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Original article (Orijinal araştırma)

Local isolate of *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) from *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae: Spilomelinae) includes cry1, cry3 and cry4 genes and their insecticidal activities

Cydalima perspectalis (Walker, 1859) (Lepidoptera: Crambidae: Spilomelinae)'ten izole edilen cry1, cry3 ve cry4 genlerini içeren yerel *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) bakterisine ait insektisidal aktivite

Mehtap USTA[1*]

Abstract

Cydalima perspectalis (Walker, 1859) (Lepidoptera: Crambidae: Spilomelinae) is a primary pest on boxwood. The larvae of this pest cause damage by eating the leaves. They prevent the plant from performing photosynthesis, gnaw the bark of the shoots, damage the cambium layer and thus dry the boxwood shoots and branches. The boxwood moth, which is an exotic species in Türkiye, was first found in gardens and parks in Istanbul in 2011 and subsequently in Artvin and Düzce in 2015, and Bartın in 2016. The aim of this study is to develop a control method for this pest by considering the damage of the species. In this context, bacteria were isolated from the larvae collected from Artvin in May 2021 and bacteria screening was conducted to obtain an effective isolate. At the end of this isolation, *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) was found. When cry gene analysis of this bacterium was performed, it was determined that it contained cry1, cry3 and cry4 genes. The insecticidal activity of this bacterium was tested on *C. perspectalis* and a mortality rate of 85% was obtained.

Keywords: *Bacillus thuringiensis,* biological control, cry genes, *Cydalima perspectalis,* microbiology

Öz

Cydalima perspectalis (Walker, 1859) (Lepidoptera: Crambidae: Spilomelinae) şimşir üzerinde birincil zararlıdır. Bu zararlının larvaları yaprakları yiyerek zarar verir. Bitkinin fotosentez yapmasını engeller, sürgünlerin kabuğunu kemirir, kambiyum tabakasına zarar verir ve böylece şimşir sürgünlerini ve dallarını kuruturlar. Türkiye'de egzotik bir tür olan şimşir güvesi ilk olarak 2011 yılında İstanbul'da bahçe ve parklarda bulunmuş ve daha sonra; 2015 yılında Artvin ve Düzce'de, 2016 yılında Bartın'da yapılmıştır. Bu çalışmanın amacı, türün zararını göz önünde bulundurarak bu zararlı için bir kontrol yöntemi geliştirmektir. Bu kapsamda Mayıs 2021'de Artvin ilinden toplanan larvalardan bakteri izolasyonu yapılmış ve etkili bir izolat elde etmek için bakteri taraması yapılmıştır. Bu izolasyon sonunda *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) bulunmuştur. Bu bakterinin cry gen analizi yapıldığında cry1, cry3 ve cry4 genlerini içerdiği belirlendi. Bu bakterinin böcek öldürücü aktivitesi *C. perspectalis* üzerinde test edilmiş ve %85'lik bir ölüm oranı elde edilmiştir.

Anahtar sözcükler: *Bacillus thuringiensis*, biyolojik mücadele, cry genleri, *Cydalima perspectalis,* mikrobiyoloji

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Introduction

Cydalima perspectalis (Walker, 1859) (Lepidoptera: Crambidae: Spilomelinae) originates from East Asia (China, Japan and Korea) and is an exotic species in Türkiye (Öztürk et al., 2016). It was first found in Europe in 2007 in Germany. Its presence and damage have been determined in southern Russia (Mally & Nuss, 2010; Wan et al., 2014; Santi et al., 2015) and it was reported that can cause damage to Buxus sempervirens L. (Buxales: Buxaceae) in Georgia in 2015 (Matsiakh et al., 2016). In Türkiye, it was observed to cause damage in gardens and parks in Sarıyer, Istanbul in 2011 (Hizal, 2012). Than It was observed in Düzce (Öztürk et al., 2016) and Artvin (Anonymous, 2011) in 2015, and in Bartın in 2016 (Toper Kaygın & Taşdeler, 2018; Yıldız et al., 2018). It was also reported to have caused intense damage in Artvin in 2016-2017 (Göktürk, 2017). *Cydalima perspectalis* feeds on *B. sempervirens* and *Buxus microphylla* (Sieb. & Zucc.,1965) (Buxales: Buxaceae) in Europe (Leuthardt et al., 2010). The pest has also been reported to feed on *Buxus sinica* (Rehder & E.H.Wilson, 1959) (Buxales: Buxaceae) (Wan et al., 2014). Also, *Euonymus japonicus* Thunb. (Linne, 1880) (Celastrales: Celastraceae) and *Ilex purpurea* Hassk. (Sims, 1819) (Aquifoliales: Aquifoliaceae) are host plants in Asia (Muus et al., 2009). *Buxus sempervirens* occurs in Artvin, Rize, Trabzon, Osmaniye, Kastamonu, Bolu, Karabük, Denizli, Kocaeli, Hatay and Kahramanmaraş Provinces of Türkiye; *Buxus balearica* Lam. (Buxales: Buxaceae) occurs naturally in Adana, Antalya and Hatay (Anonymous, 2021a, b), and is also grown as an ornamental in parks and gardens in many provinces.

Damage caused by *C. perspectalis* in *B. sempervirens* has been reported in Türkiye (Toper Kaygın & Taşdeler, 2018). Boxwood blight disease caused by *Cylindrocladium buxicola* Henricot (Hypocreales: Nectriaceae) (Henricot & Culham, 2002) has previously been reported on boxwood in areas where damage appears. This pathogen was detected in Artvin and Trabzon Provinces for the first time in 2011. Destructive effects have been reported in the infested regions. About 90% defoliation of boxwood can be caused by this fungal disease (Lehtijärvi et al., 2014, 2017).

In Türkiye, damage caused by the boxwood moth, the reduction of the natural boxwood population, and the damage to the forest ecosystem in large devastated forest areas is of great concern. Boxwood contributes to the livelihood of the local people and therefore to the economy of the country with its valuable wood and shoots in the regions where it grows. In addition, since it is a preferred species in landscaping, it is seen as an undesirable and alarming situation by the public to be damaged or even killed by boxwood moth. It is hoped to be able to prevent the spread of *C. perspectalis*, especially in the Black Sea Region, using biological methods to combat this pest.

Considering the damage caused by the pest, this study aimed to determine a local isolate for control. In this context, the effect of the bacteria obtained from the pest and the crystal proteins of this bacteria on the pest was determined and the most effective material was determined and the difference between them investigated.

Materials and Methods

Sample collection

Samples of *C. perspectalis* larvae were collected from central Artvin, Türkiye. About 75 larvae were collected from the boxwood trees at the sampled locations. Collected *C. perspectalis* larvae were placed in sterile tubes and brought to the laboratory condition. Collected larvae samples were obtained in May 2021.

Bacterial isolation

Before starting the bacterial isolation, surface sterilization was performed with 70% alcohol. After surface sterilization, the larvae washed with sterile distilled water, homogenized and in sterilized nutrient broth (NB) using a glass tissue grinder and filtered through cheesecloth. The filtered homogenates were

diluted to 1 × 10^{-5} and 0.1 ml spread on agar medium (nutrient agar, NA) (Thiery & Frachon, 1997). The homogenate was heated to 80°C for 20 min before spreading onto plates to isolate bacteria in the genus *Bacillus*. Petri dishes were incubated at 30°C for 2 days. Colonies obtained after 2-3 days were expected to be *Bacillus*. The isolates were determined according to the color and morphology of the colonies. Identification of bacterial colonies was made according to morphological, biochemical and molecular methods (16S rRNA).

Analysis of scanning electron microscope and physiological and morphological properties of isolate

Selection of bacterial isolates was made according to Bergey's Manual of Systematic Bacteriology via morphological, physiological and molecular methods according to Sneath (1986). The phenotypic characterization was determined by looking at the colony morphology on NA. The optimum pH value of the bacteria was obtained after 16 h at 30°C (Ben-Dov et al., 1995).

For the SEM image, the bacterial sample was first incubated for 24 h at 30°C on NA. The collected samples were dropped on filter membrane and air dried. Bacteria samples were coated with gold before SEM. Then fixed in phosphate buffered saline (PBS). Five µl of the bacterial solution was taken and dried on a SEM sample set (Carl Zeiss, Jena, Germany). The voltage of the microscope was adjusted to be 5 to 10 kV.

Molecular identification of bacterium

Genomic DNA isolation from bacterial sample was done according to Sambrook et al. (1989). Genomic DNA isolation from bacterial sample was done according to Sambrook et al. (1989). PCR amplification of 16S rRNA gene of bacterial isolate was performed with the following universal primers (William et al., 1991); UNI 16S-L: 5-ATTCTAGAGTTTGATCATGGCTCA-3 as forward and UNI 16S-R: 5-ATGGTACCGTGTGTGACGGGCGGTGTGTA-3 as reverse. PCR conditions were adjusted according to William et al. (1991). Reactions were totally in 50 µl; 1 µl of template DNA was mixed with 5 µl reaction buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM (each) with primer and 0.5 U with Taq DNA polymerase. Amplification was performed with 30 cycle programs (each cycle consisting of denaturation at 94°C for 3 min, annealing at 55°C for 60 s and extension at 72°C for 3 min), followed by a final extension step at 72°C for 5 min, by using thermal cycler (BioRad, Hercules, CA, USA). The reaction was made in total of 50 ml. Each PCR reaction was performed using the negative control (without DNA template). PCR product was analyzed on a 1.2% agarose gel. The visible band on gel was sent for sequencing. Sequencing of the amplified 16S rRNA samples was done by SenteBiolab (Ankara, Türkiye) using universal primers. The resulting sequences were analyzed for BLAST using the NCBI GenBank database.

Determination of cry genes

Screening of cry genes by PCR was done according to the method of Nishiwaki et al. (2007). The primer list of cry genes is given in Table 1. The reaction was made in total of 50 ml. Each PCR reaction was performed using the negative control (without DNA template). Amplified PCR products were visualized with a 1.2 % agarose gel. PCR products were transferred into to pJET1.2 Blunt Vector (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to *Escherichia coli* (Migula,1895), (Enterobacterales: Enterobacteriaceae) DH10β cells. Vectors containing cry genes of *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) were selected via white colonies. All white colonies growing on the Petri dish indicate clones that are include cry genes. Sequence analyzes were performed by checking each one for confirmation. Purification of recombinant plasmids were done by the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and sequence analyzes were performed by SenteBiolab.

Table1. Primers used for cry gene sequencing (after Ben-Dov et al., 1995)

Primer	Primer	Amplicon length (bp)	Tm (°C)
cry1Fw	CATGATTCATGCGGCAGATAAC	277	55
cry1Rv	TTGTGACACTTCTGCTTCCCATT		
cry2Fw	GTTATTCTTAATGCAGATGAATGGG	701	52
cry2Rv	CGGATAAAATAATCTGGGAAATAGT		
cry3Fw	CGTTATCGCAGAGAGATGACATTAAC	604	54
cry3Rv	CATCTGTTGTTTCTGGAGGCAAT		
cry4Fw	GCATATGATGTAGCGAAACAAGCC	439	59
cry4Rv	GCGTGACATACCCATTTCCAGGTCC		

Expression of *cry* genes in *Escherichia coli*

Recombinant pJET 1.2 Blunt vectors containing cry1, cry 3 and cry 4 genes were cut with appropriate enzymes (BamHI and NcoI) and cloned into pET-28a+ expression vector. These generated recombinant vectors were transferred into *E. coli* BL21 (DE3) component cells for construct plasmids (pET-cry1, pET-cry3 and pET-cry4). These were incubated for 16 h at 37°C by spreading on LB agar medium containing antibiotic (kanamycin). One each was selected from the colonies growing in Petri dishes and incubated at 37°C in liquid medium (LB broth) supplemented with kanamycin. When the optical density (OD; 600 nm) reached 0.6, expression was induced with 1 mM isopropyl β-D-1-thiogalactoside. After incubation of the cells for 120 min at 37°C, they were centrifuged at 5,000 x g for 5 min at 4°C. Since the expressed proteins contain His-tag, their purification was performed according to the MagneHis Protein Purification System (Promega) manual. Protein concentrations were obtained according to procedures outlined by Bradford (1976). Purified cry proteins were screened by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% PAGE).

Insecticidal activities

For insecticidal activity, firstly, the bacterial isolate was incubated in NB at 30ºC for 72 h (for sporulation). After incubation, the bacterial sample was centrifuged at 3,000 rpm for 10 min (Ben-Dov et al., 1995). The resulting pellet was suspended in sterile PBS (or sterile H_2O). The density of the cells was adjusted to OD 600 nm and five concentrations were applied. (0.945, 1.80, 2.84, 3.78 and 5.67 x 10^9 cfu/ml) (Moar et al., 1995). The boxwood leaves that used during the application were obtained from the boxwood trees, which are the natural environment where insects are also collected. When these leaves were brought to the laboratory, surface disinfection was performed with 70% ethanol before application. Boxwood leaves prepared in small pieces were wetted with bacterial suspensions and dried at room temperature. (Hernández et al., 2005). Petri dishes (16 cm) were used in the bioanalysis. Samples of *C. perspectalis* larvae were collected in central Artvin. The insects used were collected from the boxwood trees in the central Artvin. Since the larvae were obtained from the natural environment, so no pesticides had been applied and the larvae were used directly in the assay. Third instar *C. perspectalis* larvae were used to determine the insecticidal activities of bacterial isolates. During the collection of the larvae to be used in the application, only the third instar larvae were collected with the guidance of the entomologist. For the bacterial isolate, 10 larvae were tested. Experiments were performed in triplicate. Sterile PBS was used as control. The Petri dishes were incubated at room temperature and death recorded daily for 10 days. The insecticidal activities of cry proteins were determined according to Sun et al. (2004).

Crystal enzyme solutions with five concentrations (500, 750, 1000, 1500 and 2000 U/ml) enzyme activity were prepared from His-tag purified cry enzymes (Binod et al., 2007). Small boxwood leaves with a diameter of 5 mm were prepared, soaked with cry proteins for 1 min, dried at room temperature and given to previously starved larvae. The larvae were starved for 8 h and then fed on leaves coated with enzyme

solutions. The control groups were fed with the same nutrient impregnated with the elution solution.10 larvae were tested for each protein isolate. Experiments were performed in triplicate and deaths recorded daily for 10 days (Ding et al., 2008).

Statistical Analysis

Mortality data were corrected by Abbott's formula (Abbott, 1925). Lethal concentrations (LC50) for the bacterial isolate (Finney, 1952) and cry proteins against third-stage larvae of hosts were calculated by probit analysis using MS Excel.

Results

In this study, *B. thuringiensis* was isolated from *C. perspectalis*. The colony color was creamy and optimum pH was pH 5 for bacterial isolate. The 16S rRNA partial gene sequence generated in this study has accession number OK513177. Also, for identification scanning electron microscopy photography is shown in Figure 1.

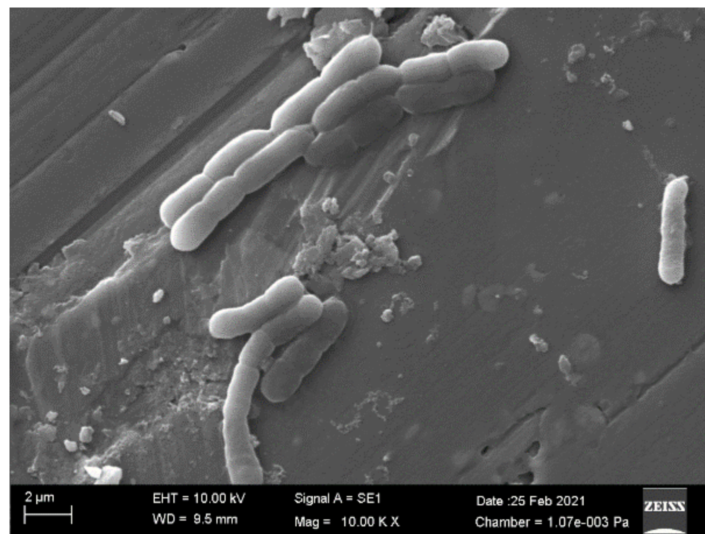

Figure 1. Scanning electron microscope image of the bacterium.

The PCR amplification of cry genes (cry1, cry2, cry3 and cry4) of the bacterium was scanned. According to amplification results only cry1, cry3 and cry4 genes were found. According to these results, expected sizes were obtained as indicated in Table 1 (Figure 2). The bands obtained were for cry1 (277 bp), cry3 (604 bp) and cry4 (439 bp) (Figure 2).

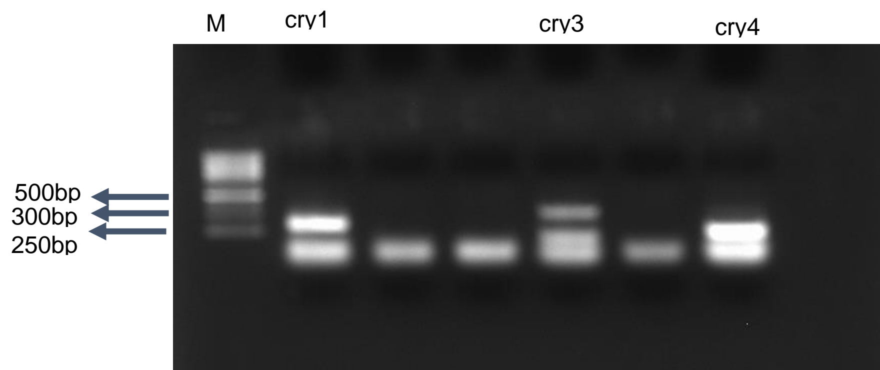

Figure 2. PCR amplicon of cry genes of *Bacillus thuringiensis.*

The proteins of cry1, cry3 and cry4 were coding approximately 130, 75 and 70 kDa, respectively (Figure 3).

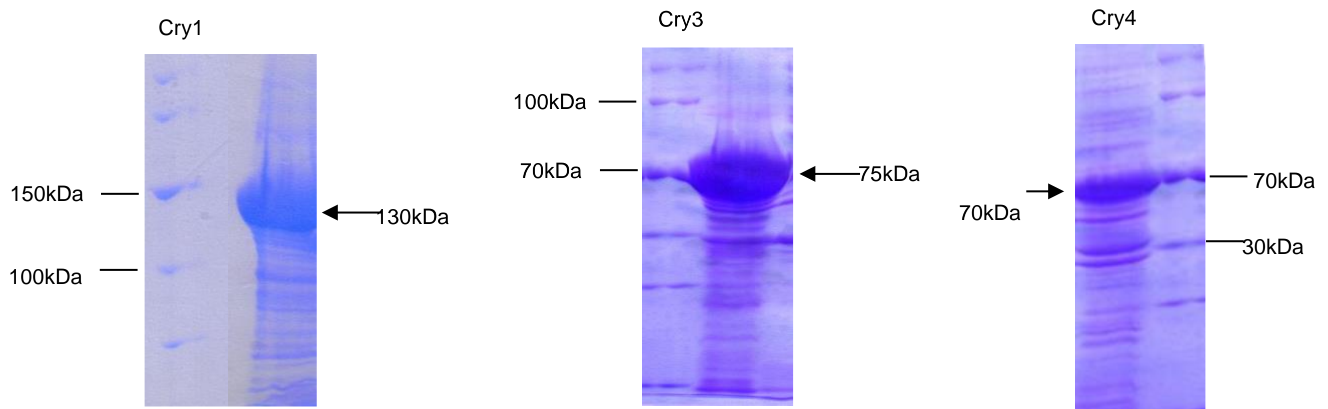

Figure 3. SDS-PAGE analysis of cry1, cry3 and cry4 proteins.

The insecticidal activity study found that the bacterial isolate has 85% insecticidal activity on *C. perspectalis* larvae.

The insecticidal activity of cry proteins was evaluated against the larvae of *C. perspectalis*. Test results showed that, cry1, 3 and 4 proteins have 75, 50 and 45% insecticidal activities against *C. perspectalis* larvae, respectively (Figure 4). The mortalities of all doses of insects infected with cry proteins are shown in Figure 5. The highest mortalities with cry1, cry3 and cry4 treatment were 85, 70 and 60% for *C. perspectalis*, respectively, at the 2000 U/ml. Also, mortality of all doses of insects infected with *B. thuringiensis* as shown in Figure 6. The highest mortality with bacterium treatment was 90% for *C. perspectalis* at 3.78 x 10^9 cfu/ml.

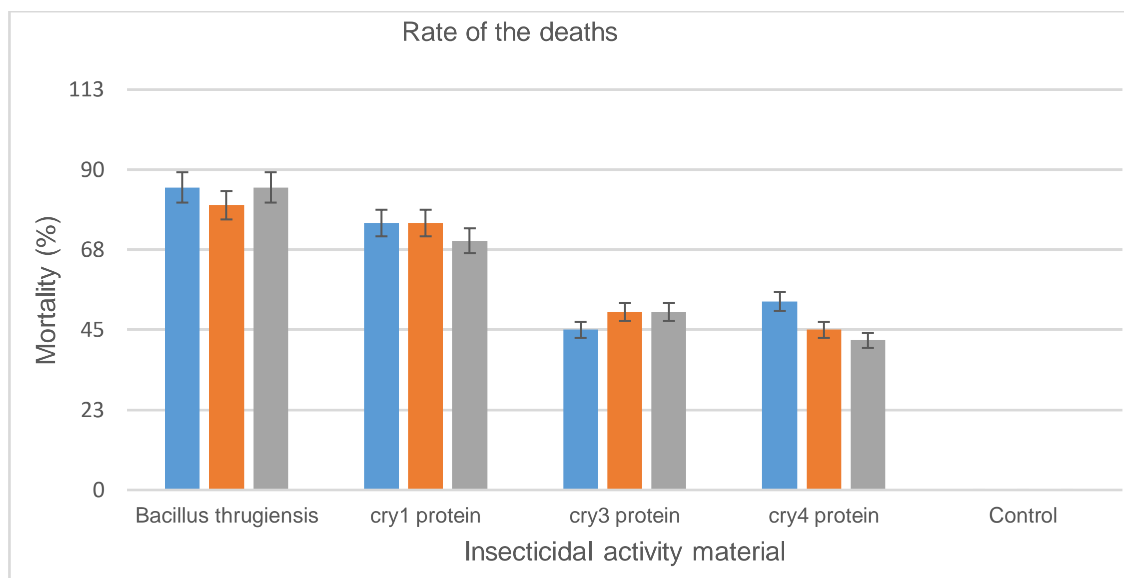

Figure 4. Insecticidal activity of *Bacillus thuringiensis* and cry proteins. Blue bars are the ratios in the first iteration, orange bars the ratios in the second iteration, and gray bars the ratios in the third iteration).

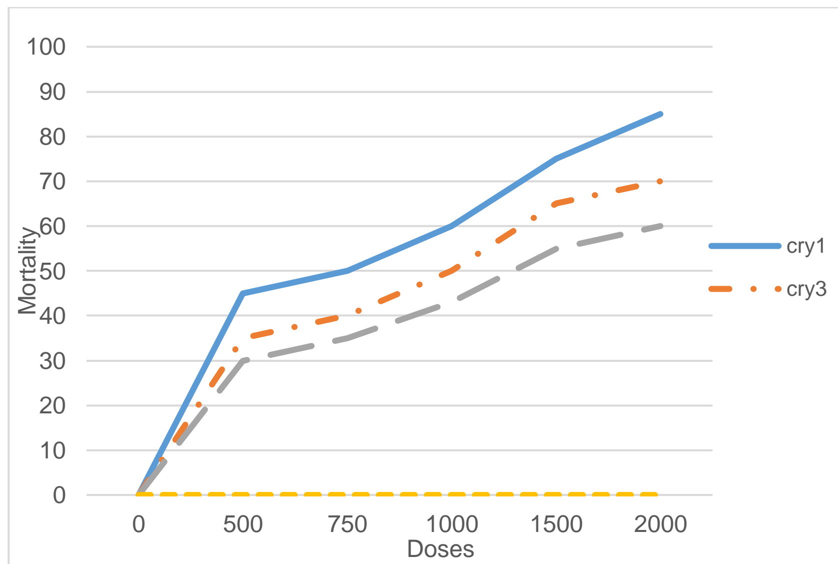

Figure 5. Mortality of insect larvae resulting from cry proteins.

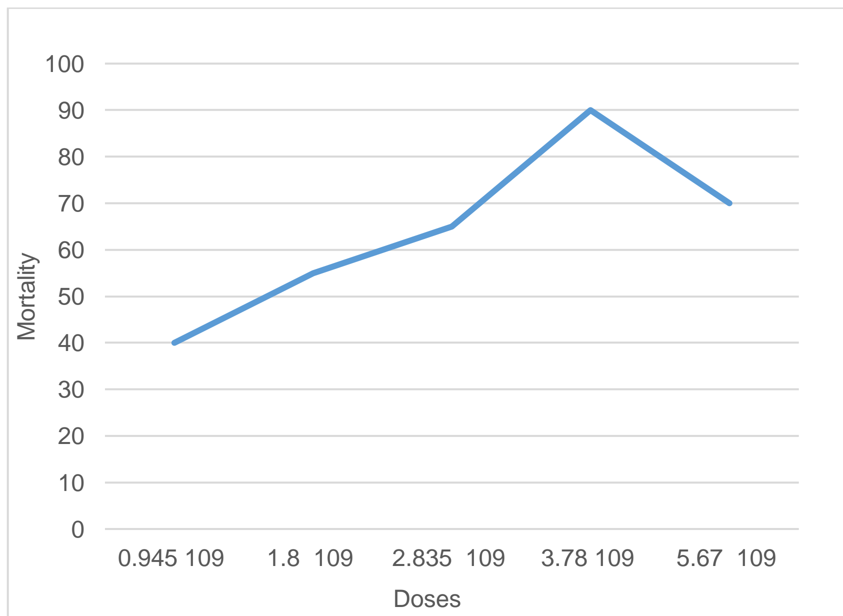

Figure 6. Mortality of insect larvae resulting from *Bacillus thuringiensis*.

The LC_{50} values calculated by probit analysis are given in Table 2. For the proteins, the LC_{50} was lowest cry3 and cry4 proteins and highest cry1 protein for *C. perspectalis.* The LC_{50} for bacterial isolate was higher than each of the individual proteins.

Table 2. Median lethal concentration (LC_{50}) of bacterial isolate and cry proteins assayed against *Cydalima perspectalis*

Isolate	LC 50 U/ml	df1	*df2*	X^2	SS	F	Slope ± SE
Bacillus thuringiensis	4070	1	3	0.92	0.554	3.01	0.325 ± 0.525
Cry1	3580	1	3	0.87	0.492	2.79	0.304 ± 0.507
Cry3	1020	1	3	0.54	0.213	1.58	0.236 ± 0.452
Cry4	1000	1	3	0.42	0.188	1.00	0.226 ± 0.423

Discussion

The first detection of *C. perspectalis* in Türkiye was in 2011 in parks and gardens located in the Sarıyer District of Istanbul (Bahçeköy, Emirgan, Hacıosman, Zekeriyaköy) (Hizal, 2012). Türkiye has favorable conditions for the spread of *C. perspectalis*, especially in the western and northern regions (especially Artvin), as the average temperature is above 30°C, and boxwood species, which are also the host of the pest, are widely used in parks and gardens.

There are various methods for control of *C. perspectalis*, including biological control with *B. thuringiensis*. This bacterium is the most important entomopathogenic bacterium. The crystal proteins/genes of this bacterium is ingested by the caterpillars during feeding, passes into the stomach, and then the active toxin crystal dissolved in the midgut passes into the blood, preventing skin formation and causing their death.

In this study, *B. thuringiensis* was isolated from *C. perspectalis.* PCR amplification of cry genes (cry1, cry2, cry3 and cry4) of the bacterium was scanned. According to amplification results only cry1, cry3 and cry4 genes were found. The cloning of the crystal protein gene (cry) of *B. thuringiensis* was first reported by Schnepf & Whiteley (1981). Since then, more than 120 cry genes have been cloned, characterized, and proteins classified based on amino acid sequence similarity (Crickmore et al., 2018). Cry1 proteins are effective against Lepidoptera species, cry3 proteins against Coleoptera species and cry4 proteins against on Diptera species. Cry genes are expressed and their products (proteins) accumulate to form a crystal inclusion, which can usually constitute 20 to 30% of the dry weight of cells (Schnepf et al., 1998). Few studies have reported a detailed characterization of Bt strain collections in terms of cry gene content. In this study, cry1 protein (90%) gave the highest mortality. In a study on Alper et al. (2014) the cry1 protein gave the highest mortality (36%), cry3 protein (1%) and cry4 protein (4%) gave lower mortality. In another study at Uribe et al. (2003) cry1 gave highest mortality again. And also, they scanned the samples for cry1 because of prevalence. In a study from Bravo et al. (1998) the cry1 genes were the most frequently found in the Mexican strain collection. A high frequency of cry1 genes seems to be common to all *B. thuringiensis* strains. As seen in the literature cry1 is commonly found to give high mortality. The second abundant genes and proteins were cry3 and cry4. In this study, *B. thuringiensis* isolated had cry1, cry3 and cry4 genes and also proteins. In a study of Brazilian *B. thuringiensis* they observed frequencies of the cry genes in *B. thuringiensis* isolates obtained from stored grain (48), compared to other isolates (180) in their collection. Using specific primers for *cry1*, 77% of the isolates from grain were positive but only 41% of the others.

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming bacterium that produces insecticidal crystal proteins. Bt exerts its insecticidal activity in the orders Lepidoptera, Coleoptera, Hymenoptera, Diptera, Hemiptera, Orthoptera, and Mallophaga and among nematodes, mites, and protozoa, and by producing parasporal crystals that are toxic to a wide variety of insect species (Schnepf et al., 1998). The fact that insecticidal products obtained from *B. thuringiensis* bacteria do not cause infection in humans, non-target organisms and beneficial insects has increased the effective use of these products in the control of harmful insects (Lacey et al., 2001). *Bacillus thuringiensis* derivative products constitute 95% of the world biopesticide market. Many commercial companies have introduced and are promoting products of *B. thuringiensis* to the market. By 1998, more than 200 products of *B. thuringiensis* origin are used against pests only in the USA (Schnepf et al., 1998, Sanahuja Solsona et al., 2011). In addition, many *B. thuringiensis*-derived products are susceptible to synthetic chemical pesticides, obtained at a lower cost. Some other species belonging to the genus *Bacillus* are also used in the control of harmful insects. *Paenibacillus popilliae* (Dutky, 1940) Pettersson et al.,1999 (Paenibacillales: Paenibacillaceae) is used in the control of some species belonging to the family Scarabaeidae, while *Lysinibacillus sphaericus* Ahmed et al.,2007 (Bacillales: Planococcaceae) is used in the control of mosquito larvae (Jackson & Klein, 2006). *Paenibacillus popilliae* needs to be produced *in vivo*, and lower than expected levels of infection in many

field applications reduces the potential of this bacterium to be used in large areas (Klein & Kaya, 1995). Although it is more resistant to various factors, its biggest disadvantage is that the host spectrum is narrow (Lacey & Undeen, 1986, Charles et al., 1996, Nicolas et al., 1994). Also, some fly species are resistant to this bacterium (Rao et al., 1995, Nielsen-Leroux et al., 1997).

In conclusion, the results presented here show cry proteins (especially cry1) and the bacterium can use for control the *C. perspectalis*. Considering the effects of cry proteins in the literature and considering the harmful effect of the insect, these proteins have the potential to be developed as biopesticides and used against pests. Apart from laboratory application, it can be used against both *C. perspectalis* and other Lepidoptera by developing both bacteria and proteins as biopesticides, supported by field application.

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