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Nematicidal activity of native *Bacillus thuringiensis* against the root knot nematode, *Meloidogyne incognita* (Kofoid and White)

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Abstract

In this study, potential of the native *Bacillus thuringiensis* Berliner was investigated against the root knot nematode, *Meloidogyne incognita* (Kofoid and White) under laboratory and greenhouse conditions. Fourteen out of 50 *Bt* isolates obtained from root zone of vegetable crops of north western zone of Tamil Nadu, India, were found to be encoded with nematicidal *cry* genes, confirmed by gene-specific PCR. Nine different Cry protein profiles were obtained from native *Bt*. Spore-crystal mixtures of 6 isolates out of 14 (at 0.5%) showed 100% inhibition to J2 juveniles emergence from egg masses of *M. incognita* within 72 h of treatment. Analysis by SEM revealed that fluffy egg masses were observed in untreated samples due to hatching, whereas egg masses were covered by gelatinous matrix, when treated with spore-crystal mixture of nematicidal *Bt*. Two (BC and BD) of the 6 isolates were selected based on their highest nematicidal activity against J2 of *M. incognita* with LC_{50} values of 0.12 and 0.23 μg/ml of protein. The spore-crystal mixtures of isolates BC and BD when applied to tomato plants under greenhouse conditions exhibited the enhanced biocontrol potential by suppressing number of egg masses, reduction of female population and decreased root gall index, when compared to control and chemical treatments.

Keywords: *Bacillus thuringiensis*, nematicidal *cry* genes, *Meloidogyne incognita*, Biological control

Background

Meloidogyne spp. are the most devastating plant-parasitic nematodes (PPNs) causing serious economic damage to agricultural and horticultural crops. The genus has 98 species among which *M. incognita* causes root galls in almost all vascular plants (Jones et al. 2013). *Meloidogyne* spp. are very difficult to control owing to their quick generation time, high reproduction rate, entophytic and sedentary nature (Engelbrecht et al. 2018).

The uses of chemical nematicides are under scrutiny now because of growing awareness of their harmful effects on soil, water, environment and non-targeted organisms. Besides, *Meloidogyne* spp. have developed resistance to many of these nematicides. Hence, there is a lot of emphasis on finding a sustainable alternative to chemical nematicides for the control of *Meloidogyne* spp.

Biological control as an environmental friendly alternative in integrated pest management strategy is gaining momentum recently (Pocurull et al. 2020; Radwan et al. 2012; Siddiqui and Futai 2009). *Bacillus thuringiensis* (*Bt*), a gram positive soil bacterium possessing nematicidal crystal proteins, is being used widely to control PPNs. For instance, *Bt*CR371 was evaluated and found effective against a model nematode, *Caenorhabditis elegans* under in vitro and in vivo conditions (Zuckerman and Dicklow, 1993). Mohammed et al. (2008) studied the biocontrol efficacy of *Bt* against *M. incognita* and

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observed a reduction of egg masses and number of eggs over untreated samples. *Bt*DB27 produced 2 novel protoxins namely, Cry21Fa1 and Cry21Ha1, which exhibited synergistic action towards *Caenorhabditis elegans* (Iatsenko et al. 2014). Scientists also explained the mechanism of nematode control by *Cry* proteins. The crystal proteins of different molecular weights are known to enter via stylet of nematodes, cause pores in the esophagus and thereby kill them. Naturally isolated *Bt* often acts as an effective location-specific control agent. The reason might be the bacterium and the nematode might coevolved in that particular ecosystem. The toxicity of location-specific *Bt* is always found to be effective and more novel proteins can be expected out of them.

North western zone of Tamil Nadu, India, one of the highly vegetable growing regions of the nation, with severe nematode infestation, was selected for the present study where the crop yield losses due to *M. incognita* were more than 60%.

In this study, native *Bt* was isolated to control *M. incognita*. The efficacy of spore-crystal mixture (SCM) of the native *Bt* isolates was evaluated under lab and greenhouse conditions.

Materials and methods

Isolation, screening and identification of *Bacillus thuringiensis*

The *Bt* was isolated from 5 soil samples collected from each of the 15 different blocks of the north western zone of Tamil Nadu state, India, and stained with Coomassie Brilliant Blue (CBB) (0.133% CBB in acetic acid 50%) for identification of crystal morphology as described by (Ramalakshmi and Udayasuriyan 2010). Twenty-four hours old broth culture of crystal positive isolates were prepared as glycerol stocks and stored at − 70 °C for further studies. Molecular identification, using 16SrRNA gene amplification by PCR was performed to identify the crystal positive isolates. The phylogenetic relationships of isolates are given in Fig. (S3).

Preparation of spore-crystal mixtures (SCM) and SDS-PAGE

The spore-crystal mixtures were prepared from *Bt* isolates as described earlier (Ramalakshmi and Udayasuriyan 2010). Five microlitres of SCM were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

Screening for nematicidal *cry* genes by PCR

The total genomic DNA from *Bt* isolates was extracted, using appropriate methods (Sambrook and Russell 2001). The nematode-specific *cry* gene primers were used to identify the *cry5*, *cry6*, *cry14* and *cry21* gene families in *Bt* isolates (Porcar and Juárez-Pérez 2003). The 25 μl PCR reaction mixture consisted of 1X Taq buffer, 2.5 mM MgCl2, 0.5 μM forward and reverse primers, 0.25 mM dNTP mixtures and 3 U of Taq polymerase (all from Fermentas, USA). PCR condition includes 95 °C for 5 min (initial denaturation), 35 cycles of 95 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min (primer extension), followed by 72 °C for 10 min (final extension). Amplification was performed in Biorad T100 PCR. Four reference strains of *Bt*, 4 M1, 4Q1, 4E1 and 4BG1 obtained from *Bacillus* Genetic Stock Centre (BGSC), the Ohio State University, Columbus, Ohio 43210, were used as a positive control for screening of native *Bt* isolates by PCR.

Root knot nematode and maintenance of egg masses

The egg masses of root knot nematode (RKN), *M. incognita* were obtained from the Department of Nematology, TNAU, Coimbatore, and maintained in pots having tomato plants.

In vitro inhibition of *M. incognita* egg masses by SCM of *Bacillus thuringiensis*

The *Bt* isolates were grown in T_3 broth till more than 95% cell lysis. After lysis, the different crystal protein suspensions 0.1, 0.2, 0.3, 0.4 and 0.5% with sterile water were prepared. The egg masses were collected from the pot culture experiment and washed with sterile distilled water before exposure to the crystal protein suspension. Three egg masses were released into each concentration of suspension in 6 mm dia petriplates. The sterile water with egg masses was used as control. The number of hatched J_2 was recorded every 24 h up to 3 days under binocular light microscope (model Olympus); the images were recorded in Cannon camera 20 MP colour CMOS attached to the Olympus microscope. All the treatments were carried out with three replications.

Scanning electron microscope analysis

The virulence of *Bt* isolates against *M. incognita* egg masses was confirmed by SEM analysis. The unhatched egg masses of *M. incognita* were observed under SEM after 3 days incubation with different concentrations of SCM of *Bt* isolates, reference strains and control. Sample preparation was made by the modified method (Orion et al. 1994; Wergin and Orion 1981). Briefly, the egg samples were fixed at 4 °C in closed tubes containing 1.25% glutaraldehyde and 1.25% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) for 4 h. The samples were treated serially for 15 min with 50, 70, 90 and 100% ethanol, and butanol. Then the samples are vacuum freeze-dried for 24 h. The samples were coated by carbon grid with platinum coating observed under a SEMFEI-Quanta 250.

In vitro inhibition of J_2 juveniles by SCM of *Bacillus thuringiensis*

The spore-crystal mixtures from nematicidal positive *Bt* isolates were further tested against *M. incognita* (J2) stage. Known concentration of 5 μg/ml of SCM was transferred into glass plates containing 100 nos of surface sterilised infective juveniles (J2). Four replications of each treatment were made to assess the juvenile mortality. The dead juveniles were counted from each treatment up to 48 h. Juveniles not showed any movement upon addition of water were considered as dead ones. Juveniles released in sterile water served as control. The experiments were performed at 30 ± 0.5 °C.

Greenhouse experiment

A pot culture experiment was conducted under greenhouse conditions for testing the biocontrol efficacy of *Bt* against *M. incognita*. Sterilised cocopeat mixture obtained from Orchard, TNAU, Coimbatore, was prepared and filled in 96 well portrays. Tomato seeds (PKM 2 variety obtained from HC and RI, TNAU, Coimbatore) were planted in portrays and maintained for better establishment. Tomato seedlings after 25 days were transferred to earthen pots of 10 kg capacity which were filled by steam sterilised pot mixture. *M. incognita* was inoculated at 1 J2/g of soil around the roots of tomato plants by making 3 holes around the plant and covered with sterilised soil. Ten millilitres of spore-crystal mixture of isolates BC and BD along with reference strain (4 M1) were applied near root zone. As a standard check Carbofuran 3G at 1 kg a.i/ha and an untreated control were included. Regular watering was done with tap water passed through 325 mesh sieve. The experiment consisted of 4 treatments with 3 replications in a completely randomised block design. Pots were placed at room temperature of 35 ± 2 °C under controlled conditions in the greenhouse.

Observations on shoot and root length, shoot and root weight were recorded after 60 days of planting. Plants were carefully uprooted and observation on gall index, number of females, number of egg masses and number of eggs per egg mass were observed. Population of nematode in soil was assessed by (KOPPENHõFER et al. 1998; Schindler 1961).

Statistical analysis

Data were pooled and analysed in the SPSS 16 software. Duncan's multiple range tests were performed for treatment with spore-crystal mixture. The *P* value < 0.005 was kept as cutoff value for significance. The LC_{50} values were determined by probit analysis using SAS 8.0.

Results and discussion

Screening of nematicidal *Bt* against *M. incognita*

Fifty native isolates were identified as *Bt* based on the presence of crystalline inclusions. The types of crystalline inclusion present in 50 isolates are presented in fig. (S1). All the 50 isolates were subjected to PCR for confirmation of nematicidal *cry* genes, using specific primers and simultaneously the spore-crystal mixtures of all the 50 isolates were run on SDS-PAGE to identify the molecular weight of the *cry* protein. Crystal protein profiles of 50 isolates were depicted in Fig. (S2). Out of 50 isolates, 14 harboured nematicidal genes and showed amplification of expected size of *cry5*, *cry6*, *cry14* and *cry21* genes either in combination or alone (Fig. 1 and Table 1). All the 14 isolates showed variation in their molecular weight of the protein (Table 1 and Fig. 2).

Biological control of insect's pests is known to be very old practice (Laznik et al. 2012). Several studies were attempted on *Bacillus* sp. based nematode control. However, *Bt* possessing nematicidal *cry* genes are more efficient in controlling specific nematodes when compared to *Bacillus* sp. Moreover, native isolates of *Bt* might harbour novel cry genes for nematode management. Furthermore, studies pertaining to *Bt*-based nematode control are limited. Iatsenko et al. 2014 extracted 2 novel protoxins *viz*., Cry21Fa1 and Cry21Ha1, from native *Bt* against *C. elegans*. Similarly, the native *Bt* BRC-XQ12 harbouring CryEa11 was active against pine wood needle nematode, *Bursaphelenchus xylophilus* (Huang et al. 2018). Guo and co-workers (2008) reported that most of the nematicidal *Cry* proteins are large proteins (90 to 140 kDa), except *Cry6* (Guo et al. 2008). In the present study, 43-135 kDa proteins in native *Bt* isolates were reported. The two *Bt* isolates shared the same *cry5* amplification profile with ~ 130 kDa protein and had the highest nematicidal activity against pinewood nematode (Wang et al. 2012). The same result was noticed in the present study also.

Fourteen isolates harbouring nematicidal *cry* genes were screened by bioassay against egg masses and 2nd stage juveniles (J2) of *M. incognita* for identification of potential isolate. The result revealed that 6 isolates were effective in inhibiting the emergence of J2 juveniles from egg masses of *M. Incognita* (Figs. 3 and 4). All the 6 isolates at 0.5% of SCM inhibited the emergence of J2 juveniles by 100%. Inhibition percentage of 80–90% of J2 juvenile emergence was observed at 0.3–0.4% SCM of all isolates (Fig. 4). The lowest inhibition was noticed in the concentration of 0.1–0.2% among the isolates. Microscopic images confirmed the inhibition of emergence of J2 by spore-crystal mixture of native isolates of *Bt* when compared to control (Fig. 3.)

Similarly, mortality of J2 was observed under microscope and the results are presented in Fig. 5. It was

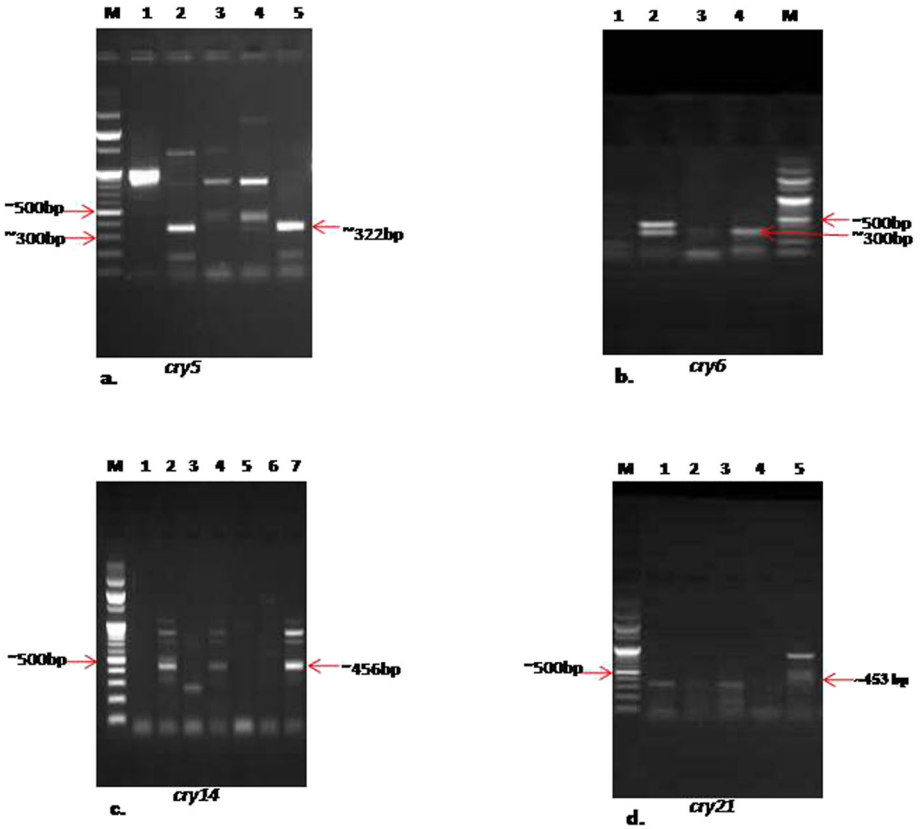

Fig. 1 PCR screening of nematicidal genes in native *Bacillus thuringiensis* isolates. **a** *cry*5at 322 bp (M, marker; lanes 1 to 5, native *Bt* isolates), **b** *cry*6 at 302 bp (M, marker; lanes 1 to 4, native *Bt* isolates), **c** *cry*14 at 456 bp (M, marker; lanes 1 to 7, native Bt isolates), **d** *cry*21 at 453 bp (M, marker; lanes 1 to 5, native Bt isolates)

Table 1 Nematicidal cry gene profiles and molecular weight of crystal proteins of native isolates of *Bacillus thuringiensis*

No	*Bt* isolates	Molecular weight of crystal protein(s) (kDa)	*Cry* gene
1	B7	~ 135	*cry*5 and cry6
2	BC	~ 135	*cry*5
3	BB	~ 135 and ~ 95	*cry*5, *cry*6 and *cry*21
4	CB2	~ 135 and ~ 65	*cry*5, *cry*6, *cry*14 and *cry*21
5	CA	~ 135	*cry*5
6	TE2	~ 135	*cry*5
7	CE	~ 135	*cry*5 and *cry*6
8	TE1	~ 95	*cry*5
9	BD	~ 135	*cry*5
10	TC	~ 95 and ~ 45	*cry*6 and *cry*14
11	Ba1	~ 95	*cry*6
12	CD	~ 43	*cry*14
13	CB2	~ 65	*cry*14
14	BA	~ 43	*cry*21

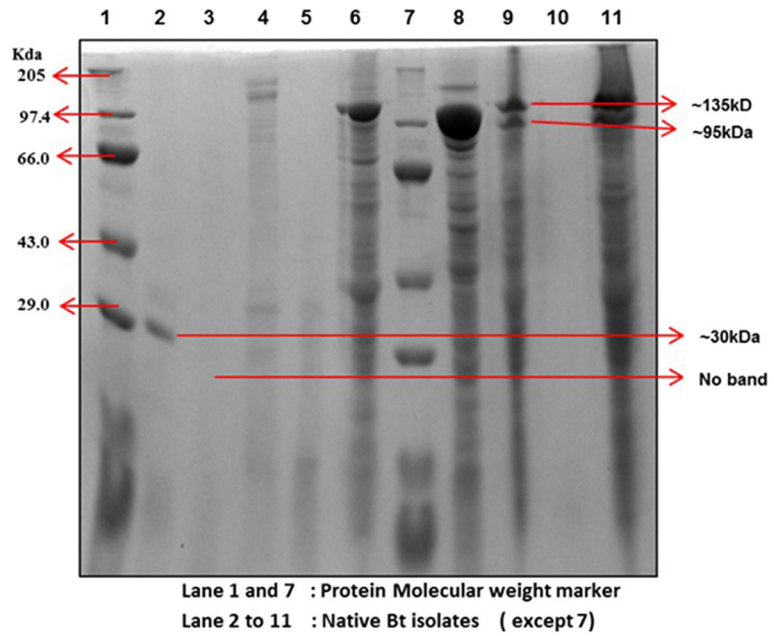

Fig. 2 Cry protein molecular weight variations among native isolates of *Bacillus thuringiensis*

evident from the figure that the spore-crystal mixture-treated juveniles were dead observed as straight ones, and no movement were noticed even after disturbance with the needle, whereas untreated J2 were found live by their curved and comma-shaped movement. (Fig. 5).

SEM analysis of changes in egg structure

The SEM analysis of egg mass treated with SCM of native isolates of *Bt* showed that the eggs were embedded in a gelatinous matrix with smooth egg shells, whereas the egg shells were fluffy owing to hatching in untreated samples (Fig. 5). From the SEM results, it is evident that the proteins inhibited the emergence of J2 from egg masses.

LC_{50} value

The spore-crystal mixture of 6 isolates were further analysed on J2 of *M. incognita* and LC_{50} values were found out. The result revealed that lethal concentration varied from 0.12 to 0.23 μg of protein (Fig. 6). The isolate BC recorded lowest LC_{50} value of 0.12 μg of protein/millilitre, followed by the isolate BD (0.13 μg of protein/millilitre). The highest LC_{50} value of 0.23 mg of protein/millilitre was recorded in isolate CD2 (Fig. 6). The 2 isolates with low LC_{50} values, namely BD and BC, were selected for greenhouse experiment using host plants. The LC_{50} value obtained in this study was much lower than already reported Cry5Ba protein whose LC_{50} value was146.05 μg of protein/millilitre (Peng et al.,

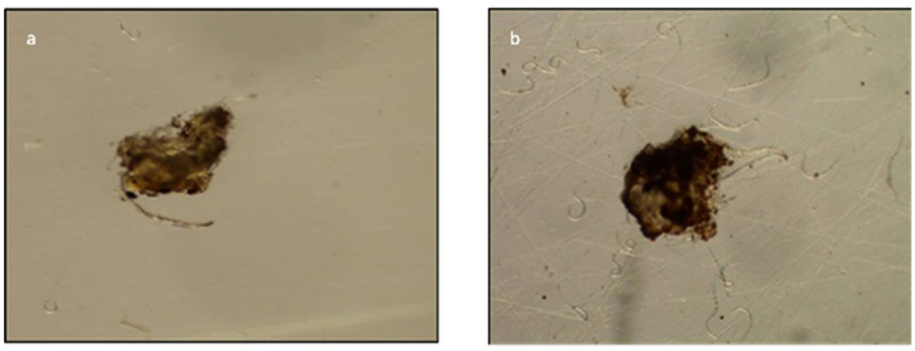

Fig. 3 In vitro inhibition of emergence of J2 juveniles from egg masses of *Meloidogyne incognita* by spore-crystal mixture of native *Bacillus thuringiensis* isolates. **a** Unhatched egg masses of *M. incognita* treated with spore-crystal mixture. **b** Release of J2 juveniles in untreated egg masses of *M. incognita*

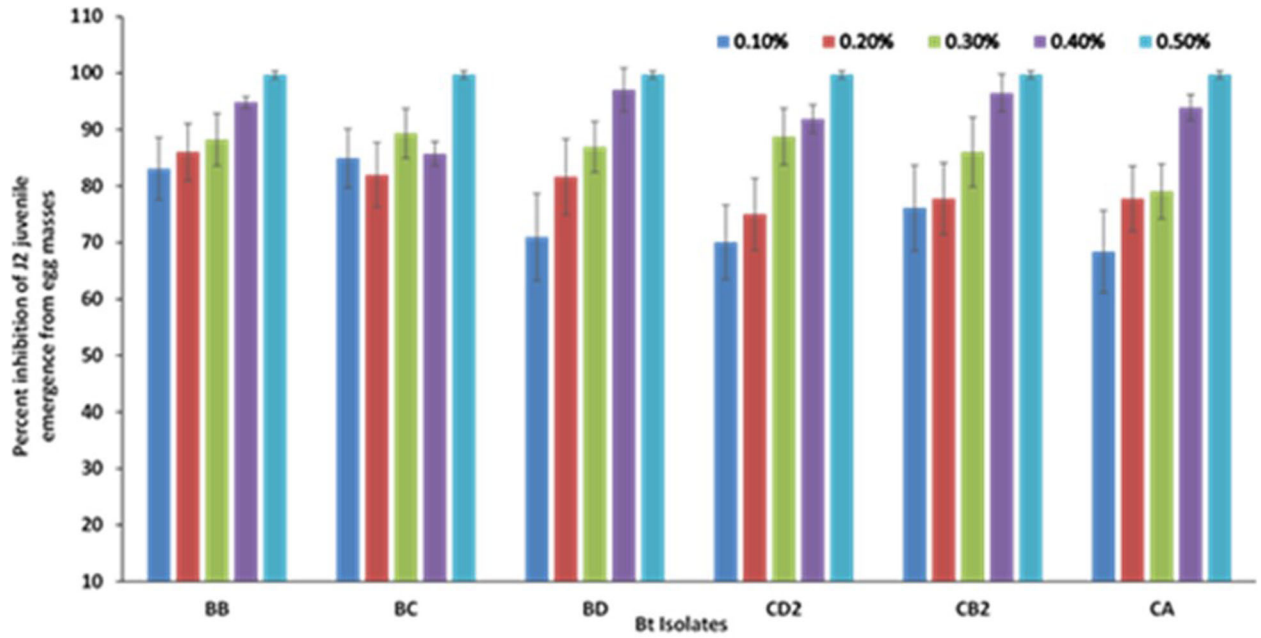

Fig. 4 Screening of *Bacillus thuringiensis* native isolates against emergence of J2 Juvenile from egg masses of *Meloidogyne incognita* at different concentrations of spore-crystal mixtures

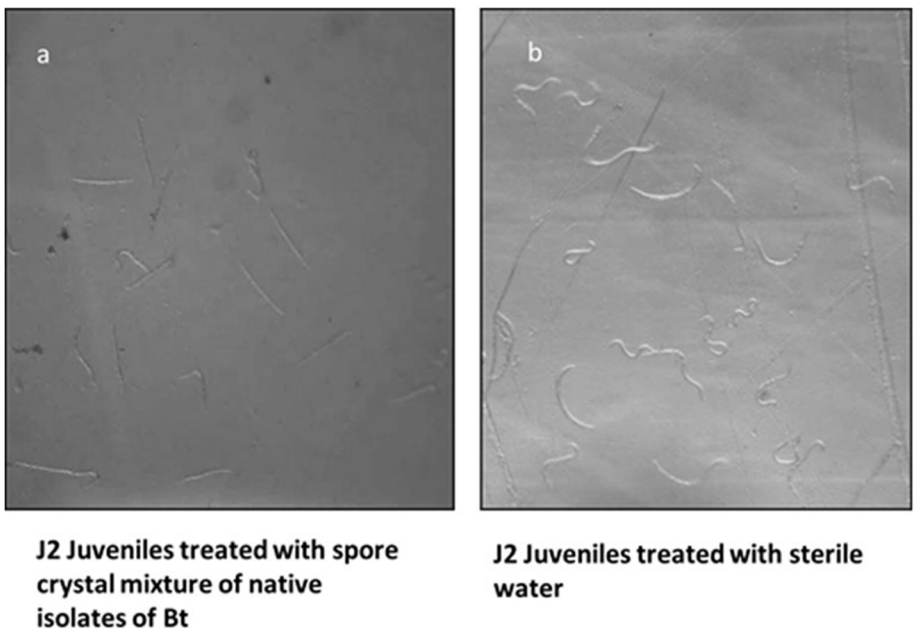

Fig. 5 Mortality of J2 juveniles by spore-crystal mixtures of native isolates of *Bacillus thuringiensis*. **a** Dead J2 juveniles (no movement) after treatment with spore-crystal mixture of native isolates of *Bt*. **b** Live juveniles with movement were observed in control

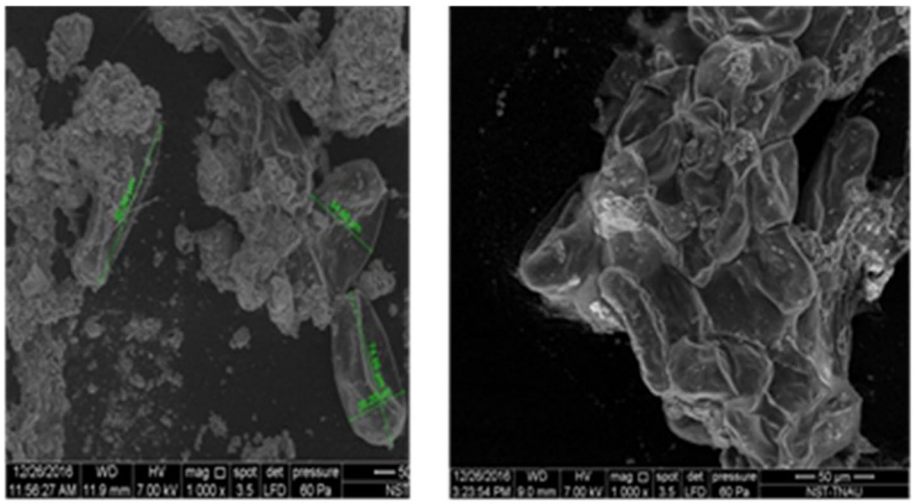

Fig. 6 SEM analysis of changes in egg masses treated with spore-crystal mixture of native isolates of *Bacillus thuringiensis*. **a** Untreated egg mass. **b** Egg mass treated with spore-crystal mixture. **c** Inhibition of hatching of egg masses by spore-crystal mixture

2011) (Fig. 7). Similarly, the LC_{50} value of this study could be compared to Huang et al. (2018) where in purified Cry1Ea11 had LC_{50} of 32.53 and 23.23 μg of protein/millilitre after 24 and 48 h, respectively.

Greenhouse experiments

Biocontrol potential of the 2 native *Bt* isolates (BC and BD) was evaluated for their effective control of root knot nematode under greenhouse conditions. The results were compared by chemical nematicide, carbofuran and with reference strain 4MI harbouring *cry5* nematicidal gene. Parameters such as number of egg masses/5 g root, number of eggs/egg mass, number of adult females/5 g root, final nematode population/kilogram soil and root gall index were evaluated. As shown in Table 2, the 2 native *Bt* isolates exhibited superior biocontrol potential of root knot nematode over the reference strain and chemical control. The highest percent decreased of the number of egg masses per 5 g of root was observed for the isolate BC to a tune of 79.35, followed by BD (76.09%). The percent decrease of reference strain 4MI was 66.66 and the lowest percent decrease of egg masses was noticed in the treatment with carbofuran with a value of 31.50.

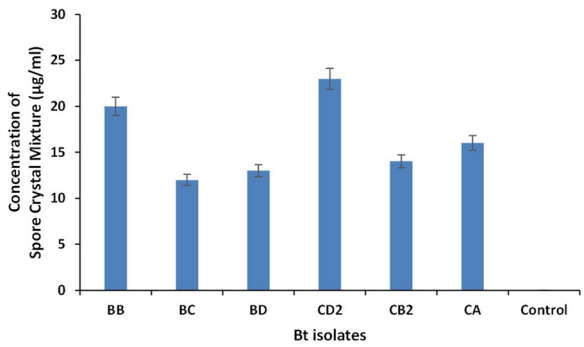

Fig. 7 LC_{50} values of spore-crystal mixtures of *Bacillus thuringiensis* isolates against J2 juveniles of *Meloidogyne incognita* under in vitro conditions

Similarly, the results on number of female population per 5 g of root also followed the same trend. The highest percent reduction of number of female population per 5 g of root was observed for isolate BC (68.14), followed by the isolate BD with a percent reduction of 64.53. The percent reduction of reference strain 4MI was 52.72%. Reduction percent of 25.45 was observed for treatment with chemicals. Final nematode population per kilogram of soil was estimated and the isolate BD had the lowest number nematode population with 73.15% decreased over control, followed by isolate BC, which had percent decrease of 71.96%.The lowest values of percent reduction (40.92) in final nematode population were recorded at the treatment with carbofuran. Root gall index was found to be low (1.66) in the native isolates of *Bt* and high of 3.33 was noticed in reference strain 4MI and in treatment with carbofuran. Bio efficacy of cry6 proteins on nematode *M. hapla* in pot culture experiments with tomato host plant was evaluated by Yu et al. (2015). The results indicated that egg masses were reduced to 62.3

Table 2 Biocontrol potential of native *Bacillus thuringiensis* against root knot nematode *Meloidogyne incognita* in tomato

Treatments	No. of egg masses/5 g root	%decrease	No. of eggs/egg mass	%decrease	No. of adult females/5 g root	% decrease	Final nematode population/ kilogram soil	% decrease	Root gall index	% reduction
Isolate BD	7.33 ± 2.15	76.09 ± 9.85	140.00 ± 11.25	48.40 ± 5.42	13.00 ± 2.53	64.53 ± 15.28	143.00 ± 12.36	73.15 ± 8.56	1.66 ± 0.25	61.66 ± 13.25
Isolate BC	6.33 ± 1.78	79.35 ± 12.36	135.66 ± 13.25	50.00 ± 6.25	11.66 ± 1.78	68.14 ± 16.56	149.33 ± 13.68	71.96 ± 13.36	1.66 ± 0.25	61.66 ± 12.36
Reference strain 4 M1	10.22 ± 3.38	66.66 ± 17.36	152.33 ± 15.66	43.85 ± 6.32	17.33 ± 3.52	52.72 ± 11.56	165.33 ± 16.68	68.96 ± 15.23	3.00 ± 0.35	30.71 ± 4.85
Soil application of Carbofuran at 1 kg a.i/ha	21.00 ± 5.20	31.50 ± 4.32	218.33 ± 20.63	19.53 ± 3.25	27.33 ± 4.56	25.45 ± 6.85	314.67 ± 25.63	40.92 ± 23.56	3.00 ± 0.35	30.71 ± 4.85
Control	30.66 ± 6.82	0	271.33 ± 25.65	0	36.66 ± 6.25	0	532.66 ± 35.68	0	4.33 ± 0.56	0

and 67.3% over control. The final population of nematode in soil was by 52.4 and 59.5% and the gall index values were reduced by 46.7 and 66.7%.

Conclusion

The present study demonstrated biocontrol efficiency of 2 native isolates of *Bt* for efficient control of root knot nematode. The study also investigated the changes in egg mass treated with spore-crystal mixture of native isolates of *Bt* by SEM analysis. The LC_{50} values of the 2 native isolates were much lower than other isolates. Molecular weight of 135 kDa and nematicidal *cry5* gene(s) were noticed in these isolates. Pot culture experiment showed that the 2 isolates were better than reference strain and chemical in controlling egg mass, nematode population and root gall index of tomato plants. The crystal proteins of native isolates of *Bt*, BC and BD, could be potential candidates for a biocontrol agents in controlling root knot nematode.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s41938-020-00293-2.

Additional file 1: Fig. S1. Types of crystalline inclusions in native isolates of *B. thuringiensis*. **Fig. S2.** Crystal protein profiles of native isolates of *B. thuringiensis*. **Fig. S3.** Phylogenetic relationship of native isolates of *Bacillus thuringiensis*

Abbreviations

PCR: Polymerase chain reaction; SEM: Scanning electron microscope; PPNs: Plant-parasitic nematodes; *Bt*: *Bacillus thuringiensis*; SCM: Spore-crystal mixture; LC_{50}: Lethal concentration 50; CBB: Coomassie Brilliant Blue; 16srRNA: 16 s ribosomal RNA; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RKN: Root knot nematode; TNAU: Tamil Nadu Agricultural University; J2: Second stage *juvenile*; BGSC: Bacillus Genetic Stock Centre; BC and BD: Name of the *Bt* isolates

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Authors' contributions

All authors read and approved the final manuscript. Dr. A. Ramalakshmi: conceived the idea, proposed the work and designed and wrote the manuscript. Dr. R. Sharmila: conducted the experiments and assisted manuscript preparation. Dr. M. Iniyakumar: contributed SEM analysis and reviewed manuscript. Dr. V. Gomathi: overall supervision and critical suggestion on the manuscript

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Availability of data and materials

All data analysed in this study are available from corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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