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Insecticidal activity of isolated bacteria from *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae)

Hyphantria cunea (Drury) (Lepidoptera: Arctiidae)'den izole edilen bakterilerin insektisidal aktivitesi

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Abstract

The fall webworm (*Hyphantria cunea)* is a polyphagous pest with numerous host plants. In the present study, the bacterial flora of *H.cunea* was investigated to identify new organisms that can be used as microbial control agent against the pest. Six bacteria were isolated and cultured from *H. cunea*. Some morphological, biochemical and other phenotypic characteristics (with API 20E, API 50 CH, API Staph and API Coryne kits) of bacterial isolates were determined. In addition, 16S rRNA gene region was sequenced. As a result of the studies conducted, bacterial isolates were identified as *Lysinibacillus sphaericus* (Abk1), *Bacillus amyloliquefaciens* (Abk2), *Staphylococcus sciuri* (Abk4), *Kocuria palustris* (Abk6), *Arthrobacter arilaitensis* (Abk7) and *Microbacterium oxydans* (Abk8). All bacterial isolates were tested for 12 days against third-fourth instar larvae of *H. cunea*. The highest insecticidal activity was obtained from *L. sphaericus* (Abk1) with 30% after application ($p<0.05$). These results indicate that *L. sphaericus* (Abk1) can be taken into account in the microbial pest control of *H. cunea.* In the future, further studies will be conducted by using pathogenicity enrichment strategies of *L. sphaericus* (Abk1) (ex. combining with other entomopathogens or insecticides) in order to increase the effectiveness on *H. cunea.*

Özet

Amerikan Beyaz Kelebeği, *Hyphantria cunea* (Drury) sayısız konukçu bitkisi olan polifag bir zararlıdır. Bu çalışmada, *H. cunea*'nın bakteriyal florası zararlıya karşı mikrobiyal kontrol ajanı olabilecek yeni organizmaları identifiye etmek için araştırıldı. *H. cunea*'dan altı bakteri izole edilerek saflaştırıldı. Bakteriyal izolatların morfolojik, biyokimyasal, diğer fenotipik özellikleri **(**API 20E, API 50 CH, API Staph ve API Coryne) belirlendi. İlave olarak, 16S rRNA gen sekans analizi de yapıldı. Yapılan çalışmalar sonucunda izolatlar; *Lysinibacillus sphaericus* (Abk1), *Bacillus amyloliquefaciens* (Abk2), *Staphylococcus sciuri* (Abk4), *Kocuria palustris* (Abk6), *Arthrobacter arilaitensis* (Abk7) ve *Microbacterium oxydans* (Abk8) olarak tanımlandı. Bütün bakteriler *H. cunea*'nın üçüncü-dördüncü gömlek larvalarına karşı oniki gün boyunca test edildi. En yüksek insektisidal aktivite %30 ile *L. sphaericus* (Abk1)'dan elde edildi ($p<0.05$). Bu sonuçlar, *L. sphaericus* (Abk1)'un, *H. cunea*'nın mikrobiyal mücadelesinde dikkate alınabileceğini göstermiştir. Gelecekte, *L. sphaericus* (Abk1)'un patojenite zenginleştirme stratejileri (diğer entomopatojelerle veya insektisitlerle kombine edilmesi gibi) kullanılarak *H. cunea* üzerindeki etkinliğinin artırılması yönünde çalışmalar yapılacaktır.

INTRODUCTION

The fall webworm, *Hyphantria cunea* (Drury) is a pest polyphagous Lepidoptera feed on the number of host plants from wide ranges of forest and fruit trees to several agricultural crops. Native to North America, *H. cunea* spread of trees different areas in Europe and Asia.

Several some studies have been performed to determine the different enthomopathogens in *H. cunea* (Yamanaka et al. 1985; Yaman et al. 2002; Albayrak Iskender et al. 2012). However, *H. cunea* is a very harmful pest species around the world, sustainable method for controlling of this pest still have not been. In the current study, we investigated bacterial flora of *H. cunea* to identify new candidate organisms as a possible microbial control agent against the pest.

MATERIALS AND METHODS

Collection of insects

H. cunea larvae were collected from Georgia, in April-May 2012. The collected larvae carefully were placed in sterile plastic boxes and immediately were transported to the laboratory.

Isolation of bacteria

The larvae were separated to dead and live, surface sterilized in 70% alcohol and then washed three times with sterile distilled water and homogenized in nutrient broth media by using a glass tissue grinder. Suspensions were diluted and 0.1ml suspension was plated on nutrient agar. Plates were incubated at 30ºC for 2-3 days. After the incubation period, the plates were examined and bacterial colonies were selected. The colonies determined were purified by subculture on the plates.

Identification of bacterial isolates

Some morphological (cell morphology, endospore formation and mobility) and biochemical properties (gram reaction, oxidase, catalase and reduction of nitrate) of bacterial isolates were examined using standard protocols (Harley and Prescott 2002). The commercially available API 20E, API 50 CH, API Staph kits and API Coryne kit (bioMe´rieux) also was used to determine some other phenotypic properties according to the manufacturer's instructions.

16S rRNA gene sequencing

Bacterial colonies were inoculated into nutrient broth and incubated approximately 18 h at 30 ^{0}C in order to extract DNA from bacterial isolates. At the end of incubation time, the bacterial cells were collected by centrifuging from the culture medium. Then, genomic DNA was isolated with Genomic DNA Purification Kit (Promega, Germany) in accordance with the manufacturer's recommendations. 16S rRNA gene sequences of the bacterial isolates were performed with the following universal primers: UNI16S-L: 5'-ATT CTA GAG TTT GAT CAT GGC TCA -3' as forward and UNI16S-R: 5'ATG GTA CCG TGT GAC GGG CGG TGT GTA-3' as reverse. PCR conditions were adjusted according to the instructions of Weisburg et al. (1991). The amplified 16S rRNA gene product was sent to RefGen Biotechnology Laboratory (Ankara, Turkey) for sequencing. The sequences obtained were used for identification of the isolates and phylogenetic analyses.

Phylogenetic relationship of the bacterial isolates

The sequences obtained were used to perform BLAST searches using the NCBI GenBank database to confirm isolate identification Altschul et al. (1990). Evolutionary relationships of the eleven bacterial isolates were evaluated. Cluster analyses of the sequences were performed using BioEdit (version7.09) with Clustal W followed by neighbor joining analysis on aligned sequences performed with MEGA 6.0 software (Tamura et al. 2013). Reliability of dendograms was tested by bootstrap analysis with 1000 replicates using MEGA 6.0.

The insecticidal effects of bacterial isolates

Third-fourth instar larvae of *H. cunea* were used for the insecticidal assay of bacterial isolates. All of the bacterial isolates were tested as overnight cultures after removing the growth medium. Bacterial isolates were incubated for 18 hours (72 h for sporulation) at 30ºC in the nutrient broth medium. After incubation, bacterial cells were centrifuged at 3000 rpm for 10 min (Ben-Dov et al. 1995). The pellet was resuspended by adding sterile PBS. The optical density of the cells was adjusted to 1.89 at OD (optical density)$_{600}$ (Moar et al. 1995). Fresh mulberry leaves were inoculated by dipping them into the bacterial suspensions and placed in a sterile plastic box (150ml). The control group was treated with sterile PBS. Ten third-fourth instar larvae were placed into each box and fresh mulberry leaves was provided in each day for twelwe days. These boxes were incubated at 26±2ºC and 60% RH during 12 h L: 12 h D photoperiod. Mortality was recorded twelwe days later. At least thirty larvae were assayed for each isolate. All experiments were repeated three times.

Mortalities were corrected according to Abbott's formula (Abbott 1925). The data were subjected to ANOVA and subsequently to LSD multiple comparison test to compare isolates against the control group and to determine differences among isolates using SPSS 15.0 statistical software.

RESULTS

In this study, 6 bacterial isolates; *Lysinibacillus sphaericus* (Abk1), *Bacillus amyloliquefaciens* (Abk2), *Staphylococcus sciuri* (Abk4), *Kocuria palustris* (Abk6), *Arthrobacter arilaitensis* (Abk7) *and Microbacterium oxydans* (Abk8) from *H. cunea* were isolated and characterized. All isolates were Gram positive. The colony colours of four isolates were cream, that of the other two isolates were

yellow. Two isolates had the shape of cocci; one isolate was bacil-coccus; the other isolates were bacilli.

All isolates were catalase positive. Oxidase and reduction of nitrate tests varied depending on the isolate. Some morphological and biochemical characteristics of bacterial isolates are summarized in Table 1.

Table 1. Some morphological and biochemical characteristics of bacterial isolates

Isolates	Abk1	Abk2	Abk4	Abk6	Abk7	Abk8
Colony color	Cream	Cream	Cream	Yellow	Cream	Yellow
Shape	Bacil	Bacil	Cossus	Cossus	Bacil-cossus	Bacil
Gram stain	+	+	+	+	+	+
Spor stain	+	+	-	-	-	-
Motility	+	+	-	-	-	+
Catalase test	+	+	+	+	+	+
Nitrate reduction	-	+	+	+	-	-
Oxidase test	+	+	+	-	-	-

Symbols: +, positive; -, negative

Phylogenetic tree was constructed by using Neighbor Joining method (Figure 1). The similarities between isolates were ranged between 98%- 99% compared to other species.

Based on studies, isolates were identified as *Lysinibacillus sphaericus* (Abk1), *Bacillus amyloliquefaciens* (Abk2), *Staphylococcus sciuri* (Abk4), *Kocuria palustris* (Abk6), *Arthrobacter arilaitensis* (Abk7) *and Microbacterium oxydans* (Abk8). These identifications were also confirmed by phylogenetic analysis of the bacterial isolates and their closely related species based on the 16S rRNA sequence (Figure 1).

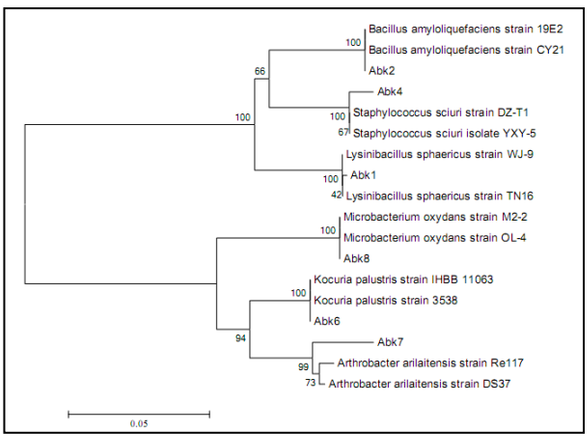

Fig. 1. Neighbor-joining tree of bacterial isolates from *H. cunea* and their closely related 12 bacterial species. The dendrogram was constructed by MEGA 6.0 software based on the partial sequences of the 16S rRNA gene. Bootstrap values shown next to nodes are based on 1000 replicates. The scale on the bottom of the dendrogram shows the degree.

API test results are listed in Tables 2, 3, 4 and 5, respectively.

Table 2. The results of API 20E test system of bacteria isolated from *H. cunea*

Tests	Abk1	Abk2	Abk3	Abk4	Abk5	Abk6
β-galaktosidase	-	-	-	-	±	±
Arginine dihydrolase	-	-	-	-	-	-
Lysine decarboxylase	-	-	+	-	-	-
Ornithine decarboxylase	-	-	+	-	-	-
Trisodium citrate	+	+	+	-	-	±
H_2S(sodium thiosulfate)	-	-	+	-	-	-
Urease	-	-	-	±	-	-
L-tryptophan	-	-	-	-	-	-
Indole	-	-	±	-	-	-
VP (sodium pyruvate) test	-	+	±	-	±	±
Gelatinase	+	+	+	-	±	+
Glucose fermentation	-	+	+	+	-	±
D-mannitol fermentation	-	+	+	-	-	±
Inositol fermentation	-	+	+	-	-	-
D-sorbitol fermentation	-	+	+	-	-	-
L-rhamnose fermentation	-	-	+	-	-	±
D-saccharose fermentation	-	+	+	+	-	-
D-melibiose fermentation	-	+	-	-	-	-
Amygdaline fermentation	-	+	-	±	-	±
L- arabinose fermentation	-	+	-	±	-	-

+positive,-negative, ± weak positive

Table 3. The results of API 50CH test system of Abk1 and Abk2 isolates

Tests	Abk1	Abk2
Glycerol	+	+
Erythritol	-	-
D-Arabinose	-	-
L-Arabinose	-	+
D-Ribose	-	+
D-Xylose	-	+
L-Xylose	-	-
D-Adonitol	-	-
Methyl- βD-xylopyranoside	-	-
D-Galactose	-	±
D-Glucose	-	+
D-Fructose	-	+
D-Mannose	-	+
L-Sorbose	-	-
L-Rhamnose	-	-
Dulcitol	-	-
Inositol	-	+
D-Mannitol	-	+
D-Sorbitol	-	+
Methyl- αD-mannopyranoside	-	-
Methyl- αD-glucopyranoside	-	+
N-Acetylglucosamine	+	-
Amygdalin	-	+
Arbutin	-	+
Esculin-ferric citrate	±	+
Salicin	+	+
D-Cellobiose	-	+
D-Maltose	-	+
D-Lactose (bovine origin)	-	+
D-Melibiose	-	+
D-Saccharose	-	+
D-Trehalose	-	+
Inulin	-	±
D-Melezitose	-	±
D-Raffinose	-	+
Starch	-	+
Glycogen	-	+
Xylitol	±	-
Gentiobiose	-	±
D-Turanose	-	-
D-Lyxose	-	-
D-Tagatose	±	-
D-Fucose	-	-
L-Fucose	-	-
D-Arabitol	-	-
L-Arabitol	-	-
Potassium gluconate	-	-
Potassium 2-ketogluconate	-	-
Potassium 5-ketogluconate	-	-

+ positive, - negative, ±: weak positive

Table 4. The API STAPH test results of Abk4 and Abk6 isolates

Tests	Abk4	Abk6
Glucose	+	+
Fructose	+	+
Mannose	+	-
Maltose	+	-
Lactose	+	-
Trehalose	+	+
Mannitol	+	-
Xylitol	-	+
Melibiose	-	-
Nitrate to nitrite	+	+
Alkaline phosphatase production	+	-
Acetyl methyl carbinol production	±	-
Raffinose	-	-
Xylose	-	-
Saccharose	+	+
Acid from alpha-methylglucoside	-	-
Acid from N-acetylglucosamine	+	-
Arginine dihydrolase ADH	-	-
Urease	-	±

+ positive, - negative, ±: weak positive

Table 5. The API Coryne test results of Abk7 and Abk8 isolates

Tests	Abk 7	Abk8
NIT (Nitrate reduction)	-	-
PYZ (pyrazinamidase)	+	+
PYRA (pyrrolidonyl arylamidase)	±	-
PAL (alkaline phosphatase)	+	+
βGUR (β-glucuronidase)	-	-
βGAL (β-galactosidase)	±	+
αGLU (α-glucosidase)	±	+
βNAG (N-asetyl-β-glucosaminidase)	-	±
ESC (Esculin)	-	+
URE (urease)	-	-
GEL(gelatine)	±	+
GLU (glucose)	-	±
RIB (ribose)	-	-
XYL (xylose)	-	-
MAN (mannitol)	-	±
MAL (maltose)	-	±
LAC (lactose)	-	-
SAC (sucrose)	-	-
GLYG (glycogen)	-	-

+ positive, - negative, ±: weak positive

16S rRNA gene sequence analysis results of isolates are given in Table 6. The 16S rRNA partial gene sequences generated in this study have been deposited with the GenBank database under the accession numbers KF704369, KF704370, KU200945, KF704371, KU200946 and KU200947, respectively.

Table 6. Conclusion identification and GenBank Accession numbers of bacterial isolates according to the partial 16S rRNA gene sequence.

Isolates	GenBank	Conclusion	16S rRNA similarity(%)	Accesion numbers
Abk1	KF704369	*Lysinibacillus sphaericus*	99	KC211301, JQ415988
Abk2	KF704370	*Bacillus amyloliquefaciens*	99	FJ705346, JX907998
Abk4	KU200945	*Staphylococcus sciuri*	99	KR476410, KP400531
Abk6	KF704371	*Kocuria palustris*	99	KR085940, KP345961
Abk7	KU200946	*Arthrobacter arilaitensis*	99	NR074608, DQ361101
Abk8	KU200947	*Microbacterium oxydans*	99	KF358264, HQ202812

All these bacteria were tested against third-fourth instar larvae of *H. cunea*. The highest insecticidal activity 30% was obtained from *L. sphaericus* (Abk1) ($p<0.05$), whereas insecticidal activity was not obtained from *K. palustris* (Abk6) within twelwe days. Insecticidal activity of the remaining isolates (Abk2, Abk4 and Abk7 the same, Abk8) were as follows; 16.6%, 13.3% and 10%, respectively. The insecticidal activity of the isolates on *H. cunea* adults are shown in Figure 2.

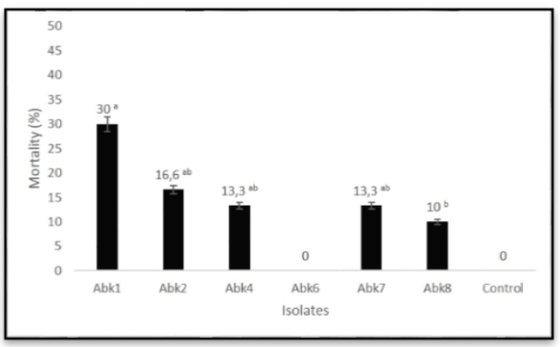

Fig. 2. Mortality of bacterial isolates from *H. cunea* on this pest within twelwe days. Abk1, *Lysinibacillus sphaericus*; Abk2, *Bacillus amyloliquefaciens*; Abk4, *Staphylococcus sciuri*; Abk6, *Kocuria palustris*; Abk7, *Arthrobacter arilaitensis*; Abk8, *Microbacterium oxydans.*

DISCUSSION

In the current study, we determined the culturable bacterial flora of *H. cunea* to identify new candidate organisms as a possible biocontrol agent against pest. Bacteria that belong to *Lysinibacillus sphaericus* (Abk1), *Bacillus amyloliquefaciens* (Abk2), *Staphylococcus sciuri* (Abk4), *Kocuria palustris* (Abk6) and *Microbacterium oxydans* (Abk8) with insects has previously been demonstrated (Leroy et al. 2011, Tranchida et al. 2011, Gupta et al. 2012, Huang et al. 2012, Chandel et al. 2013, Ozsahin et al. 2014). However, to our knowledge, this is the first documentation of *Arthrobacter arilaitensis* (Abk7) from any insects.

Yaman et al. (2002) isolated and identified four different bacterial isolates including *Bacillus thuringiensis, Escherichia freundii, Micrococcus* sp. and *Streptococcus* sp. from *H. cunea* two-third instar larvae. They also showed insecticidal activities of these isolates. In this study, however, *L. sphaericus* (Abk1), *B. amyloliquefaciens* (Abk2), *S. sciuri* (Abk4), *K. palustris* (Abk6), *A. arilaitensis* (Abk7) and *M. oxydans* (Abk8) were reported for the first time as bacterial flora of *H. cunea* third-fourth instar larvae.

Isolate Abk1**,** *L. sphaericus,* is a naturally occurring, mesophilic, soil bacterium, toxic to mosquito larvae. *L. sphaericus* was isolated from dead larvae of *Culex pipiens* and *Palomena prasina* in the previous studies and investigated on the pathogenicity of the isolated hosts. (Tranchida et al. 2011, Ozsahin et al. 2014) The insecticidal property of this organism is due to two proteins produced during sporulation. These proteins are the binary toxins, which accumulate as crystal inclusions and mosquitocidal toxins (Mtx proteins) are produced during vegetative growth of the bacteria. The bacteria are not harmful to humans and other animals, *L. sphaericus* is an ideal insecticide. *L. sphaericus* (Abk1), was found to be causing the highest pathogenicity rates (30%) within twelwe days in larvae of *H. cunea.*

Isolate Abk2, *B. amyloliquefaciens* is an important source of alpha-amylase and protease for industrial applications. This bacterium was isolated from the gut of house flies, *Musca domestica*, in the previous studies (Gupta et al. 2012). Geetha et al. were reported for the first time mosquitocidal activity of *B. amyloliquefaciens*. But, we found that it has 16.6% insecticidal effect on larvae of *H. cunea.*

Isolate Abk4, *S. sciuri* is coccus, Gram-positive and nonmotile. *S. sciuri* was isolated from *Acyrthosiphon*

pisum in the previous studies (Leroy et. al. 2011). In the present study, we found that it has 13.3% insecticidal effect on larvae of *H. cunea.*

Isolate Abk6 *K. palustris* is Gram-positive, non-motile bacterium was isolated from the *H. cunea.* The genus *Kocuria* is commonly found in the environment; it is part of the flora of the skin and mouths of healthy humans (Szczerba 2003). This bacterium was isolated from the gut of *Culex quinquefasciatus*, in the previous studies (Chandel et al. 2013). According to our results, *K. palustris* Abk6 had no insecticidal activity on *H. cunea* as the control group*,* indicating this bacterium is not an insect pathogen.

Isolate Abk7, *A. arilaitensis* is an aerobic, Gram-positive, non spore-forming and non-motile. *Arthrobacter* species are metabolically, ecologically diverse and commonly are found among bacterial populations in soils (Mongodin et al. 2006). This is the first report of *A. arilaitensis* from insects. But, *A. arilaitensis* also did not show high mortality against larvae *of H. cunea* as Abk4 (13.3%).

Isolate Abk8, *M. oxydans* is rod, Gram-positive, motile. *M. oxydans* was isolated from the gut of *Holotrichia parallela* and *Culex quinquefasciatus*, in the previous studies (Huang et al. 2012; Chandel et al. 2013). In this study, the lowest insecticidal activity 10% was obtained from *M. oxydans.*

Since importing strain-based biopesticide/bacterial agents from foreign countries is an expensive and time-consuming process, development of these agents on the local scale and promoting local industrial capability is very important (Suryadi et al. 2016). Yadav et al. improved a production system with medium formulation to breed potential bacterial agents using local and low-cost ingredients (2011).

In conclusion, in this study, *L. sphaericus* (Abk1), *B. amyloliquefaciens (*Abk2), *S. sciuri* (Abk4), *K. palustris* (Abk6), *A. arilaitensis* (Abk7*) and M. oxydans* (Abk8) were reported for the first time as bacterial flora of *H. cunea. L. sphaericus* (Abk1*)* was found to be causing the highest pathogenicity (30%). The results indicate that *L. sphaericus* (Abk1) may be take notice of microbial control agent for *H. cunea*. In the future, using strategies to the pathogenicity enrichment of *L. sphaericus* (Abk1) will be carried of the work increase the effectiveness on *H. cunea*.

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