Isolation and insecticidal activity of some bacteria from the hazelnut beetle (*Balaninus nucum* L.)

Kazım Sezen and Zihni Demirbağ*

Department of Biology, Faculty of Arts and Science, Karadeniz Technical University, 61080-Trabzon, Turkey

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Abstract

The hazelnut beetle (*Balaninus nucum* L., Coleoptera: Curculionidae) is the single greatest source of damage to hazelnut fruits throughout the world. It causes approximately 30–40% of the total economic damage to hazelnut products per year in Turkey. In this study, we investigated the bacterial flora of the hazelnut beetle collected from the vicinity of Trabzon (Turkey) during 1995–1997 and tested them for insecticidal activities. The number of total bacteria in larvae (1.8×10^9 cell/larva) and adults (1.41×10^{11} cell/adult) was identified. Based on colony colour and morphology, five isolates were identified. According to morphological, physiological, biochemical and molecular characters of the isolates, they were identified as *Bacillus thuringiensis*, *Pseudomonas fluorescens*, *Micrococcus luteus*, *Serratia marcescens* and *Escherichia coli*. The insecticidal activities of these bacterial isolates on third-stadium larvae of *B. nucum* were investigated. The highest insecticidal effect determined on *B. nucum* within three days was 100% with *S. marcescens*. The effects of the rest of the isolates are 45% for *B. thuringiensis*, 20% for *P. fluorescens*, 30% for *M. luteus* and 25% for *E. coli*.

Key words: Hazelnut beetle, *Balaninus nucum*, bacterial flora, insecticidal activity, microbial control

INTRODUCTION

The main purpose of most agricultural studies is to increase the yield of products per hectare. With regard to production and export, Turkey is first among all hazelnut producing countries. However, it lags far behind many of them in terms of amount harvested per unit area. A main reason for this is that hazelnut has many agricultural pests which can not be effectively controlled. The most important of these pests is the hazelnut beetle, *B. nucum* (Martin, 1949; Piskornik, 1992).

Studies on bacterial pathogens of curculionids have been neglected. However, many authors during diagnostic work have isolated and identified various bacteria from several insects (White, 1924; Stutzer and Wsorrow, 1926; Ivanov, 1966b). In most cases non spore-forming bacteria have been isolated, and in only a few cases spore-forming bacteria of *Bacillus* genus have been observed (Ivanov, 1966a; Paillot, 1933; Weiser, 1966).

This is the first study on the bacterial flora of *B. nucum*. In this study we isolated and identified bacteria from the hazelnut beetle and determined the insecticidal effects of these bacterial isolates against the hazelnut beetle as possible microbial control agents.

MATERIALS AND METHODS

Collection of larvae and adults. Adults and larvae of the hazelnut beetle were collected from the vicinity of Trabzon, in Turkey during the period of 1995–1997. Adults were collected weekly from pre-determined fields beginning in March. Collection was performed at sunset and sunrise. The insects were collected by the stroke method with a 3×4 m cloth (Martin, 1949). Larvae were collected daily from the beginning of July. Collected insects were brought to the laboratory in bottles, the cover of which was perforated to permit air flow.

Quantitative analysis of bacteria from larvae and adults. Firstly, living larvae and adults were surface sterilized with 70% alcohol. The sus-

*To whom correspondence should be addressed at: Tel: +90-462-325 3223, Fax: +90-462-325 3195, E-mail: zihni@risc01.bim.ktu.edu.tr

pension which was provided from larvae and adults bodies were mixed separately in 5 ml of sterilized phosphate buffer solution (PBS, pH: 7.4), and filtered twice through two layers of cheesecloth to remove debris (Poinar, 1978). The suspension was diluted to 10^{-8} (Christine and Ted, 1992). Each suspension (100 μ l) was plated on nutrient agar. Plates were incubated at 30°C for 24 h. After incubation, the total number of bacteria in larvae and adults was determined.

Isolation of bacteria. Living larvae and adults of field collected *B. nukum* were surface sterilized with 70% alcohol. In some cases, attempts were made to isolate bacteria from hemolymph; in other cases the insect body was triturated and a loop suspension was streaked on nutrient agar. Plates were incubated at 30°C for 2–3 days. Isolates were separated based on colour and morphology of the colonies. Then, pure cultures of bacterial colonies were prepared, and these cultures were identified by various tests.

Identification of bacterial isolates. The identification procedure of isolated bacteria was according to "Bergey's Manual of Systematic Bacteriology 1 and 2" (Palleroni, 1986; Sneath, 1986).

Tests such as utilization of organic compounds, spore formation, NaCl tolerance and protein profile (Belduz et al., 1993; Bradford,

1976) were performed for all isolates.

Bioassay of bacterial isolates. A diet was prepared from equal amounts of fresh leaves and fruits of *Coryllus* sp. L. The diet was placed into individual glass containers (80 mm in diameter), each containing a single bacterial isolate. Containers with diet were sterilized to prevent contamination. Each bacterial isolate prepared in PBS was applied to the surface of the diet individually.

All bacterial isolates were incubated in nutrient broth medium at 30°C for 18 h. After incubation, the density was measured as 1.89 at OD₆₀₀ (Ben-Dov et al., 1995). Five milliliters of this culture was centrifuged at 3,000 rpm for 10 min. The pellet was resuspended in 5 ml of sterilized PBS. Ten third-stadium larvae were placed on the diet in containers. Containers were kept at $26 \pm 2^\circ\text{C}$ and 60% RH on a 12 : 12 h photoregime (Mitchell and Smith, 1985). The mortality of larvae was recorded every 24 h and all dead larvae were removed from containers. At least 90 larvae were assayed for each bacterial isolate (1, 2, 3, 4 and 5).

RESULTS

Total bacterial number for larvae and adults

We determined the number of bacteria in larvae and adults by counting the number of colonies on the plates which were inoculated with diluted bacterial suspensions. The total

Table 1. The morphological characteristics of bacterial isolates

Isolate number	1	2	3	4	5
Colour and shape of colonies	Cream, concentric, round	Cream, smooth, round	Yellow, concentric, round	Red, smooth, round	Cream, concentric, round
Turbidity when grown in NB ^a	Turbid	Turbid	Turbid	Turbid	Turbid
Shape of bacteria	Bacil	Bacil	Coccus	Bacil	Bacil
Gram stain	+	—	+	—	—
Capsule stain	+	—	—	—	—
Spore stain	+	—	—	—	—
Spore shape	Ellipsoid	ND ^b	ND	ND	ND
Spore form	Terminal	ND	ND	ND	ND
Diameter (μ m)	ND	ND	0.9–1.2	ND	ND
Length (μ m)	4–6	1.3–3.2	ND	1–1.5	2–4
Wideness (μ m)	0.8–1	0.5–1	ND	0.6–0.8	0.6–1.1
Motility	+	+	—	+	+

^aNB: nutrient broth, ^bND: no data.

Table 2. The physiological and biochemical characteristics of the bacterial isolates

Isolate number	1	2	3	4	5
Nitrate reduction	+	+	—	+	+
Catalase test	+	+	+	+	+
Starch test	+	—	—	—	—
Oxidase test	+	+	+	+	—
Hydrolysis of gelatine	+	+	+	+	—
Hydrolysis of urea	+	—	—	—	—
Citrate utilisation	—	+	—	+	—
Propionate utilisation	—	+	ND ^a	—	ND
Indole test	—	—	—	—	+
Methyl red test	—	—	—	—	+
Voges proskauer test	+	+	—	+	—
Growth in 1% NaCl	+	+	+	+	+
Growth in 2% NaCl	+	+	+	+	+
Growth in 5% NaCl	+	+	+	+	+
Growth in 7% NaCl	—	+	+	—	+
Growth in 12% NaCl	ND	+	+	—	ND
Growth in 20% NaCl	ND	ND	+	ND	ND
Growth with lysozyme present	+	+	—	+	+
Growth at 28°C	+	+	+	+	+
Growth at 30°C	+	+	+	+	+
Growth at 37°C	+	+	+	+	+
Using of L-arginine	+	+	+	+	—
Hydrolysis of tween 80	W ^b	+	—	+	—
Tyrosinase production	—	ND	ND	ND	ND
Hydrolysis of phenyl alanine	—	ND	—	ND	+
Growth at pH>7 MVRP broth	+	ND	ND	ND	ND
Growth at pH<6 MVRP broth	W	ND	ND	ND	ND
Glucose fermentation	+	+	—	+	+
Arabinose fermentation	—	+	—	—	+
Xylose fermentation	—	+	—	+	+
Mannose fermentation	+	+	W+	+	+
Sucrose fermentation	W+	+	+	+	—
Esculine fermentation	+	+	—	+	—
Optimum pH	6–8	6–8	7–8	8–9	7–8
Optimum growth °C	30	30	30	30	30
Growth +4°C	ND	+	ND	+	ND
Growth +41°C	ND	—	ND	—	ND
Anaerobic growth	+	+	—	+	+

^aND: no data, ^bW: weak growth.

number of bacteria was found to be $1.8 \times 10^9 \pm 0.164$ cell/larva ($n=20$) and $1.41 \times 10^{11} \pm 0.703$ cell/adult ($n=10$).

Isolation of bacteria

A total of five isolates were finally selected and characterized for morphology, spore formation, nutritional features, and physiological and biochemical characteristics (Tables 1 and

2). In addition, the isolates were investigated for total protein profiles. On agar plates, three isolates (1, 2, 5) were cream, flat or convex colonies, smooth or concentric after one day of incubation at 30°C. Yellow pigment was produced only by a third isolate and red pigment was produced only by a fourth isolate.

Morphological characteristics of bacterial isolates

Enrichments and purification procedures carried out for both larvae and adults of *B. nukum* allowed the isolation of one spore forming bacterium (isolate 1) and four non-spore forming bacteria. The morphological characteristics of the bacteria are indicated in Table 1. Four of the isolates (isolates 1, 2, 4 and 5) were rod-shaped, occurring singly or in pairs, commonly 1–6 μm in length. The remaining isolate (isolate 3) was coc-shaped, characteristically dividing into more than one plane to form regular or irregular clusters or packets, measuring 0.9–1.2 μm in diameter. Two isolates (1 and 3) were gram positive and the rest were gram negative. Motility was observed in all isolates except the third isolate.

Physiological and biochemical characteristics of bacterial isolates

The physiological and biochemical characteristics of the isolates are reported in Table 2. While the catalase test was positive in all isolates, the oxidase test was negative in isolate 5. All isolates tolerated different values of NaCl: 5, 12, 20, 5 and 7% respectively. The isolates grew optimally in nutrient broth medium where pH values were 6 to 9. Most isolates were facultatively anaerobic, and only one (isolate 3) was aerobic. Growth was observed for all isolates with all substrates studied. The isolated bacteria are listed in Table 3.

SDS-PAGE protein profiles

SDS-polyacrylamide gel electrophoresis of total proteins revealed that hazelnut beetle isolates tested showed great variability in their protein profiles (data not shown). There was

Table 3. Bacteria isolated from *B. nukum* larvae and adults

Source	Isolated bacteria	Frequency of isolation or identification
Larva	<i>P. flourescens</i>	8 (53.3%)
Larva	<i>M. luteus</i>	1 (6.6%)
Adult	<i>B. thuringiensis</i>	4 (26.6%)
Adult	<i>S. marcescens</i>	2 (13.3%)
Adult	<i>E. coli</i>	1 (6.6%)

Table 4. The insecticidal effects of isolates on *B. nukum* larvae within three days

Isolates	First day	Second day	Third day	Total (%)
Control (PBS)	—	—	—	—
<i>B. thuringiensis</i>	—	5	40	45
<i>P. flourescens</i>	—	—	20	20
<i>M. luteus</i>	—	—	30	30
<i>S. marcescens</i>	—	40	60	100
<i>E. coli</i>	—	—	25	25

approximately 90–100% variability among the isolates in terms of protein profiles.

Bioassay

A number of bioassays were carried out using larvae of *B. nukum* as the test insect which was fed with isolated bacteria. The effects of the isolates on *B. nukum* larvae are shown in Table 4. Table 4 shows a comparison of the pathogenicity of these bacteria when administrated to larvae of *B. nukum* by feeding.

Among all assays, the highest insecticidal effect determined on *B. nukum* within three days was 100% with *Serratia marcescens* (isolate 4). *Bacillus thuringiensis* (isolate 1), *Pseudomonas fluorescens* (isolate 2), *Micrococcus luteus* (isolate 3) and *Escherichia coli* (isolate 5) infected *B. nukum* larvae at different ratios, 45, 20, 30 and 25% respectively.

DISCUSSION

There has recently been an increasing interest in finding an effective and safer biological control agent against hazardous insects. To date, there has been no study on bacterial isolates and biological control agents of *B. nukum*.

In the present study, we determined five bacterial isolates from *B. nukum*. Based on morphological, nutritional, physiological, biochemical and molecular characteristics, the isolates were determined to be *B. thuringiensis* (isolate 1), *P. fluorescens* (isolate 2), *M. luteus* (isolate 3), *S. marcescens* (isolate 4) and *E. coli* (isolate 5).

Polyacrylamide gel also showed differences between total protein profile of the bacterial isolates. Cato et al. (1982) have suggested that different species have different protein profiles.

Our study showed that all of the isolates have approximately 90–100% difference from each other in terms of protein profiles.

In the current study, it was determined that, following one week of bioassay, larvae showed different signs before death; generally, the larvae displayed sluggishness and lost of appetite. In particular, larvae infected with *S. marcescens* (isolate 4) displayed the red colour sign (data not shown). In addition to the other major symptoms, infection decreased larval survival. The average duration of larval life span healthy individuals at $26 \pm 2^\circ\text{C}$ was 12 ± 2 days, whereas the average life span for infected larvae at $26 \pm 2^\circ\text{C}$ was 3 ± 2 days. In all bioassays, the highest insecticidal effect determined on *B. nuncum* larvae within three days was 100% with *S. marcescens*. The effects on the rest of the samples were 45% with *B. thuringiensis*, 20% with *P. fluorescens*, 30% with *M. luteus* and, 25% with *E. coli*. Lipa and Wiland (1972) found that the effect of a natural form of *S. marcescens* was 100% on *Agrotis* sp., and also observed that the effect of *E. coli* was 21% on *Agrotis segetum* larvae. The tests on pathogenicity of *B. thuringiensis* to *B. nuncum* showed that the mortality of larvae fed heavy doses of spores was not very high. As discussed by Burgerjon and Martouret (1971) many species of larvae did not exhibit any toxæmia from *B. thuringiensis* due to the fact that the pH in their gut is alkaline.

Consequently, it was determined that *S. marcescens* in particular, can be used as a biological control agent against *B. nuncum* larvae. Future studies will be conducted with the aim of finding a biological control agent against *B. nuncum* using this pesticide or other newly improved pesticides. The present study has contributed significantly to the literature on bacterial isolates and biological control of hazelnut beetle.

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