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Development of a biopesticide formulation using an indigenous *Bacillus thuringiensis* Isolate and its efficacy against fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

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Abstract
Invasion of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), in India in 2018, resulted in heavy yield losses in maize and poses a great threat to the cultivation of other allied crops. In this study an attempt was made to develop *Bacillus thuringiensis* (*Bt*), biopesticide formulation(s) and evaluated against the FAW. A native *Bt* isolate, T15 was used for the preparation of formulation. The colony of the T15 isolate was creamy white in color, flat and irregularly shaped with undulated margins. The T15 isolate produced bipyramidal parasporal inclusions and it was positive for the presence of lepidopteran toxic *cry1, cry 1Ac, cry2, cry2Ab and vip3A* genes. The liquid formulations were prepared by adding various ingredients with the *Bt* isolate, after lysis stage of the culture and evaluated against the fall armyworm. The biopesticide formulation prepared using *Bt* culture broth + Pungam oil 3% + Glycerol 6% + Triton X-100 0.1% performed well against the neonates of the fall armyworm with 96.67% mortality under laboratory conditions.

Keywords: *Bacillus thuringiensis*, formulation, fall armyworm, bioassay

Introduction
The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is a serious agricultural pest that attacks more than 353 host plants (Montezano *et al.*, 2018) [19]. Previously, the FAW existed only in the Americas, but in 2016 it invaded into African countries and into India (Shivamogga, Karnataka) during June 2018 (Chormule *et al.*, 2019) [5]. In Tamil Nadu, it was first reported in November, 2018 at Erode and Karur (Srikanth *et al.*, 2018) [25]. *Bacillus thuringiensis* (*Bt*) based biopesticides will be a good alternative to chemical insecticides.

Bt is a Gram-positive, spore-forming bacterium, which produces parasporal inclusions (or crystals) during stationary and sporulation phases and the inclusions comprising crystal proteins are toxic to insects. Insecticidal activity of this bacterium is primarily due to the expression of crystalline proteins encoding insecticidal genes during sporulation (Schnepf *et al.*, 1998) [24]. When crystalline inclusions are consumed by lepidopteran larvae, they get solubilized in the midgut and releasing δ-endotoxins. The enzyme midgut protease activates the protoxins, and these active toxins interact with the midgut epithelium to destroy membrane integrity, which finally results in death of the larvae (Gill *et al.*, 1992) [9]. *Bt* is varied worldwide, and each isolate generates toxins that are unique to a particular group of insect pests without affecting higher species or the environment (Schnepf *et al.*, 1998) [24]. Biopesticides are produced using *Bt* toxins, and transgenic *Bt* plants are developed to manage different insect pests using the genes encoding insecticidal proteins (Romeis *et al.*, 2006) [22].

As the development of transgenic plants requires huge budget and releasing of the transgenic plants depends on the policies of the particular country, development of a *Bt* biopesticide formulation will be much useful for counteracting the disadvantages of the chemical insecticides. To understand how the biopesticide, pest, and environment interact, formulation development uses a multidisciplinary approach that includes microbial-plant ecology and pathology (Deacon, 1991; Whipps, 1997; Butt *et al.*, 2001) [6, 28, 4]. *Bt* has been the most widely used commercial biopesticide in the biological control industry and it accounts for 90% among all biopesticides sold worldwide (Yang *et al.*, 1998; Glare & Callaghan, 2000) [29, 10]. In this study, we chose an indigenous *Bt* isolate T15 for the preparation of formulation(s) and evaluated against the fall armyworm, *S. frugiperda*.

Materials and Methods

Bt isolate and insect culture

The native *Bt* isolate, T15, was obtained from the collection of *Bt* isolates maintained at Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore. T_3 medium (3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 8.9 g of disodium hydrogen phosphate, 6.9 g of sodium dihydrogen phosphate, 100 µl of 0.05 g of manganese chloride diluted in 1 ml of water for 1 litre of medium and a pH 6.8-7.0 was maintained) was used to revive and maintain the *Bt* culture. The larvae of maize fall armyworm *S. frugiperda*, were collected from the maize field and mass cultured in the laboratory. The larval culture was maintained on artificial diet and kept at a temperature of 25 °C and a relative humidity of 75±5% (Tefera, 2010) [26]. The experiment was conducted at Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulam (8.706º N latitude; 77.855º E longitude; 40 m above MSL).

Colony and crystal morphology

The spore-crystal mixture from the T15 *Bt* isolate was harvested to study the crystal morphology. A loopful of *Bt* isolate was inoculated into 5 ml of T_3 broth and incubated at 30 °C for overnight. After 14-18 hours of incubation, 1 percentage (250 µl) of cell culture was transferred to 25 ml of T_3 broth and incubated at 30 °C and 180 rpm for 48-60 hours. After incubation, a drop of inoculum (at lysis stage) was taken and smeared on sterile glass slide, air dried and heat-fixed. Then, 100 µl of Coomassie Brilliant Blue stain was added and incubated for 1 minute (0.133g Coomassie Brilliant Blue G250 in 100 ml of 50% GAA). The bacterial colony was observed for its colour, surface and margin morphology shape under bright field microscope.

PCR Screening for *cry* and *vip* Genes

Genomic DNA was isolated as per the protocol given by (Kalman *et al.*, 1993) [11] from the test *Bt* isolate (T15) and from the reference strain (HD1). The extracted DNA was assessed by loading on 0.8% agarose gel and verified using agarose gel electrophoresis (Sambrook *et al.*, 1989) [23]. PCR was carried out to find out the presence of lepidopteran specific *cry / vip* genes. The primers used for detecting the presence of *cry* and *vip* genes are given in Table 1. HD1 *Bt* strain was used as positive standard for *cry1* and *cry2* genes and 4G1 *Bt* strain was used as positive standard for *cry9* gene.

Table 1: Primers used for PCR screening of lepidopteron toxic *cry/vip* genes

S. No.	Primer sequence	*Cry/vip* gene	Amplicon size	Reference
1.	FP: 5' CATGATTCATGCGGCAGATAAAC 3' RP: 5' TTGTGACACTTCTGCTTCCCATT 3'	*cry1*	~280 bp	(Ben-Dov *et al.*, 1997) [2]
2.	FP: 5' GTTATTCTTAATGCAGATGAATGGG 3' RP: 5' GAGATTAGTCGCCCCTATGAG 3'	*cry2Aa*	~500 bp	
3.	FP: 5' GTTATTCTTAATGCAGATGAATGGG 3' RP: 5' TGGCGTTAACAATGGGGGGAGAAAT 3'	*cry2Ab*	~550 bp	
4.	FP: 5' CGGTGTTACTATTAGCGAGGGCGG 3' RP: 5' GTTTGAGCCGCTTCACAGCAATCC 3'	*cry9*	~350 bp	(Ben-Dov *et al.*, 1999) [1]
5.	FP: 5' ACATCCTCCCTACACTTTCTAATAC 3' RP: 5' TCTTCTATGGACCCGTTCTCTAC 3'	*vip3A*	~680 bp	(Espinasse *et al.*, 2003) [8]
6.	FP: 5' GGAAACTTTCTTTTTAATGG 3' RP: 5' TTATTAAAATCACCATTTTTA 3'	*cry1Ac*	~ 1606 bp	(Reddy *et al.*, 2012) [21]
7.	FP: 5' CGGATGCTCATAGAGGAGAA 3' RP: 5' CTCCTTGTACGCTGTGACAC 3'	*cry1Ab*	~ 2116 bp	
8.	FP: 5´ACCTTTATTTGCACAGGCA 3' RP: 5'AATATCTGAAAAACGAGCTC 3'	*cry2*	~ 1249bp	(Mendoza *et al.*, 2012) [17]
9.	FP: 5' GCAGACACCCTTGGTCGT 3' RP: 5' TGGCAACGCCCTCCCGAT 3'	*cry2Ac*	~ 841bp	

Preparation of *Bt* formulation

One loopful of pure culture from one colony was inoculated into 100 ml T_3 broth. The culture was kept at 30 ºC for 48 hours, in an incubator-cum-shaker for 200 rpm. Using a bright field microscope, the culture was monitored for lysis after 48 hours. When the culture attained 90% of lysis, the sporulated culture was used for preparation of different *Bt* formulations. The culture broth was used for the preparation of formulation of *Bt* biopesticide using different ingredients as given in Table 2.

Bioassay

Bioassay studies were carried out by diet surface contamination method to know the insecticidal activity of the *Bt* formulation prepared using the indigenous *Bt* isolate (T15). A known quantity of 0.75 ml diet was taken up in sterilized micro centrifuge tubes and pressed firmly. The diet surface was coated with 15µl of *Bt* formulation and dried under laminar airflow chamber. In each micro centrifuge tube, single neonate larva was released on the contaminated diet. Three replications were maintained and for each replication, twenty larvae were maintained (one larva per tube). The larval mortality was observed at 7 days after treatment. The mortality data were subjected to ANOVA and statistically analysed (Table 2).

Results and Discussion

Colonies of the T15 *Bt* isolate were creamy white in color, fried egg type, irregularly shaped with undulated margins and flat surface. It was in line with the finding of Navya *et al.* (2021) [20]. The T15 isolate had bipyramidal parasporal inclusions. Bernhard *et al.* (1997) [3] and Mahadeva Swamy *et al.* (2013) [13] found the abundance of bipyramidal form of the crystal structure in their test *Bt* isolates and reported that *Bt* isolates were effective against lepidopteran larvae. Manikandan *et al.* (2016) [15] had already reported that the T15

Bt isolate had *cry1*, *cry1A*, *cry1Aa*, *cry1Ac* and *vip3A* genes. In this present study, it was confirmed that T15 isolate had *cry1*, *cry1Ac*, *cry2*, *cry2Ab* and *vip3A* genes.

In the bioassay studies, the mortality ranged from 53.33 to 96.67%. The formulation made up of *Bt* culture broth + Pungam oil 3% + Glycerol 6% + Triton X-100 0.1% recorded the highest mortality (96.67%) followed by the formulation with *Bt* culture broth + Groundnut oil 3% + Glycerol 6% + Triton X-100 0.1% (80.00%). There was no mortality in untreated control (Table 2).

Table 2: Evaluation of *Bt* formulation prepared in different combinations using different ingredients against neonate larvae of *S. frugiperda*

S. No.	Treatment		Mean mortality (%)
1.	*Bt* culture broth		63.33 (52.78)^{c}
2.	*Bt* culture broth + Glycerol 6% + Triton X-100 0.1%		66.67 (54.78)^{bc}
3.	*Bt* culture broth + Wheat flour 2% + Soybean flour 2% + Wessons salt mixture 1%		60.00 (50.85)^{c}
4.	*Bt* culture broth + Wheat flour 2% + Soybean flour 2% + Wessons salt mixture 1% + Glycerol 6% + Triton X-100 0.1%		66.67 (54.78)^{bc}
5.	*Bt* culture broth + Caesin enzymatic hydrolysate 1% + Wessons salt mixture 1%		53.33 (46.92)^{c}
6.	*Bt* culture broth + Caesin enzymatic hydrolysate 1% + Wessons salt mixture 1% + Glycerol 6% + Triton X-100 0.1%		60.00 (50.85)^{c}
7.	*Bt* culture broth + Pungam oil 3% + Glycerol 6% + Triton X-100 0.1%		96.67 (83.67)^{a}
8.	*Bt* culture broth + Groundnut oil 3% + Glycerol 6% + Triton X-100 0.1%		80.00 (63.93)^{b}
9.	Untreated control		0.00 (0.27)^{d}
	CV= 10.97	CD (0.05) = 9.592	SED = 4.56

In table, figures in parentheses are *arc* sine transformed values

In a column, means followed by a common letter (s) are not significantly different by LSD (p = 0.05).

Kaviyapriya *et al.* (2019) [12] found that the indigenous *Bt* isolate T29 displayed bipyramidal and cuboidal crystal inclusions and recorded 100% mortality against the invasive pest Fall armyworm *S. frugiperda* in the bioassay study (leaf dip coating method) using spore-crystal mixture of the *Bt* isolate which had the *cry1*, *cry2Aa* and *vip 3A* genes. Maheesha *et al.* (2021) [14] analysed nine native *Bt* isolates for toxicity against the fall armyworm *S. frugiperda* in Tamil Nadu, India. In a leaf dip bioassay, two indigenous *Bt* isolates T350 and T532 reported 100% mortality against neonate.

Monika *et al.* (2017) [18], identified a *Bt* isolate AUG-5, which was found to be the most effective against wide range of lepidopteran insects. Different mediums were assessed for the growth and development of *Bt*. The neonates of the tobacco caterpillar, *Spodoptera litura*, and cotton bollworm, *Helicoverpa armigera*, were highly susceptible to the spore-crystal complexes of Medium II (2% Wheat flour, 2% Soybean meal, 1% Wesson salt mixture) and medium III (1% Casein enzymatic hydrolysate, 0.5% Yeast extract, 0.5% NaCl and 1% Wesson salt mixture), respectively.

Devi *et al.* (2005) [7] standardized wheat bran-based medium for *Bt* culturing found considerable rise in the spore count, toxin content and scaled up for *Bt* production in plastic tubs with aeration at intervals of 8 hours and the maximum lysis of *Bt* cells was achieved after 60 h at 30 °C incubation. In laboratory bioassays, the *Bt* product found to be very effective at 0.1% concentration, causing complete mortality after three days against the castor semilooper, *Achaea janata* L.

Marzban *et al.* (2021) [16] formulated a wettable powder formulation using *Bt* isolate KD2 and by addition of 3% w/v Triton X100, Tensiofix LX, and Tensiofix BCZ in three different treatments. The produced technical powder was transformed into a wettable powder formulation by the addition of moisturisers, spreaders, filler ingredients and UV light protection components. The findings of the biological testing of the formulations revealed that Belthirul had the highest mortality rate on *H. armigera* larvae, at 78%, while Biolep had the lowest, at 53%. Out of nine formulations tested, Triton X 100 + Kaolin and Kaolin + LX formulations showed the best results.

Vimala Devi *et al.* (2021) [21] prepared *Bt* formulation in a freely-flowing WDG with a high concentration of the active ingredient and biodegradable adjuvants. Using a binder (guar gum), a wetting agent (Tween-80), and a water-soluble carrier (starch), the DOR *Bt*-127, a native strain of *B. thuringiensis var. kurstaki*, was produced into a water dispersible granule (WDG) formulation. This formulation has a high concentration of protein at 134.7 mg/g and a high potency of 74,200 SU/mg against larvae of 9 days old *S. litura*. In pot culture studies and field trials, Bt-127 WDG formulation was as effective as commercial *Btk* formulation against 7- 9 days old larvae. The Bt-127 WDG formulation showed promising results against early as well as older instars.

In this study, the formulation containing, *Bt* culture broth + Pungam oil 3% + Glycerol 6% + Triton X-100 0.1% recorded 96.67% mortality against neonates of *S. frugiperda*, when compared to *Bt* culture broth alone which recorded 63.33% only.

Conclusion

The formulation prepared by addition of Pungam oil 3%, Glycerol 6%, Triton X-100 0.1% in *Bt* culture broth has performed well and can be taken up for large scale level and has to be tested at field level against other major lepidopteran insect pests.

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