UDC 579

Selection of *Bacillus thuringiensis* against Pathogenic Nematodes Attacking Pepper Tree

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Received 26.11.2019
Revised 10.03.2020
Accepted 30.04.2020

Abstract–Nine strains belonging to the *Bacillus* genus have been isolated from soil samples in Tien Phuoc district, Quang Nam province. They were capable of surviving at 42°C, and the B1, B4, B7 and B9 strains could produce toxic crystals at this temperature. B7 had the most promising characteristics in terms of spore-forming ability. The result of the 16S rRNA gene sequencing showed that B7 belongs to the *Bacillus thuringiensis* species, which is known to effectively control root-knot *Meloidogyne* sp. nematodes attacking pepper tree. This study was aimed at evaluating the inhibitory effect of the bacterial isolate under study on root-knot eggs and juveniles. It was shown that the highest inhibitory activity of the cultures of the bacterial strains under study was observed at their concentration of 10^9 cells/mL; in this case, up to 89.67% of nematode eggs and 100% of juveniles J2 were killed after 10 h of treatment.

Key words: *Bacillus thuringiensis, Meloidogyne* sp., pepper tree, root-knot, nematodes

doi: 10.21519/0234-2758-2020-36-3-57-62

Pepper tree (*Piper nigrum* (L.)) originated from India has been cultivated for 6,000 years. This is a long-term industrial tree with high economic value grown in many places in the world. Vietnam is now one of the world's leading pepper exporters with the current sown area of pepper tree about 100,000 ha, concentrated in Binh Duong, Binh Phuoc, Dong Nai, Gia Lai, Dak Lak and Ba Ria and Vung Tau provinces.

However, pepper production suffers from severe losses due to yellow leaves (a slow-death disease) caused by root-knot nematodes [1]. *Meloidogyne* ssp. has been considered as the most harmful group of parasitic nematodes in agriculture; they are widely spread all over the world, live parasitically on most crops in different climatic areas and cause serious economic damage to pepper plants.

The main current method for nematode inhibition is the treatment with specific drugs, such as Furadan, Marshal, Oncol, Nokap, and Vimoca. However, the use of chemicals leads to drug resistance, ecological imbalance and presence of drug residues in agricultural products. Therefore, it is necessary to select an indigenous microorganism with the potential ability to control nematodes in order to reduce losses and improve plant productivity and product quality.

Bacillus thuringiensis is widely used to control various agricultural pathogens around the world; it accounts for about 53% of the global market for biological pesticides with an annual turnover of $ 210 million. Studies on nematode control by *B. thuringiensis* are not numerous in Vietnam. Therefore, the present research was devoted to the selection of indigenous representative of the *B. thuringiensis* species with the promising ability to inhibit pathogenic nematodes on pepper tree with the common goal of diversifying biological resources and contributing to sustainable agricultural development.

MATERIALS AND METHODS

Isolation of Bacteria

10 g of soil was diluted in 90 ml of 0.85% NaCl, homogenized on a shaker for 10 min and then heated at 80 °C for 10 min in water bath to eliminate vegetative

Abbreviations: LB – lysogeny broth; VP – Voges–Proskauer reaction.

cells and non-spore-forming cells. The samples were serially diluted to the concentration of 10^6 cells/mL and cultivated on nutrient LB agar at 28 ºC for 24 h [2]. The identification of the *Bacillus* genus was based on a morphological method [3]. The colonies were examined under a microscope for sporulation and crystal formation, and those that were believed to be *Bacillus thuringiensis* were isolated and kept on slant agar in tubes at 4 ºC for further characterization.

The determined biochemical characteristics and conducted tests were as follows: oxidase and catalase activities, VP, citrate utilization assay, gelatin hydrolysis test, test for the ability of sugar (glucose, sucrose and mannose) usage, and evaluation of growth characteristics at 5 °C and 42 °C and NaCl concentration of 10% [4].

Screening of the Isolates based on Spore Density Determination

The suspected *B. thuringiensis* was streaked on LB agar and then cultured at 28 ºC for 72 h to collect biomass, which was pretreated by heating at 80 °C for 10 min and then serially diluted to the density of 10^{-9} cells/mL. Each dillution (100 µl) was streaked on LB agar. The plates were incubated at 28 ºC for 24 h, and then the number of conlonies was counted. The quantity of spores was calculated according to the formula:

$$\text{CFU} = n \cdot a \cdot 10,$$

where CFU is number of spores per ml; n is average number of colonies in 100 µl of a dilution; and a is cell concentration in a dilution.

16S rRNA Gene Analysis and Sequencing

DNA extraction and sequencing of 16S rRNA gene was carried out via the Sanger method. BLAST NCBI analysis was then used to search for sequence matching.

Activity of *B. thuringiensis* Strain against Root-Knot Nematodes

Cultivation of parasitic nematode *Meloidogyne* sp. This nematode was isolated from the swollen roots of pepper plants, identified and propagated on tomato roots as described by López-Pérez, et al. [5].

To this end, tomato seeds (sensitive to *Meloidogyne* ssp.varieties) were subjected to surface sterilization with 70% etanol for 5 min and then washed in 1% sodium hypochlorite solution for 15 min to eliminate infectious agents. After that, the tomato seeds were washed many times with distilled water to completely remove NaCl. The seeds were grown in disinfected soil trays until the appearance of two true leaves; then they were transferred into pots with 250 cm^3 of soil. When the roots appeared, 200 juveniles J2 of *Meloydogyne* ssp. were transferred into soil, and the parasite was collected after 30–60 days.

For this purpose, the infected tomato roots were washed many times with water and cut to small fragments of about 1–2 cm. Eggs and juveniles J2 were extracted by shaking of root fragments in NaCl (0.5%) and the suspension was passed through a filter with a pore diameter of 25 µ and 40 µ, respectively [6].

Evaluation of the *Bacillus thrungiensis* activity against root-knot *Meloidogyne.* ssp. nematodes. To assess the inhibition of the emergence of juveniles from egg mass, the method described by Chahal [7] was used. Eggs (30 pieces) were placed in vials containing 4 mL of the whole broth culture of the selected *B. thrungiensis* strain (10^5, 10^7, 10^9 cells/ mL) and incubated at 30 °C. The juveniles emerging from the eggs were counted every 24 h for 10 days. Distilled water was used instead of culture broth in a control sample.

The ability to kill juveniles was evaluated according to the method described by Yap Chin Ann [8]. Whole culture of the selected *Bacillus thrungiensis* strain (10^5, 10^7, 10^9 cells/mL) was introduced in an amount of 300 µl into Eppendorf tubes (1.5 mL) containing 50 µg/mL of antibiotic (ampicillin) and incubated at 30 ºC. The number of dead (immobile) juvenile J2 nematodes was counted after 2h, 6 h and 10 h of incubation via optical microscopy.

Statistical Analysis

All experiments were carried out in triplicates. The obtained results are shown as averages with standard deviations. The excel software (2010) was used to analyze the obtained data. The test of significance was set to $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and Selection of *Bacillus* sp. Bacteria Producing Toxic Crystals

Ten strains (designated from B1 to B10) that were able to grow at 42 ºC were isolated from soil samples collected from different areas in Tien Phuoc district, Quang Nam province, Vietnam. Their morphology, and the ability to produce spore and crystals are reflected in Table 1. The results show that nine strains belong to the *Bacillus* genus, and four strains, including B1, B4, B7 and B9, are able to produce toxic crystal.

Fig. 1 show that the isolates under study had a wide variety of crystal forms, from pyramidal to spherical, and different sizes. Differences in the crystal shape and structure are due to different protein compositions of crystals. The abundance of crystal shapes may result from a large number of various toxic genes, which determine the broad spectrum of *Bacillus* activity against parasitic nematodes [2].

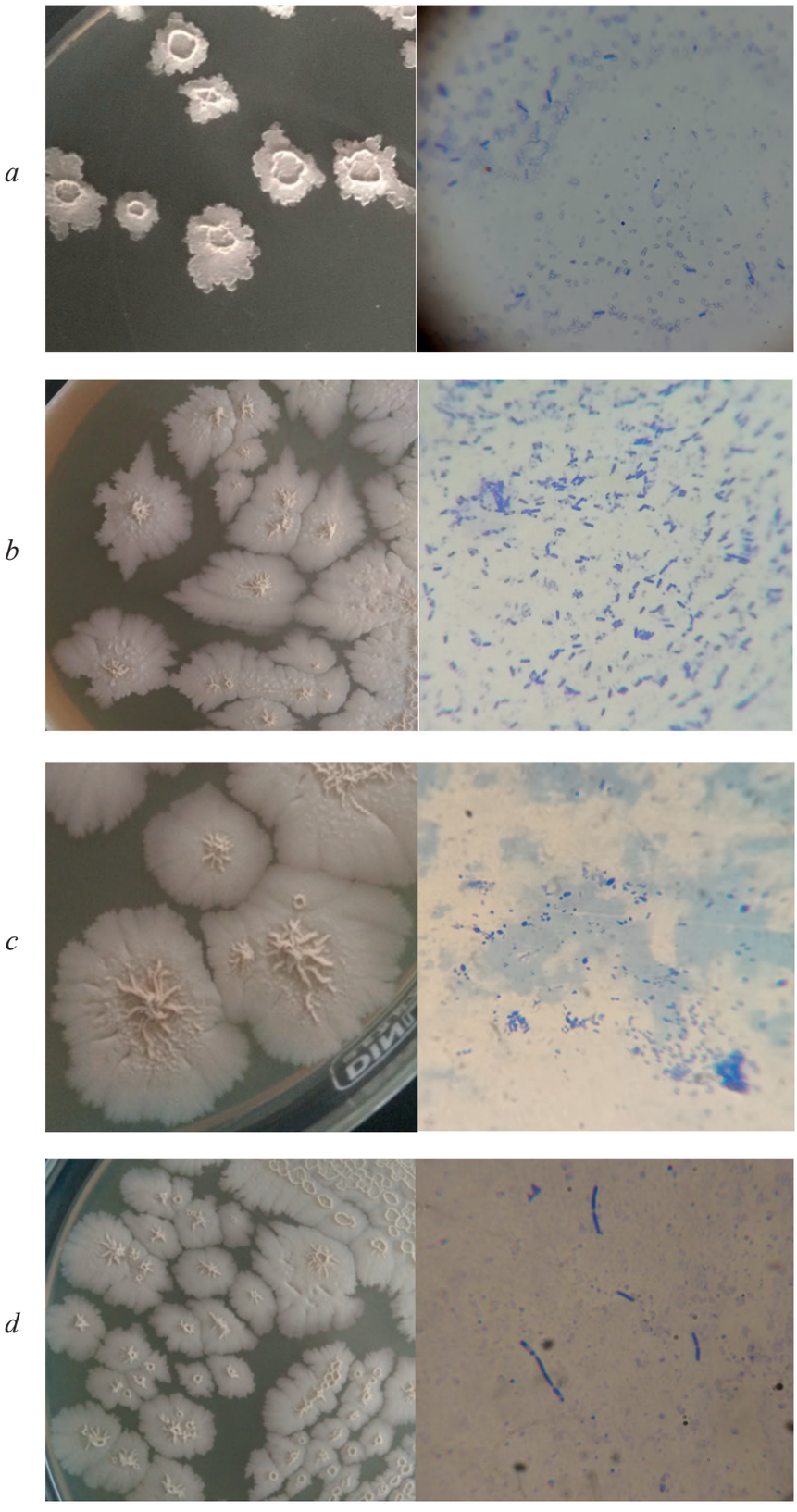


Fig. 1 Cell and colony morphology of *Bacillus* ssp. strains: (*a*) – B1, (*b*) – B4, (*c*) – B7, (*d*) – B9

Table 1

Mophology, ability of sporulation and crystal production at 42 °C of isolated strains

Strain	Colony morphology	Cell morphology	Sporulation	Crystal formation
B1	Wrinkled, rounded, milky white, concave in the middle, wide border	Rod, individually arranged, centric spores, spherical crystals	+	+
B2	Teardrop shape, with mucus	Rod, string-arranged, eccentric spores	+	–
B3	Rounded, milky white, with mucus	Rod, individually arranged, eccentric spores	+	–
B4	Milky white, knob in the middle, wide border, rough surface	Rod, individually arranged, centric spores, spherical crystals	+	+
B5	Light yellow, rounded, slightly mucus	Rod	–	–
B6	Opalescent, smooth surface, smooth edge	Rod, individually arranged, centric spores	+	–
B7	Flower shape, milky white, knob in the middle, wide border, rough surface	Rod, individually arranged, centric spores, spherical and rhombic crystals	+	+
B8	Milky white, knob in the middle, bird feather-shaped, blurred edge	Rod, individually arranged, eccentric spores	+	–
B9	Milky white, concave knob in the middle, wide border	Rod, individually arranged, centric spores, spherical crystals	+	+
B10	Opalescent, dry, agar adhesion	Rod, centric spore	+	–

Biochemical Characteristics of the Selected Strains

The biological characteristics of the B1, B4, B7 and B9 strains are represented in Table 2.

All of the isolates turned to be gram-positive and motile strains; they also have functioning catalase and oxidase, are capable of assimilating citrate, hydrolyzing gelatin, utilizing glucose and sucrose, and are VP-positive. All the four strains could grow well at 42 °C, but not at 5 °C; they were also able to grow on a medium with 10% NaCl. These results were consistent with those reported by Claus and Berkeley [3], which confirms once again that B1, B4, B7 and B9 belong to the *Bacillus* genus.

Number of Spores after 24 h Cultivation

According to Federici (1998), *Bacillus thuringiensis* is a unique species among other representatives of the *Bacillus* genus that is able to produce toxic crystals (δ-endotoxin) against nematodes. Cells of this species synthesize plasmid proteins with insecticidal activity during sporulation. These proteins crystallize into large multi-sided inclusions that account for up to 30% of the dry cell weight [9]. Therefore, each bacterial cell after destruction releases a spore and a poisonous crystal, and we can judge the bacteria growth rate and content of toxic crystals by the number of spores in the culture broth [2]. The results of the assessment of the spore number are shown in Table 3.

Table 2

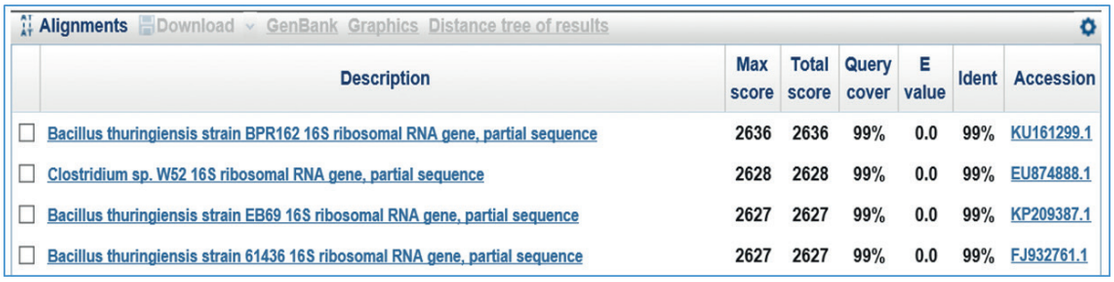
Biochemical, cultural and physiological characteristics of the *Bacillus* ssp. strains B1, B4, B7 and B9

Test	B1	B4	B7	B9
Gram staining	+	+	+	+
Oxidase test	+	+	+	+
Catalase test	+	+	+	+
Citrate utilization	+	–	+	+
Gelatin hydrolysis	+	+	+	+
VP	+	+	+	+
Growth at 5 °C	–	–	–	–
Growth at 42 °C	+	+	+	+
Growth in 10% NaCl	–	–	–	–
Glucose utilization	+	+	+	+
Sucrose utilization	+	+	+	+
Mobility	+	+	+	+

Table 3

Spore content after 24 h cultivation of the studied bacteria

Strain	Spore number, · 10^9/ml
B1	1.23±0.07
B4	4.82±0.04
B7	7.18±0.01
B9	3.18±0.03



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus thuringiensis strain BPR162 16S ribosomal RNA gene, partial sequence	2636	2636	99%	0.0	99%	KU161299.1
Clostridium sp. W52 16S ribosomal RNA gene, partial sequence	2628	2628	99%	0.0	99%	EU874888.1
Bacillus thuringiensis strain EB69 16S ribosomal RNA gene, partial sequence	2627	2627	99%	0.0	99%	KP209387.1
Bacillus thuringiensis strain 61436 16S ribosomal RNA gene, partial sequence	2627	2627	99%	0.0	99%	FJ932761.1

Fig. 2. B1 strain identification as *Bacillus thuringiensis* via the BLAST NCBI search

Table 3 shows that B7 had the highest growth rate and the greatest spore number ($7.18 \cdot 10^9$ spores/ml) among other four strains.

Identification of the Selected Strain

Based on 16S rRNA gene analysis, the strain designated as B7 was phylogenetically characterized, and its closest relative was identified via the BLAST search (NCBI). Thus, the B1 strain was identified as belonging to the Bacillaceae family; B1 showed a 99% sequence similarity with its closest relative, *Bacillus thuringiensis* (Fig. 2).

Bacillus thuringiens Activity against *Medoilogyne* sp.

The inhibitory efficiency of the studied *Bacillus thuringiens* strain against *Medoilogyne* sp. eggs is represented in Table 4.

The obtained results indicate that *Medoilogyne* sp. eggs at the age of 10 days after hatching were sensitive to the exotoxin produced by the selected *B. thuringiensis* B7 strain. The level of emerging juveniles varied, depending on the bacteria concentration, and the highest level of inhibition (89.67%) was achieved with the greatest content of *B. thuringiensis* cells (10^9 cell/ml). The B7 strain has a lower activity against *Medoilogyne* sp. eggs compared to the results obtained by Chahal [7]. It was shown in [7] that none of *Medoilogyne incognita* juveniles emerged from eggs in the presence of the whole *B. thuringiensis* culture broth. This could be explained by the ability of *B. thuringiensis* to kill nematode eggs via dehydration of a gelatinous matrix surrounding the eggs [10]. Production of exotoxin by *B. thuringiensis* has already been reported [11]. According to Chigaleichik (1976) [12], chitinase produced by *B. thuringiensis* hydrolyzed chitin (N-acetyl-glycosamine polymer), a component of the chitin-protein layer of the egg shell and gelatinous matrix of egg masses, and thus affected the egg permeability. Therefore, chitinase activity and exotoxin could be involved in the killing of eggs.

The results represented in Table 5 show that the selected *Bacillus thrungiensis* strain was able to kill

Table 4

Activity of *Bacillus thuringiens* B7 strain under study against *Medoilogyne* sp. eggs

Bacteria concentration, cells/ml	Emergence of juveniles J2/30 eggs	Inhibition, %
10^5	9.10±0.25	69.67
10^7	5.40±0.15	82.00
10^9	3.10±0.10	89.67
Control (distilled water)	30	0.00

Table 5

Activity of *Bacillus thuringiens* B7 strain against *Medoilogyne* sp. juveniles J2

Bacteria concentration, cells/ml	Death rate of juveniles J2 in whole broth of *B. thrungiensis* after		
	2 h	6 h	10 h
10^5	35	54	76
10^7	50	68	87
10^9	76	95	100
Control (distilled water)	0.0	0.0	0.0

juveniles J2 at all tested concentrations (10^5, 10^7 and 10^9 cells/ml). However, the highest activity against juveniles reaching 100% after 10 h of treatment was observed at a bacteria concentration of 10^9 cells/ml. As was said, the abundance of crystal shapes of the selected *Bacillus thuringiensis* strain could be associated with a variability of its toxin gene, which plays the key role in the nematode-killing activity [13].

CONCLUSIONS

The present study identified the B7 strain as *Bacillus thuringiensis*. It was shown to be effective in controlling the pepper tree infection with root-knot nematodes *Meloidogyne* sp. The highest ability of this isolate to kill *Meloidogyne* sp. eggs and juveniles

J2 (100% and 89.67%, respectively, after 10 h treatment) was observed at a bacteria concentration of 10^9 cells/ml.

ACKNOWLEDGEMENTS

We are grateful to the Danang University of Science and Education and Duy Tan University for supporting this study.

REFERENCES

1. Trinh T.T.T. Incidence and effect of meloidogyne incognita (*Nematode Meloidogyne*) in black pepper in Vietnam. Doctoral thesis, Catholic Universty of Leaven, 2010.

2. Nguyen Thien Phu, Tran Thanh Thuy. The islolation of selection of *Bacillus thurigiesis* species with insecticidal activeness against diamondback moth (Plutella xylos). *Ho Chi Minh city university of education – Journal of science. 2013,* 51: 49–58.

3. Claus D., Berkeley R. C. (1986). *W. Genus Bacillus Cohn 1872, 174 AL. Bergey's manual of systematic bacteriology.* Baltimore, Md: Williams and Wilkins: 1105–1139.

4. Aksoy, H. M., I. Saruhan, I. Akca et al. Isolation and Characterization of Bacillus thuringiensis Isolated from Soil and their Possible Impact on Culex pipiens Larvae. *Egyptian Journal of Biological Pest Control.* 2015, 25(2), 439–444.

5. J.A. López Pérez, M. Escuer, M.A. Díez Rojo et al. Host range of Meloidogyne arenaria (Neal,1889) Chitwood, 1949 (Nematoda: Meloidogynidae) in Spain. *Nematropica*. 1991, 41, 130–140.

6. R.S. Hussey, K.R. Barker. Comparison of methods for collecting inocula of Meloidogyne spp. including a new technique. *Plant Disease Reporter*. 1973, 57, 1025–1028.

7. Chahal P.P.K., Chahal V.P.S. Control of Meloidogyne incognita with *Bacillus thuringiensis*. In: Wright R.J., Baligar V.C., Murrmann R.P. (eds) Plant-Soil Interactions at Low pH. *Developments in Plant and Soil Sciences*. 1991, 45 677–680.

8. Yap Chin Ann. Screening for Nematicidal Activities of Bacillus Species Against Root Knot Nematode (*Meloidogyne incognita*). *American Journal of Experimental Agriculture*. 2013, 3(4), 794–805.

9. Federici, B.A., Bauer, L.S. Cyt1Aa protein of *Bacillus thuringiensis* is toxic to the cottonwood leaf beetle, Chrysomela scripta, and suppresses high levels of resistance to Cry3Aa. *Appl. Environ. Microbiol*. 1998, 64, 4368–4371.

10. Bird A.F., Soeffky A. Changes in the ultrastructure of the gelatin matrix of Meloidogyne incognita during dehydration. *Journal of Nematology*. 1972, 4, 166–168

11. McConnell E., A. G. Richards. The production by *Bacillus thuringiensis* Berliner of a heat-stable substance toxic for insects. *Canadian Journal of Microbiology*. 1959, 5, 161–168.

12. Chigaleichik A.G. (1976). Chitinase of *Bacillus thuringiensis*. *Mikrobiologiya*. 1976, 45, 966–972.

13. Prakai Thaphan, Suttipun Keawsompong, Jariya Chanpaisaeng. Isolation, toxicity and detection of cry gene in *Bacillus thuringiensis* isolates in Krabi province, Thailand. *Songklanakarin J. Sci. Technol*.2008, 30 (5), 597–601.

14. Uribe, D., Martinez, W., and Cerón, J. Distribution and diversity of cry genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Columbia. *Journal of Invertebrate Pathology*. 2003, 82, 119–127.