia marcescens, Enterococcus mundtii, K. pneumoniae* and *P. paralactis* are recently reported (Devi *et al.*, 2022) to be of augmenting the mortality of *S. litura*. This present study attempts to assess the mortality of *S. litura,* which is imposed by the action of *Bt*- toxin along with various host gut microbiota.

Materials and Methods

Isolation of *Bacillus thuringiensis* (*Bt*) from soil sample

20 g soil sample collected from a tubular soil sample after removing the 2-3 cm of the top layer soil. Suspend the soil samples 1 g in 10ml 0.85% NaCl., Heated with shaking at 80˚C for 12 min, then placed on ice for 5 min. Serial dilutions were conducted in 1% saline. Plate aliquots of 100µl of spreded onto nutrient agar (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar). Incubate plates at 30˚C for 48h. The dishes were then inverted and stored for 48 h in an incubator at 28 ◦C. Stain the culture with amino black and Ziehl's carbol fuchsin and

examine under a standard light microscope for preliminary identification. Colonies of Bt characteristics were inoculated in a nutrient broth presence of penicillin G (100 mg/L), which act as the selective medium, and then placed in a rotating incubator for approximately 48 h at 28°C and 180 rpm. The colonies that grew in the medium were observed using a phase contrast microscope (100 × magnification) to confirm the presence of parasporal material (protein crystals) and culture centrifuged 8000 rpm to collect supernatant, stored in 4°C for larvae mortality study (Lobo *et al.*, 2018).

PCR conformation of *cry* genes types

The whole genomic DNA was extracted from the broth culture of a single bacterial colony and utilised as a template to amplify the cry1, cry1Ac, and cry2. The amplified PCR products were sequenced using the appropriate primers. The forward sequences were edited using the BioEdit program, and the presence of the cry toxin in our isolate was verified using BLASTn.

Cryt1primer (F-CTGGATTTACAGGTGGGGATAT), (R-T GAGTCGCTTCGCATATTTGACT) cry1Ac primer (F-TGTAGAAGAGGAAGTCTATCCA) (R- TATCGGTTTCTGGGAAGTA) cry2primer (F-GTTATTCTTAATGCAGATGAATGGG) (R-AGATTAGTCGCCCCTATGAG) The following was the PCR program's execution: One minute at 95°C; thirty cycles at 95°C for one minute; one minute at each of the annealing and extension temperatures of 52°C and 72°C; and five minutes at 72°C as a last stage. Final amplified product maintained in 4°C cry1, (bp-558) cry1Ac (bp-272), and cry2 gene (bp-498) PCR amplified product conformed on 0.8% agarose gel with EtBr (0.5 µg ml-1) in 1 X TAE buffer at 80 volts. PCR amplicons size were compared using a 100bp DNA ladder (500µg ml-1) used as a molecular weight standard marker (Cerqueira *et al.*, 2016).

Collection of egg mass and rearing of larvae

Golden brown color of *Spodoptera litura* egg mass collected in caster leaves of caster plant in PMT College campus. Pale greenish with dark markings larvae were emerged from egg mass, larvae fed with caster leave steriled by 5% sodium hypochloride and washed in autoclaved distil water and reared until 4th instar larvae and to form adult. Adult identified with forewings are brown in colour with wavy white marking, hind wings are white in colour with a brown patch along the margin (Thakur *et al.*, 2015)

Larvae dissection and collection midgut to culture of bacteria

To isolate the gut bacteria, ten healthy larvae were selected and starved for 24 h. The starved larvae were surface washed with 70% (v/v) ethanol followed by 5% (v/v) Sodium Hypochlorite (NaOCl) solution followed by thorough rinsing with sterilized distilled water. The larvae were dissected with the help of solid wax in petri dish, larva head and hindgut region pierced with help of sharp pin to fix the larva to solid wax. Dorsal region opened by surgical blade, removed midgut chopped, transferred to 5 ml eppendorp tube contain normal saline and, sonicated to homogenous. 100 µl of the diluted Homogenous mixer were spread plated on Luria Bertani agar plates (10g tryptone; 5g peptone; 5g NaCl; 15g NaCl in 1L of MQ water). The plates were incubated in a 30±2°C and the bacterial CFUs were enumerated in plated serial dilution on LB agar. The dominant frequently appearing gut associated bacteria were identified by bacteriological properties and 16S rRNA gene sequencing. The colonies were differentiated based on their size, colour and morphology, and a single isolate of each morphotype was transferred to a new plate. After three repeated streaking the purified isolates were preserved in LB of 70% glycerol stocks at -20°C for further study (Singh *et al.*, 2023).

DNA isolated from midgut culturable bacteria using the GTE method (Glucose Tris EDTA method). Polymerase Chain Reaction (PCR) for the genomic DNA isolated from each bacterial isolate was to amplify the 16s rRNA genes. A forward 27F (5′-AGA GTT TGA TCC TGG CTC AG3′) and reverse 1492R (5'GGT TAC CTT GTT ACG ACT T 3') 16S rRNA primers were used. Total reaction mixture of 25µl was prepared to have the concentration of 1µM for forward and reverse primer each, 25 ng DNA template, 200 µM dNTP (each), 1.5 U Taq polymerase, Taq buffer (1X), and DNase free water for make up the final volume. PCR conditions followed were as initial denaturation for 5 min 94ºC, followed by 30 cycles of annealing at 55ºC for one min, extension at 72ºC for 2 min, and final extension at 72ºC for 7 min. The genes were amplified in a Gencycler-G96G thermocycler (Biosystems The amplification was observed on 0.8% agarose gel with EtBr (0.5 µg ml-1) in 1 X TAE buffer at 80 volts. PCR amplicons size were compared using a 100bp DNA ladder (500µg ml-1) used as a molecular weight standard marker. The gel was visualized on a UV trans-illuminator using Gel Doc. The amplified products were subjected to Sanger sequencing. The raw data were obtained to forward sequence. The sequences were trimmed was made using BioEdit. The obtained sequences were subjected to BLAST (Basic Local Alignment Search Tool) in NCBI, the closely related bacterial species were retrieved from GenBank to compared to identify gut microbiota (Woo *et al.*, 2008)

Mortality assay for *Bt* toxin wih midgut microbiota

Fifty 4th instar healthy larvae were opted for each midgut microbiota from the colony and reared in plastic boxes for 2 days with the treated leaves. A solution of 5 mg/mL ofloxacin, 5 mg/mL vancomycin were sprayed on caster leaves fed to larvae to kill the presence of gut bacteria in first day. In second day, 8 hours freshly cultured each microbiota mixed with Bt toxin supernatant in 1:1 ratio, smeared on fresh sterile caster leaves, fed to to each group larvae. Control group treated with sterile caster leaves alone. Observed mortality for 24 hour, All treatments were repeated thrice (Xia *et al.*, 2020)

RESULTS AND DISCUSSION

Sub-culture of soil bacterial colonies show *Bt*-like phenotype such as lack of pigmentation, wavy edges and a circular form for single colony isolation again on fresh plates and incubated. Native *Bt* isolates from the soil that can potentially be used for the development of biocontrol tools to kill *S. litura*. Soil is a natural sources of *Bt* spores and is mostly the preferred sample for the isolation of *Bacillus* species. *Bt* strains have significantly high larvicidal efficacy against cotton worm isolated from soil samples (El-Kersh *et al.*, 2016). In our isolate, cry1, cry2 both gene conformed in agarose gel in comparion of a 100bp DNA ladder (500µg ml-1) used as a molecular weight standard marker. Four Bt-like phenotype taken for PCR amplification. One of four conformed cry1 and cry2 in PCR amplification and taken to further to extract *Bt* toxin extraction.

An investigation into the characteristics of *Bacillus thuringiensis* isolates with strong insecticidal activity against *Spodoptera frugiperda* reveals that the frequencies and profiles of cry genes vary globally. Furthermore, they revealed that the most commonly identified genes *Bt* collections based on area were cry1D, cry1G, cry1B, and cry1E in Brazil's (Cerqueira *et al.*, 2016).

A total of 7 dominant isolates frequently appearing were successfully collected from the gut of the 4th instar larvae, 7 bacteria were isolated using pure culture method and subjected to 16S rRNA forward sequencing. The sequences obtained were analyzed using BLAST. The 7 species *Enterococcus mundtii, Enterococcus gallinarum, Acinetobacter sp., Bacillus sp., Enterobacter cloacae, Escherichia coli* strain, *Pseudomonas aeruginos* were identified based on their similarities with other sequences.

Table 1. Mortality of Larvae among both Control and various Intervention groups

PARAMETERS	Control	Intervention (s)						
		Group.1	Group.2	Group.3	Group.4	Group.5	Group.6	Group.7
	Bt-Toxin (n=50)	*Bt* toxin + *Enterococcus mundtii* (n=50)	*Bt* toxin + *Enterococcus gallinarum* (n=50)	*Bt* toxin + *Acinetobacter sp* (n=50)	*Bt* toxin + *Bacillus sp* (n=50)	*Bt* toxin + *Enterobacter cloacae* (n=50)	*Bt* toxin + *E. coli* (n=50)	*Bt* toxin + *Pseudomonas aeruginosa* (n=50)
No. Mortality among Larvae	4	9	10	30	32	36	10	18
Mortality (%)	8	18	20	60	64	72	20	36
Survival (%)	92	82	80	40	36	28	80	64

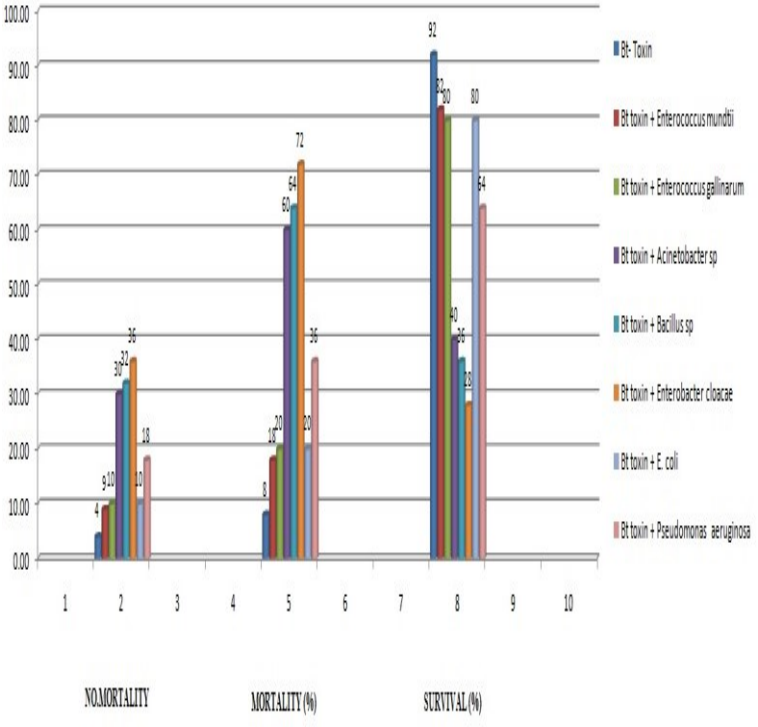

Figure 1. Percentage mortality of *Spodoptera litura* treatment with *Bt* toxin

Table: 2. ANOVA Results

Group	Mortality (Mean ± SD)	ANOVA	
		F-Test	Significance (p-Value)
Control (*Bt*- Toxin)	0.08±0.274	16.539	≤ 0.001
Group.1 (*Bt* toxin + *Enterococcus mundtii)*	0.18±0.388		
Group.2 (*Bt* toxin + *Enterococcus gallinarum)*	0.20±0.404		
Group.3 (*Bt* toxin + *Acinetobacter sp)*	0.60±0.495		
Group.4 (*Bt* toxin + *Bacillus sp)*	0.64±0.485		
Group.5 (*Bt* toxin + *Enterobacter cloacae)*	0.72 ±0.454		
Group.6 (*Bt* toxin + *E. coli)*	0.20±0.404		
Group.7 (*Bt* toxin + *Pseudomonas aeruginosa)*	0.36 ±0485		

Among all the *Bt*-toxin with gutbiota combinations, the *Bt*-Toxin + *E. cloacae* combination yielded the highest mortality (36[72%]), with the next highest mortality (30[60%]) observed on the *Bt*-toxin + *Bacillus* sp group. The least mortality was observed among observed among the *Bt* toxin + *E. mundtii* group **(**Table 1 and Figure 1**)**. There was a statistically significant difference between groups as determined by one-way ANOVA (F [7,392] = 16.539, $p \leq 0.001$) **(**Table 2).

Inoculation of *Spodoptera litura* larvae with *E. mundtii* and *Enterococcus gallinarum*, *E. coli* with *Bt toxin* significantly reduced susceptibility to *Bt* toxin because these can degrade *Bt* toxin followed that increase the resistance against toxin (Li et al., 2022). *Pseudomonas aeruginosa* is a well-known bacterial pathogen of many insects including adult grasshoppers. *Aeruginosa* was the most frequently isolated bacterium from larval. On investigating midgut bacteria of *Spodoptera littoralis* larvae, Bacillus sp has 61% biocontrol efficacy and P. *aeruginosa* had a low mortality effect (36%) on *S. littoralis* larvae under laboratory condition (Ramachandiran et al., 2018). We investigated the culturable bacteria of midgut of *S. litura* to exhibit highest mortality rate to *Acinetobacter sp* 60%, *Bacillus sp* 61%, *Enterobacter cloacae* 63% respectively.

The pH of larvae midgut is usually alkaline, which activates the inactive form of *Bt* Toxin, which enters during the treatment, into active form. The active form, in turn, increases the pore size of the gut epithelial cells, which eventually results in the lysis of the gut tissues (Bravo et al 2007). The earlier work by in their study with the Egyptian Cotton Leaf worm, *Spodoptera littoralis*, reported the various molecular factors underlying the combined toxicity of gutbiota along with the *Bt* toxin (Caccia *et al.*, 2016).These factors could convert the symbionts into pathobionts. Various recent reports suggest the interaction of *Bt*-Toxin with gut biota in mediating the host mortality are reported (Paddock *et al.*, 2021). The effect of the Bt-protoxin Cry1AC along with the gut biota is documented with *Plutella xylostella*. In addition reported that *Bt*-toxin and Gut biota cellular factors such as Antimicrobial peptides (AMPs) and Dual Oxidases (DUOX) could generate Reactive Oxygen Species (ROS) which could in turn lead to host mortality (Li *et al.*, 2020).

The maximum mortality of larvae with the combined treatment of *Bt*-toxin and gut biota *E. cloacae* observed in the present study is also reflected in previous studies with *S. litura* with infections by *E. cloacae* (Thakur *et al.*, 2015; Devi *et al.*, 2022)

CONCLUSION

Biocontrol-based approaches are well preferred to conventional insecticides and pesticides by virtue of their safe and eco-friendly nature. This present study observed *E. cloacae* and *Bacillus sp.* to be the possible candidate microbes to be used along with *Bt*-toxin Cry1, Cry2 for the management of *S. litura*. As discussed earlier *E. cloacae* is already reported to be a promising microbe when used along with *Bt*-toxin for agriculturally important crop management. This study anticipates similar studies with the *Bt*-Gut biota interaction, which could give insight scientific host-pathogen cellular interaction with molecular regulations.

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