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Original article (Orijinal araştırma)

Susceptibility of different *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) populations to indigenous *Bacillus thuringiensis* strains[1]

Farklı *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) popülasyonlarının yerel *Bacillus thuringiensis* suşlarına duyarlılığı

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Abstract

Tomato leafminer, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) is one of the most important tomato pests worldwide and causes 100% product loss if not controlled. Chemical insecticides, which have been overused for many years, have induced resistance in the pests and made it difficult to control their populations in the field. The use of biological agents that express insecticidal proteins, such as *Bacillus thuringiensis*, is an alternative to conventional insecticides to suppress pest populations. In this study, to recover novel *B. thuringiensis* strains from soil samples, a survey was conducted in Bilecik province in 2021. Thirteen local *B. thuringiensis* strains were isolated and the susceptibility of three different field populations (Samsun, İzmir, and Bilecik) of *T. absoluta* to these strains was evaluated. *Bacillus thuringiensis* B3 (*Bt*-B3) strain, which contains lepidopteran-active toxin genes, was more virulent for all *T. absoluta* populations tested. In addition, Samsun population was more sensitive to the B3 strain than İzmir and Bilecik. The LC_{50} values of *Bt*-B3 were determined to be 13.28, 26.06 and 24.24 ppm for Samsun, İzmir and Bilecik populations, respectively. Sequencing of the 16S rRNA gene region confirmed that the isolate was *B. thuringiensis*, while electron microscopy revealed that the isolate produced bipyramidal, cubic and spherical insecticidal proteins. The results of this study indicate that the isolate *Bt*-B3 appears to be a promising biocontrol agent for integrated pest management of *T. absoluta* in Türkiye.

Keywords: *Bacillus thuringiensis*, biocontrol, *cry* genes, *Tuta absoluta*

Öz

Domates güvesi, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) dünya çapında en önemli domates zararlılarından biridir ve mücadele edilmediği takdirde %100 ürün kaybına neden olur. Uzun yıllardır aşırı kullanılan kimyasal insektisitler, zararlıda direnç oluşturmuş ve popülasyonlarını kontrol etmeyi zorlaştırmıştır. *Bacillus thuringiensis* gibi insektisidal proteinleri eksprese eden biyolojik savaş etmenlerinin kullanımı, zararlı popülasyonlarını baskılamak için geleneksel insektisitlere bir alternatiftir. Bu çalışmada, toprak örneklerinden yeni *B. thuringiensis* suşları elde etmek için 2021 yılında Bilecik ilinde bir sürvey yapıldı. On üç yerel *B. thuringiensis* suşu izole edilmiş ve üç farklı tarla popülasyonunun (Samsun, İzmir ve Bilecik) bu suşlara duyarlılığı değerlendirilmiştir. Lepidopteran-aktif toksin genleri içeren *B. thuringiensis* B3 (*Bt*-B3) suşu, test edilen tüm *T. absoluta* popülasyonlarında daha virülent bulundu. Ayrıca Samsun popülasyonu, B3 suşuna İzmir ve Bilecik popülasyonuna göre daha duyarlıydı. *Bt*-B3'ün LC_{50} değerleri Samsun, İzmir ve Bilecik popülasyonları için sırasıyla 13.28, 26.06 ve 24.24 ppm olarak belirlendi. İzolatın 16S rRNA gen bölgesinin sekanslanması, *B. thuringiensis* olduğunu doğrularken, elektron mikroskopisi izolatın bipiramidal, kübik ve küresel insektisidal proteinler ürettiğini ortaya koydu. Çalışma sonuçları, *Bt*-B3 izolatının Türkiye'de *T. absoluta*'nın entegre zararlı mücadelesi için umut verici bir biyolojik savaş etmeni olduğunu göstermektedir.

Anahtar sözcükler: *Bacillus thuringiensis*, biyolojik savaş, *cry* genleri, *Tuta absoluta*

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Introduction

The tomato leafminer, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) is known to be one of the most destructive pests affecting tomato production in the Mediterranean region. *Tuta absoluta* is an oligophagous pest that damages several species of Solanaceae plants such as potato, eggplant, tobacco, common bean, and various wild solanaceous plants (Desneux et al., 2011; Zhang et al., 2021).

The larvae can decimate both yield and fruit quality by feeding on leaves, stems and fruit. The pest has been responsible for losses of 80-100% in tomato plantations in the greenhouse and open field. Yield losses of up to 100% have been reported in severe outbreaks, and even with the use of conventional pesticides, losses can still exceed 5% (Terzidis et al., 2014). The main approaches currently used to control *T. absoluta* are based on synthetic chemical pesticides. Unfortunately, some of them do not provide the desired effect, as resistance develops due to intensive use. Resistance to avermectins, pyrethroids, diamides, benzoylureas, organophosphates, oxadiazines, semicarbazones and spinosyns have been reported worldwide (Siqueira et al., 2001; Silva et al., 2016; Langa et al., 2022). The pest may also develop resistance to other insecticides over time. In addition, the adverse effects of chemicals on non-target organisms limit the use of its predators, -as biological control strategies (Arnó & Gabarra, 2011). Therefore, integration with other control strategies such as biological and biotechnological methods is necessary instead of chemical insecticides.

An alternative to chemical insecticides is insect-pathogenic microorganisms such as *Bacillus thuringiensis* (*Bt*), which produce insecticidal crystal proteins during the sporulation phase. *Bt* strains may have one or more *cry* genes, providing the strains to express one or more Cry proteins. The crystal proteins shown to have lethal effect against different orders of insects such as Lepidoptera, Coleoptera, and Diptera. To date, local *Bt* isolates and *Bt*-based biopesticides have been tested to suppress *T. absoluta* populations under laboratory, greenhouse and open field conditions (Giustolin et al., 2001; Sandeep Kumar et al., 2020; Aynalem et al., 2021; Buragohain et al., 2021).

It is important to continue the search for novel *Bt* strains that are effective to lepidopteran insects such as *T. absoluta* because their virulence is not fully same in different ecological conditions, as there are insect populations that are resistant to *Bt*. Therefore, the aim of this study was to isolate novel *Bt* strains containing lepidopteran-active *cry* genes and to assess the susceptibility of different field populations of *T. absuluta*.

Materials and Methods

Isolation of *Bacillus thuringiensis* from soil samples

Soil samples were collected from different locations in Bilecik, Türkiye, during the summer of 2020. After scraping the surface material, the samples were collected from a depth of 15 cm below the surface using a soil corer and placed in an autoclaved glass jar (Eski & Gezgin, 2022). The samples were transferred to the laboratory for isolation of *B. thuringiensis*.

Bacillus thuringiensis isolation was conducted according to the procedure described by Santana et al. (2008). Five grams of samples were preheated in an incubator at 80°C for 3 hours. One gram of the soil sample was mixed in 10 ml of 0.85% NaCl by vigorous shaking for 2 minutes and serially diluted tenfold with phosphate saline buffer. Dilutions were then incubated at 80°C for 12 minutes and a 0.1 ml sample was spread on tryptic soy agar plates. After incubation at 30°C for 2 days, *Bt*-like colonies were picked up (cream-colored and looking like fried eggs on the plate) and purified by serial spreading.

Presence of insecticidal crystal proteins

Potential *Bt* isolates were grown in terrific broth medium (Estruch et al., 1996) at 200 rpm and 30°C until the complete lysis of cells. Then, spore and crystals were collected by centrifugation at 12000 × g for 5 min. The pellet containing spores and crystals was washed with cold NaCl (0.5 M) to remove extracellular components and finally resuspended in sterile distilled water. The spore-crystal mixtures of each isolate were stained with amido black and Ziehl's carbol fuchsin (Smirnoff, 1962) and checked for the presence of crystal under a phase contrast microscope. In addition, the spore-crystal mixtures were stored at 4°C to be used for screening tests.

Cry gene profile of isolates

Total DNA was extracted from 100 mg wet weight of bacteria (approximately 10^9 bacterial cells) using the Zymo DNA isolation kit (Zymo Research, Irvine, CA, USA). *Cry* genes were amplified by PCR using *cry* gene specific oligonucleotide primers (Table 1) (Jain et al., 2012). The 25 µl of PCR mixture contained 200 µM of each dNTP, 0.2 µM concentration of each primer, 0.4 U *Taq* DNA polymerase, 2.5 µl 10× PCR buffer, 1 µl total DNA template. Reactions were set for 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 49-60°C (depended on each pair of primer) for 30 s and extension at 68°C for 30 s. PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer and visualized under UV transilluminator.

Table 1. Oligonucleotide primers used in *cry* gene screening

Primer (product size)	Primer sequence (5' -> 3')	T_m (°C)
cry1 (277 bp)	CATGATTCATGCGGCAGATAAAC (f) TTGTGACACTTCTGCTTCCCATT (r)	55
cry2 (701 bp)	GTTATTCTTAATGCAGATGAATGGG (f) CGGATAAAATAATCTGGGAAATAGT (r)	52
cry3 (604bp)	CGTTATCGCAGAGAGATGACATTAAC (f) CATCTGTTGTTTCTGGAGGCAAT (r)	54
cry4 (439 bp)	GCATATGATGTAGCGAAACAAGCC (f) GCGTGACATACCCATTTCCAGGTCC (r)	59
cry5 (474 bp)	TTACGTAAATTGGTCAATCAAGCAAA (f) AAGACCAAATTCAATACCAGGGTT (r)	52
cry7 (420 bp)	AAGCAGTGAATGCCTTGTTTAC (f) CTTCTAAACCTTGACTACTT (r)	49
cry9 (359 bp)	CGGTGTTACTATTAGCGAGGGCGG (f) GTTTGAGCCGCTTCACAGCAATCC (r)	60
cry11 (305 bp)	TTCCAACCCAACTTTCAAGC (f) AGCTATGGCCTAAGGGGAAA (r)	51

f: forward primer, r: reverse primer.

Screening experiments

The spore-crystal mixture used in the bioassays was prepared as described above and dried using a freeze dryer according to the manufacturer's instructions. The freeze-dried spore-crystal mixtures were then resuspended in sterile distilled water at a concentration of 50 ppm and used in bioassays.

Susceptibility of three different field populations (Samsun, İzmir and Bilecik) of *T. absoluta* to spore crystal mixtures was evaluated using the leaf-dip bioassay as described in Insecticide Resistance Action Committee Test Method No. 22. Tomato leaf disks (3 cm) were immersed in the bacterial suspension and allowed to dry on a wire mesh. When the leaf surface was completely dry, these discs were placed in Petri dishes (60 mm) containing moistened cotton. Then, one second instar *T. absoluta* larva was placed in each Petri dish. At least thirty-two larvae were used for the bioassays, and the tests were replicated three times for each application. Sterile distilled water was used in the control group. The bioassay was performed at 25°C, 65% RH, and a photoperiod of 14:10 h (L:D) for 3 days. The mortality rate was recorded daily.

Concentration response experiments

As a result of the screening tests, *Bt*-B3 isolate, which had a high effect on all the three *T. absoluta* populations, was used in concentration response experiments. Six different spore-crystal suspensions of the isolate were prepared at concentrations of 60, 50, 40, 30, 20 and 10 ppm, and bioassays were performed as indicated in the screening tests.

Statistical analysis

Mortality data was corrected using the Abbott's formula (Abbott, 1925) and subjected to analysis of variance, followed by comparison of means with Tukey's test. Data normality and homogeneity of variance were checked using the Shapiro-Wilk test and Bartlett's test, respectively. Lethal concentrations needed to kill 50% and 90% of the larvae were calculated using Probit analysis. SPSS Statistics 24 software package (IBM, Armonk, NY, USA) was used as the statistical tool.

Detailed characterization of *Bt*-B3

The crystal structures of *Bt*-B3 isolate, which had the highest virulence in the tested populations and whose spore crystal presence was detected by phase contrast microscopy, were determined by electron microscopy, and molecular characterization was performed by amplification of the 16S rDNA gene region.

The morphology of insecticidal crystal proteins of *Bt*-B3 was examined under the scanning electron microscope. Twenty microliters of the spore crystal mixture were transferred on a stub and dried at 37°C for 24 h. The dried stub was coated with platinum dust using an automatic sputter coater (Quorum Technology SC7620-CF). The coated spore-crystal mixture was examined with a Zeiss Evo LS10 (Tokyo, Japan) and photographed at various magnifications.

Total DNA previously extracted from *Bt*-B3 was amplified by 16S rRNA gene specific primers (UNI16S-F and UNI16S-R) (Weisburg et al., 1991). The 25 µ of PCR mixture contained: 200 µM of each dNTP, 0.2 µM concentration of each primer, 0.5 U of *Taq* DNA polymerase (NEB,), 2.5 µl of 10× reaction buffer and 50 ng of DNA template. Amplification was performed using a thermal cycler, with the following program: 95°C for 30 s, 1 cycle; 95°C for 30 s, 55°C for 30 s, 68°C for 1 min, 30 cycles; 68°C for 5 min, 1 cycle. The PCR product was loaded on 1.0% agarose gel and visualized under UV light. The amplified fragment was sent for sequencing at Ficus Biotechnology (Ankara, Turkey).

The obtained sequence was compared with NCBI nucleotide database using BLAST tool and submitted to the GenBank database. The phylogenetic trees based on partial 16S rDNA sequences were inferred using the neighbor-joining (NJ) algorithm and 1000 bootstrap replicates in the MEGA X (Kumar et al., 2018).

Results

Isolation of *B. thuringiensis* isolates

Forty soil samples were collected from 24 different locations in Bilecik, Turkey, and a total of 64 *Bacillus*-like colonies were isolated. Spore crystal staining of the isolates revealed that 13 of them had insecticidal crystal proteins that distinguished *Bt* from all other sporulating bacteria. Since the *Bt* index was defined as the ratio between the number of *Bt* colonies identified and the total number of *Bacillus*-like colonies examined, the *Bt* index was 0.203. Phase-contrast microscopy examination revealed that M5, M6, P4, P6, Y5, B3, O5, O6, G4, G8, S3, S6, and S10 formed crystals.

PCR analysis was performed to identify *cry* genes encoding toxin proteins produced during the sporulation phase. Among the eight *cry* gene primers used in the present study, *cry1* and *cry2* were the predominant genes observed in seven *Bt* strains. The other *cry* genes such as *cry3* and *cry4* were observed in four strains each. However, the *cry5* gene was found in only one isolate (O5) (Table 2).

Table 2. *Cry* gene contents of indigenous *Bt* isolates

Bt isolates	*cry* genes							
	*cry*1	*cry*2	*cry*3	*cry*4	*cry*5	*cry*7	*cry*9	*cry*11
M5	+	+	-	-	-	-	+	-
M6	+	+	-	-	-	-	-	-
P4	-	-	+	-	-	-	-	-
P6	-	+	-	-	-	-	+	-
Y5	-	+	-	+	-	-	-	-
B3	+	+	-	+	-	+	-	+
O5	-	-	+	-	+	-	-	-
O6	-	-	+	-	-	-	-	+
G4	+	-	-	+	-	-	-	-
G8	+	-	-	+	-	-	-	-
S3	+	+	-	-	-	-	-	-
S6	-	-	+	-	-	+	+	-
S10	+	+	-	-	-	-	-	-
Frequency (%)	53.8	53.8	30.8	30.8	7.7	15.4	23	15.4

Screening test and concentration response experiments

The insecticidal activities of thirteen *Bt* isolates were tested on three different populations (İzmir, Bilecik and Samsun) of tomato leafminer. In the laboratory experiments, it was found that all *Bt* isolates showed pathogenicity on the Samsun population of the pest and their virulence ranged from 30% to 90% ($F=19.96$; $df=12,26$; $p < 0.05$). Accordingly, the most effective isolate was *Bt*-B3, which killed 90% of the larvae, while the least effective isolate was S6 with 30% (Figure 1A). It was also found that the virulence of the isolates in the İzmir and Bilecik populations was lower than the effect in the Samsun population. The virulence varied between 20-75% ($F=13.72$; $df=12,26$; $p < 0.05$) and 25-96% ($F=373.78$; $df=12,26$; $p < 0.05$) in the İzmir and Bilecik populations, respectively. B3 was the most effective isolate in both the İzmir (75%) and Bilecik (96%) populations (Figure 1B, C).

Concentration response experiments were performed with *Bt*-B3 isolate, which showed the highest effect in all the three populations. *Bt*-B3 at a concentration of 60 ppm showed mortality rates of 95%, 85%, and 81% in Samsun, İzmir and Bilecik populations of the pest, respectively. *Bt*-B3 had the highest toxicity in Samsun population with the lowest LC_{50} value ($F=147.8$; $df=2,6$; $p < 0.05$) (Table 3).

Table 3. Lethal concentrations (LC_{50} and LC_{90}) of *Bt*-B3 isolate against three different field populations of *T. absoluta*

Insect population	LC_{50} (FL, 95%)	Slope ± SE	LC_{90}	df	X^2	*p* value
İzmir	26.06 (17.95-34.73) b	2.5±0.21	100.28	4	12.29	0.015
Samsun	13.28 (2.52-20.77) a	2.5±0.22	60.06	4	19.41	0.001
Bilecik	24.24 (20.76-27.69) b	2.0±0.21	121.31	4	2.43	0.656

Lowercase letters represent statistical differences between LC_{50} values according to Tukey's multiple comparison test ($P<0.05$). FL: fiducial limit, SE: standard error, df: degree of freedom, X^2: Chi-square.

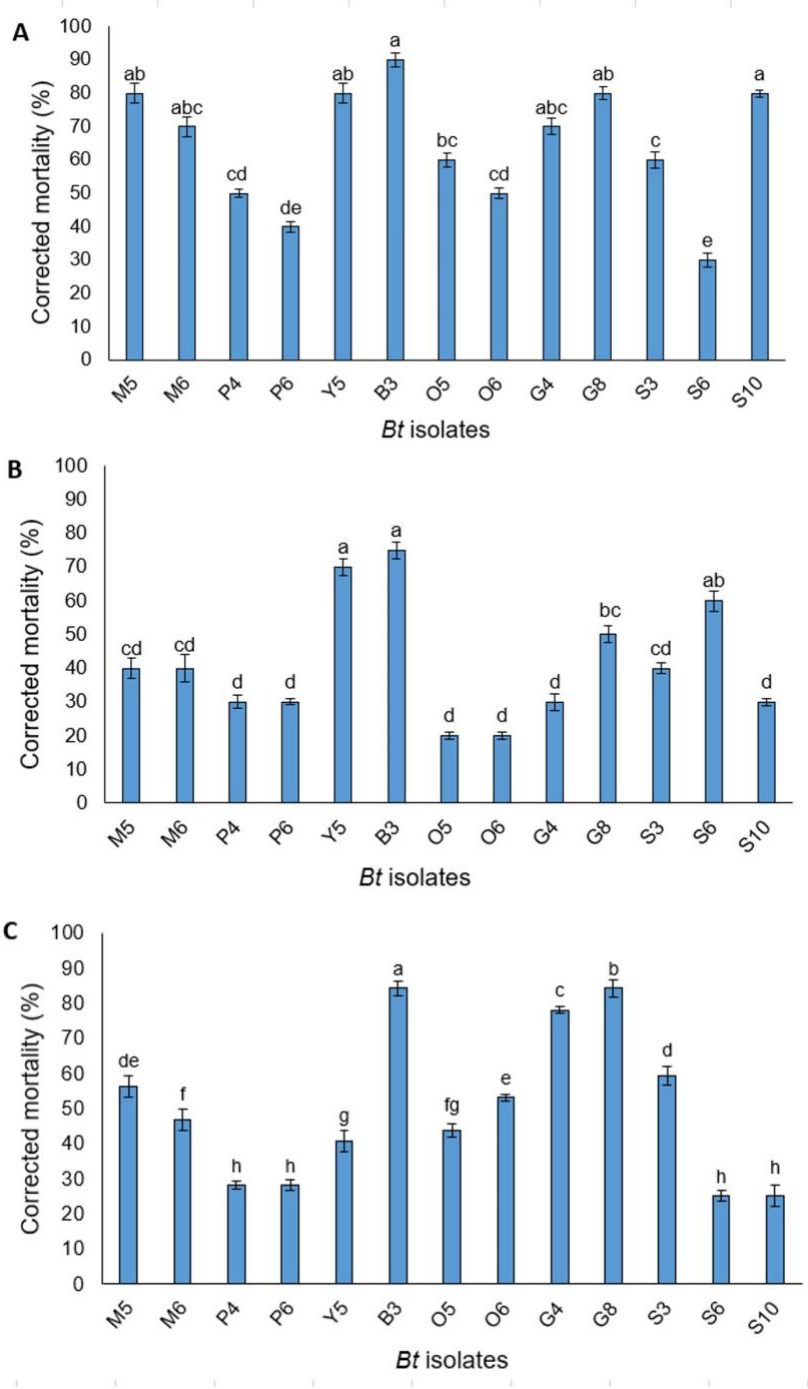

Figure 1. Corrected mortality rates of Samsun (A), İzmir (B), and Bilecik (C) populations of *T. absoluta* exposed to native *Bt* isolates with 50 ppm spore-crystal mixture 72 hours after treatment. Different letters represent statistically significant differences between mortality rates according to Tukey's multiple comparison test ($P<0.05$). Mortality indicates the mean of three replicates. The bars show the standard deviation of the mean values.

Detailed characterization of *Bt*-B3

The spore crystal morphology of *Bt*-Se13 isolates was characterized using SEM. The SEM images showed that bipyramidal, cubic, and spherical crystal proteins were present (Figure 2).

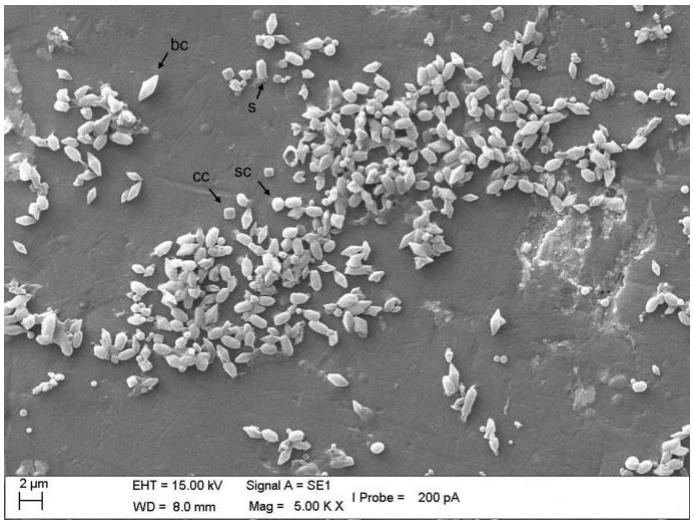

Figure 2. Scanning electron microscopy of *Bt*-B3 isolate. s: endospore; bc: bipyramidal crystal; cc:cubic crystal; sc:spherical crystal.

We also sequenced approximately 1.350 bp of the 16S rRNA gene of the *Bt*-B3 isolate for molecular identification and nucleotide sequence homology search was carried out using BLASTn (http://www.ncbi.nlm.nih.gov). The *Bt*-B3 isolate was found to have the highest homology (99%) with other known *B. thuringiensis* isolates. The sequence was submitted to the NCBI GenBank database with accession number OM732508. Phylogenetic analysis also showed that *Bt*-B3 isolate clustered with reference *B. thuringiensis* isolates (Figure 3).

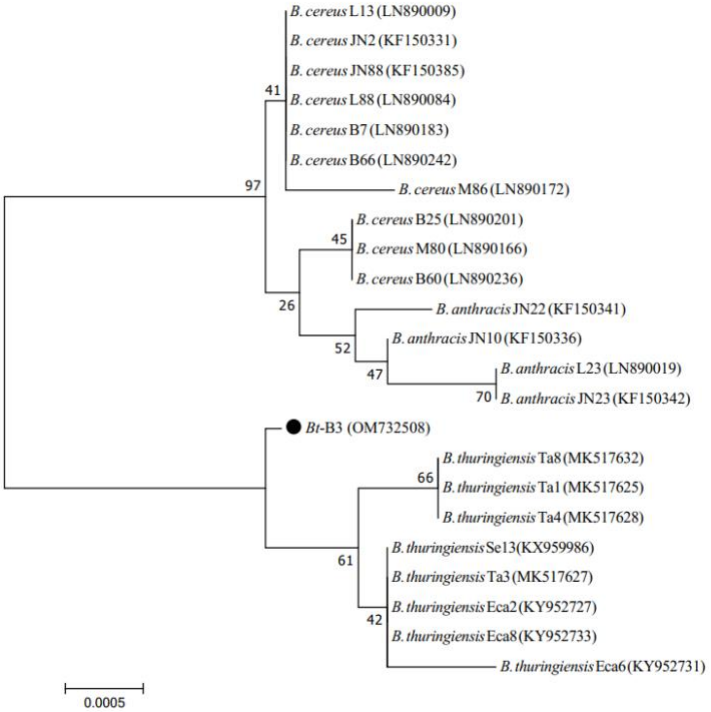

Figure 3. Phylogenetic analysis of *Bt*-B3 isolate inferred from neighbor-joining analysis with *p*-distance model of 16S rRNA sequence.

Discussion

Native *Bt* isolates have novel insecticidal genes with broader toxicity and might be more effective than exotic strains. Therefore, soil screening for novel and effective *Bt* strains is one of the global strategies for pest control. In this study, 13 indigenous *Bt* strains were isolated from forty soil samples (*Bt* index=0.325). Previous studies reported variable frequency of isolation of *Bt* from soil samples, ranging from 3 to 85% (Ramalakshmi & Udayasuriyan, 2010; Hassan et al., 2021). Abo-Bakr et al. (2020) studied 16 Egyptian soil samples and isolated 16 *Bt* strains from 56 *Bacillus*-like colonies (*Bt* index=0.28). On the other hand, Djenane et al. (2017) isolated 180 colonies, of which only 16 isolates were identified as *Bt* (*Bt* index=0.08). The variation of *Bt* index in soil samples could be related to the chemical properties of the soil, such as macro/micronutrients, soil moisture, and soil oxygenation, which may affect *Bt* growth and toxin production (Polanczyk et al., 2009).

Since the aim of this study was to determine the effective isolates against *T. absoluta*, the *cry1*, *cry2*, and *cry9* genes known to be effective against Lepidoptera pests were identified in the isolates (Rosas-García et al., 2008; Salama et al., 2015). In this study, *Bt*-B3 isolate was found to possess *cry1*, *cry2*, *cry9* and *cry11* genes. We also found a high frequency (53.8%) of *cry1* and *cry2* genes in our *Bt* collection, which was similar to that described in other reports (Bravo et al., 1998; Wang et al., 2003), while *cry5* gene had the lowest frequency (7.7%) (Table 2). Thammasittirong & Attathom (2008) found that 81.3% of strains harbored *cry1* genes, 80.6% *cry2* genes, and 37.3% *cry9* genes in Thailand collection. In another report, Rashki et al. (2021) found that *cry1* genes were more abundant in Iranian *Bt* strains. On the other hand, *cry9* genes were more abundant (47.8%) than *cry1* and *cry2* genes (6.5 and 2.1%) in Brazilian collection (Pinto & Fiuza, 2003). It is clear that the occurrence, distribution and diversity of *cry* genes are variable and depend on the geographical region.

The strong insecticidal activity of the *Bt* strains seems to be due to the combined properties of several Cry proteins that combine to form an inclusion body. In our study, scanning electron microscopy showed that *Bt*-B3, the most effective isolate in all the populations tested, had spherical, cubic, and bipyramidal insecticidal Cry proteins.

While *Bt*-B3 had the highest virulence in the Samsun population with 90%, the lowest virulence was observed in the İzmir population with 74%. It is believed that the difference in insecticidal efficacy between the populations is due to the development of resistance in insects. Izmir is the first region where the pest was seen in Turkey in 2009, and chemical insecticides have been used for control since then. Thus, the insecticides used to prevent losses from the pest may have led to the development of resistance over time. In the screening tests, isolate *Bt*-B3 had the highest effect on all populations tested among the isolates. Therefore, concentration experiments with *Bt*-B3 were performed on populations and LC_{50} values of 13.28, 26.06, and 24.24 µg/ml were determined for the Samsun, İzmir, and Bilecik populations, respectively. The concentration-response experiments showed once again that the Samsun population was more sensitive to the *Bt*-B3 isolate.

Several studies have been conducted on the efficacy of *Bt* or *Bt*-based biopesticides on populations of *T. absoluta*. Sandeep Kumar et al. (2020) tested the *Bt* strains (4D1, 4D4, 4G1) on the laboratory reared population and the LC_{50} values for the second larval stage were 6.10, 6.62 and 8.18 µg/ml, respectively. However, Sabbour & Soliman (2014) found that the LC_{50} values of the commercial product Dipel and the strains *Bt* kurstaki HD-73 and HD-234 were 140, 109, and 90 µg/ml, respectively, on the larvae of *T. absoluta* under laboratory conditions. The differences in LC_{50} values are directly related to the virulence of the isolates and the susceptibility of the insect population. Previous studies have investigated the susceptibility of some conventional insecticides such as indoxacarb, metaflumizone, spinosad, chlorantraniliprole, and λ-cyhalothrin to different field populations of the pest (Yalçın et al., 2015; Bala et al., 2019; Prasannakumar et al., 2021). However, there is no study on the susceptibility of different field populations of *T. absoluta* to local *Bt* strains.

Our results suggest that *Bt*-B3 containing lepidopteran active *cry* genes may be an effective biological control agent to be used in integrated control of the pest. To achieve high efficacy of the spore-crystal mixture under greenhouse and field conditions, the *Bt*-B3 isolate should be formulated in further studies to overcome the adverse effect of the environment such as UV radiation, rain, and temperature, and then tested against *T. absoluta* under greenhouse and field conditions.

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