Potential of standard strains of *Bacillus thuringiensis* against the tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract

**Background:** The tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the key pests of tomato worldwide, causing an estimated crop loss of 80 to 100%. This pest has developed resistance to several pesticides due to overuse, resulting in control failures in the field. The use of biological insecticides as *Bacillus thuringiensis* that expressed insecticidal proteins can be an alternative tool by insecticides to suppress the pest population.

**Main body:** Laboratory study investigated the efficacy of standard *Bacillus thuringiensis* (*Bt*) strains (4D1, 4D4, 4G1, 4K5 and 4XX4) against *T. absoluta*. Bioassay was conducted using tomato leaf discs treated with spore crystal lysates prepared from the standard strains, and mortality data was subjected to concentration-mortality probit analysis. The LC50 values for *Bt* 4D1, *Bt* 4D4 and *Bt* 4G1 were 6.10, 6.62 and 8.18 μg/ml for the 2nd instar; 9.90, 10.20 and 11.12 μg/ml for the 3rd instar; and 19.82, 23.16 and 24.54 μg/ml for the 4th instar, respectively, while the *Bt* 4K5 and *Bt* 4XX4 were not toxic to *T. absoluta*.

**Conclusion:** This study suggests that *Bt* strain 4D1 is effective against different larval instars of the pest and can be used in its management.

**Keywords:** *Tuta absoluta*, *Bacillus thuringiensis*, Potential, LC50, Bioassay

Background

Tomato pinworm *Tuta absoluta* (Meyrick, 1917) (Gelechiidae: Lepidoptera) is a tomato pest in South America and recently introduced to India (Shashank et al., 2015). This pest was first reported in 1914 in Peru, and now it is a common pest found in South America (Dilip and Srinivasan, 2019). Since 2006, *T. absoluta* had invaded Europe, Africa and Asian countries where it has caused significant economic losses of 80–100% both under greenhouse and field conditions (Urbaneja et al., 2013). *T. absoluta* is one of the most devastating tomato pests because it feeds on foliage, stems, fruits and flowers. Larvae infest all stages of plant growth causing wounds which facilitate the invasion of secondary pathogens (Hatice et al., 2017). The pest species has high reproductive potential with 12 generations in a year and female can lay up to 260 eggs (Ayalew, 2015).

During the last few decades, tomato productivity has been increased worldwide. Heavy reliance on...
chemical pesticides provide ephemeral benefits, often with adverse side effects and not viable (Hernandez et al., 2011) and, in some instances, actually worsen farmer’s overall pest problems, and this pest became resistance to pesticides (Sandeep et al., 2020a). Thus, the major challenge is to increase and sustain crop productivity with less use of pesticides.

Variety of management tactics are used to reduce the pest infestations. The first option is to reduce the pest population through cultural practices, i.e. deep ploughing and trap crops, in order to safeguard the main crop. But chemical management is the most viable method for pest control. Farmers apply huge quantity of insecticides to manage insect pests; consequently, these insects have developed resistance to insecticides (Manivannan et al., 2019). The failure to control this pest may have a strong economic impact, and its recent history of introductions has increased the need for studies to develop strategies for its biological control,

by the use of Bacillus thuringiensis (Bt) that express insecticidal proteins (Gonzalez et al., 2011).

*B. thuringiensis* (Berliner), a species of gram-positive sporulating soil bacteria that forms insecticidal crystal (CRY) proteins during sporulation phase of its growth cycle, is the major source for the control of insect pest. The crystals contain one or more endotoxins known as cry proteins, which vary at different Bt strains. Cry and Cyt genes are named by cloning and sequencing from many cry proteins. Each of the Bt strains can carry one or more crystal toxic genes, and therefore, strains of the organism may synthesize one or more crystal proteins, and about 323 holotype crystal proteins are documented as toxic to insects of different orders viz. Lepidoptera, Coleoptera and Diptera (Crickmore, 2017). These crystal proteins are sequestered in bacteria as crystalline inclusions, mediates specific pathogenicity against insects (Schnepf et al., 1998).

*B. thuringiensis* strains are very effective against all larval stages of *T. absoluta* (Joel et al., 2011; Molla et al., 2011 and Azra et al., 2015). Cry proteins are highly specific and very effective against the tomato pinworm (Sandeep et al., 2020b and Dakshina and Gary, 2003) and narrow specific to lepidopterans (Hernandez et al., 2011 and Muhammad et al., 2019). Bt has been characterized as being highly specific against several insect orders including Lepidoptera, Diptera and Coleoptera (Xin Zhang et al., 2018). It has been found to be a very effective, environmentally safe insect-specific biopesticide (Palma et al., 2014). With this background, the present study was undertook to evaluate the potential of 5 standard *Bt* strains (4D1, 4D4, 4G1, 4K5 and 4XX4) against *T. absoluta* under laboratory conditions.

### Materials and methods

Five standard *B. thuringiensis* viz. 4D1 (BGSC HD1), 4D4 (BGSC HD73), 4G1 (BGSC HD8), 4K5 (BGSC LM79) and 4XX4 (BGSC YBT-1518) were obtained from *Bt* collection deposits at Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore. These strains were originally obtained from Bacillus Genetic Stock Centre, Ohio University, and Columbus, Ohio, USA. All *Bt* strains were subcultured with four side streaking method on Luria Bertani Agar Media plates and incubated at 30 °C for 24 h. Then a single colony was taken from each culture and inoculated in 15 ml test tube containing 5 ml LB broth individually. The test tubes were incubated at 30 °C for 24 h with 200 rpm in a shaker. The cultures were stored in sterile 50% glycerol at −20 °C.

### Isolation of spore crystal toxins and cry protein solubilization of Bt strains

The spore-crystal mixture of strains were prepared by acetone-lactose co-precipitation method as described by Dulmage et al. (1970). The resulting spore crystal powder was stored at 4 °C for further use. *Bt* culture from glycerol stock was plated in LB agar and incubated for overnight at 30 °C. From this culture, a loop was inoculated in to 1.5 ml Eppendorf tube containing sterile water (1 ml) and incubated at 70 °C for 1 h to kill other bacteria present in the culture. After 1 h, sterile water with *Bt* was poured into a test tube containing 5 ml of Plain LB Broth and incubated for 12 h at 30 °C. From this overnight culture, 1.25 ml was used for inoculating 125 ml LB Broth in a 250-ml conical flask and incubated at 30 °C in an incubated shaker with 200 rpm for 72 h. After 72 h, 6 g of sodium chloride was added to each flask and incubated for 3 h at the same conditions to release the cell contents into the broth. The sporulated broth culture was transferred to refrigerated centrifuge at 4 °C and spore crystal mixture was isolated.

The LB broth containing spore-crystal mixture was centrifuged at10,000 rpm for 10 min at 4 °C. The pellet was washed once with 20 ml of ice-cold Tris-EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)], once with 20 ml of ice-cold 0.5 M NaCl, followed by 2 more washes with 20 ml of Tris-EDTA buffer with 0.5 mM PMSF by centrifuging at the same speed and time (Ramalakshmi and Udayasuriyan, 2010). The final pellet was solubilized in a solubilizing buffer [50 mM Na2CO3, pH 10.5 mM (DTT) dithiothreitol] at 30 °C for 4 h by shaking and then centrifuged at 10,000 rpm, for 15 min at 4 °C. The supernatant containing solubilized protoxin was removed and stored at −20 °C for further use.
This contains pure Cry proteins and their concentrations were estimated as described by (Lowry et al., 1951).

**In vitro bio-assay of Bt strains against Tuta absoluta**

Laboratory experiments were conducted at Horticul- ture College & Research Institute, Periyakulam, Tamil Nadu Agricultural University. *T. absoluta* larvae collected, from leaves, stalks and fruits, were packed in plastic bags and brought to the laboratory. Larvae were immediately transferred into a larval rearing cage (45 × 45 × 45 cm) with mesh on all the 4 sides, glass top and wooden bottom. Adult cages (30 × 30 × 30 cm) were used for oviposition only, where leaves of tomato were provided daily as substrate. Adults of *T. absoluta* were fed by 10% sugar solution, while larvae were fed by tomato leaves, cultivated under greenhouse conditions without any insecticide application. The populations were reared in the laboratory at 25 ± 0.5 °C, with a relative humidity of 75 ± 5% and a 12:12 L:D photoperiod.

Potential activity of standard Bt strains was tested on *T. absoluta* by leaf-dip bioassay method (Dakshina and Gary, 2003). Leaves from 2-month-old pot-cultured tomato plant grown in a greenhouse were used for assay. The healthy tomato (PKM 1) leaves (leaf discs of 1.5 cm diameter) were first washed by distilled water containing 0.02% Triton X-100 thoroughly, air-dried and dipped in *Bt* toxin suspension of different strains, whose protein content was previously quantified by Lowry et al. (1951) method. Each leaf disc was dipped for 10 s, allowed to air-dry for a period of 1 h and transferred to clean Petri dishes (6 × 1.5 cm) over a moist filter paper to maintain turgidity of leaves. Single-dose 5-day bioassays with a concentration of 2.5 μg/ml were performed by 10 *T. absoluta* larvae (2nd, 3rd and 4th instars separately). Ten larvae were released per plate on the leaf discs overlaid on filter paper, using a fine camel hair brush. The concentrations of *Bt* strains were prepared separately for 4D1, 4D4 (2.5–15 μg/ml) and 4G1, 4K5 and 4XX4 (2.5–25 μg/ml). Forty larvae per treatment were used and each treatment which replicated with 4 subsets. A treatment without *Bt* protein (treated with 0.02% Tween 20) served as control.

**Data analysis**

Larval mortality was assessed on 3rd, 4th and 5th days of exposure. Larvae were withdrawn carefully from galleries of tomato leaves and disturbed with a fine camel hair brush; they were considered dead if unable to move the length of their body. Bioassays were conducted under completely randomized design in laboratory conditions. Corrected mortality percentages were worked out by using Abbott’s formula (Abott, 1925) and subjected to probit analysis (Finney, 1971) from EPA Probit Analysis Program (version 1.5).

**Results and discussion**

The results of probit regression analysis of concentration-response mortality data for the bioassays of *Bt* strains against *T. absoluta* were recorded. The slope values of different larval instars varied significantly, indicating variability in the susceptibility to *Bt* strains among the larval stages. *T. absoluta* showed variable responses to *Bt* strains as reflected in the LC50 values for 2nd, 3rd and 4th larval instars. *Bt* strains showed toxicity to the 3 larval instars of pinworm. Based on the concentration mortality response to *Bt* strains (4D1, 4D4 and 4G1), LC50 values were 6.10, 6.62 and 8.18 μg/ml for the 2nd instar (Table 1); 9.90, 10.20 and 11.12 μg/ml for the 3rd instar (Table 2); and 19.82, 23.16 and 24.54 μg/ml for the 4th instar (Table 3), respectively. The susceptibilities of different larval instars of tomato pinworm to *Bt* strains were presented in Figs. 1, 2 and 3. At LC50 concentration, 50% mortality was observed on the 3rd day of treatment for 4D1 and 4D4 and 5th day for 4G1 in all the instars tested. Bioassay with 4K5 and 4XX4 strains of *Bt* did not show toxicity against the *T. absoluta*, as there was no difference between the treatment and control. In both control and treatments, larvae fed the same area of leaf tissue (mesophyll) over 5 days. Based on the present study, it is evident that all the 3 larval instars of the pest were susceptible to the *Bt* strains. The results indicated that susceptibility of larvae decreased with larval developmental stage. Variations in susceptibility of tomato pinworm depend on the age of the insect and susceptibility decreased with increase in the age of the insect.

### Table 1 Toxicity of 4D1 to 2nd, 3rd and 4th larval instars of tomato pinworm Tuta absoluta

| Larval stage | Slope | SE* | χ²a | LC₅₀ (μg/ml) | Confidence limits (95%) | LC₉₅b (μg/ml) | Confidence Limits (95%) |
|--------------|-------|-----|-----|-------------|--------------------------|--------------|------------------------|
|              |       |     |     |             | Lower limit | Upper limit | Lower limit | Upper limit |
| 2nd instar   | 2.61  | 0.50| 3.164| 6.10        | 4.23        | 7.65        | 25.93      | 18.54 | 25.76 | 41.43 | 131.94 |
| 3rd instar   | 2.36  | 0.46| 1.99 | 6.62        | 4.70        | 8.30        | 32.74      | 22.00 | 23.16 | 24.54 | 74.86 |
| 4th instar   | 2.33  | 0.52| 0.27 | 8.18        | 5.74        | 10.43       | 41.43      | 25.76 | 19.82 | 23.16 | 24.54 |

*aStandard error

bChi-square
The use of Bt became a vital component in the integrated pest management (IPM), and it has been accepted throughout the world. Already, Bt proved to be the best alternative to the pesticides (Roh et al., 2007 and Gonzalez et al., 2011). Different types of agricultural pests were subtle to Bt toxins, and they are essential to notice novel Bt strains to control T. absoluta. The results of the present study exposed a high mortality of the 3 larval instars of T. absoluta that were fed on Bt treated leaves, having value in developing IPM to control tomato pinworm.

Cry proteins (Cry1, Cry2, and Cry9) were highly toxic and specific for lepidopteran insect pests. Cry toxins active against coleopteran insects were Cry3, Cry7, Cry8 and Cry11a (Crickmore, 2017), and Cry5, Cry6, Cry12, Cry13, Cry14 and Cry21 were highly specific to the Nematodes (Guo et al., 2008). HD1 was known to produce 7 different proteins viz. Cry1Aa, Cry1Ab, Cry1Ac, Cry1D, Cry2Aa, Cry2Ab and Cry9D that were toxic to lepidopteran insects. HD73 and HD8 produce Cry1Ac and Cry9 proteins, respectively, which were also lepidopteran toxic (Nayan et al., 2018). Bt strain 4K5 did not show any mortality on tomato pinworm as it produces Cry3A, which is highly toxic and specific to coleopteran (De Souza et al., 1993). No reports were available on toxicity of Bt 4XX4 against lepidopteran insects, which produce Cry6Aa2, Cry55Aa1 and Cry5Ba2 proteins, and highly specific to nematodes and not toxic to T. absoluta (Manivannan et al., 2019).

Earlier reports by Hernandez et al. (2011) reported that INTA Mo9-5, INTA 7-3 and HD1 were highly effective against T. absoluta with mean LC50 of 8 ppm. Among all the tested Bt isolates (strains), only KG52, KG55 and KG8 showed 100% mortality rate in the 2nd instar of T. absoluta on the 7th day after treatment compared to standard reference strain HD1 (95%) (Gowtham et al., 2018).

Theoduloz et al. (1997) reported Scrobipalpuloides absoluta (currently T. absoluta) was highly susceptible to native Bt strains (121e, 66b, 72a, 104a) and Kurstaki of Chile with LC50 values of 6.1, 18.5, 39.6, 16.4 and 19.2 µg larva-1. Narmen and Hassan (2013) recorded 80 to 93.3% mortality rate of 4th instar larvae produced by Bt strains (B1, B2, B3 and B4), as against 13.3% mortality by B12 isolate and Protecto; a commercial formulation of Bt at 2 g/l concentration showed the highest mortality from 96.7 to 100%. The present finding agrees with the findings of Higuchi et al. (2000), who evaluated the potential of Bt strains (HD1, 84-F-51-46, 93-Y-18-1, 84-F26-3 and 94-F(M)633-2) against Plutella xylostella, (Lepidoptera: Gelechiidae) where the LC50 values recorded 0.21, 2.81, 13.1, 9.85 and 6.52 µg/ml, respectively.

The present findings agree with Mohan et al. (2008), who reported the toxicity of Bt strains (Bt kurstaki HD-1, Bt kurstaki HD-73, Bt aizawai HD-137, Bt tolworthi HD-125, Bt galleriae HD-8 and Bt japonensis T23 001) to the populations of Plutella xylostella (collected from Havelbagh, Darim and Gwaldam). They concluded that Bt HD-1 was highly toxic to P. xylostella for all 3 populations, followed by Bt HD-8 and Bt HD-73. Bt HD-137 and Bt T23 001 were moderately toxic to populations from all the 3 locations. However, Bt HD-125 was non-toxic against diamond back moth. They revealed that the LC50 values for Bt strains (Bt kurstaki HD-1, Bt

### Table 2: Toxicity of 4D4 to 2nd, 3rd and 4th larval instars of tomato pinworm Tuta absoluta

| Larval stage | Slope  | SE*  | χ²b | LC50 (µg/ml) | Confidence limits (95%) | LC95 (µg/ml) | Confidence limits (95%) |
|--------------|--------|------|-----|--------------|-------------------------|--------------|-------------------------|
|              |        |      |     | Lower limit  | Upper limit             | Lower limit  | Upper limit             |
| 2nd instar   | 3.60   | 0.94 | 0.70| 9.90         | 7.40                    | 11.87       | 28.34                   |
| 3rd instar   | 3.04   | 0.78 | 0.62| 10.20        | 7.74                    | 12.51       | 35.38                   |
| 4th instar   | 2.98   | 0.76 | 0.32| 11.12        | 8.79                    | 13.84       | 39.58                   |

*Standard error

### Table 3: Toxicity of 4G1 to 2nd, 3rd and 4th larval instars of tomato pinworm Tuta absoluta

| Larval stage | Slope  | SE*  | χ²b | LC50 (µg/ml) | Confidence limits (95%) | LC95 (µg/ml) | Confidence limits (95%) |
|--------------|--------|------|-----|--------------|-------------------------|--------------|-------------------------|
|              |        |      |     | Lower limit  | Upper limit             | Lower limit  | Upper limit             |
| 2nd instar   | 2.40   | 0.75 | 0.57| 19.82        | 15.64                   | 34.83       | 95.52                   |
| 3rd instar   | 3.47   | 1.42 | 0.50| 23.16        | 18.42                   | 75.15       | 68.98                   |
| 4th instar   | 2.81   | 0.77 | 1.07| 24.54        | 19.99                   | 39.08       | 94.14                   |

*Standard error

*Chi-square
kurstaki HD-73, Bt aizawai HD-137, Bt tolworthi HD-125, Bt galleriae HD-8 and Bt japonensis T23-001) were 0.04, 0.33, 13.30, - , 0.27 and 4.25 mg AI/L; 0.50, 1.13, 7.60, - , 1.17 and 7.91 mg AI/l; and 0.34, 1.71, 4.62, - , 0.43 and 5.11 mg AI/l, respectively, against P. xylostella.

Sabbour and Soliman (2014) evaluated the efficacy of dipel (2×), Bt kurstaki HD-73 and Bt kurstaki HD-234 against T. absoluta larvae under laboratory, greenhouse and field trials. LC 50 values recorded were 140, 109 and 90 μg/ml for dipel, Bt kurstaki HD 73 and Bt kurstaki 234, respectively, under laboratory conditions. They recorded LC 50 of 166, 122 and 102 μg/ml under greenhouse condition for dipel, Bt kurstaki HD 73 and Bt kurstaki 234, respectively. Under field trials, the lowest infestation was recorded in HD 73, followed by HD 234 and dipel, respectively.

Similarly, obtained findings are not in accordance with Azra et al. (2015), who tested the LC 25 and LC 50 values of Bt and Spinosad separately and in combination against 1st, 2nd and 3rd larval instars of T. absoluta. The LC 50 values for Bt treatments were recorded as 2386.75, 2109.97 and 2757.65 μg/ml. For Spinosad, the results were recorded as 1283.91, 1339.86 and 2253.18 ppm, respectively. LC 25 values for Bt and spinosad against 3 larval instars of T. absoluta were 985.44, 1368.20 and 1914.57 ppm and 436.26, 643.78 and 1526.94 ppm, respectively. They concluded that spinosad was more toxic against T. absoluta than Bt. Their results showed that the combination of both spinosad and Bt was very effective against T. absoluta than their individual treatments.

![Fig. 1](image1.png) Mortality of different larval instars of Tuta absoluta caused by 4D1 at LC 50

![Fig. 2](image2.png) Mortality of different larval instars of Tuta absoluta caused by 4D4 at LC 50
In Egypt, Sabbour (2014), recorded LC50 values of 243.9 μg/ml and 211 μg/ml against T. absoluta under laboratory and greenhouse conditions, respectively, for Bt var. kurstaki. Earlier studies have shown that Bt 4XX4 was toxic only to nematodes (De Souza et al., 1993 and Guo et al., 2008 and Manivannan et al., 2019), as that of Cry5, Cry6, Cry12, Cry13, Cry14 and Cry21 (Yu et al., 2015).

**Conclusion**

The present study confirms that Bt proteins are host specific. Specificity makes Bt proteins safer to non-target organisms including predators and parasitoids, which provide pesticide-free tomato yield with fruit quality and safety.

**Abbreviations**

T. absoluta: Tuta absoluta; Bt: Bacillus thuringiensis

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**Authors’ contributions**

BV performed the idea of this article. SKJ and BV wrote the manuscript. JJ and MS contributed the material and helped in the maintenance of Tuta absoluta, while all authors equally did the bioassay experiments. The authors read and approved the final manuscript.

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