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Research Article

ANTIBACTERIAL AND NEMATICIDAL EFFECTS OF *BACILLUS THURINGIENSIS* STRAINS ON ROOT-KNOT NEMATODE-*MELOIDOGYNE INCOGNITA*

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ABSTRACT. This work represents to investigate the capacity to synthesize antibacterial substances and nematicidal effect from *Bacillus thuringiensis* (*Bt*) strains isolated from agricultural soil samples of Turkey. Isolated *Bacillus* strains were identified based on 16S rRNA sequence analysis. The four *Bt* isolates showed 100 % sequence identity with *Bt*. These strains were named as *Bt* A1, A14, A15 and A17. The *Bt* strains were tested for resistance to ampicillin, streptomycin, bacitracin, penicillin, tetracycline. While all *Bt* strains were sensitive to tetracycline and streptomycin, they showed resistance to ampicillin, penicillin and bacitracin. These strains were also analyzed using the agar diffusion method in terms of their antibacterial activities to some pathogen bacteria. It has been found that *Bt* A14 isolate was effective against both of some gram negative and positive bacteria. Especially, it was observed to be the effective against *K. pneumoniae* and *Y. enterocolitica* as gram positive bacteria. In addition, supernatants and pellets of *Bt* strains were investigated for the root-knot nematode *Meloidogyne incognita*. Both of them were found as a good nematicidal agents.

Keywords: *Bacillus thuringiensis, antibacterial substance, crystal, nematicidal activity, Meloidogyne incognita*

INTRODUCTION

Bacillus thuringiensis (*Bt*) is an about 1 µm wide and 5 µm long, soil-dwelling, spore forming gram positive bacteria [1, 2]. It produces crystal proteins (Cry proteins) and uses it to remove predators, insects and pathogens [3]. The Cry proteins (or endotoxins) collect during sporulation, producing crystalline inclusions of several morphologies [4, 5]. Crystals are digested by insects and dissolved in the midgut. These are activated by midgut proteases and bind to specific receptors in the insect cell membrane [6], it leads to cell degradation and thus death of the insect. Due to the specificity of their membrane receptors, they show specific activity against the insect orders Lepidoptera, Coleoptera and Diptera [7-10]. Additionally, this bacterium synthesized some molecules such as chitinases [11, 12], acyl homoserine lactonase [13, 14], lipopeptides [15, 16] and antibiotics [17] can act in synergy with Cry and Vip proteins as an antimicrobial agent against several pathogenic bacteria.

Root-knot nematodes cause significant yield losses in vegetable growing areas in the world. The nematodes damage plants by forming root galls that uptake of nutrients and water [18]. In this way, high yield losses occur by the nematodes. Many methods including chemicals that harmful to humans of control are used to prevent and reduce the

damage caused by nematodes in plants [19]. Moreover, *Verticillium lecanii* (Hypocreales: Hypocreaceae) strains were studied for *Meloidogyne incognita* (Tylenchida: Meloidogynidae) as biocontrol agent. According to the study, the strains significantly reduced egg production of the nematode [42]. But, it cannot say that there is successfully method against the nematodes. With the spread of this nematode in our country, especially tomato production is threatened. For this reason, it is necessary to determine new methods of struggle against root-knot nematodes in tomato production.

It is an important subject of the applications in the biological control of phytopathogenic insects, especially nematicidal potential of some bacterial strains. The aim of this is to use instead of chemical pesticides with a new alternative, that is clean and safe for environment. This work represents to isolate of *Bt* in agricultural soils of Turkey and to determine the some biological properties of these isolates. It is also aimed to determine the effects of *Bt* cell free extract as antibacterial substance and pellet as crystal-spore complex on the root-knot nematode-*M. incognita*, 1949 (Tylenchida: Heteroderidae), which is an environmentally friendly alternative within the framework of biological control.

MATERIALS AND METHODS

Materials

In a previous study, it was screened *Bacillus* species from agricultural soil samples in Turkey [20] and *Bt* strains were identified by 16S rRNA analysis. Antibacterial activity was evaluated against *Yersinia enterocolitica* (ATCC 9610), *Salmonella typhimurium* (ATCC 14028), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027) and *Klebsiella pneumoniae* (ATCC 13883). All strains were taken from Bursa Uludag University, Faculty of Medicine, Department of Microbiology.

Identification of Bacillus sp. strain using 16S rRNA sequencing

Bacillus genomic DNA was extracted for the bacterial identification and phylogenetic analysis (Qbiogene, Montreal, PQ, Canada). The sequence analysis were performed using ABI 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were checked with the GenBank database (NCBI) by BLAST [21]. The 16S rRNA sequencing of four strains were aligned with other *Bacillus* species by CLUSTAL W program. The phylogenetic analysis was done by MEGA 6.0 software, the phylogenetic tree was created using the neighbor-joining method [24].

Medium and Metabolite Production

A loopful of *Bt* strains was used to inoculate to LB medium enriched with salts (g/L) 2 g glucose, 0.002 g $FeSO_4$, 0.3 g $MgSO_4$, 0.02 g $ZnSO_4$ and 0.02 g $MnSO_4$, pH 7.2. After at 30 ºC for 96 h incubating, *Bt* samples were centrifuged at 9000 x g, +4 ºC and 15 min and cell free supernatant as antibacterial substance were filtred by 0.45 µM pore size syringe filter. The pellets as spore/crystal mixture was washed with 0.1 M NaCl solution and sterile distilled water and then resuspended it. Pellets were made as films on slides and fixed by heating, then stained with Coomassie Brilliant Blue [25]. Slides were observed by using light microscope. Both pellet and supernatant were stored at -20 ºC for further use.

Antibiotic Resistance and Sensitivity

The antibiotic resistance of identified *Bt* strains was tested against commercial antibiotics ampicillin (Amp), streptomycin (Str), bacitracin (Bac), penicillin (Pen), tetracycline (Tet) using 96 well plate microdilution assay of the Clinical Laboratory Standards Institute (CLSI) [26] and minimal inhibitory concentrations (MICs) of antibiotics against 4 of selected *Bt* strains were determined. Stock solutions of antibiotics were prepared, transferred in 100 µl aliquots to a 96-well plate. $1x10^6$ colony forming units per mililitres (CFU/ml) of *Bt* suspensions were pipetted into the wells in same volume. Medium containing *Bt* suspension was used as positive control. The microplates were incubated at 37 °C for 24 h and optic density at 600 nm were spectrophotometrically read. MICs of antibiotics were recorded. Experiments were performed in triplicate.

Antibacterial Activity Assay

The agar well diffusion assay were used for antibacterial activity of *Bt* substances [27]. For antibacterial activity against seven test pathogens including *Staphylococcus aureus* (ATCC 25923), *Yersinia enterocolitica* (ATCC 9610), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 25922) were used. Test pathogens were growth in Muller- Hinton Broth (MHB) for 24 h and then adjusted to a concentration of $1x10^6$ CFU/ml for adding to Muller Hinton agar (MHA). The wells were filled with 100 µl of the filtered antibacterial substance. All the plates were incubated at 37 °C for 24 h and then diameters of inhibition zones were measured and recorded in millimeters (mm).

To corroborate the proteinaceous *Bt* A14 that showed the inhibition, *Bt* A14 was treated with protease and proteinase K. 90 µl of samples were incubated with 10 µl of enzyme (1 mg/ml) and protease treated sample was incubated at 37 °C and proteinase K treated sample was incubated at 42 °C for 2 h. Then, activity was determined by using well diffusion method [28].

Rearing of Meloidogyne incognita

Egg clusters belongs to *M. incognita* from the roots of the tomato plant were placed in 2 cm diameter egg cups in petri dishes and 10 ml of distilled water was given to each petri dish. The pies were incubated at 24 °C and kept for 24 h. After that, the hatching vessels were removed from the petri dishes and the 2nd term larvae that had just hatched from the egg in the water were used in the study [29, 30].

Nematicidal Activity of Bt Strains Against M. incognita

Larvae of the nematode that can be found into soil were treated with the following cell free extract as antibacterial substance and pellet as crystal-spore complex of *Bt* strains: A1, A14, A15 and A17. All experiments were performed in 24-well tissue culture plates and replicated in triplicates with 100 second stage nematode larvae for each replicate. Distilled water was applied to 100 larvae as control in triplicate. The nematodes were transferred into each well then 1 ml of *Bt* antibacterial substance and crystal spore complex were added into same wells. The plates were then covered with parafilm and incubated at 24 °C for 1-day-old wells. After the incubation, dead and alive nematode larvaes were counted and mortality rates were calculated.

Statistical Analysis

The data of the experiments were analyzed by ANOVA and then by a Least Significant Difference (LSD) test at the significance level of $p<0.05$ using JMP® 7.0 software.

RESULTS AND DISCUSSION

Identification of Bacillus thuringiensis

Biochemical and morphological identification of *Bacillus* species were studied previously (datas not shown) [20, 31]. After this study, isolates were identified regarding to 16S rRNA gene sequencing. The four isolates (A1, A14, A15 and A17) showed 100% sequence identity with *Bacillus thuringiensis Bt*407 (Fig. 1).



Fig. 1. *Phylogenetic relationship between Bacillus sp. strains regarding to their 16S rRNA gene sequence analysis. Bar, 0.005 changes per nucleotide position.*

Antibacterial activity of Bt isolates

The newly isolated *Bacillus thuringiensis* strains were tried for resistance to Amp, Str, Bac, Pen and Tet. All *Bt* isolates were susceptible to at least two antibiotics (Tet and Str). All *Bt* isolates were found resistance to Amp, Pen and Bac (Table 1). In a study, it was found that the most of the *B. thuringiensis* and *B. cereus* isolates were found resistant to oxacillin, ampicillin, amoxicillin, penicillina and ceftriaxone, while were susceptible to ciprofloxacin, chloramphenicol, gentamicin, levofloxacin, gatifloxacin, tetracycline , moxifloxacin, rifampicin, vancomyci, linezolid, streptomycin and tigecycline[32]. These antibiotics are effective on the cell wall of bacteria and prevent bacterial growth by preventing wall formation. *Bt* isolates, which are considered to be non-pathogenic in humans and widely used in pest control, have been reported to be resistant to β-lactams (such as ampicillin and penicillin) [33].

Table 1. *Antibiotic susceptibility test of Bt strains. Amp, ampicillin; Str, streptomycin; Pen, penicillin, Bac, bacitracin; Tet, tetracycline*

	MICs of strains (µg/ml)			
Strains / Antibiotics	***Bt* A1**	***Bt* A14**	***Bt* A15**	***Bt* A17**
Amp	>256	>256	>256	≥256
Str	32-64	64	64-128	32
Pen	>256	>256	>256	>256
Bac	>256	>256	>256	>256
Tet	≤2	≤2	≤2	≤2

The *Bt* isolates were also evaluated to produce the antibacterial substances in LB medium enriched with salts and the maximum level of antibacterial activity occurred at 96 h (data not shown). After 96 h, filtered supernatants of *Bt* strains were used as antibacterial substances. *Bt* antibacterial substances were tested for their antibacterial efficiency against to seven test bacteria, two Gram (+) bacteria (*S. aureus* and *E. faecalis*) and five Gram (-) bacteria (*E. coli, Y. enterocolitica, K. pneumoniae, P. aeruginosa* and *S. typhimurium)*. Among the four *Bt* isolates, *Bt* A14 isolate was active against all tested pathogens, except *P. aeruginosa* (Table 2). It is important to note that assays with *Bt* A14 antibacterial substance did not inhibite protease and proteinase K.

It have reported that *Bacillus thuringiensis* isolates were tested against to four pathogenic bacteria, two Gram (+) bacteria (*S. aureus* variant (SM) and variant (RM)), and two Gram (-) bacteria (*P. aeruginosa* and *E. coli*). It was found that among the 137 *B. thuringiensis* isolates, 10 isolates were effect against to *P. aeruginosa,* 20 isolates against to *E. coli,* 27 isolates against to *S. aureus* and 30 isolates against to *S. aureus* S [32, 34]. In addition, another studies indicated that inhibitory effect of *Bt* endotoxins against some pathogenic bacteria such as *S. sonnei, E. coli, E. faecalis,* S. *aureus, P. aeruginosa, S. typhimurium, K. pneumoniae* and *Salmonella sp.* [33, 34]. Differently from these study, among of 4 *Bt* isolates, only one isolate (*Bt* A14) was found as active against tested bacteria, except *P. aeruginosa* in our study.

Table 2. *Inhibition zones of antibacterial Bt substances against tested pathogens. Results were reported as means ± standard deviation (n = 3)*

Bt Antibacterial Substances / Test bacteria	Diameters of inhibition zones (mean of 3 trials) (mm)			
	A1	A14	A15	A17
P. aeruginosa	-	-	-	-
Y. enterocolitica	-	15±1	-	-
K. pneumoniae	-	15±2	-	-
S. typhimurium	-	14±1	-	-
S. aureus	-	10±1	-	-
E. coli	-	12±1	-	-
E. faecalis	-	11±1	-	-

Production of Crystal Spore Complexes

Additionally, *Bt* isolates were evaluated to produce the crystal-spore complexes in LB medium enriched with salts and the maximum level of crystal and endospore occurred at 96 h. After 96 h, pellets of *Bt* strains were used as endotoxins. All sporangia got ruptured, and *Bt* endospores were released completely in 96 h for all *Bt* strains. The commassie brillant blue (CBB) staining enabled to differentiate the endospores from parasporal crystals by light microscopy (Fig. 2). These are similar crystals and spores in another *Bt* strains and also in the reference studies [35, 36].

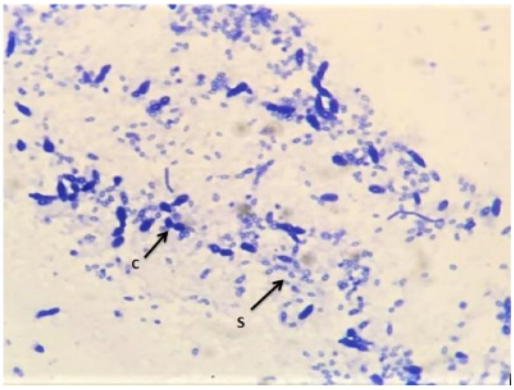

Fig. 2. *Light microscopy images of Bt A14 crystals (C) and spores (S) produced in LB medium enriched with salts*

Nematicidal activity of Bt isolates

M. incognita as endoparasitic nematode is obligate plant parasites and it feeds in the root, and Cry proteins must be digested to be effective, it was decided to test the ability of *Bt* crystal spore complexes to control *M. incognita*.

The most mortality rate on the nematode larvae was determined as 78 % by *Bt* A1 antibacterial substance, but there were no statistically difference among *Bt* A1, *Bt* A14 and *Bt* A15 antibacterial substances. However, *Bt* A15 caused the lowest mortality rate 21 % (Fig. 3). Moreover *Bt* A1, *Bt* A14, *Bt* A15 and *Bt* A17 crystal-spore complexes, caused more than 60 % mortality on the nematode. According to the results, used *Bt* antibacterial substances and crystal-spore complexes have significantly more effective than control ($F=104.15$, $df=10; 32$, $p<0.0001$) (Fig. 3). In a study, Kiewnick and Sikora (2006) indicated that effect of *Paecilomyces lilacinus* for controlling the *M. incognita* on tomato and nematode population of between 58 and 74 % [37]. Similarly, *Bt* toxins were tested for their nematicidal activities on tomato. The results showed that the both cell free supernatant (CFS) of the *Bt*7N and crude strain reduced the number of *M. incognita* number of eggs by 76% and 84 % and egg masses by 77 % and 78 % respectively [38]. In another studies, Bt-ToIr65 was reduced the nematode population and prohibition of hatching of *M. javanica* egg in vitro [43] and *Bt* isolates spore crystal mixtures were showed nematicidal effects in egg mass, root gall index of tomato plants and *M. incognita* population [44].

Bt strains are widely used for biological control of nematodes [8, 39, 43]. They produces a number of virulence factors that have to pathogenic properties. They include exotoxins, collagenase, chitinase and extracellular proteases that makes *B. thuringiensis* an effective insectisides [8, 40, 41]. Additionally, *Meloidogyne* spp. are very difficult to control because of their entophytic and immobile nature, fast generation times and high

reproduction rate [44]. Consequently, it is no doubt detected that all *Bt* substances have the nematode control potential in the framework of biological control.

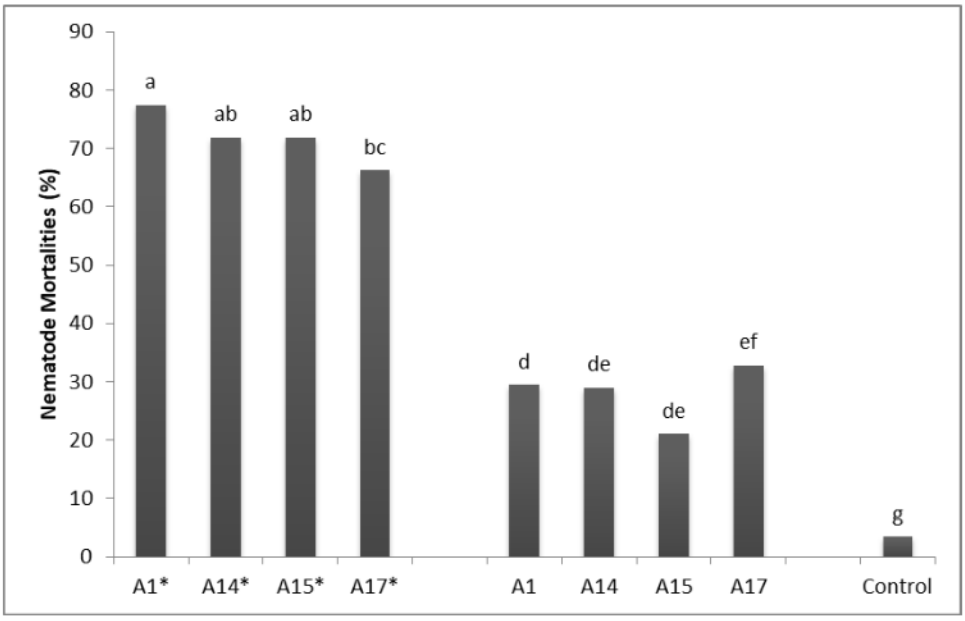

Fig. 3. *Mortality rates (%) of used Bt antibacterial subtances and crystal spore complexes on root-knot nematode Meloidogyne incognita. A*: Bt antibacterial substances, A: Bt crystal spore complexes*

CONCLUSION

Bt has been used commercially for biological control of harmful pests for the last 50 years Here, it was reported some biological properties of *Bt* strains isolated from agricultural soil samples of Turkey. Especially, new *Bt* isolates had potential in agricultural pest control. And also, isolate *Bt* A14, exhibited an antibacterial activity against some pathogenic bacteria. Thus, these newly isolated *Bt* strains may have the potential to be used as biocontrol agents. More studies are required on characterization of antibacterial substances and crystals of *Bt* isolates in order to improve their biocontrol efficiency and thus provide benefitical methods on nematodes management. The benefits of *Bt* isolates and the identification of antimicrobials are subject to further studies.

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