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Original article (Orijinal araştırma)

Isolation of *Bacillus zhangzhouensis* OBB Liu et al. (Caryophanales: Bacillaceae) from native *Polyphylla fullo* (L., 1758) (Coleoptera: Scarabaeidae) larvae, PCR-based detection of *cry1* gene and evaluation of its biological control potential[1]

Yerel *Polyphylla fullo* (L., 1758) (Coleoptera: Scarabaeidae) larvalarından *Bacillus zhangzhouensis* OBB Liu et al. (Caryophanales: Bacillaceae)'nin izolasyonu, *cry1* geninin PCR tabanlı tespiti ve biyolojik kontrol potansiyelinin değerlendirilmesi

Özlem BAKİR BOĞA[2*] **Esabi BAŞARAN KURBANOĞLU[2]**

Abstract

Bacillus zhangzhouensis OBB Liu et al. (Caryophanales: Bacillaceae) isolate was obtained from *Polyphylla fullo* (L., 1758) (Coleoptera: Scarabaeidae) larvae in the Microbiology Laboratory of the Biology Department of the Faculty of Science of Atatürk University in 2024. Additionally, the presence of the *cry1* gene was identified. After endospore staining of the isolate, the presence of crystal protein was detected by phase contrast microscopy and SEM analysis. As a result of PCR, only the presence of the cry1 gene was detected and confirmed. The total protein contents were compared with those of *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) by performing SDS-PAGE analysis using a crystal protein and spore mixture. *B. zhangzhouensis* OBB showed bands ~ 250 kDa and ~ 80 kDa, while *B. thuringiensis* showed bands corresponding to ~70 kDa and ~45 kDa. Probit analysis was used to determine the LC_{50} value of the isolates, and the Abbott method was used to determine the mortality percentages of the larvae. Spore-crystal mixtures of *B. thuringiensis* and *B. zhangzhouensis* OBB isolates were tested against *P. fullo* larvae at doses of 1000, 2000, and 4000 ppm. The highest mortality rate was determined in the spore-crystal mixture of *B. zhangzhouensis* OBB isolate at 4000 ppm dose.

Keywords: *Bacillus zhangzhouensis* OBB, biopesticides, *cry* genes, *Polyphylla fullo*, SDS-PAGE

Öz

Bacillus zhangzhouensis OBB Liu et al. (Caryophanales: Bacillaceae) izolatı, 2024 yılında Atatürk Üniversitesi Fen Fakültesi Biyoloji Bölümü Mikrobiyoloji Laboratuvarında *Polyphylla fullo* (L., 1758) (Coleoptera: Scarabaeidae) larvalarından elde edilmiştir ve *cry1* geninin varlığı tanımlanmıştır. İzolatın endospor boyaması yapıldıktan sonra faz kontrast mikroskobu ve SEM analizi ile kristal protein varlığı tespit edilmiştir. PCR sonucunda sadece cry1 geninin varlığı tespit edilmiştir ve doğrulanmıştır. Kristal protein ve spor karışımı kullanılarak SDS-PAGE analizi ile *Bacillus thuringiensis* (Berliner, 1915) (Bacteria: Bacillaceae) ile arasındaki total protein içerikleri karşılaştırılmıştır. *Bacillus zhangzhouensis* OBB ~250 kDa ile ~80 kDa arasında bantlar gösterirken, *B. thuringiensis,* 70 kDa ve ~45 kDa'ya karşılık gelen bantlar göstermiştir.İzolatların LC_{50} değerini belirlemek için probit analizi, larvaların ölüm yüzdelerini belirlemek için ise Abbott yöntemi kullanılmıştır. *B.thuringiensis* ve *B. zhangzhouensis* OBB izolatlarının spor-kristal karışımları (1000, 2000 ve 4000 ppm) dozlarında *P. fullo* larvalarına karşı test edilmiştir. En yüksek ölüm oranı *B. zhangzhouensis* OBB izolatının 4000 ppm dozunda spor-kristal karışımında belirlenmiştir.

Anahtar sözcükler: *Bacillus zhangzhouensis* OBB, biyopestisitler, *cry* genleri, *Polyphylla fullo*, SDS-PAGE

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Introduction

With the urbanization brought about by rapid population growth, agricultural areas are decreasing day by day and the amount of agricultural products per capita is declining. Türkiye, which used to be a self-sufficient country in terms of agricultural products, now imports agricultural products from many countries. One of the most important reasons for this is that pest control in economically important plants cannot be done consciously and completely (Bülbüloğlu, 2000). There are around 30,000 beetle species in the Scarabaeidae family (Insecta: Coleoptera) worldwide. *Polyphylla fullo* (L., 1758) (Coleoptera: Scarabaeidae) is one of the most significant members of this family. For *P. fullo* larvae, the term "Manas bug" is often used. *P. fullo* is one of the most damaging pests of orchards, vineyards, ornamental plants, grass, potatoes, peppers, tomatoes, and a number of other crops in Türkiye. It also drastically lowers the production of plants and forests (Anonymous, 2008). April and May saw the emergence of *P. fullo* adults from the soil as the temperature warmed. After nightfall, adults fly to the trees, mate, and deposit their eggs in the dirt of overgrown and uncultivated gardens. After hatching from the eggs, the larvae dwell in groups, feed on the grass roots, and shed their skins to become second-stage larvae after two months. Larvae in their second stage consume a substantial amount of food. In the fall, they hibernate deep in the ground. A feeding that can cause serious harm occurs from March or April until the first part of June. After that, they undergo a skin change and develop into third-stage larvae. During their one-year lifespan, the third-stage larvae do considerable harm. They pupate in a dirt nest that is 15-35 cm below the earth's surface in July. The pupae remain in the nest until the next spring, maturing into adults in the following autumn, in September. After hatching in this manner, an individual matures into an adult three years later and gives birth to offspring once every three years. Adult insects ruin things by eating flowers and foliage. They can leave behind leafless fruit and forest trees in heavily populated areas (Anonymous, 2008, 2011a). They target the root systems of several crops, such as ornamental plants, grass, grape vines, and young fruit trees. While older larvae nibble at tree roots, causing the plants to dry up and die, younger larvae feed on the roots of herbaceous plants (Borror et al., 1981). In Türkiye, the damage rate of these insects ranges from 50% to 80%, depending on the crop and soil type (Erler & Ates, 2015). Considering the damage they cause, controlling pests is very important for optimizing product yield. In addition, chemical pesticides registered worldwide are used against these insects. Due to the negative effects of chemical pesticides, including their non-biodegradability, persistence, and toxicity, an environmentally friendly approach is being adopted for crop pests (Buss, 2006). As an alternative to the traditional synthetic pesticides currently used for pre-harvest and post-harvest management of crop pests and diseases, biopesticides have garnered significant attention in the field of pest control in recent years (Kour et al., 2020; Yadav et al., 2020). Since microbial pesticides are an ecologically benign way to reduce pest populations in the agricultural business, they are very useful for both farmers and researchers (Kour et al., 2019a, b, c). Compared to readily accessible chemicals, the use of biopesticides to control insect pests is more cost-effective, ecologically friendly, and efficient (Thakur et al., 2020). According to Koul & Dhaliwal (2003), biopesticides protect crops over their whole growth phase, do not harm plants, and manage a significant number of insect pests. Biopesticides do not damage people or the environment; they only affect the pests they are intended to kill (Lengai & Muthomi, 2018). These days, biopesticides are widely employed in organic agriculture (Seiber et al., 2014) and play a significant role in the agricultural market (Nawaz et al., 2016).

The most important of these methods is biological control. Bacteria, viruses, fungi, nematodes, organisms belonging to the protozoa group and agents developed with recombinant techniques constitute the elements used in biological control. Among these, soil group bacteria are the most promising biological control agents (Bülbüloğlu, 2000). Today, many microorganisms are used in the biological control of insects. Although more than 100 bacterial species have been defined as insect pathogens so far, only the *Bacillus* species are commercially preferred as control agents. Bacteria of the genus *Bacillus* are microorganisms that attract attention due to their metabolic properties such as antibiotic, enzyme, and toxin

production, their industrial importance, and their easy production (Rosovitz et al., 1998). In addition, their sporulation abilities and diversity of metabolic activities provide significant advantages that facilitate their spread across diverse environments (Wipat & Harwood, 1999). *Bacillus* species produce peptide antibiotics used in the pharmaceutical industry, such as tyrocidine (*Bacillus brevis* Burmeister), subtilin, and bacilicin (*Bacillus subtilis* G.). *Bacillus thuringiensis*, *Bacillus sphaericus* Meyer & Neide and *Bacillus popilliae* Dutky species are pathogenic to various insect larvae and are used as biological control agents (Rosovitz et al., 1998). *Bacillus thuringiensis* (Bt) Berliner (Bacteria: Bacillaceae) is a bacterial species naturally found in soil and is an agricultural agent of economic importance. Bt is one of the most successful microbial insecticide agents and has been used and researched for years, especially in agriculture and forestry, in the biological control of pests such as mosquitoes, which are effective virus carriers, especially in agriculture and forestry, thanks to the proteins they produce in vegetative form and during sporulation. Bt has great commercial importance, especially because it is effective against many agricultural pests. Insecticidal proteins are insecticidal crystal proteins (Cry), vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip). Of these, extensive research has been done on Cry proteins, and various plant or crop varieties have been developed. Genes encoding insecticidal proteins are usually carried on plasmids. Plasmid transfer among these isolates provides gene and toxin diversity in Bt isolates (de Maagd et al., 2001). In addition, the collection of Bt samples from different geographical regions is important in finding new insecticidal genes and proteins. Studies in the literature focus on Bt isolation, characterization and bioactivity from different geographies of the world. New Bt collections are obtained to investigate the insecticidal activities of these isolates. Effective strains need to be found for the production of new commercial products (Baranek et al., 2015; Boukedi et al., 2016).

When it comes to controlling certain insect species from the Lepidoptera, Diptera, and Coleoptera orders, Bt is a safe organism that targets specific species. However, this study is the first to discover the cry gene in *Bacillus zhangzhouensis* OBB Liu et al. (Caryophanales: Bacillaceae), is the first. While there are entomopathogenic nematode studies on *P. fullo* insects in the literature, no entomopathogenic bacteria studies have been reported. In addition, the application of Cry proteins to *P. fullo* larvae and adults has unique value in terms of using a locally sourced strain and investigating the potential of this local strain to be used as a bioinsecticide. Furthermore, the collection of local samples is important for finding new insecticidal genes and proteins. The discovery of strains that can form the active ingredient of new commercial products also increases the unique value. Since the study is naturally of biological origin, it will not harm the environment or humans. This study aimed to screen *cry* genes in *B. zhangzhouensis* OBB isolate from *P. fullo using* PCR method, to detect cry proteins by SEM analysis, to determine the protein profile by SDS-PAGE, and examine its insecticidal activity. In this way, the potential used on Coleoptera will be determined. The effective *B. zhangzhouensis* OBB isolate we found will be an important alternative in breaking the resistance mechanism developed against both chemical pesticides and existing Bt preparations.

Materials and Methods

Sample collection

Polyphylla fullo larvae from the study were gathered in high concentrations from a range of urban and peri-urban locations in Türkiye, such as fields, gardens, and vineyards. Tokat, Türkiye; latitude: 40°18'50"N; longitude: 36°33'15"E. The field-collected larvae were stored in plastic containers filled with dirt. The larvae were kept in six 11-liter plastic containers and were regularly supplemented with food in the form of fruits. In September 2023, the larvae samples were gathered (Figure 1).

Figure 1. *Polyphylla fullo* larvae collected from dried tree roots in Tokat/Türkiye.

Isolation of bacteria from *Polyphylla fullo*

The dead larvae of *P. fullo* were used to isolate bacteria. 70% ethyl alcohol was used to surface sterilize *Polyphylla fullo* larvae. After removing the insects' guts, the samples were cleaned two or three times with sterile distilled water to remove any residual alcohol. The intestines were crushed using 10 milliliters of phosphate buffer (PB, 50 mM, pH 7.4). 10 grams of Mueller Hinton, 10 grams of yeast extract, 3 grams of K_2HP0_4, 2 grams of glucose, and 0.5 grams of trehalose were added to one liter of MYPGP medium (Sharpe et al., 1970). 30 milliliters of MYPGP medium were mixed with 500 microliters of crushed intestinal sample, and the mixture was cultured for 48 hours at 30°C. Subsequently, 50 µl of MYPGP agar medium was added to the bacterial culture, which had been diluted to a concentration of 10^{-9}. The colony was selected and inoculated into new MYPGP agar medium following an overnight incubation period at 30°C (Kang et al., 2012).

Gram and endospore staining of isolates of *Bacillus zhangzhouensis* OBB

Gram staining (Claus, 1992) and spore staining (Reynolds et al., 2009) characteristics of the isolate were determined.

Scanning Electron Microscopy

Isolates of *B. zhangzhouensis* OBB were incubated for seven days at 30°C while being shaken at 250 rpm in T3 medium. For ten minutes, the cell cultures were centrifuged at 4000 rpm. Three times, the pellets were reconstituted in sterile distilled water. After being fixed for 12 hours at 4°C in 2.5% glutaraldehyde, the cells were rinsed with sterile distilled water. Sterilized distilled water was used to dissolve them. SEM images were taken at Bilecik Seyh Edebali University Central Research Laboratory (Suludere et al., 1992).

Molecular identification of isolate

Genomic DNA Miniprep (BioBasic) was used to isolate the genomic DNA of the bacteria. In accordance with William et al., 1991, PCR was used to analyze the 16S rRNA sequences of the bacterial isolates from which genomic DNA was extracted. Universal primers were used to sequence the 16S rRNA gene. Applied Biological Materials' Taq 2X PCR Master Mix (G888) was utilized. The following ingredients were used to create a total of 25 µl of reaction mixture: One microliter of a 10 µM forward primer, one microliter of a 10 µM reverse primer, one microliter of a 5 ng/µl DNA solution, twenty-five microliters of PCR Taq 2X master mix, and twenty-two microliters of clean water. After that, the samples were put through a reaction cycle in a Sensoquest thermocycler. PCR stages were performed according to Mehtap et al., 2022. A 1.2% agarose gel was used to visualize the results of the polymerase chain reaction (PCR). The band in the gel was sent to MACROGEN in the Netherlands for sequencing. Confirmation was made by comparing with similar species registered in the NCBI database.

***Bacillus zhangzhouensis* OBB phylogeny**

BioEdit software was used to assemble the sequence (Hall et al., 2011). The isolate's sequencing was submitted into the GenBank database. To compare the sequences with the RefSeq database, the NCBI GenBank nBLAST search engine (http: //www.ncbi.nlm.nih.gov) was utilized. Phylogenetic analysis was performed using the isolate's sequence and those of closely related species. Phylogenetic tree of isolates was created with Neighbor-Joining analysis using Mega 11.0 program and performed with 1000 replicates with Bootstrap method (Figure 3).

PCR analysis of *Bacillus zhangzhouensis* OBB isolates *cry* genes

To find *cry* genes (Ben-Dov et al., 1995), PCR was conducted using the primers specified in Table 1.

Table 1. Primers used to screen for the presence of *cry* genes

Gene	Primer sequences (5' -> 3')	Tm (°C)
cry1 (277 bp)	CATGATTCATGCGGCAGATAAAC (f) TTGTGACACTTCTGCTTCCCATT (r)	59
cry2 (701 bp)	GTTATTCTTAATGCAGATGAATGGG (f) CGGATAAAATAATCTGGGAAATAGT (r)	55
cry3 (604bp)	CGTTATCGCAGAGAGATGACATTAAC (f) CATCTGTTGTTTCTGGAGGCAAT (r)	59
cry4 (439 bp)	GCATATGATGTAGCGAAACAAGCC (f) GCGTGACATACCCATTTCCAGGTCC (r)	59

SDS-PAGE Analysis

Bacillus zhangzhouensis OBB and *B. thuringiensis* isolates were used. *Bacillus thuringiensis* isolates were used as controls. The isolates were cultured in T3 medium for ten days and centrifuged at 14000 rpm, and +4°C. Following centrifugation, the larger proteins (Cry) stay in the pellet. The pellet is used directly in SDS after being repeatedly cleaned with pure water. Protein concentrations were measured using the methods Bradford (1976) described. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% PAGE) was used to test for purified cry proteins (Mehtap, 2022).

Preparation of Spore-Crystal Proteins

100 milliliters of liquid sporulation medium T3 (3 g L^{-1} tryptone, 2 g L^{-1} tryptose, 1.5 g L^{-1} yeast extract, 0.005 g L^{-1} $MnCl_2$, 6 g L^{-1} NaH_2PO_4, and 7.1 g L^{-1} Na_2HPO_4) were used to cultivate the activated isolate. It was incubated for seven days at 200 rpm and 30 °C in an incubator that was constantly shaking. Following the incubation period, the samples were centrifuged for 20 minutes at 15000 rpm. The pellet that resulted

from this process (spore-crystal protein) was then rinsed twice in 20 milliliters of sterile distilled water and centrifuged for 10 minutes at 15000 rpm (Travers et al., 1987). The spore-crystal protein mixture was precipitated by centrifugation, and then it was dried for 24 hours at 60°C to create a powder.

Bioassays

Spore-crystal suspensions of *B. zhangzhouensis* OBB and *B. thuringiensis* isolates were applied at 1000 ppm, 2000 ppm, and 4000 ppm rates. Ten larvae were tested for the bacterial isolate. The experiments were performed in triplicate. The experiments were performed on tomatoes grown in 30 cm diameter pots (Figure 2). The setups were arranged with three repetitions for six different applications. Tomato seedlings were planted in nine pots to form the groups and were prepared for the experiment after growing for 8-10 days. Only water was used in the control group. Effectiveness tests of different concentrations were conducted. Mortality data were calculated using the Abbott formula (Abbott, 1925). Lethal concentrations (LC_{50}) were calculated for the bacterial isolate (Finney, 1952).

Figure 2. Application of spore-crystal mixture to tomato seedlings.

Statistical Analysis

SPSS 22.0 was used for statistical analysis. It was analyzed with the Probit Analysis-MSChart 2.0 program to calculate the LC_{50} value. All data were shown as mean±standard deviation (Mean±SD). Data of other tests were subjected to one-way analysis of variance (ANOVA) and evaluated using the LSD test ($p<0.05$).

Result and Discussion

16S rRNA, and BLAST data indicated that *B. zhangzhouensis* OBB bacterium had identified. *B. zhangzhouensis* is a Gram-positive, rod-shaped, aerobic bacterium. Its colonies have an oval endospore in the middle and are spherical, creamy white, and translucent. The bacterium has catalase-positive and oxidase-positive results. Based on the findings of the BLAST analysis, a phylogenetic tree was created using the NCBI database (Figure 3).

The isolates' motility traits, endospore structure, cell shape, and Gram staining were all investigated. Gram staining produced purple bacilli, which were identified as Gram-positive under a light microscope. To diagnose the presence of spores, endospore staining was performed. Under a microscope, spores stained with malachite green were identified. Coomassie Brilliant Blue was used to stain the parasporal crystal proteins of the isolates, which were then observed under an Olympus CKX41 phase contrast microscope (Figure 4). Electron microscopy also revealed the existence of crystal protein (Figure 5).

To ascertain whether crystal proteins were present, light and electron microscopy were used to analyze the spore-crystal combination produced from the *B. zhangzhouensis* OBB strain (Figure 5). The crystals were found to be square, cubic, and bipyramidal in shape based on electron microscope examinations (Figure 5). Multiple crystal proteins can be synthesized by subspecies. All three of these isolates from the reference strain *Btk* HD-1 produced bipyramidal crystals. While *Cry2* creates cuboidal crystals that exhibit lepidopteran toxicity, *Cry1* toxins are linked to the formation of bipyramidal crystals and toxicity against lepidopteran insects, including *S. frugiperda* (Monnerat et al., 2007). Bipyramidal, cubic, flat rhomboid, spherical, and composite forms are all possible for crystal proteins (Yu et al., 2015). The Cry proteins varied among the isolates, according to a microscopic examination. Bipyramidal and other crystal forms were found in the samples during examination. The diversity of *cry* genes is further supported by these studies.

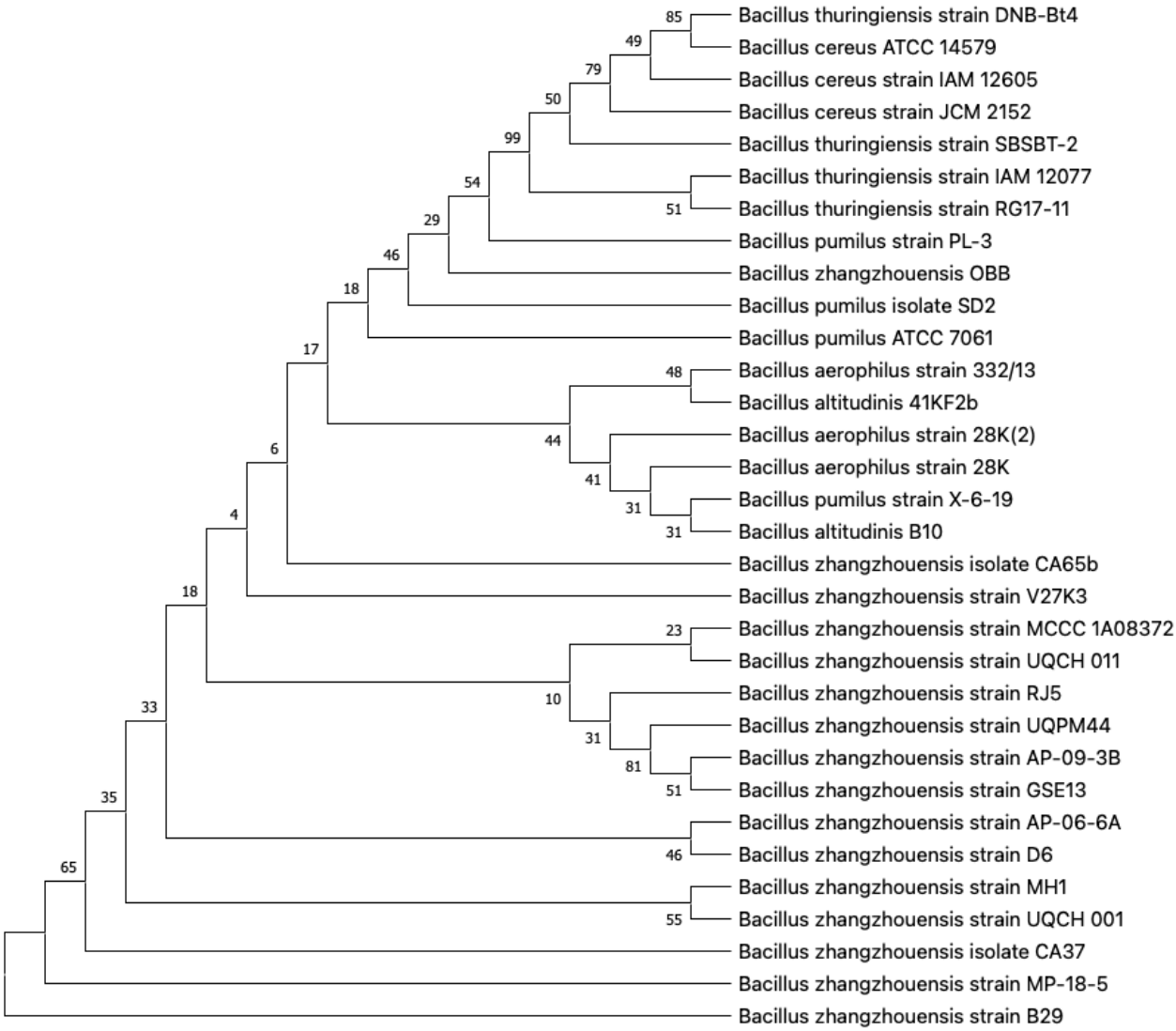

Figure 3. Phylogenetic tree of the 16S rRNA gene region of the isolate.

Figure 4. Endospore structure of *Bacillus zhangzhouensis* OBB bacteria under phase contrast microscope (100x).

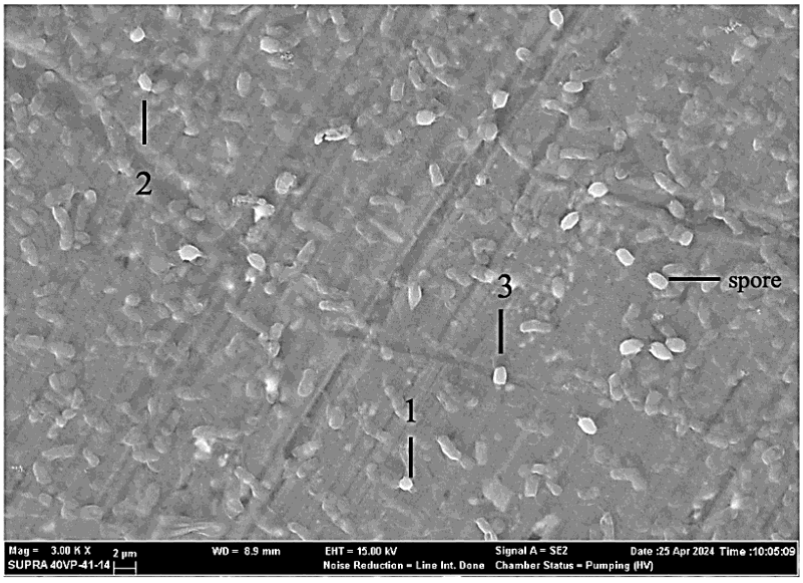

Figure 5. Using scanning electron microscopy (SEM) investigation of several crystal morphologies, the *Bacillus zhangzhouensis* OBB isolate was observed: (1) spherical crystal, (2) bipyramidal crystal and (3) square crystal.

The PCR study of the toxin genes of the *B. zhangzhouensis* OBB strain's toxin genes revealed that the strain carries the *cry1* gene. The *cry1* gene in *B. zhangzhouensis* OBB was shown to produce a 277 bp band on 1% agarose gel (Figure 6). In the literature, it is seen that many researchers have investigated the *cry* gene content and distribution in Bt collections in different parts of the world. In the general results obtained, *cry1* is the most abundant gene group, followed by the *cry2* genes (Baig &Mehnaz, 2010). Jain et al. (2017) found that the *cry1* gene (100%) was the most abundant among the Bt isolates they obtained from India. Bozlağan et al. (2010) obtained 60 Bt isolates from soil samples collected from different regions of Kayseri and showed that 28% of them were *cry1* positive. In the study conducted by Alper et al. (2014) in which the *cry* gene contents of 288 Bt isolates from fig orchards were screened, *cry1* (36%) was found to be the most common, while the rates of *cry2 (*3%) and *cry3* (1%) were lower. In another study, 24 of 44 soil isolates were *cry1*, and 14 were *cry2*. It was determined that these isolates contain these genes (Lone et al., 2017).

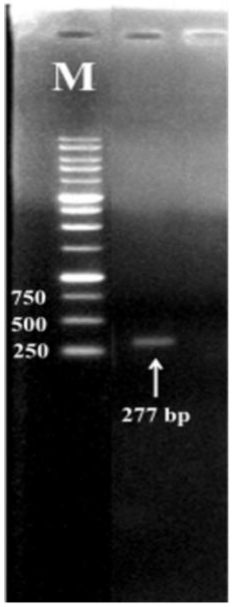

Figure 6. Visualization of *cry1* gene of *Bacillus zhangzhouensis* OBB in 1.2% agarose gel as a result of PCR *(*M: Marker (Ecotech-1 kb DNA Ladder).

Spore-crystal mixtures of *B. thuringiensis* and *B. zhangzhouensis* OBB bacteria were applied at doses of 1000, 2000, and 4000 ppm. The highest mortality rate on *P. fullo* larvae was determined to be 90% effective at 4000 ppm spore-crystal concentration of *B. zhangzhouensis* OBB. The lowest mortality rate was calculated as 55% at 1000 ppm spore crystal concentration of *B. thuringiensis* isolate (Figure 7). The effect of bacterial isolates on the larvae of the pest was determined by probit analysis for 4000 ppm concentration and it was determined that *B. zhangzhouensis* OBB had the highest LC_{50} value of 6,11. The lowest LC_{50} value of bacterial isolates on the pest was *B. thuringiensis* with 4.87 for 4000 ppm concentration (Table 2).

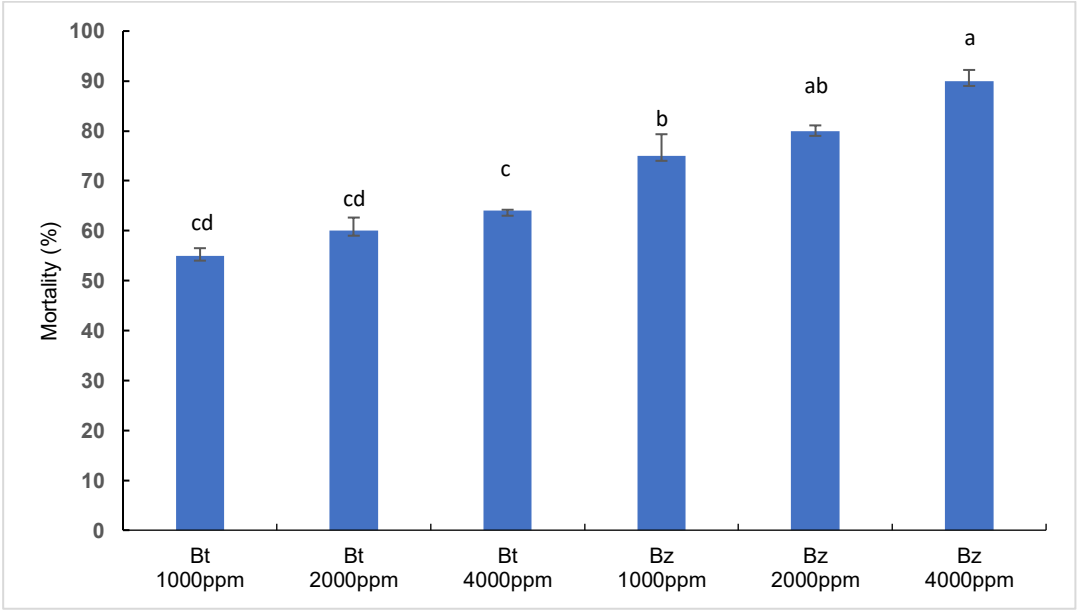

Figure 7. Lethal effect of spore-crystal mixture of *Bacillus thuringiensis* (Bt) and *Bacillus zhangzhouensis* OBB (Bz) isolates at doses of 1000, 2000, 4000 ppm on *Polyphylla fullo* larvae.
* Means followed by different letters within a column are statistically different ($p<0.05$) from each other.

Table 2. Probit analysis results of isolates

Isolates	N	Slope±SD	LC_{50}(Confidence interval, 95%)	X_2	df	p
Bacillus thuringiensis	30	0,80 ± 0,055	4,87 (4,52-5,43)	6,62	3	,0001
Bacillus zhangzhouensis OBB	30	0,90 ± 0,117	6,11 (5,73-6,75)	0,50	3	,0001

Figure 8 illustrates the distinction between control and applications. Given that the *cry1* gene was the most commonly found gene in this collection and that the Cry1 protein has particular insecticidal properties against Lepidoptera (Tarekegn & Teferra, 2023), one could wonder if the presence of the cry protein gene in the related *B. zhangzhouensis* OBB was caused by a different insect ecology brought about by different geographic and climatic conditions. Saadoun et al. (2001) reported that the LC_{50} values of Bt isolates isolated from soil ranged between 4.60 to 8.65 spore-crystal conc./ml for *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) larvae and 5.30 to 6.74 spore-crystal conc./ml for *C. pulex*. In the study conducted by Wang et al., it was determined that spore toxin mixtures of Bt isolates carrying *cry1* genes were 71-83% effective against *Helicoverpa virescens* (Fabricus, 1777) and *Helicoverpa zea* Boddie, 1850 (Lepidoptera: Noctuidae) larvae (Wang et al., 2003). The highest mortality rate was determined in the spore-crystal mixture at the dose of 4000 ppm. It shows that the *B. zhangzhouensis* OBB isolate is more lethal than other isolates. According to the results, the *B. zhangzhouensis* OBB isolates that were acquired for this investigation can be employed as biological control agents.

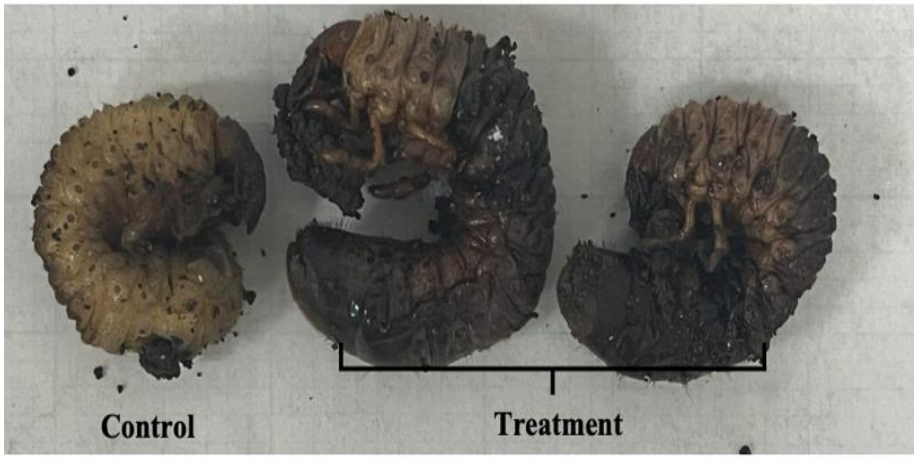

Figure 8. Difference between water only (control) and 10 days after application of *Bacillus zhangzhouensis* 4000 ppm.

SDS-PAGE was used to compare the spore-crystal mixture in Bt and *B. zhangzhouensis* OBB isolates. *B. zhangzhouensis* OBB displayed bands between 250 kDa and 80 kDa, whereas Bt displayed bands corresponding to 70 kDa and 45 kDa (Figure 9). Similarly, the Bt strain isolated from *Balaninus nucum* showed the presence of *cry1* and *cry2* genes and their corresponding Cry1 and Cry2 proteins with ~130 kDa and ~65 kDa in SDS-PAGE analysis (Kati et al., 2007). The spore crystal mixture collected from 80 Bt species isolated from the *Sichuan basin* showed protein bands ranging from 40 to 130 kDa belonging to the major Cry protein family in SDS-PAGE analysis (Zhu et al., 2009). Boonmee et al. (2019) characterized 511 Bt isolates in Thailand and detected the presence of lepidopteran toxic genes using PCR and found that the molecular mass of the proteins was between ~65 and ~130 kDa in SDS-PAGE analysis. The spore-crystal suspension of the isolates was analyzed using SDS-PAGE, and we found that the electrophoretic bands were highly varied. Several proteins were expressed by *B. zhangzhouensis* OBB. This is thought to be because Bt usually produces more than one parasporal crystal and therefore protein profiles may differ among strains. In addition, the profile of the same Cry protein in different studies may differ due to environmental conditions. This is because the expression of certain cry genes is affected by certain environmental conditions and its turned on or off (Sevim et al., 2012).

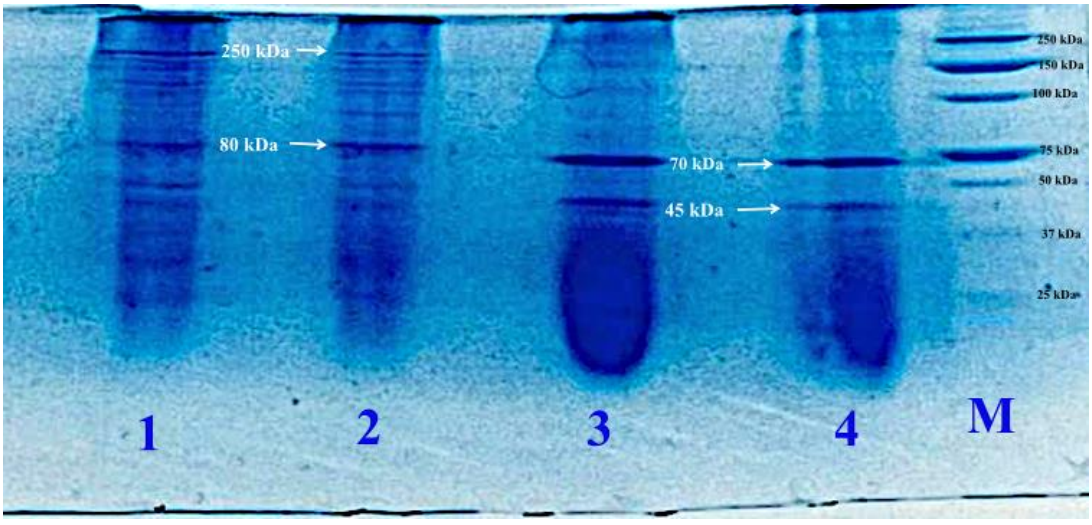

Figure 9. Comparison of spore-crystal mixture with SDS-PAGE analysis; 1-2: *Bacillus zhangzhouensis* OBB, 3-4: *Bacillus thuringiensis.*

Conclusion

According to current knowledge, the cry gene is only found in *B. thuringiensis* and some other bacteria. We were able to identify the cry protein in *B. zhangzhouensis* OBB strain. Since the cry1 gene is the most common gene in this collection and the cry1 protein has specific insecticidal activities against Lepidoptera, different geographical and climatic environments may have created a different ecology in insects, which in turn led to the presence of the cry protein gene in *B. zhangzhouensis.* It was determined that the mortality rate of *B. zhangzhouensis* OBB bacteria was much higher than *B. thuringiensis.* Considering the effects of cry proteins and the harmful effects of the insect reported in literature, these proteins have the potential to be developed as biopesticides and used against pests. In addition, the use of harmful pesticides and chemicals causes various health and environmental problems; we think that it will be an alternative to existing *Bacillus* isolates to overcome this problem.

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