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Molecular Characterization and Nematicidal Activity of Indigenous *Bacillus thuringiensis* isolate T210

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ABSTRACT: *Bacillus thuringiensis* (*Bt*), renowned for its insecticidal properties, has emerged as a promising solution in pest management, addressing concerns over environmental impact, regulatory restrictions, and insect resistance to synthetic insecticides. This study focuses on the indigenous *Bt* isolate T210, which exhibits nematicidal activity against the model nematode *Caenorhabditis elegans*. The investigation begins with a comprehensive characterization of native *Bt* isolate T210, including colony morphology, protein profiling and toxicity analysis. T210 exhibited creamy white colour colonies with an irregular shape, fried egg-like surface pattern, flat elevation, and undulated margins. Microscopic examination reveals presence of bipyramidal, cuboidal, and spherical-shaped crystals in T210. Protein profiling through SDS-PAGE analysis highlights diverse protein bands with molecular weights around 135 kDa, 115 kDa, 50 kDa, 46 kDa, and 42 kDa. These findings suggest the presence of various toxic proteins in the *Bt* isolate T210, potentially contributing to its nematicidal activity. PCR screening for major insecticidal genes, including various *cry* and *vip* genes, yields negative results, indicating the absence of insecticidal genes screened in T210. This suggests the presence of novel genes with unique properties. The nematicidal activity of T210 is evaluated through *in vitro* bioassays, revealing 100 % larval mortality in *C. elegans* at 500 µg/ml of crude protein. Probit analysis of crude protein from T210, against *C. elegans* revealed the LC50 value of 323.79 µg/ml.

Keywords: *Bt* isolate, *Caenorhabditis elegans,* Molecular characterization, Nematode, T210.

INTRODUCTION

Bacillus thuringiensis (*Bt*), known for its insecticidal properties, has been successfully employed in pest management for years (Raymond *et al*., 2010). Due to environmental concerns, current regulations, and insect resistance to synthetic insecticides, alternative methods for pest management are gaining popularity. *Bt* offers a solution for these challenges in pest management. *Bt* is a Gram-positive microorganism found in soil, insect cadavers, plants, and aquatic environments (Lambert *et al.,* 1992; Bravo, 1998; Gupta *et al.,* 2021; Pawar *et al.,* 2022a). Its toxicity primarily stems from the production of crystal (Cry) and cytolytic (Cyt) proteins encoded by *cry* and *cyt* genes, along with vegetative insecticidal proteins (*vip*) produced during the growth or sporulation phase of *Bt* (Schnepf *et al*., 1998; Pawar *et al*., 2022b). Additionally, *Bt* also produces Secreted Insecticidal Protein (Sip) toxins (Syed *et al*., 2020). Cry proteins, a type of bacterial endotoxin, are classified into 16 distinct classes, with Cry, Cyt, and Vip3 being the three retaining their original names as *Bt* toxins. The Cyt and Vip3 classes maintain their original names. The Cry class retains the traditional 3-domain structure, encompassing proteins with or without extended C-termini, and variations containing additional regions like beta-trefoil domains (Crickmore *et al*., 2021).

An analysis of *B. thuringiensis* genomic sequences, specifically YBT-1518, has unveiled the presence of numerous potential virulence factors containing M4 motifs. Notably, within this strain, a novel mosaic structured protease named Bmp1 has been identified. Intriguingly, M4 motif exhibits a low degree of similarity to other sequences that have been published previously. Furthermore, it has been observed that Bmp1 is not only toxic to *Caenorhabditis elegans* but also that its ability to degrade intestinal tissue enhances the toxicity of the crystal protein Cry5Ba against *C.*

elegans. This discovery suggests a potentially unique mechanism for enhancing toxicity in nematode system (Luo *et al*., 2013).

Various plant pathogens, including fungi, bacteria, viruses, and nematodes, give rise to diverse plant diseases, leading to reduced crop yields worldwide (Ab Rahman *et al*., 2018; Ali *et al*., 2022). Plant-parasitic nematodes represent significant pests, causing agricultural losses of approximately USD 157 billion annually on a global scale (Jin *et al*., 2018). *Aphelenchoides besseyi* is a destructive plant parasitic nematode causing significant damage to crops, particularly rice (Cheng *et al*., 2013). It spreads *via* infected seeds, causing rice white tip disease and yield losses ranging from 10 to 50 % in heavily infested areas (Wang *et al*., 2014; Lubis *et al.,* 2020). Similarly, *Meloidogyne* spp. are also considered as highly destructive plant-parasitic nematodes (PPNs) that inflict significant economic harm on agricultural and horticultural crops (Yang *et al*., 2021). Within the genus, there are 98 species, with *M. incognita* being particularly notable for inducing root galls in nearly all vascular plants (Jones *et al*., 2013). *C.elegans* is a tiny, free-living nematode known for its bacterivorous lifestyle. It has been selected as a model organism in the study of "small metazoans" with the aim of gaining insights into development and as a potentially valuable system for understanding parasitic nematodes. This choice is rooted in the belief that by studying this relatively simple organism, researchers can uncover fundamental principles of biology that may have broader applications, including in the field of parasitic nematology (Burglin *et al*., 1998).

In our study, we conducted a molecular and morphological analysis of an indigenous *Bt* isolate T210 toxic to nematodes. This finding not only advances our understanding of nematicidal proteins but also lays the foundation for future investigations into the isolate's potential nematicidal effects against diverse agricultural parasitic nematodes. This research contributes valuable insights to the field and paves the way for further in-depth analyses of its nematicidal properties.

MATERIALS AND METHODS

Managing Nematode Cultures: Acquiring and Maintenance Protocols

The nematode model organism, *C. elegans* N2 wild strain were obtained from the Department of Zoology at Bharathiyar University, Coimbatore, Tamil Nadu, India. Nematode culture was maintained on NGM (Nematode Growing Media) agar plates (Brenner, 1974) and stored at a temperature of 24 ±1°C (Maniatis *et al*., 1982). The culture plates were sub cultured regularly with seven days interval using the agar chunk method (a piece of agar from NGM plates which having ~100 no's of nematodes). *C. elegans* is cultivated monoxenically in laboratory settings, using the *E. coli* strain OP50 as its food source. To establish the *E. coli* OP50 culture, a starter culture was derived from glycerol stocks available at Department of Plant Biotechnology, Centre for plant molecular biology and biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The OP50 *E. coli* culture was grown on LB agar media, composed of 10 g Bacto-tryptone, 5 g Bacto-yeast, 10 g NaCl, 20 g agar, and water to make up to 1 litre with a pH of 6.8-7.0. OP50 culture was initiated in 5 ml of LB broth from the purified single colony and incubated in 37ºC for overnight with 180 rpm. The resulting *E. coli* OP50 culture was then ready to be used for seeding NGM plates. To seed NGM plates, the *E. coli* OP50 grown cultures were added using the pour plate method, with the inoculation of 100 µl culture and spread using a glass rod and allowed the bacterial lawn to grow overnight at 37°C (Stiernagle and Theresa 1999).

***Bt* culture.** The native *Bt* isolate, T210, along with the standard *Bt* strains YBT-1518 and HD1, were sourced from the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The bacterial cultures were revived and maintained on T3 agar media.

Morphological characterization of *Bt* isolate. The visual observations were carried out to examine morphological characters of *Bt* isolate T210 by observing colony colour, surface texture, and margin appearance. The colonies were observed on T3 agar plates that had been grown at 30°C for 24 hours. For the detailed study of parasporal crystalline inclusions, culture smears were prepared on a glass slide, heat-fixed and stained with 0.133% Coomassie brilliant blue G250 stain for 1 minute. Subsequently, the stained glass-slides were gently rinsed with running tap water, and blot dried. The stained glass-slides were observed under bright field microscopy at 1000x magnification (iScope, Euromex).

Culture condition and protein isolation for toxicity analysis. Spore crystal mixtures were isolated from T210, YBT-1518, and HD1 using established protocols (Ramalakshmi and Udayasuriyan 2010). Briefly, a single colony from each *Bt* culture was selected and inoculated into test tubes containing 5 ml of T3 broth and incubated overnight at 30 °C with 200 rpm in a shaking incubator (Orbitek, Scigenics Biotech). From the overnight grown cultures, 1 % inoculum (250 µl) was transferred to 25 ml of T3 broth in a 250 ml conical flask and incubated at 30°C with 200 rpm for 48 hours. After 48 hours, once more than 90% of the cells were observed to be lysed, the culture was subjected to centrifugation at 4°C for 15 minutes at 7500 rpm in a sterile 50 ml falcon tube to pellet the lysed cultures. The resulting pellet was then re-suspended in 500 µl of Sterile Distilled water with 1mM phenylmethyl sulfonyl fluoride (PMSF). The suspended mixture was transferred to 1.5 ml tubes and stored at -20°C for further use.

Protein profiling. Protein profiling was determined using spore crystal mixtures derived from T210, YBT-1518, and HD1 by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) following the method of Laemmli (1970). The SDS-PAGE analysis was carried out using 10% separating gel and 4% stacking gel composition. To prepare the protein

samples for SDS-PAGE, spore-crystal mixtures were mixed at a 2:1 ratio with 2X loading buffer (0.25M Tris HCl (pH 6.8), 8% SDS, 40% glycerol, and 0.5 bromophenol blue). Prior to loading, the samples were boiled for 2 minutes in the water bath and loaded into the well. To estimate the molecular weight of the protein, a three-color pre-stained protein marker ranging from 10 to 315 kDa (PG-PMT 2962, Genetix Biotech Asia Pvt Ltd) was used.

PCR screening of *cry, cyt* and *vip* genes. The bacterial genomic DNA was extracted from the overnight grown *Bt* cultures following the method of Sambrook and Russell (2001). The concentration of the isolated DNA was quantified using a Nano-Drop Spectrometer (Genova Nano, Jenway), while agarose gel electrophoresis (1%) was performed to evaluate the integrity of the DNA. For PCR amplification, each PCR mixture comprised of 1 µl (30 ng) of template DNA, 1µl of each primer (10 pmol), and 10 µl of 2X PCR Master Mix (TaKaRa; EmeraldAmp GT PCR Master Mix), containing dNTPs, Taq polymerase, and PCR buffer. The total volume was adjusted to 20 µl using sterile double-distilled water. PCR amplification was done in a thermal cycler (Mastercycler® nexus GX2, Eppendorf) with temperature cycles specified in Table 1. The amplified products were resolved in agarose gel with EtBr staining and visualized using a gel documentation system (Bio-Print imaging device, Vilber, France).

Synchronizing of nematodes for bioassay. Synchronization is done to ensure the availability of uniform *C. elegans* life stage in subsequent experiments. To synchronize *C. elegans* to get same life stage, gravid adults were placed on 90-mm NGM plates and subjected to an extensive washing procedure (Lewis *et al*., 1995). The plates were washed multiple times with 2 ml of sterile water in microfuge at 5000 rpm for 1 minute. After removing the supernatant, the settled worms were washed with additional 2 ml of water, followed by another centrifugation step. The supernatant was carefully removed using a pipette. Subsequently, 700 µl of sterile water, 200 µl of 5% bleach, and 100 µl of 5N NaOH were added to the tubes. The tubes were vortexed for 4 minutes, with intermittent examination under a Stereomicroscope every minute to monitor the extent of lysis. Once the complete lysis of the worms and egg release were achieved, 1 ml of M9 buffer was added to stop the bleaching process. Centrifugation at 8000 rpm for 3 minutes was performed, followed by careful removal of the supernatant. The washing procedure was repeated 5 times, adding 1 ml of M9 buffer each time. Subsequently, the mixture of pellet with M9 buffer was divided into aliquots and placed on a 35 mm NGM agar plates. Seeded NGM agar plates were incubated for 12 hours to facilitate hatching.

Bioassay and quantification of *Bt* protein. The protein concentration of the spore-crystal mixtures was estimated using Bradford protein assay with Bovine serum albumin (BSA) as standard (Bradford *et al*., 1976). For bioassay, 24-well plates were used. Each well was comprised of 40 µl of *E. coli* OP50 with an OD_{600} of ~1.3, along with 140 µl of S medium and 30 µl of crude protein. The preliminary bioassay was conducted using crude protein at 500 µg/ml. Different concentrations of crude protein ranging from 200, 400, 600, 800 and 1000 µg/ml for T210 and 40, 60, 80, 100 and 120 µg/ml for YBT-1518 was used to analyse the LC_{50} against *C. elegans*. Ten L4 stage *C. elegans* nematodes were carefully selected and released into each well using a platinum pick. To inhibit bacterial growth, 1 µl of chloramphenicol (10 mg/ml) was added, and the final volume was adjusted to 400 µl using S medium. The entire setup was maintained at a temperature of 20°C. Each treatment was replicated three times, and control was concurrently maintained. Worm viability was assessed based on their movement under a brightfield microscope (iScope, Euromex). Moving worms were classified as live worms. In cases where worms were immobile, a gentle touch with a platinum pick was done to evaluate their responsiveness. Worms that remained motionless after three to four touches were considered deceased. These observations were conducted on the third day to establish the LC_{50} value, providing insights into the toxicity of T210 against the L4 stage of *C. elegans* nematodes.

Statistical analysis. Statistical analysis was done to calculate the LC_{50} and LC_{95} values for each treatment, accompanied by the determination of standard error values. The mean percentage of larval mortality data was arc sine transformed prior to analysis. The LC_{50} value was determined using the 'Probit analysis in Excel' method developed by Srinivasan (2004).

RESULTS AND DISCUSSION

The molecular characterization of the Indigenous *Bt* isolate T210 holds significant promise for discovering novel genes with enhanced pesticidal efficacy. This achievement marks a substantial milestone in advancing pest management within agriculture. In this investigation, the native *Bt* isolate T210 underwent a comprehensive analysis. This analysis involved characterizing toxin genes, and assessing toxicity against the model nematode *C. elegans*.

Morphological characterization of *Bt* isolate. Initial identification involved studying colony morphology, with the presence of crystalline protein inclusions observed during sporulation. *Bt* isolate T210 was found to be creamy white in colour with fried egg-like surface pattern. The colony morphology of *Bt* typically exhibits creamy white colonies with an irregular shape, uneven margin and flat elevation (Maheesha *et al*., 2021; Karuppaiyan *et al*., 2022). The colonies exhibited a flat elevation with undulated margin. Under phase contrast microscope, T210 displayed bipyramidal, cuboidal and spherical shaped crystals. These findings were in accordance with the earlier report by Yamamoto *et al.* (1993).

Profiling of *Bt* proteins. The SDS-PAGE analysis of spore crystal mixtures derived from T210 revealed banding patterns with molecular weights approximately around ~135 kDa, ~115 kDa, ~50 kDa, ~46 kDa, and ~42 kDa. While standard strain YBT-1518 produced

different protein bands at 54 kDa and 45 kDa, whereas HD1 produced at 135 kDa and 65 kDa (Fig. 1). Notably, the ~130 kDa protein band was correlated with Cry14 (132 kDa) as per findings of (Kahn *et al*., 2021). The presence of protein bands revealed the possibility of corresponding genes presence in T210 based on earlier reports such as Vip2 (~45 kDa) (Syed *et al*., 2020), Spp1 (~50 kDa) (Nishiwaki *et al*., 2007), Cry35 (~44 kDa) (Schnepf *et al*., 2005), and Cry21 (130 kDa) (Iatsenko *et al*., 2014). Both Cry14 and Cry21 proteins demonstrated nematocidal activity (Kotze *et al.*, 2005).

PCR screening analysis. The *Bt* isolate T210 did not show any amplification with the gene specific primers for *cry1*, *cry2*, *cry3*, *cry4*, *cry9*, *cry10*, *cry11*, *cyt1*, *vip1*, *vip2*, and *vip3*. These findings collectively imply the absence of lepidopteran, dipteran, and coleopteran toxic genes in the indigenous *Bt* isolate T210. The absence of these specific genes in T210 suggests distinct properties in comparison to the other isolates, making it a valuable subject for further investigation and analysis. Based on the earlier findings, presence of protein bands at ~135 kDa indicated the presence of Cry14 or Cry21 or both. The research revealed that toxic proteins like Cry14A and Cry21A may present in T210 which displayed nematocidal activity, with Cry14A being the most potent against *C. elegans* (Wei *et al*., 2003).

Nematicidal activity of T210 against *Caenorhabditis elegans*. As the crude protein isolated from T210 did not show any mortality in four species of lepidopteran insects *Spodoptera frugiperda*, *S. litura*, *Helicoverpa armigera*, and *Plutella xylostella*, (data not shown). Toxicity assay was performed on the model organism *C. elegans* to evaluate T210's effectiveness against nematodes. In the preliminary assay with crude protein of T210, 100 per cent *C. elegans* worm mortality was observed at 500 µg/ml. While, the negative control didn't show any worm mortality after 72 hr and also had a greater number of *C. elegans* progenies (Fig. 2, 3). For the toxicity analysis of T210 against *C. elegans*, the probit analysis was conducted at varying concentrations of spore crystal mixtures (200, 400, 600, 800 and 1000 µg/ml) and compared with standard isolate YBT-1518, which harbours three nematicidal crystal protein genes (*cry55Aa1*, *cry6Aa2*, and *cry5Ba2*) and multiple potential virulence factors (Wang *et al*., 2014). Probit analysis revealed that the LC_{50} value of T210 was 323.79 µg/ml, with fiducial limits ranging from 213.04 to 492.11 µg/ml. In comparison, the reference strain YBT-1518 exhibited a much lower LC_{50} of 42.40 µg/ml, with fiducial limits ranging from 31.10 to 57.80 µg/ml (Table 2; Fig. 4).

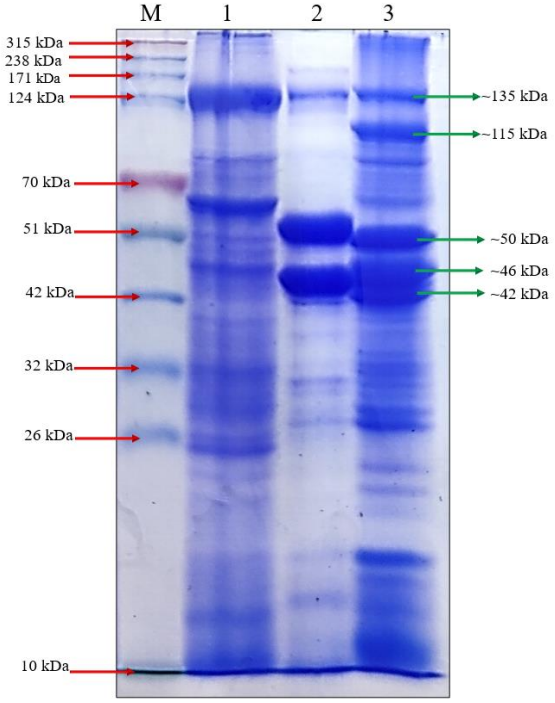


Fig. 1. Protein profile of *Bacillus thuringiensis* isolate T210. Lane M- protein ladder, Lane 1- HD1, Lane 2- YBT-1518, Lane 3- T210.

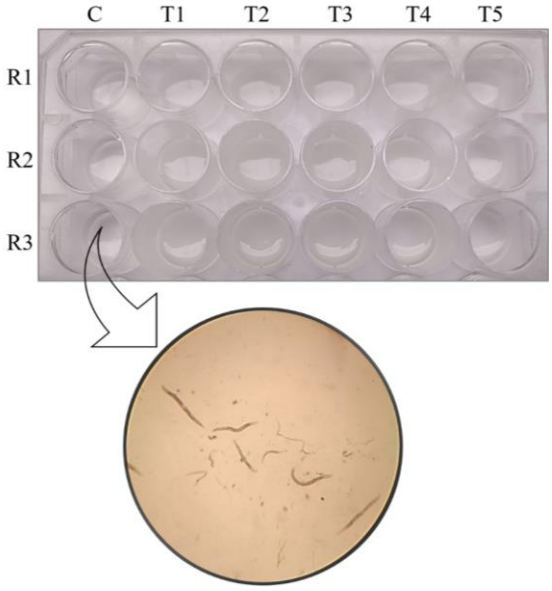
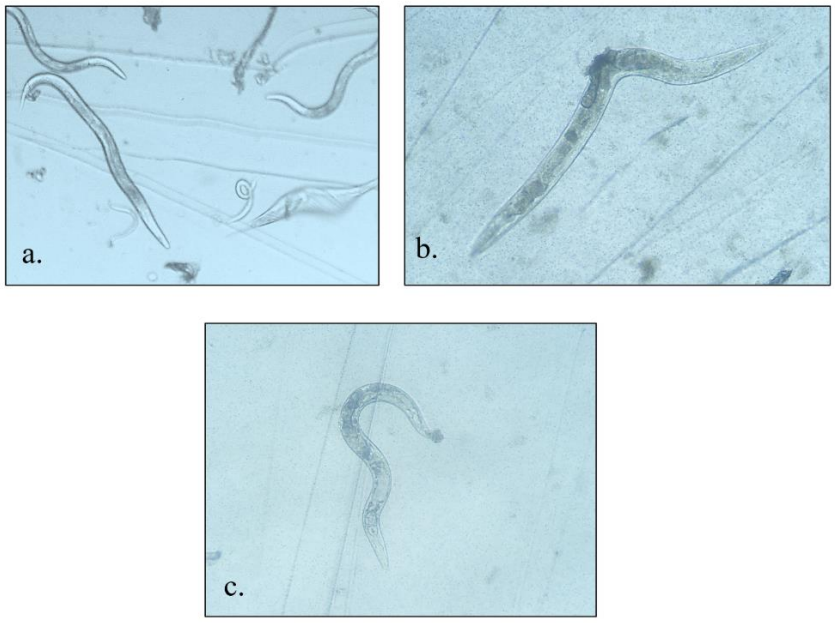


Fig. 2. *In vitro* bioassay with spore-crystal mixtures of *Bt* isolates against model nematode *C. elegans*, (Well, C – Control; Well, T1 to T5: 200, 400, 600, 800 and 1000 µg/ml of T210).



a. Negative control (BSA- 1 mg/ml); b. Postive control (YBT-1518- 250µg/ml); c. – Indigenous *Bt* isolate T210 (500µg/ml)

Fig. 3. Nematicidal activity of *Bacillus thuringiensis* isolate T210 against *C. elegans.*

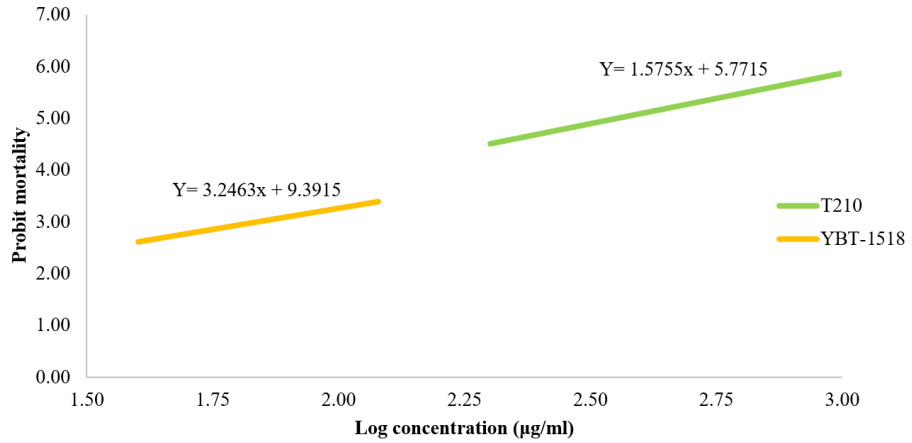


Fig. 4. Probit analysis of *Bt* isolatesYBT-1518 and T210 for toxicity against *C. elegans.*

Table 1: Primers and PCR conditions used.

Target genes	Primer sequence (5' -3')	Product size	PCR conditions
cry1	FP: CATGATTCATGCGGCAGATAAAC RP: TTGTGACACTTCTGCTTCCCATT	276bp	94ºC for 2 min; 94ºC for 40 s; 62ºC for 40 s; 72ºC for 1 min; 72ºC for 7 min; 30 cycles
cry2	FP: GTTATTCTTAATGCAGATGAATGGG RP: CGGATAAAATAATCTGGGAAATAGT	690bp	94ºC for 2 min; 94ºC for 1 min; 60ºC for 40 s; 72ºC for 40 s; 72ºC for 10 min; 35 cycles
cry3	FP: CGTTATCGCAGAGAGATGACATTAAC RP: CATCTGTTGTTTCTGGACGCAAT	612bp	94ºC for 2 min; 94ºC for 45 s; 52ºC for 45 s; 72ºC for 45 s; 72ºC for 10 min; 30 cycles
cry4Aa	FP: GAACTGGGTATGGCACTCAAC RP: CTCACAACGATTAGACCCTTC	777bp	94ºC for 2 min; 94ºC for 1 min; 60ºC for 45 s; 72ºC for 45 s; 72ºC for 10 min; 35 cycles
cry4Ba	FP: GCGAGGTTTCCCATGTCTAC RP: GTTGTAGGGTGGAATTGTTATC	347bp	94ºC for 2 min; 94ºC for 1 min; 57ºC for 45 s; 72ºC for 1 min; 72ºC for 10 min; 35 cycles
cry8	FP: ATGAGTCCAAATAATCTAAATG RP: TTTGATTAATGAGTTCTTCCACTCG	370bp	94ºC for 2 min; 94ºC for 40 s; 49ºC for 40 s; 72ºC for 40 s; 72ºC for 10

			min; 30 cycles
cry9	FP: CGGTGTTACTATTAGCGAGGGCGG RP: GTTTGAGCCGCTTCACAGCAATCC	402bp	59ºC for 40 s; 61.5ºC for 40 s; 72ºC for 1 min; 72ºC for 7 min; 72ºC for 1 min; 25 cycles
cry10Aa	FP: ATTGTTGGAGTTAGTGCAGG RP: AATACTTTGGATGTGTCTTGAG	995bp	94ºC for 5 min; 94ºC for 45 s; 60ºC for 45 s; 72ºC for 50 s; 72ºC for 10 min; 38cycles
cry11Ba	FP: TACAGGATGGATAGGGAATGG RP: TAATACTGCCATCTGTTGCTTG	608bp	94ºC for 5 min; 94ºC for 45 s; 60ºC for 45 s; 72ºC for 50 s; 72ºC for 10 min; 38 cycles
vip1	FP: GGATCCGATGAAAAATATGAAGAA RP: GTCGACTTATCTAGATTTGTTAGGT	2.3 kb	94ºC for 5 min; 94ºC for 1 min; 58ºC for 1 min; 72ºC for 2.5 min 72ºC for 10 min; 35 cycles
vip2	FP: GGATCCGATGAAAAGAATGGAGGG RP: GTCGACTTAATTTGTTAATAATGTTG	1.3 kb	94ºC for 5 min; 94ºC for 1 min; 57ºC for 1 min; 72ºC for 1.5 min; 72ºC for 10 min; 35 cycles
vip3	FP: ATGAACAAGAATAATACTAAATTAAGC RP: TTACTTAATAGAGACATCGTAAAAA	2.3 kb	94ºC for 5 min; 94ºC for 1 min; 55ºC for 1 min; 72ºC for 2.5 min; 72ºC for 10 min; 35 cycles
cyt1	FP: AACCCCTCAATCAACAGCAAGG RP: GGTACACAATACATAACGCCACC	520bp	94ºC for 2 min; 94ºC for 45 s; 52ºC for 45 s; 72ºC for 45 s; 72ºC for 10 min; 35 cycles

Table 2: Toxicity of indigenous *Bacillus thuringiensis* isolate T210 against *C. elegans.*

Bacterial isolate/strain	Regression equation	χ^2	LC50 (µg/ml)	Confidence limits (50%)		LC95 (µg/ml)	Confidence limits (95%)	
				LL	UL		LL	UL
T210	Y= 1.5755x + 5.7715	3.178	323.7	213.04	492.11	4240.8	1189.68	15116.61
YBT-1518	Y= 3.2463x + 9.3915	4.796	42.4	31.10	57.80	171.7	103.09	285.96

This demonstrates that T210's toxicity is notably seven-fold lower than that of reference strain YBT-1518, showcasing the differences in their biological effects and potency. This demonstrated YBT-1518's superiority over indigenous *Bt* isolate T210 and confirmed T210's nematocidal activity. Efficacy of crude protein against the model nematode *C. elegans* would be an appropriate avenue for further studies on parasitic nematodes. Further studies on T210, such as whole genome sequence analysis and expression of toxic proteins, would provide a better understanding of the presence of toxic genes and proteins within this indigenous *Bt* isolate. This could potentially lead to the discovery of a new novel nematicidal toxic gene, contributing to the protection of millions of agricultural crops.

CONCLUSIONS

The presence of diversified proteins and their corresponding genes in T210 opens the door to further research into their potential for protecting agricultural crops against crop pests. *In vitro* bioassay study confirmed the toxicity potential of the *Bt* isolate T210 against nematodes, however discovering the new nematicidal toxic genes present in T210 is a compelling avenue for future exploration. Comprehensive analyses, such as whole genome sequencing and toxic protein expression, could provide invaluable insights into the protection of crops and, ultimately, benefit agriculture on a large scale.

FUTURE SCOPE

Further studies on this nematode toxic *Bt* isolate (T210), such as whole genome sequence analysis, expression of toxic proteins, toxicity evaluation against plant parasitic nematodes would provide a better understanding of the presence of novel toxic genes and proteins within this indigenous *Bt* isolate. This could potentially lead to the discovery of a new novel nematicidal toxic gene, contributing to the protection of millions of agricultural and horticultural crops.

Author contributions. Conceived and designed the analysis – CB, VB, and GR; Collected the data – CB; Contributed data or analysis tools – RR,MJ, EK, MM; Performed the analysis – CB, VB and GR; Manuscript writing and correction – CB, GR, VB.

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Conflict of Interest. None.

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