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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 LC_{50} 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, LC_{50}, 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

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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 undertaken 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 Na_2CO_3, 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 Horticulture 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. Bioassay was conducted under completely randomized design in laboratory conditions. Corrected mortality percentages were worked out by using Abbott's formula (Abbott, 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 LC_{50} 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), LC_{50} 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 LC_{50} 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[a]	χ^{2b}	LC_{50} (μg/ml)	Confidence limits (95%)		LC_{95}[c] (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	50.77
3rd instar	2.36	0.46	1.99	6.62	4.70	8.30	32.74	22.00	74.86
4th instar	2.33	0.52	0.27	8.18	5.74	10.43	41.43	25.76	131.94

[a]Standard error
[b]Chi-square

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

Larval stage	Slope	SE[a]	χ^{2b}	LC_{50} (μg/ml)	Confidence limits (95%)		LC_{95} (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	20.18	75.64
3rd instar	3.04	0.78	0.62	10.20	7.74	12.51	35.38	23.40	114.97
4th instar	2.98	0.76	0.32	11.12	8.79	13.84	39.58	25.30	144.73

[a]Standard error
[b]Chi-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 Cry1Ia (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 *Bt* strains ZCUJTL11 and ZCUJTL39 showed 3 times higher in biological activity against *T. absoluta* 2nd instar larvae when compared to strain *Bt var. kurstaki* HD1, with LC_{50} values of 2.40, 5.53 and 6.4 μg/ml, respectively. Alejandro et al. (2004) reported that INTA Mo9-5, INTA 7-3 and HD1 were highly effective against *T. absoluta* with mean LC_{50} of 8 ppm. Among all the tested *Bt* isolates (strains), only KGS2, KGS5 and KGS8 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 LC_{50} 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 LC_{50} 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 Hawalbagh, 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 LC_{50} values for *Bt* strains (*Bt kurstaki* HD-1, *Bt*

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

Larval stage	Slope	SE[a]	χ^{2b}	LC_{50} (μg/ml)	Confidence limits (95%)		LC_{95} (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	46.26	1811.28
3rd instar	3.47	1.42	0.50	23.16	18.42	75.15	68.98	36.91	1831.69
4th instar	2.81	0.77	1.07	24.54	19.99	39.08	94.14	51.49	669.80

[a]Standard error
[b]Chi-square

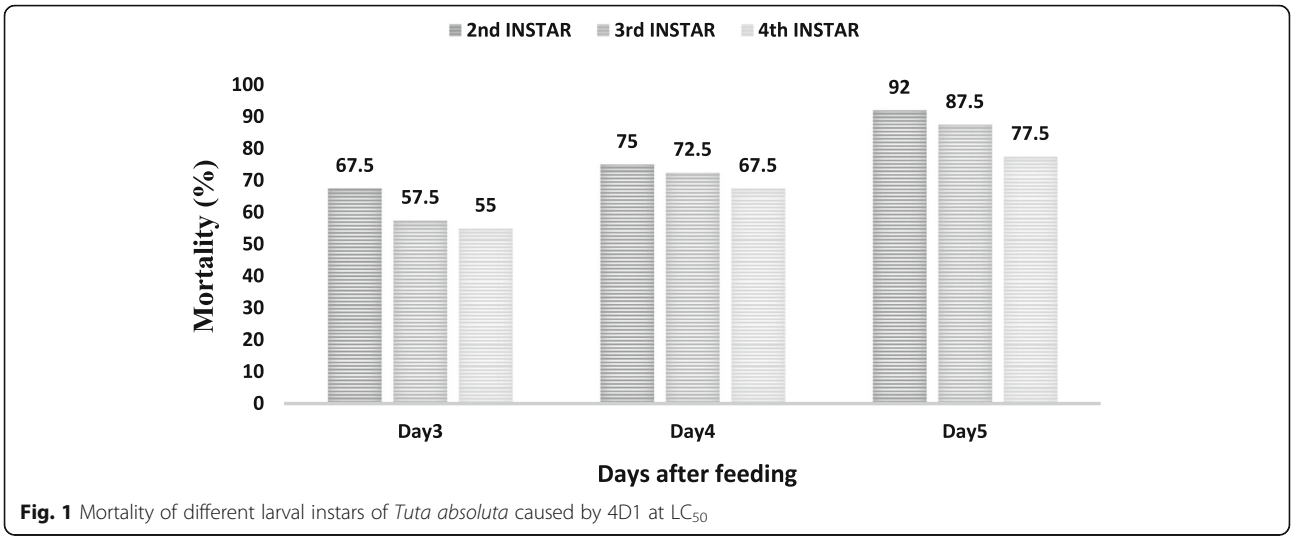

Fig. 1 Mortality of different larval instars of *Tuta absoluta* caused by 4D1 at LC_{50}

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.

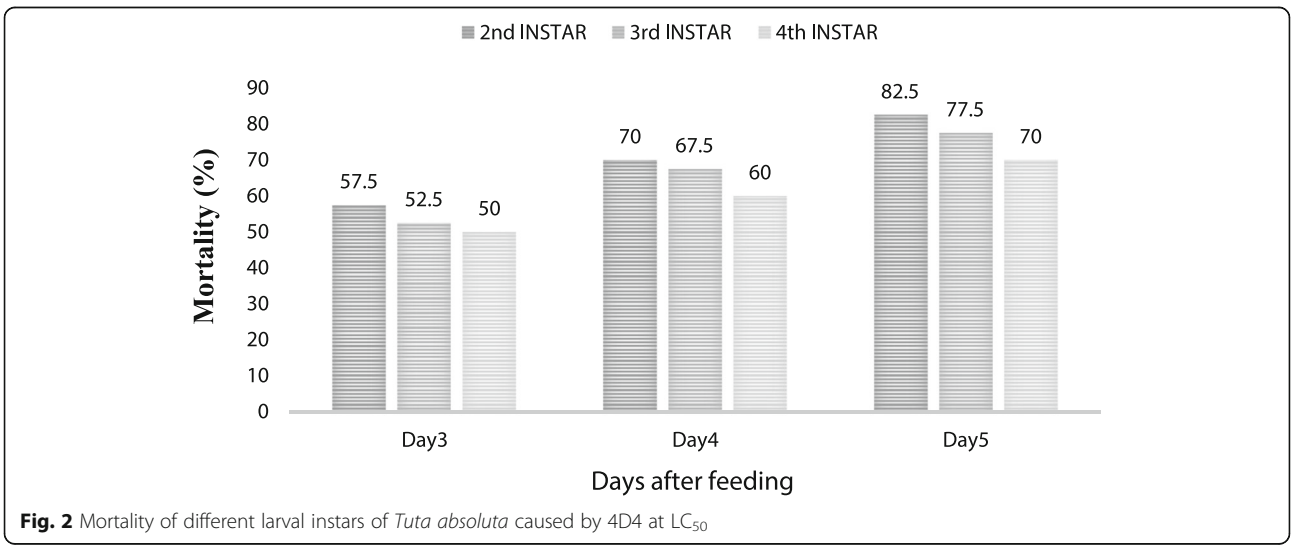

Fig. 2 Mortality of different larval instars of *Tuta absoluta* caused by 4D4 at LC_{50}

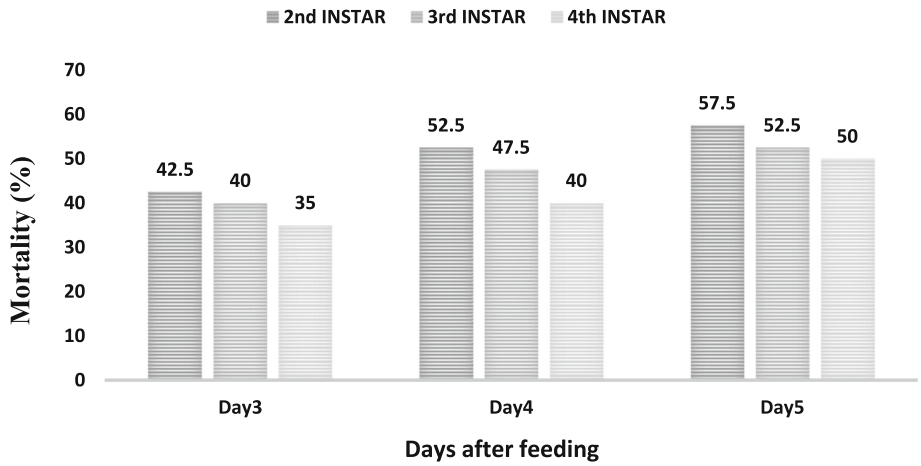

Fig. 3 Mortality of different larval instars of *Tuta absoluta* caused by 4G1 at LC_{50}

In Egypt, Sabbour (2014), recorded LC_{50} 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 participated in writing the manuscript and statistical analysis. MT, SI and SP 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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Competing interests

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