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Toxicity of *Bt* crystal protein Cry55Aa against pest of tomato and model nematode, *Caenorhabditis elegans*

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Abstract
Bioefficacy of Cry55Aa protein was tested against a model nematode, *Caenorhabditis elegans* and tomato pests *viz. Helicoverpa armigera, Spodoptera litura, Tuta absoluta* and *Meloidogyne incognita.* When tested against *Caenorhabditis elegans* in lawn feeding method, 10.00 percent mortality was observed with severe inhibition of growth on surviving nematodes and live worms looked very sick and did not produce progeny. Bioassay on tomato against *H. armigera, S. litura, T. absoluta* showed that the Cry55Aa is not effective against these insects. When the protein was tested against root knot nematode, *Meloidogyne incognita*, a decrease in Gall Index was observed. Besides, significant reduction in number of galls, number of females, number of egg mass and number of eggs per egg mass was also observed.

Keywords: Cry55Aa, bioassay, insect, nematode, tomato

Introduction
Tomato, *Solanum lycopersicum* (*Lycopersicon esculentum* Mill.) is the most important vegetable grown worldwide. Globally, it is the third major vegetable crop, after potato and sweet potato. In India, it is grown in an area of 7.89 lakh ha with a total production of 19.759 million tonnes [1]. The average productivity of tomato crop is low (17.8 q / ha), due to number of production constraints which include occurrence of pest and diseases. Nearly 200 species of pests are reported to infest tomato in fields [2]. Among them, fruit borer (*Helicoverpa armigera* Hubner), tobacco caterpillar (*Spodoptera litura* Fab.), pin worm (*Tuta absoluta* Meyrick) and root knot nematode (*Meloidogyne incognita*) pose serious threat to crop production.

Tomato fruit borer *Helicoverpa armigera* is a polyphagous pest infesting tomato, cotton, chilli, okra, pigeon pea, gram, cabbage, *etc.* [3] and causing yield loss ranging from 20 to 60 per cent. *S. litura* larvae cause 30 to 50 per cent crop loss due to foliage and fruit damage [4]. Tomato pin worm is another key pest in the field and greenhouse where it can cause yield loss up to 100 per cent [5].

The root knot nematode is a sedentary endoparasite which thrives within the tissue and has a vast geographical distribution and forms (root knots). Though 90 species of *Meloidogyne* were identified, *M. incognita, M. javanica, M. hapla* and *M. arenaria* are economically important because they cause yield loss up to 40% [6]. Nematode infected tomato plants exhibit symptoms such as poor growth, yellowing of leaf, wilting, poor root development and yield loss [7].

Through variety of management techniques such as deep ploughing and trap crops, are used to reduce the pest infestations, chemical management is the most widely used method. Farmers apply huge quantities of insecticides to manage insect pests which resulted in pests developing of resistance to insecticides, [8] detrimental pesticide residues in fruits and negative effect on the biodiversity. To overcome these problems, expression of insecticidal protein genes in host plant was successfully demonstrated by many workers [9-11]. Cry toxins are specific to insects and nematodes [12, 13]. About 323 holotype crystal proteins are documented as toxic to insects of different orders *viz.* Lepidoptera, Coleoptera and Diptera and nematodes [14]. These are biodegradable crystal proteins and so referred as environmentally safe alternative to chemical pesticides.

The present study was undertaken to evaluate the toxicity of the protein encoded by cry55Aa which was earlier cloned at our centre, against pest of tomato *viz*. fruit borer, tobacco caterpillar, pin worm, root knot nematode and model nematode, *Caenorhabditis elegans.*

Materials and Methods

Gene construct

The gene *cry55Aa* was originally cloned from an indigenous *Bacillus thuringiensis* isolate T44. The recombinant *Escherichia coli* strain BL21 (DE3) harbouring pET29a vector with *cry55Aa* gene insert (Accession no: HG764207.1) was used in this study. The integrity of the plasmid was confirmed by PCR analysis with gene specific primers and restriction digestion analysis.

Expression of Cry55Aa in *E. coli* cells

Two *E. coli* strains *viz*. BL21 harbouring pET29a with *cry55Aa* gene and pET29a vector without the insert were grown in 5 ml of LB broth containing kanamycin 50 mg/l overnight at 37 ºC. About 250 µl of overnight-grown culture was used for inoculating 25 ml of LB broth with kanamycin 50 mg/l and the culture was incubated at 37 °C shaker till OD_{600} of ~0.6 was reached. In each flask, 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce protein expression and the cells were further grown at 37 °C till the OD_{600} of ~1.3 is reached. Then, cultures were harvested by centrifugation and washed with 1X TE buffer **(**10.0 mM Tris-Cl, 1.0 mM EDTA, pH 8**)**. The pellet was dissolved in 5 ml of TE buffer containing 1 mM PMSF (phenyl methyl sulfonyl fluoride) and sonicated using ultra-sonic liquid processor (Sonics and Material Inc., USA). Sonication was done with off pulsar mode for one min at 20 amplitude for four times with a time interval of one min. The broken cells were pelleted by centrifugation at 7000 rpm for 15 min. The pellet was dissolved in the TE buffer and washed twice in the same buffer. The final product was suspended in 200 µl of 1% SDS and an aliquot of 5 µl was used for separation in SDS-PAGE. This crude protein was used for further studies, after quantification by Lowry's method (1951) [15].

Toxicity analysis of Cry55Aa crude protein against pest of tomato

Toxicity studies on the Cry55Aa protein obtained from the recombinant *E. coli* BL21 (DE3) strain was conducted against *S. litura, H. armigera* and *T. absoluta* by leaf coating and surface-diet contamination method and in *M. incognita* by pot culture assay and in *C. elegans* by lawn feeding method**.**

Culture maintenance

The insect cultures of *S. litura* and *H. armigera which* were procured NBAIR, Bangalore, India and field collected *T. absoluta* were maintained in artificial diet [16] at 27 ºC temperature, 70% relative humidity. Initial *M. incognita* culture was collected from infested tomato fields of Coimbatore, Tamil Nadu and multiplied in potted tomato plants maintained in greenhouse condition. The *C. elegans* N2 strain worms were maintained in laboratory conditions on *E. coli* (OP50) lawns using nematode growth medium, on 50 mm plastic Petri plates.

Surface-diet contamination method

The *in vitro* bioassay against *S. litura, H. armigera* and *T. absoluta* were performed by exposing neonate larvae to semisynthetic diet dispersed into 1.8 ml cryovial (Tarson®; 1cm dia.) smeared with 10 µl crude Cry55Aa protein. One larva per cryovial was used and in total thirty larvae were used in each treatment with seven replications. Simultaneously, BL21 carrying pET29a alone and BL21 cells without vector were used as control. The larval mortality was recorded after five days of exposure.

Leaf coating method

Healthy leaves of uniform size (leaf discs of 1 cm diameter) from tomato plants (PKM1) grown in greenhouse were washed with 0.02% Triton X-100 solution followed by rinsing with distilled water and blot dried. The crude Cry55Aa protein was coated on the upper surface of the leaf. Treated leaf-bits were kept in a Perti dish containing moist filter paper to maintain turgidity of leaves. Five neonate larvae of *S. litura, H. armigera and T. absoluta* were released per plate on the leaf disc overlaid on filter paper using a fine camel hair brush. Thirty larvae per treatment (6 plates) was used and each treatment was replicated seven times. BL21 carrying pET29a without insert and BL21 without vector were used as control. Bioassay was carried out at 24-26 °C. Larval mortality was recorded at 24 h interval and cumulative mortality was recorded on 5th day.

Bioassay against *M. incognita*

Bioassay with *M. incognita* was performed on potted plants. Pots of 10 cm diameter were filled with pot mixture (sterile sand: soil: vermiculite @ 1:1:1) and kept in greenhouse (at 25± 2 ºC). Tomato seedlings cv. PKM1 were transplanted in these pots for further experiments. One week after transplanting 10 ml of lysed and sonicated extract from 10 ml of crude BL21 culture (OD~1.3 at 600nm) was inoculated into each pot. Approximately 500 J2 stage *M. incognita* were inoculated to each pot by pouring the suspension (2–4 cm below the soil surface) around the plants. Two cultures of BL21 were used with water as control. Each treatment was replicated eleven times. Observation were made on number of galls, number of egg mass, number of females in roots and number of eggs per egg mass, on 45th day after initial treatment. Soil has been completely sterilized and discarded after the experiment. Root gall index was determined based on scale (1 to 5), with no gall, 1 to 25, 26 to 50, 51 to 75 and >76 representing Gall Index of 1, 2, 3, 4, and 5 respectively [17].

Toxicity analysis against model nematode, *C. elegans*

For *in vitro* bioassay, 100 µl of overnight grown culture (OD~1.3 at 600 nm) of *E. coli*, BL21 harbouring pET29a+*cy55Aa* and pET29a without insert and *E. coli* OP50 were spread on enriched nematode growth medium and incubated at 37 ºC for 48 hrs. After 48 hrs of incubation, five numbers of L4 stage *C. elegans* were released on each plate and totally thirty nematodes per treatment were used. Experiment was performed for three days at 20 °C. Each treatment was replicated seven times. Observations were made on conditions of worms, their appearance and mortality.

Results and discussions

Bioassay with Cry55Aa protein against insect pests of tomato

Artificial diet assay and leaf coating assay on all three lepidopteran insects showed that Cry55Aa was not effective

against lepidopteran insects as there was no difference between the treatment and control (Table 1). In both control and treatments, equal area of leaf tissue was consumed by the larvae over a period of five days**.** Earlier studies have also shown that Cry55Aa protein was toxic only to nematodes [18, 19], as that of Cry5, Cry6, Cry12, Cry13, Cry14 and Cry21 [19-21]. However, no reports are available on toxicity of Cry55Aa against lepidopteran insects.

Bioassay of Cry55Aa protein against root knot nematode, *M. incognita*

Cry55Aa protein significantly decreased the Gall Index compared to controls. The number of female nematodes in these galls was reduced by 25.45 per cent compared to control. Similarly, number of egg masses present these galls was 42.76 per cent less than the control. The average number of eggs present in egg masses was also significantly less than the control (Table. 2). The number of eggs in each egg mass was reduced by 31.81 per cent over control. The present finding is comparable with the nematode specific Cry14 which reduced *Meloidogyne* infestation in tomato significantly in pot culture experiments [20]. Peng *et al*., [18] have reported that the Cry55Aa was toxic to *hapla* based on a laboratory experiment. Another nematode specific Cry protein, Cry6Aa2 was also shown to inhibit hatching of eggs of root knot nematode *M. hapla* [21]. The **c**ombination of Cry5B, Cry6A with Cry55Aa has synergetic toxicity effect against *M. incognita* [19].

Normally Cry proteins are very sensitive to environmental factors such as chemical, physical and biological factors, which can degrade the Cry protein activity in soil. However, previous studies reported that Cry proteins was easily adsorbed by clay or humic acids in soil due to their opposite charge, remain more stable and possess longer activity [22, 23]. Root knot nematode (RKN) spent most of the life cycle in root. Once they penetrate into plant roots, it is not exposed to Cry55Aa toxin. So, timing of application is vital for effectiveness of biocontrol, and prophylactic soil application of Cry proteins could provide protection from RKNs.

Bioassay of Cry55Aa protein against model nematode, *C. elegans*

In quantitative lawn bioassay with *C. elegans*, L4 worms were fed on lawns of *E. coli* BL21 expressing Cry55Aa and BL21 without insert and *E. coli* OP50 as control. BL21 expressing Cry55Aa showed 10.00 per cent mortality and surviving worms exhibited severe inhibition of growth and development. No progeny was produced by the remaining alive worms, which were looking very sick and pale. There was no mortality of worms fed on lawns of *E. coli* BL21 carrying vector pET29a without insert and on *E. coli* OP50 (Table 3). The present findings are in accordance with previous reports on toxicity of Cry5B protein on growth and progeny development in *C. elegans* [24]**.**

Table 1: Bioassay of Cry55Aa protein against insect pests of tomato

Treatment detail	Per cent mortality on 5th day					
	Leaf coating			Artificial diet		
	H. armigera	*S. litura*	*T. absoluta*	*H. armigera*	*S. litura*	*T. absoluta*
BL21 expressing Cry55Aa protein	8.57 (14.41)	8.57 (15.84)	10.00 (17.00)	7.14 (11.81)	5.71 (9.22)	5.71 (9.22)
BL21 carrying pET29a without insert	5.71 (9.22)	8.57 (14.41)	8.57 (15.84)	5.71 (9.22)	7.14 (11.81)	7.14 (13.25)
BL21 without pET29a vector	8.57 (14.41)	10.00 (17.00)	8.57 (14.41)	8.57 (14.41)	7.14 (13.25)	8.57 (14.41)
CD (P=0.5)	NS	NS	NS	NS	NS	NS

Mean of seven replications. Values in parentheses are arcsine transformed.

Table 2: Effect of Cry55Aa on *Meloidogyne incognita* infestation on tomato (PKM1)

Treatment	Number of galls	PIC*	Gall Index	Number of egg mass	PIC	Number of females	PIC	No. Eggs/egg mass	PIC
BL21 expressing Cry55Aa protein	41.92 (6.68)^{b}	26.35	3	19.46 (4.40)^{b}	42.76	71.90 (8.45)^{b}	25.45	145.45 (12.04)^{b}	31.81
BL21 carrying empty vector pET29a	56.92 (7.74)^{a}	0.17	4	34.00 (5.91)^{a}	1.62	96.45 (9.81)^{a}	0.90	213.33 (14.59)^{a}	2.64
Water	57.02 (7.81)^{a}		4	34.56 (5.95)^{a}		97.33 (9.83)^{a}		219.13 (14.74)^{a}	
CD (P=0.5)	0.38			0.37		0.39		0.52	

*PIC: Per cent inhibition over control. Mean of eleven replications. Values in parentheses are arcsine transformed. Means in a column followed by same superscripts are not significantly different at $P \leq 0.05$

Table 3: Bioassay of Cry55Aa protein against *C. elegans* by lawn feeding method

Treatment detail	Per cent mortality	Percentage of sick worms	Percentage healthy worms
BL21 expressing Cry55Aa protein	10.00 (14.41)^{a}	61.42 (51.29)^{a}	28.57 (31.83)^{b}
BL21 carrying empty vector pET29a	0.00 (4.04)^{b}	0.00 (0.28)^{b}	100 (89.71)^{a}
E. coli OP50	0.00 (0.28)^{b}	0.00 (0.28)^{b}	100 (89.71)^{a}
CD (P=0.5)	9.18	2.65	5.21

Mean of seven replications. Values in parentheses are arcsine transformed. Means in a column followed by same superscripts are not significantly different at $P \leq 0.05$

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Conclusion

The results presented here demonstrate that the Cry55Aa is toxic to the nematode, *C. elegans* and plant parasitic nematode, *M. incognita*. However, this toxic protein is not effective against Lepidopteran pests of tomato

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