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Original article (Özgün makale)

The isolation and characterization of bacteria isolated from *Capnodis tenebrionis* L. and their biological control potential against some insect pests

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Capnodis tenebrionis L.'den izole edilen bakterilerin izolasyonu, karakterizasyonu ve bazı böcek zararlılarına karşı biyolojik mücadele potansiyelleri

Öz: *Capnodis tenebrionis* L. (Coleoptera: Buprestidae), Türkiye'nin de dahil olduğu birçok ülkede sert çekirdekli meyve bahçelerinin en yıkıcı zararlılarından biridir. Bu çalışmada *C. tenebrionis'*in larva ve erginlerinden 21 bakteri izolatı izole edilmiş, morfolojik, fizyolojik, biyokimyasal ve moleküler özellikleri belirlenmiştir. Buna göre *C. tenebrionis*'in kültürlenebilir bakteri florası; *Bacillus cereus, B. mycoides, B. pumilus, Paenibacillus xylanilyticus, B. flexus, B. simplex, Raoultella terrigena, Enterobacter cloacae, Klebsiella oxytoca, B. safensis, B. amyloliquefaciens* ve *B. aryabhattai* olarak saptanmıştır. Bu bakteri izolatlarının farklı zararlı böcek türlerinin larvalarına karşı biyolojik mücadele potansiyelleri; Karaağaç yaprak böceği (*Pyrrhalta luteola* M.), Petek güvesi (*Galleria mellonella* L.) ve Un kurdu (*Tenebrio molitor* Ludwig) üzerinde incelenmiştir. *Bacillus cereus* izolatları bu zararlı böcekler üzerinde en yüksek etkiyi göstermiş ve hedeflenen zararlıların biyolojik mücadelesinde kullanılabilecek potansiyel bir etmen olarak saptanmıştır.

Anahtar sözcükler: *Capnodis tenebrionis*, Bakteriyel izolatlar, Biyolojik kontrol

Abstract: *Capnodis tenebrionis* L. (Coleoptera: Buprestidae) is one of the most destructive pests of stone fruit orchards in several countries, including Türkiye. In the present study, 21 bacterial isolates were isolated from the larvae and adults of *C. tenebrionis*. The morphological, physiological, biochemical and molecular characteristics of the bacterial isolates were determined. The bacterial flora isolated from *C. tenebrionis* was as follows: *Bacillus cereus, B. mycoides, B. pumilus, Paenibacillus xylanilyticus, B.*

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flexus, B. simplex, Raoultella terrigena, Enterobacter cloacae, Klebsiella oxytoca, B. safensis, B. amyloliquefaciens and *B. aryabhattai*. The biological control potential of these bacterial isolates against the larvae of several economic pest species, the elm leaf beetle (*Pyrrhalta luteola* (M.)), the honeycomb moth (*Galleria mellonella* L.), and the mealworm (*Tenebrio molitor* Ludwig), was investigated. The isolates of *B. cereus* showed the highest efficacy against these insect pests.

Key words: *Capnodis tenebrionis*, Bacterial isolates, Biological control

Introduction

Capnodis tenebrionis (L.) (Coleoptera: Buprestidae), also known as the Mediterranean flathead peach borer, is a widespread pest of stone fruit crops in the Rosaceae family. It is widespread in several regions of the arid and semi-arid Mediterranean regions (Vit 2004). The great wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), causes the losses of honeycomb and pollen. The elm leaf beetle, *Pyrrhalta luteola* (M.) (Coleoptera: Chrysomelidae), damages elms planted for shade and ornamentation in Türkiye; the trees become bare due to excessive leaf fall. In recent years, the mealworm, *Tenebrio molitor* Ludwig (Coleoptera: Tenebrionidae), has become a new invertebrate model to study microbial infections (De Souza et al. 2015), and is an alternative protein-rich food (Grau et al. 2017).

Several control methods have already been used against these pests. However, the damage is still great. Synthetic pesticides are widely used in Türkiye to control these pests. The complexity of chemical control, its negative impact on the environment, and the associated costly damage, have encouraged the search for alternative pest control strategies (Ben-Yehuda et al. 2000), especially to protect organic fruit production. The use of entomopathogenic bacteria (EPB) is one of the best potential alternatives to the use of synthetic pesticides (Muratoğlu et al. 2011).

The most used microorganisms against insect pests today are entomopathogenic bacteria. In studies conducted over the past 50 years, many entomopathogenic bacteria have been isolated from insects and some have been found to cause disease in them. Among them, *Bacillus* species, which belong to the soil bacteria, stand out. The most important of these bacteria is *Bacillus thrungiensis* (Bt). *Bacillus thuringiensis* is a spore-forming, Gram-positive, aerobic soil bacterium that produces crystalline toxins with insecticidal properties against insects of the orders Lepidoptera, Diptera and Coleoptera (Beegle & Yamamoto 1992). However, it has also been isolated from diseased and healthy insects, from leaf surfaces of plants, and from stored products (Burges & Hurst 1977; Carozzi et al. 1991; Kaelin et al. 1994).

The objective of this study was to isolate the culturable bacterial flora of *C. tenebrionis* collected in Türkiye, and evaluate their potential against some

economically important insect pests. Twentyone bacterial isolate were isolated from the larvae and adults of *C. tenebrionis*. The isolates were identified using morphological, biochemical, physiological and molecular techniques and tested against the larvae of *P. luteola, T. molitor* and *G. mellonella*.

Materials and Methods

Collection of larvae and adults

The larvae and adults of *C. tenebrionis* were collected from orchards in Amasya Province, Türkiye. The collected larvae and adults were brought to the laboratory in plastic boxes with perforated lids to allow air circulation. In the laboratory, the larvae and adults were macroscopically examined and then divided into dead larvae, live larvae and adults. For the bioassays with the three insects, *P. luteola,* were collected from the fields in Trabzon Province, and *G. mellonella* and *T. molitor* were obtained from the laboratory cultures at the Department of Biology of Karadeniz Technical University (KTU) in Trabzon, Türkiye.

Isolation of bacteria

Bacterial isolation was performed on live larvae, dead larvae and adults of *C. tenebrionis*. Insects collected from the field were sterilized with 70% ethanol for 5 minutes (Poinar & Thomas 1978) and washed 3 times with sterile distilled water. Larval and adult bodies were then homogenized in a nutrient broth, using a glass mill. The homogenate was filtered through two layers of cheese muslin cloth into sterilized tubes to remove larval debris. Then, 10, 25, and 50 µl of the larval extracts were applied to nutrient agar and incubated at 30°C for 2–3 days. The remaining mixtures were incubated at 30°C for 3–4 hours to enrich the number of bacteria that showed low concentrations. Of these mixtures, 10, 25, and 50 µl were also plated on nutrient agar and incubated for 2–3 days at 30°C. Finally, the incubated larval suspensions were heated in a water bath at 80°C for 10 min to eliminate non-spore forming bacteria. After heat inactivation, 10, 25 and 50 µl of the heated suspensions were plated on nutrient agar and incubated at 30°C for 2–3 days. Isolates were differentiated on the basis of colony color and morphology. Pure cultures of the bacterial colonies were prepared and stored in 20% glycerol at –80°C in the Microbiology Laboratory, Department of Biology, KTU, Trabzon, Türkiye.

Phenotypic and biochemical identification of bacterial isolates

Phenotypic and biochemical characterizations of bacterial isolates were performed according to Bergey's Manual of Systematic Bacteriology, Vols. 1 and 2 (Sneath et al. 1986). Colony morphology of the isolates plated on nutrient agar plates was

examined by direct observation and under a stereomicroscope. The shape of the bacterial isolates was also determined using a light microscope at 1,000× magnification. Gram staining was performed according to the procedure described by Claus (1992). Endospore staining was performed using the method of Prescott et al. (1996). Temperature tests were performed at 4, 10, 35, 40, 45 and 50°C; NaCl tolerance tests were performed with medium containing 3, 5, 7, 9, and 11% NaCl; and pH tests were performed with the medium at pHs 3, 4, 5, 6, 7, 8, 9, 10, and 11.

To determine the biochemical properties of the isolates, starch hydrolysis and oxidase tests, and the catalase test, according to Benson (1985) and Cappuccino & Sherman (1992), respectively, were performed. In addition, their growth on McConkey agar was studied.

The API 20E, API 50CH and VITEK-2 systems were also used for biochemical characterization of the bacterial isolates. Whereas the API 20E test was used for Gram-negative bacteria, the API 50CH test was used for Gram-positive bacteria. At the end of the plating of the API 50CHB test tubes, the tube with the number zero was considered the control and color-changing of the tube was considered a positive result. In the same way, reagents (TDA, JAMES, VP1-VP2, NIT1-NIT2) were added to portions of the cultures in the API 20E test tubes, the remainder was observed for direct color change, and the metabolic and biochemical properties of the isolates were determined. The API test results were evaluated using IdBactv. 1.1 software from G. Kronvall, with the media for the API20E and API 50CHB tests from bioMerieux, France.

To corroborate the results of the API tests, VITEK-2 tests (manufacturer's details) were performed; VITEK-2 is an automated microbial identification system that provides highly accurate and reproducible results with its colorimetric cards and associated hardware and software. Various metabolic activities, such as acidification, alkalinization and enzyme hydrolysis of the bacterial isolates, were determined fully automatically by using the VITEK-2 panel test system. The results were then analysed by comparison with data in its database. In addition, some biochemical properties were tested using the VITEK-2 BCL card for Gram-positive, spore-forming bacteria, and the VITEK-2 GN card for Gram-negative cocci and bacilli.

16S rRNA gene sequencing and phylogenetic tree

Partial sequencing of the 16S rRNA gene was used to confirm isolate identification. The total genomic DNA of the bacterial isolates was extracted according to the standard protocol and stored at –20°C until use. PCR amplification of the 16S rRNA genes of the bacterial isolates was performed using universal primers. UNI16SF (5'-ATTCTAGAGTTTGATCATGGCTCA-3' and UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3') primers were used as

forward and reverse primers, respectively (Weisburg et al. 1991). To determine the nucleotide sequence of the 16S rDNA gene regions, it they were sent to Macrogen (The Netherlands) for automated DNA base analysis. Sequence analysis was performed from both sides of the gene and the results were evaluated and compared with other sequences in the gene bank.

Nucleotide sequences of 16S rRNA genes were edited with BioEdit and aligned with ClustalW (Hall 1999). Phylogenetic analysis was performed using the neighbour-joining method (NJ) run by MEGA 4.0 software (Tamura et al. 2011). The NJ analysis was based on Kimura's 2-parameter test. Gaps in the alignment were treated as missing data. The reliability of the phylograms was tested by bootstrap analysis with 1,000 repetitions using MEGA 4.0. The closed circles in black represent the isolates examined in this study. Bootstrap values ≥70% are labeled. The scale at the bottom of the dendrogram indicates the degree of dissimilarity.

Crystal protein

To amplify the *cry* genes of these nine isolates, genomic DNA was extracted from the cultures using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol and the PCR reaction was performed by using 4 pairs of universal primers for the *cry1, cry2, cry3,* and *cry4* genes, as described by Ben-Dov et al. (1997) in Katı et al. (2007).

By using light microscopy, a distinction was made between crystals, spores and vegetative cells. Preparation of specimens for microscopic examination included the following steps: The bacterial smears were immersed for 3 minutes in a small vessel containing a Coomassie Brilliant Blue solution (consisting of 0.25% Coomassie Brilliant Blue, 50% ethanol and 7% acetic acid). They were then rinsed with tap water, air dried, and viewed under a light microscope (Shaerif & Alaeddinoğlu 1988).

For the SDS-PAGE gel, the spore-crystal mixture was resuspended in 1 M cold NaCl and centrifuged at 12.000 rpm for 5 minutes. The pellet was washed twice in sterilized dH_2O and resuspended with sterilized dH_2O. Protein concentrations were determined with the Bradford (1976) assay method and analyzed in 10% SDS-PAGE, as described by Laemmli (1970). Silver staining was used to detect proteins after electrophoretic separation on polyacrylamide gel, and the molecular weight of proteins was estimated by comparison with protein molecular weight standards.

Bioassays

The bacterial isolates were spread on nutrient agar plates to obtain single colonies for each isolate. The obtained single colonies were inoculated into nutrient broth medium and incubated overnight at 30°C. After incubation, the bacterial density

was measured at OD_{600} (optical density) and adjusted to 1.8×10^9 cfu/ml (Ben-Dov et al. 1995). Five milliliters of this culture were centrifuged at 3000 rpm for 10 minutes. The pellet was then resuspended in 5 mL of sterilized phosphate-buffered saline solution and used in bioassays.

The susceptibility of *P. luteola*, *T. molitor* and *G. mellonella* to each bacterial isolate was tested under laboratory conditions. Healthy third-instar larvae of *T. molitor* and *G. mellonella* were purchased and healthy second-instar larvae of *P. luteola* were collected from elm trees (species) in Trabzon. The larvae used for the bioassays were randomly selected. Fresh elm leaves, bran and semi-synthetic food were used as the diets for *P. luteola*, *T. molitor* and *G. mellonella*, respectively, in the bioassays. The semisynthetic foo was prepared as follows: 500 g of bran, 200 g of honeycomb, 150 ml of filtered honey, 150 mL of water and 300 mL of glycerol were thoroughly mixed (Bronskill 1961).

For each isolate, one milliliter of the bacterial suspension, as described above, was dribbled onto the elm leaf, bran and semisynthetic feed, and placed in individual plastic boxes, each containing a single bacterial isolate. Ten, second-third instar larvae were placed on the food material in containers and maintained at 30 ±2°C and 60% relative humidity in a 12:12 photoperiod. Larval mortality was recorded every 24 hours, with all the dead larvae removed from the containers daily. All bioassays were repeated 3 times on different occasions. Four different concentrations (1.8×10^9 cfu/ml, 1.8×10^8 cfu/ml, 1.8×10^7 cfu/ml, and $1.8x10^6$ cfu/ml), based on OD_{600} values of the bacterial isolates, were used in the dose-response experiments. The bioassays were performed as previously described for *T. molitor*. The mortality percentages were corrected in SPSS, according to the Abbott formula (Abbott 1925).

Results and Discussion

A total of 21 bacterial isolates were collected from *C. tenebrionis* were identified to species level by using phenotypic, biochemical, and molecular methods. Color and shape of colonies of the bacterial isolates were determined on nutrient agar. Based on Gram staining and endospore staining results, three of them were determined to be Gram-negative, non-spore forming, coccobacilli, and the other 18 were determined to be Gram-positive, spore-forming bacilli (Table 1).

Table 1**.** Morphological and staining characteristics of the 21 bacterial isolates collected from *Capnodis tenebrionis* in Türkiye.

Isolates	Colony color	Colony shape	Cell shape	Gram staining	Spore staining
L-1	Light cream	Smooth	Coccobacil	-	-
L-2	Cream	Smooth	Bacil	+	+
L-3	Cream	Smooth	Coccobacil	-	-
L-4	Cream	Smooth	Bacil	+	+
L-5	Cream	Rough	Bacil	+	+
L-6	Cream	Rough	Bacil	+	+
L-7	Light cream	Rough	Bacil	+	+
L-8	Light cream	Rough	Bacil	+	+
L-9	Cream	Smooth	Bacil	+	+
L-10	Cream	Rough	Bacil	+	+
OL-1	Light cream	Rhizoid	Bacil	+	+
OL-2	Light cream	Rough	Bacil	+	+
OL-3	Light cream	Smooth	Bacil	+	+
OL-4	Cream	Smooth	Bacil	+	+
OL-5	Light cream	Smooth	Bacil	+	+
E-1	Cream	Rough	Bacil	+	+
E-2	Light cream	Smooth	Coccobacil	-	-
E-3	Cream	Smooth	Bacil	+	+
E-4	Cream	Rough	Bacil	+	+
E-5	Cream	Rough	Bacil	+	+
E-6	Cream	Rough	Bacil	+	+

Tests were performed to determine the optimal ranges of the physical factors, such as pH, NaCl, and temperature, that affect bacterial growth. In the temperature tests, all isolates were able to grow at 35°C, isolates numbered L-1, L-10, and OL-5 were able to grow (but poorly) at +4°C, and isolates numbered L-1, L-10, and OL-2 were able to grow but poorly at 10°C.Only the isolate OL-1 failed to grow at 40°C. The isolates numbered L-3, L-7, L-8, L-9, OL-2, OL-3, OL-5, and E-3 could grow at 45°C, and the isolates numbered L-7 and E-3 could grow well at 50°C.

An important criterion in the identification of species is also that the bacteria cannot produce and manufacture certain enzymes, can synthesize or cannot synthesize some organic substances, or can or cannot degrade some organic substances. For this purpose, starch hydrolysis, oxidase and catalase tests were performed. The isolates ability to grow on MacConkey agar was also investigated (Table 2).

Table 2. Some biochemical characteristics of bacterial isolates collected from *Capnodis tenebrionis* in Türkiye.

Isolates	Catalase test	Oxidase test	Starch Hydrolysis test	MacConkey test
L-1	-	+	-	+
L-2	+	+	+	-
L-3	+	-	-	+
L-4	+	-	+	-
L-5	+	+	+	-
L-6	+	+	+	-
L-7	+	+	-	-
L-8	+	-	+	-
L-9	+	+	+	-
L-10	+	+	-	W+
OL-1	+	-	+	-
OL-2	+	+	+	-
OL-3	+	-	-	-
OL-4	+	-	+	-
OL-5	+	+	-	W+
E-1	+	W+	+	-
E-2	+	+	-	+
E-3	+	+	-	-
E-4	+	+	+	-
E-5	+	+	+	-
E-6	+	+	+	-

W: Weak positive

The use of the 16S rDNA gene sequences in the construction of phylogenetic trees is widely used to determine bacterial systematics; examples of the bacterial 16S rRNA samples from this study are shown in Fig. 1.

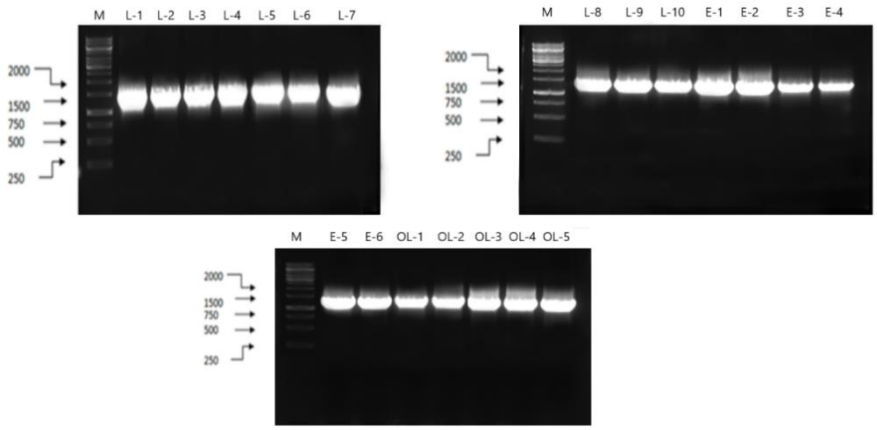

Figure 1. PCR results for 16S rRNA from bacterial isolates collected from *Capnodis tenebrionis* in Türkiye (M: Marker, Fermentas, GeneRuler 1kb).

After all characterization studies, the cultured bacterial flora from *C. tenebrionis* were determined to be: *Bacillus cereus* (E-1; MH220062, E-4; MH220065, E-5; MH220066, E-6; MH220067, OL-4; MH220081, L-2; MH220069, L-4; MH220071, L-5; MH220072, L-6; MH220073) *Bacillus mycoides* (OL-1; MH220078), *Bacillus pumilus* (L-7; MH220074), *Paenibacillus xylanilyticus*, (L-8; MH220075), *Bacillus flexus* (L-9; MH220076), *Bacillus simplex* (L-10; MH220077, OL-5; MH220082), *Raoultella terrigena* (L-1; MH220068), *Enterobacter cloacae* (L-3; MH220070), *Klebsiella oxytoca* (E-2; MH220063), *Bacillus safensis* (E-3; MH220064), *Bacillus amyloliquefaciens* (OL-2; MH220079) and *Bacillus aryabhattai* (OL-3; MH220080). The phylogenetic tree data and 16S rDNA results support each other (Figs. 2 and 3).

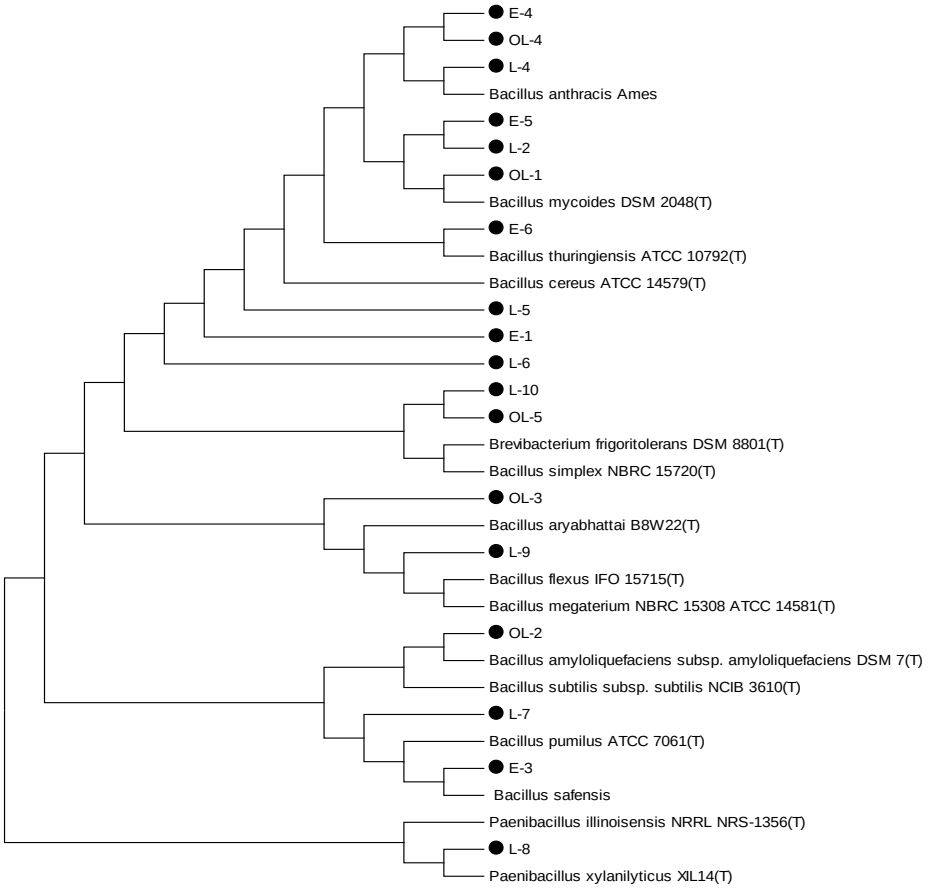

Figure 2. Neighbour-joining analysis of the 16S rDNA region of the Gram-positive isolates of bacteria from *Capnodis tenebrionis* in Türkiye yielded this phylogenetic tree.

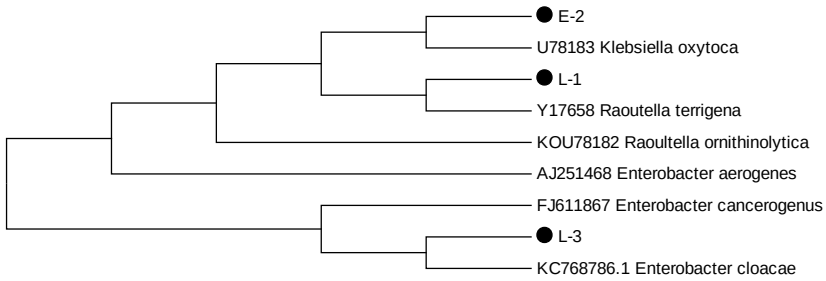

Figure 3. Neighbour-joining analysis of the 16S rDNA region of Gram-negative bacterial isolates collected from *Capnodis tenebrionis* in Türkiye yielded this phylogenetic tree.

The nine gram-negative bacterial isolates, which were thought to be *B. thuringiensis*, were amplified by PCR to detect *cry* genes encoding for crystal proteins. However, no bands of the same size were observed in comparison with the control strain.

These nine bacterial isolates were then subjected to crystal staining to detect crystal proteins. They were examined under a light microscope (type of microscope & manufacturer's details) and no crystal proteins were observed in any isolate.

Mixtures prepared to detect crystal proteins from isolates thought to be *B. thuringiensis* in order to support the results of cry-gene PCR were analyzed in 10% SDS-PAGE (Fig. 4). However, the crystal protein band was not observed in any of the isolates.

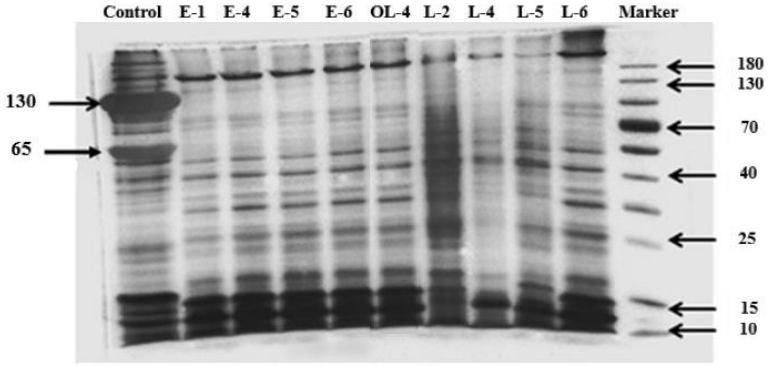

Figure 4. Protein profiles of the isolates stained with silver nitrate. (M: Marker, Control: *B. thuringiensis kurstaki*; BnBt).

To determine the insecticidal potential of these isolates, the third larval stage of *P. luteola* was used under laboratory conditions. Isolates E-5 and E-6 showed the highest efficacy with 87% mortality. This level of lethal effect was followed by that caused by L-5 with 83%, L-6, L-4, E-1 with (80%), L-2, E-4, L-4, L-7 with (77%) (Fig. 5).

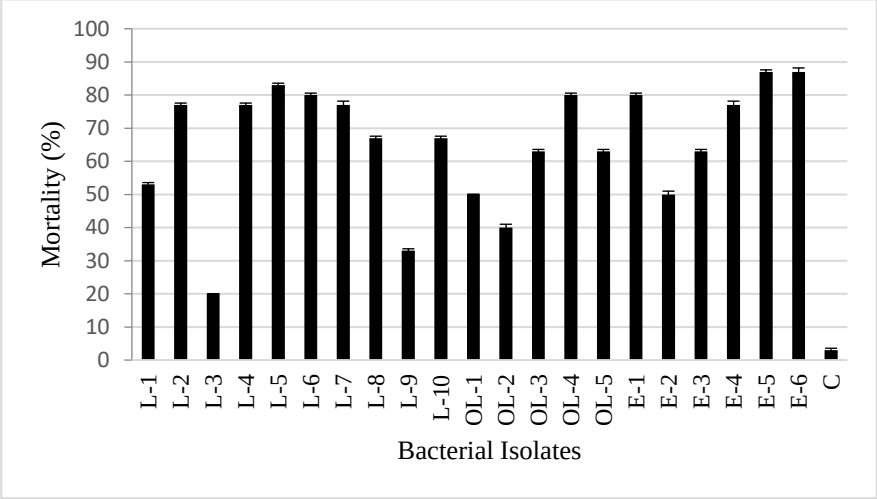

Figure 5. Insecticidal activities of bacteria isolated from *Capnodis tenebrionis* in Türkiye on *Pyrrhalta luteola*; C: Control.

For the larvae of *G. mellonella*, the isolate OL-4 caused ed a mortality of 60%, followed by the isolates E-4 and E-5 with (53%) (Fig. 6).

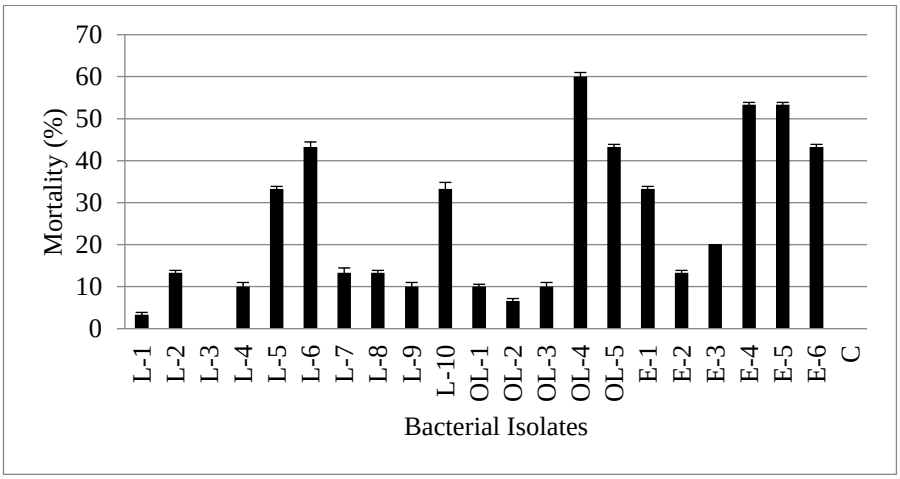

Figure 6. Insecticidal activities of bacteria isolated from *Capnodis tenebrionis* on *Galleria mellonella*; C: Control.

For *T. molitor*, the suspensions were prepared at four different dosages (1.8×10^9, 1.8×10^8, 1.8×10^7, 1.8×10^6 cfu/ml). The isolate OL-4 showed the highest lethal effect at a concentration of 1.8×10^9 cfu/mL (47%), 13% at a concentration of 1.8×10^8 cfu/mL, and 7% at a concentration of 1.8×10^7 cfu/ml. Isolates E-1 and OL-5 produced mortality of 20% and 20%, respectively, at a concentration of 1.8×10^9, 7% and 13% at a concentration of 1.8×10^8 cfu/ml, and 3% and 3% at a concentration of 1.8×10^7 cfu/ml. These tests were followed by testing of the

isolates, L-2 and E-6, which caused a mortality rates of 10% after the use of 1.8×10^9 and 1.8×10^8 bacteria, respectively. No mortality was observed for *Tenebrio molitor* at a concentration of 1.8×10^6 cfu/ml (Fig. 7, Table 3).

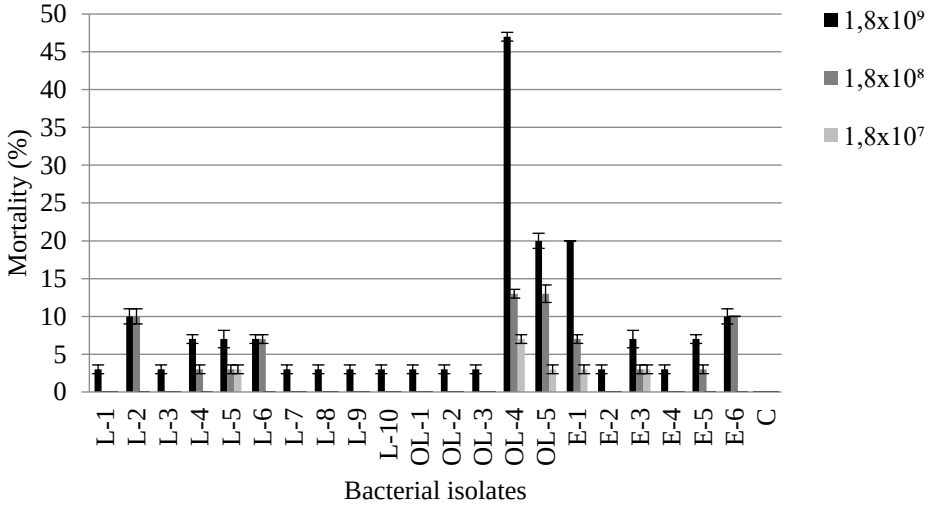

Figure 7. The dose tests of bacteria isolated from *Capnodis tenebrionis* on *Tenebrio molitor*; C: control.

Morphological and physiological methods were used for bacterial characterization, and 16S rDNA analysis was performed to confirm the identifications of the isolates. The API20E, API50CH, and the results from VITEK-2 identification systems were also used to support 16S rDNA sequence analysis. Data obtained from these assayss were used whenever data obtained from routine assays were insuficient for species identification.

Table 3. LC_{50} and LC_{95} (cfu/mL) values of OL-4, OL-5, and E-1 bacterial isolates collected from *Capnodis tenebrionis* in Türkiye, against the larvae of *Tenebrio molitor*.

Isolates	**LC_{50} (FL, 95%)**	**Slope ± SE**	**LC_{95} (FL, 95%)**	**df**	**X^2**
OL-4	$1.82x10^9$ ($0.32x10^9$ to ($10.41x10^9$)	0.709±0.386	$39.423x10^9$	1	0.478
OL-5	$34.35x10^9$ ($2.08x10^9$ to $566.71x10^9$)	0.496±0.621	$72461.38x10^9$	1	0.578
E-1	$63.41x10^9$ ($3.23x10^9$ to $1242.94x10^9$)	0.496± 0.659	$132622.21x10^9$	1	0.727

FL: fiducial limit, SE: standard error, df: degrees of freedom, X^2: Chi square.

After 16S rDNA sequence analysis and biochemical testing, isolates E-1, E-4, E-5, E-6, OL-4, L-2, L-4, L-5 and L-6 had characteristics of *B. thuringiensis*, *B.*

cereus and *B. toyonensis*. SDS gel electrophoresis and crystal staining were performed, and the isolates were found to contain no crystal protein, and the VP (Voges-Proskauer) test was positive. Thus, they were identified as *B. cereus* which can adapt to changing environmental conditions. *Bacillus cereus* is widely distributed in the soil (Vilas-Boas et al. 2002). Many of these species have been isolated from insects, such as *Prodenia eridania, Plodia interpunctella* and *Periplaneta americana* (Steinhaus 1947).

In the efficacy tests, the isolates E-5 and E-6 of this group showed the highest efficacy (87%) against the elm leaf beetle. In addition, the OL-4 and E-1 isolates caused 80% mortality. Efficacy testing on honeycomb showed that the E-4 and E-5 isolates produced 53% mortality, and the E-6 and L-6 isolates produced 43% mortality, whereas the OL-4 isolate had the highest lethal effect at 60%.

In this study, the efficacy tests on mealworm larvae, the isolate OL -4 caused the highest mortality rate of 47% at a concentration of 1.8×10^9 cfu/ml. In a different study, isolate L-4 caused 77% mortality of *P. luteola*, and *B. cereus* strain Ags1 caused 30% mortality of *Agrotis segetum* (Sevim et al. 2010).

Six *B. cereus* strains have been reported to produce several small, non-protein insecticidal exotoxins (Perchat et al. 2005; Ruiu et al. 2015). In addition, treatment of *Caenorhabditis elegans* and *Meloidogyne incognita* with the supernatant of a *B. cereus* strain culture resulted in mortality of 77.89% of *C. elegans* and 90.96%) of *M. incognita* (Gao et al. 2016).

16S rDNA sequence analysis revealed that the OL-1 isolate is similar to *B. thuringiensis*, *B. cereus*, and *B. mycoides*. Because it forms a rhizoidal colony, the VP (Voges-Proskauer) was positive and immobilized, it was determined to be *B. mycoides*.

Bacillus mycoides is a rod-shaped, chain-forming bacterium that produces unique rhizoid colonies consisting of cells connected end-to-end on agar plates. Several reports on *B. mycoides* have described its plant growth-promoting effects on sugar beet, cucurbits and tobacco (Neher et al. 2009). *Bacillus mycoides* caused 50% mortality of *P. luteola*, and 10% of *G. mellonella*. It is also showed biocontrol activity against *Cercospora* leaf spot disease (*Cercospora beticola* Sacc.) of sugar beet in both greenhouse and field experiments (Bargabus et al. 2002). In addition, *B. mycoides* (strain B38 V) was reported to have antagonistic activity against *Sclerotinia scleratiorum* (Ambrosini et al. 2015).

According to 16S rDNA analysis, the L-9 isolate could have been *B. flexus*, *B. aryabhattai* or *B. pumilus*. *Bacillus flexus* has a positive ability to hydrolyze starch, and is positive for the gelatinaseand oxidase tests. *Bacillus flexus* has been isolated several times from different lakes and seaweeds, e.g., *Ulva lactula* (Wang & Zhao 2013). In the current study, solate L-9 was found to cause 33% mortality of *P. luteola* and 10% mortality of *G. mellonella*. Studies showed that *B. flexus* inhibits

the growth of fouling bacterial strains and strongly inhibits the production of byssal filaments and their attachment in the mussel, *Perna indica* (Ramasubburayan et al. 2017).

According to the 16S rDNA sequence analysis, the OL -2 isolate was *Bacillus amyloliquefaciens*, *B. subtilis* or *B. velezensis*. Based on all of the results, the isolate was identified as *B. amyloliquefaciens* because the starch hydrolysis test was positive, and the arginine hydrolysis test was negative. *Bacillus amyloliquefaciens* is a non-pathogenic soil bacterium that exhibits certain antifungal properties (Caldeira 2007). OL-2 had a potential caused mortality of 40% and 6% for *P. luteola* and *G. mellonella*, respectively. It caused mortality of 3, 14, and 13% of *Plodia interpunctella* larvae, *Ephestia kuehniella* larvae and *Sitophilus granarius* adults, respectively (Demeli 2014).

16S rDNA analysis of the isolates OL-5 and L-10 suggestedthat they could be *Bacillus simplex* or *Brevibacterium frigoritolerans*. Considering the results of VITEK-2 testing, it was determined that these isolates were *B. simplex*. *Bacillus simplex* was isolated from *Thaumetopoea pityocampa* (İnce et al. 2008) and *Gryllotalpa gryllotalpa* (Sezen et al. 2013). The OL -5 isolate caused 63%) mortality of *P. luteola* and 43% of *G. mellonella*. Also, isolate L-10 caused 67%)mortality of *P. luteola* and 33% of *G. mellonella* but caused no deaths of *T. molitor*. *B. simplex* caused 75% mortality of *G. gryllotalpa* nymphs (Sezen et al. 2013).

Isolate L-7 was identified as *Bacillus pumilus*, based on both 16S rDNA sequence analysis and the results of the API and VITEK-2 assay testing. The negative results of the nitrate-nitrite, starch hydrolysis and indole tests confirmed that this isolate was *B. pumilus*. This species had been earlier isolated from many insect species such as *Diatraea saccharalis* (Fabricius) (Dantur et al. 2015). It caused 77% mortality of *P. luteola* and 13%to *G. mellonella*. A soil isolate of *B. pumilus* caused 95.7% mortality of *Leptinotarsa decemlineata* (Say) larvae (Coleoptera: Chrysomelidae) (Ertürk et al. 2008).

Te results of 16S rDNA sequencing suggested that the E-3 isolate could have been *Bacillus stratosfericus*, *B. pumilus*, *B. safensis* or *B. aerophilus*. Based on thethe API results, it was determined to be *G. thermoglucosidasius*. However, the VITEK-2 panel system was unable to identify this isolate. Based on all of the test results, it was identified as *B. safensis*. This species was first isolated from far-field space (Satomi et al. 2006). Later, Tomova et al. (2013) isolated it from a cave in Bulgaria. It caused 63% mortality of *P. luteola* and 20% of *G. mellonella*. It also iit caused 80% mortality of *Sesamia. nonagrioides* (Eski et al. 2015).

After considering the preliminary results from 16S rDNA analysis, and VITEK-2 and API test results, it was determined that the isolate OL-3 could be *Bacillus aryabhattai* or *B. megaterium*. However, after looking at the biochemical

properties of the isolate, it was identified as *B. aryabhattai*, based on the positive gelatinase, VP (Voges-Proskauer) and starch hydrolysis tests. In a previous study, it was isolated from *Oryctes rhinoceros* (Sari et al. 2016). In the current study, this isolate caused 63% mortality of *P. luteola* and 10% mortality of *G. mellonella*.

16S rDNA sequencing and VITEK-2 system analysis showed that isolate E-2 could be *Klebsiella oxytoca* or *Raoultella terrigena*. Based on all the results of biochemical testing, this isolate was identified as *K. oxytoca*. It had been previously isolated from *Diatraea saccharalis* (Fabricius) (Dantur et al. 2015). This isolate caused 50% and 13% mortality of the larvae of the elm leaf beetle and honeycomb, respectively.

The L-3 isolate was identified as *Enterobacter cloacae*, based on the results of API and VITEK-2 testing, and 16S rDNA sequence analysis. This bacterium had been isolated from some pests, such as *Bombyx mori* (Watanabe & Sato, 1998), *Heliothis armigera* (Hübner), *Agrotis segetum* (Lipa & Wiland 1972) and *Melolontha melolontha* (Sezen et al. 2001). It caused mortality of 20% and 40% of elm leaf beetle larvae and *M. melolontha* larvae, respectively (Sezen et al. 2001).

16S rDNA sequencing of the L-8 isolate revealed it to be *Paenibacillus xylanilyticus*, *P. illinoisensis* or *P. pabuli*. According to the results of API and VITEK-2 system testing, it was *P. polymyxa* and *P. glucanolyticus*, respectively. Considering the results of all tests, this isolate was identified as *P. xylanilyticus*. *Paenibacillus* species are found in soil, water and insect larvae (Daane et al. 2002). It was isolated from *G. gryllotalpa* and found to cause 67% mortality of *P. luteola* and 13% mortality of *G. mellonella* (Sezen et al. 2013).

Finally, isolate L-1 showed high similarity with *Raoultella terrigena*, *R. variicola* and *R. ornithinolytica*, according to 16S rDNA sequence analysis; *R. terrigena* according to the API results; and *R. ornithinolytica*, according to VITEK-2 results. It was determined to be a Gram-negative, spore-free bacterium with a low activity level. Based on all test results, it was identified as *R. terrigena*. This species has been isolated from *Dendroctonus rhizophagus* and *D. valens* (Morales-Jimenez et al. 2013). This L-1 isolate caused 53% mortality of *P. luteola*.

Conclusions

In this study, 21 bacterial isolates were isolated from the larvae and adults of *C. tenebrionis*, a destructive pest of stone fruit orchards. The biochemical, molecular, morphological, physiological, and characteristics of the isolates were determined. Isolates E-5 and E-6 (*Bacillus cereus*) had the highest lethal effect (87%) on *P. luteola*, while OL-4 (*Bacillus cereus*) had the highest lethal effect on both *G. mellonella* and *T. molitor*. Thus, these three isolates appear to have the most potential to be used against coleopteran and lepidopteran pests of agriculture. Field

testing of the isolates against pests in the field would help determine whether their laboratory success translates to field success. Separately, studies on different groups of insect pests are still needed.

References

Abbott W. S., 1925. A method of computing of effectiveness of insecticide. Journal of *Economic Entomology*, 18:265−267.

Ambrosini A., F. H. Sant'Anna, R. de Souza, M. Tadra-Sfeir, H. Faoro, S. M. Alvarenga, F. O. Pedrosa, E. M. Souza & L. M. P. Passaglia, 2015. Genome sequence of *Bacillus mycoides* B38V, a growth-promoting bacterium of sunflower. *Genome Announcement*, 3:e00245-15.

Bargabus R., N. Zidack, J. Sherwood & B. Jacobsen, 2002. Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiological and Molecular Plant Pathology*, 61:289–298.

Beegle C. C. & T. Yamamoto, 1992. Invitation Paper (C. P. Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development, *Canadian Entomologist,* 124: 587- 616.

Ben-Dov E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, R. Manasherob, A. Khameraev, E. Troitskaya, A. Dubitsky, N. Berezina & Y. Margalith, 1997. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. *Applied Environmental Microbiology*, 63:4883-4890.

Ben-Dov E., S. Boussiba & A. Zaritsky, 1995. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis. Journal of Bacteriology*, 177: 2851–2857.

Benson H. J., 1985. Microbiological Applications, a Laboratory Manuel in General Microbiology, Brock, Fourth Edition, Wm C. Brown Publishers, Dubuque, Iowa.

Ben-Yehuda S., A. Assal & Z. Mendel, 2000. Improved chemical control of *Capnodis tenebrionis* and *C. carbonaria* in stone-fruit plantations in Israel. *Phytoparasitica*, 28:27–41.

Bradford M. M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72:248–254.

Bronskill J. K., 1961. A cage to simplify the rearing of the greater wax moth, *Galleria mellonella* (Pyralidae). *Journal of the Lepidopterists' Society,* 15: 102-104.

Burges H. D. & J. A. Hurst, 1977. Ecology of *Bacillus thuringiensis* in storage moths. *Journal of Invertebrate Pathology*, 30: 131-139.

Caldeira A. T., S. S. Feio, J. M. S. Arteiro, A. V. Coelho & J. C. Roseiro, 2007. Environmental dynamics of *Bacillus amyloquefaciens* CCMI 1051 anti-fungal activity under different nitrogen patterns. *Journal of Applied Microbiology*, 104:806-816.

Cappuccino J. G. & N. Sherman, 1992. Microbiology, a Laboratory Manual, Third Edition, Rockland Community College, Suffern, New York.

Carozzi N. B., V. C. Kramer, G. W. Warren, S. Evola & M. G. Koziel, 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profilles. *Applied and Environmental Microbiology,* 57: 3057 3061.

Claus M. 1992. A standardized Gram staining procedure. *World Journal of Microbiology and Biotechnology,* 8:451–452.

Daane L. L., I. Harjono, S. M. Barns, L. A. Launen, N. J. Palleroni & M. M. Häggblom, 2002. PAH-degradation by *Paenibacillus* spp. and description of *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. *International Journal Systematic and Evolutionary Microbiology,* 52:131–139.

Dantur K. I., R. Enrique, B. Welin & A. P. Castagnaro, 2015. Isolation of cellulolytic bacteria from the intestine of *Diatraea saccharalis* larvae and evaluation of their capacity to degrade sugarcane biomass. *AMB Express,* 25 (5): 15.

De Souza P. C., A. T. Morey, G. M. Castanheira, K. P. Bocate, L. A. Panagio, F. A. Ito, M. C. Furlaneto, S. F. Yamada-Ogatta, I. N. Costa, H. M. Mora-Montes & R. S. Almeida, 2015. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. *Journal of Microbiological Methods,* 118: 182–6.

Demeli M., Z. Demirbağ & K. Sezen, 2014. Isolation and characterization of *Bacillus* from some warehouses in Trabzon. *Journal of Applied Biological Sciences,* 8(2):69-80.

Ertürk O., M. Yaman & I. Aslan, 2008. Effects of four *Bacillus* ssp. of soil origin on the Colorado potato beetle *Leptinotarsa decemlineata* (Say). *Entomological Research,* 38: 135–138.

Eski A., F. Özkan Çakıcı, M. Güllü, H. Muratoğlu, Z. Demirbağ & İ. Demir, 2015. Identification and pathogenicity of bacteria in the Mediterranean corn borer *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae), *Turkish Journal of Biology,* 39: 31–48.

Gao H., G. Qi, R. Yin, H. Zhang, C. Li & X. Zhao, 2016. *Bacillus cereus* strain S2 shows high nematicidal activity against *Meloidogyne incognita* by producing sphingosine. *Scientific Reports,* 6: 28756.

Grau T., A. Vilcinskas & G. Joop, 2017. Sustainable farming of the mealworm *Tenebrio molitor* for the production of food and feed. *Zeitschrift für Naturforschung C,* 72 (9–10): 337–349.

Hall T. A., 1999. Bio Edit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/Nt. *Nucleic Acids Symposium,* 41: 95–98.

İnce I. A., H. Katı, H. Yılmaz, İ. Demir & Z. Demirbağ. 2008. Isolation and identification of bacteria from *Thaumetapoea pityocampa* Den. And Schiff. (Lep., Thaumetapoeidae) and determination of their biocontrol potential. *World Journal of Microbiology and Biotechnology,* 24: 3005-3015.

Kaelin P., P. Morel, & F. Gadani, 1994. Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F.). *Applied Environmental Microbiology,* 60: 19-25.

Katı H., K. Sezen, R. Nalcacioglu & Z. Demirbag, 2007. A highly pathogenic strain of *Bacillus thuringiensis serovar kurstaki* in lepidopteran pests. *The Journal of Microbiology,* 45: 553–557.

Laemmli U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227: 680– 685.

Lipa J. J. & E. Wiland, 1972. Bacteria isolated from cutworms and their infectivity to *Agrotis* spp. *Acta Microbiologica Polonica,* 4: 127–140.

Morales-Jiménez J., A. Vera-Ponce de León & A. García-Domínguez, 2013. Nitrogen-fixing and uricolytic bacteria associated with the gut of *Dendroctonus rhizophagus* and *Dendroctonus valens* (Curculionidae: Scolytinae). *Microbial Ecology,* 66: 200-210.

Muratoğlu H., K. Sezen & Z. Demirbağ, 2011. Determination and pathogenicity of the bacterial flora associated with the spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae). *Turkish Journal of Biology,* 35: 19–20.

Neher O. T., M. R. Johnston, N. K. Zidack & B. J. Jacobsen, 2009. Evaluation of *Bacillus mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 for the control of anthracnose of cucurbits caused by *Glomerella cingulata* var. *orbiculare. Biological Control,* 48: 140–146.

Perchat S., C. Buisson, J. Chaufaux, V. Sanchis, D. Lereclus & M. Gohar, 2005. *Bacillus cereus* produces several non proteinaceous insecticidal exotoxins. *Journal of Invertebrate Pathology,* 90: 131–133.

Poinar G. O. & G. M. Thomas, 1978. Diagnostic Manual for the Identification of Insect Pathogens. New York, NY, USA: Plenum Press.

Prescott L. M., J. P. Harley & D. A. Klein, 1996. Microbiology. Dubuque, IA, USA: William C. Brown Publishers.

Ramasubburayan R., S. Titus, P. Kumar, G. Immanuel & A. Palavesam, 2017. Antifouling activity of marine epibiotic bacterium *Bacillus flexus* APGI isolated from Kanyakumari Coast, Tamilnadu, India. *Indian Journal of Geo-Marine Sciences,* 46 (07): 1396-1400.

Ruiu L., G. Falchi, I. Floris, M. G. Marche, M. E. Mura & A. Satta, 2015. Pathogenicity and characterization of a novel *Bacillus cereus sensu lato* isolate toxic to the Mediterranean fruit fly *Ceratitis capitata* Wied. *Journal of Invertebrate Pathology,* 126: 71–77.

Sari S. L. A., A. Pangastuti, A. Susilowati, T. J. Purwoko, E. Mahajoeno, W. Hidayat, I. Mardhena, D. F. Panuntun, D. Kurniawati & R. Anitasari, 2016. Cellulolytic and hemicellulolytic bacteria from the gut of *Oryctes rhinoceros* larvae. *Biodiversitas Journal of Biological Diversity,* 17: 78–83.

Satomi M., M. T. La Duc & K. Venkateswaran, 2006. *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *International Journal of Systematic and Evolutionary Microbiology,* 56: 1735–1740.

Sevim A., Z. Demirbağ & İ. Demir, 2010. A new study on the bacteria of *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) and the insecticidal activities. *Turkish Journal of Agriculture and Forestry,* 34: 333–342.

Sezen K., İ. Demir & Z. Demirbağ, 2001. Identification and insecticidal effects of bacteria isolated from pests of hazelnut, Proceedings of the *1st Eurasian Congress on Molecular Biotechnology,* 17-20 Sep, Trabzon, Turkey, 146–148.

Sezen K., Ş. İşçi, H. Muratoğlu, K. İnan & Z. Demirbağ, 2013. Identification and pathogenicity of bacteria from *Gryllotalpa gryllotalpa* L. (Orthoptera: Gryllotalpidae). *Turkish Journal of Biological Control,* 4 (2): 89-108.

Shaerif F. A. & N. G. Alaeddinoğlu, 1988. A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis. Journal of Industrial Microbiology,* 3: 227–229.

Sneath P. H. A., N. S. Mair, M. E. Sharpe & J. G. Holt, 1986. Regular, nonsporing gram-positive rods (Editors: Kandler O., Weiss N.,) Bergey's Manual of Systematic Bacteriology. Baltimore, MD, USA: Williams and Wilkins, 1208–1260.

Steinhaus E. A., 1947. Insect Microbiology. Comstock Publishing Co. Inc., Ithaca, New York, 768 s.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei & S. Kumar, 2011. MEGA5: Molecular evolution are genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution,* 28: 2731–2739.

Tomova I., I. Lazarkevich, A. Tomova, M. Kambourova & E. V. Tonkova, 2013. Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria. *International Journal of Speleology,* 42 (1): 65-67.

Vilas-Boas G., V. Sanchis, D. Lereclus, M. V. F. Lemos & D. Bourguet, 2002. Genetic differentiation between sympatric populations of Bacillus cereus and *Bacillus thuringiensis. Applied and Environmental Microbiology,* 68: 1414–1424.

Vit K., 2004. Fauna Europaea: Buprestidae. In: Alonso-Zarazaga M. A. (ed.) Fauna Europaea: Coleoptera. Fauna Europaea version 1.1. http://www.faunaeur.org.

Wang X. & H. Zhao, 2013. Isolation and characterization of a *Bacillus flexus* strain used in alkaline wastewater treatment. *Advanced Materials Research,* 750-752: 1381–1384.

Watanabe K. & M. Sato, 1998. Plasmid-mediated gene transfer between insect-resident bacteria, *Enterobacter cloacae,* and plant-epiphytic bacteria, *Erwinia herbicola* inguts of silkworm larvae. *Current Microbiology,* 37: 352–355.

Weisburg W. G., S. M. Barns, D. A. Pelletier & D. J. Lane, 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173: 697–703.