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The First Study on Bacterial Flora and Biological Control Agent of *Anoplus roboris* (Sufr., Coleoptera)

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The hazelnut leaf holer (*Anoplus roboris* Sufr., Coleoptera: Curculionidae) is a devastating pest of hazelnut and oak trees. It causes approximately 20-30% economic damage to hazelnut production per year in Turkey. In the present study, in order to find a more effective and safe biological control agent against *A. roboris*, we investigated the bacterial flora of the hazelnut leave holer, and tested them for insecticidal effects on it. According to morphological, physiological and biochemical tests, bacterial flora were identified as *Bacillus circulans* (Ar1), *Bacillus polymyxa* (Ar2), *Enterobacter* sp. (Ar3) and *Bacillus sphaericus* (Ar4). Insecticidal effects of bacterial isolates were performed on adult *A. roboris*. The highest insecticidal effect determined was 67% by *B. sphaericus* within eight days. The insecticidal effects of the other isolates (Ar1, Ar2 and Ar3) were determined as 33%, 47% and 47% within the same period, respectively.

Key words: *Anoplus roboris,* bacterial flora, biological control

The hazelnut leave holer, *Anoplus roboris*, (Coleoptera: Curculionidae) is among the most serious pests of hazelnut and oak trees in the Black Sea region of Turkey (Ecevit *et al.*, 1993; The Ministry of Agriculture of Turkey, 1995). This pest damages the hazelnut and oak leaves during spring and summer. It causes approximately 20~30% economic damage particularly on hazelnut production per year in Turkey. Up to now, chemical substances such as Carbaryl 85 (WP), Endosulfan (WP, EP) and Omethoate 50 (EM) have been utilized to control this pest (The Ministry of Agriculture of Turkey, 1992). However, recent concern about the hazardous effect of chemical pesticides on the environment have encouraged scientists to consider finding more effective and safer control agents. In the search for safer and more lasting methods, they have turned their attention to the possibility of using other organisms as biological control agents. Fortunately, most of the microorganisms capable of causing disease in insects do not harm other animals and plants. They are generally considered to be less toxic to the environment and can be integrated more easily into pest management systems that are based on biological control. This is one of the most important factors encouraging the use of insect pathogens as biological control agents. Suppression of pest organisms by their natural enemies is recognized as one of the most suitable long term pest management strategies for many production systems. In the last few years, 59 pathogenic bacterial species have been developed as pesticides worldwide. These various bacterial insect pathogens are being used successfully in the biological control of insects (Thiery and Frachon, 1997; Sezen and Demirbag, 1999; Sezen *et al.*, 2001).

Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling the hazelnut leave holer, *A. roboris*. Surprisingly, despite their mass occurrence and wide distribution, very little is known about the bacterial pathogens limiting their population. Studies on bacterial pathogens of the hazelnut leave holer have been neglected. In addition, nothing is known about what its natural enemies are (The Ministry of Agriculture of Turkey, 1995). For this reason, this pest is a very attractive object of microbiological control studies, as well as a target for control by introduction of biological agents.

During this study four types of bacteria were isolated from *A. roboris*. These bacteria have been identified and their pathogenicity to pests was studied.

Materials and Methods

Collection of Insects

Larvae and adults of *A. roboris* were collected from the vicinity of Trabzon, Turkey from May to September 2001. Insects were collected from leaves of *Coryllus* sp., and

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were placed individually into plastic boxes and fed on fresh leaves (Thiery and Frachon, 1997). The boxes were regularly checked and healthy larvae and adults were determined and used in assays for bacteria isolation.

Isolation of bacteria

After macroscopic examination of insects, dead, diseased and healthy larvae were distinguished. The insects were repeatedly washed with acetone to remove possible contamination. The larvae were homogenized in nutrient broth by using a glass tissue grinder. Then 0.1 ml suspension was spread on nutrient agar (Thiery and Frachon, 1997). Plates were incubated at 30°C for 2-3 days. After the incubation period, plates were examined and bacterial colonies were selected according to color and morphology of colonies. Finally, selected colonies were purified by subculturing on plates, and the cultures were identified by a variety of tests.

Identification of bacterial isolates

The identification procedure of isolated bacteria was performed according to "Bergey's Manual of Systematic Bacteriology 1 and 2" (Palleroni, 1986; Sneath, 1986), and Manual of Techniques in Insect Pathology (Thiery and Frachon, 1997). After color and shape of colonies of bacterial isolates were determined, Gram stain was performed on isolates. Based on the results of Gram stain and shape of isolates, several physiological and biochemical tests were performed for all isolates. Isolates were tested for tolerance to NaCl (grown in nutrient broth containing 2%, 5%, 7% and 12% NaCl) and heat (grown at 28°C, 30°C, 37°C, 40°C, 50°C and 60°C). We also determined whether bacterial isolates produce various enzymes and products by plating on media including different special features (Palleroni, 1986; Sneath, 1986 and Thiery and Frachon, 1997).

Isolation of spore-forming bacteria

After the suspension was prepared as explained above, it was heated at 80°C for 10 min in a water bath to eliminate the non spore-forming bacteria (Ohba and Aizawa, 1986; Lee *et al.*, 1992; Theiry and Frachon, 1997). Following this, a volume of 0.1 ml of the heat-treated suspension was spread on nutrient agar plates and incubated at 30°C for 48 to 96 h (Lee *et al.*, 1995), then the plates were examined and bacterial colonies were selected.

The insecticidal effects of bacterial isolates

Healthy adults were used for the insecticidal effects of bacterial isolates. Since the larvae of *A. roboris* are very small (1-3 mm), they were not used for bioassay. Isolates were incubated for 18 h (72 h for *Bacillus* to sporulation) at 30°C in nutrient broth. After incubation, the density of cells was set at 1.89 at OD_{600} and 5 ml of culture was centrifuged at 3.000 rpm for 10 min (Ben-Dov *et al.*, 1995). The pellet was resuspended in 5 ml for sterilized PBS and used for bioassays (Moar *et al.*, 1995). Because *A. roboris* does more damage to *Coryllus* sp. than *Quercus* sp. in the Black Sea Region and was collected from *Coryllus* sp., hazelnut leaves were used in bioassays as diet. The diet was contaminated with prepared bacterial isolates and was placed into individual glass containers (80 mm in diameter). Insects were starved for 4 h before treatment. Ten adults were placed into each container. Containers were kept at 26± 2°C and 60% RH on a 12:12 hr photoperiod (Lipa *et al.*, 1994). The mortality of insects was recorded every 24 h and all dead insects were removed from containers. Because mortality did not increase noticebly after eight days post infection, bioassays were finished at the 8th day, and living insects were destrayed. At least 30 adults were assayed for each bacterial isolate.

Results

We selected and characterized four bacterial isolates from

Table 1. The morphological characteristics of bacterial isolates

Isolate number	Ar1	Ar2	Ar3	Ar4
Color and shape of colonies	Cream, smooth, round	Cream, filamentous, round	Cream, smooth, irregularly round	Cream, concentric, round
Gram stain	+	+	-	+
Shape of bacteria	Bacillus	Bacillus	Bacillus	Bacillus
Spore stain	+	+	ND[b]	+
Spore shape	Ellipsoid	Ellipsoid	ND	Round
Spore form	Central	Terminal	ND	Terminal
Length (μm)	1.9-2.85	1.71-3.32	2.85-4.75	3.8-5.7
Width (μm)	0.8-0.95	0.66-0.95	0.76-0.95	0.95-1.14
Turbidity when grown in NB[a]	Turbid	Turbid	Turbid	Turbid
Motility	+	+	+	+
Anaerobic growth	+	+	+	+

[a]NB: Nutrient Broth
[b]ND: No Data

A. roboris by color and shape of colonies, spore formation and nutritional features (Table 1). All isolates were cream-colored round colonies. While the shape of two colonies (Ar1 and Ar3) were smooth, the others (Ar2 and Ar4) were filamentous and concentric. Also, all isolates were bacil, turbid and motile; three isolates (Ar1, Ar2 and Ar4) were Gram positive. The other characteristics are shown in Table 1. Enrichments and purification procedures carried out with larvae and adults of *A. roboris* allowed the isolation of three spore forming isolates (Ar1, Ar2, and Ar4). The shapes of two of these spores (Ar1 and Ar2) were ellipsoid and the other (Ar4) was round. Forms of spores were terminal in Ar2 and Ar4 and central in Ar1.

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

Isolate Number	Ar1	Ar2	Ar3	Ar4
Nitrate reduction	-	+	-	-
Catalase test	+	+	+	+
Starch test	+	+	-	-
Oxidase test	-	+	-	+
Hydrolysis of gelatine	-	+	+	-
Hydrolysis of urea	-	-	+	+
Citrate utilisation	-	-	+	-
Propionate utilisation	-	-	-	-
Indole test	-	-	-	-
Metyhl red test	[1]W+	-	-	-
Voges proskauer test	-	+	+	-
H_2S production	ND[2]	ND	+	ND
Growth in 2% NaCl	+	+	+	+
Growth in 5% NaCl	+	+	+	+
Growth in 7% NaCl	+	+	+	+
Growth in 12% NaCl	-	ND	W+	-
Growth with lysozyme	+	+	+	+
Growth at 28°C	+	+	+	+
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 40°C	+	+	ND	+
Growth at 50°C	-	W+	ND	-
Growth at 60°C	-	-	ND	-
Use of L-arginine	-	-	-	-
Hydrolysis of tween 80	+	+	-	+
Tyrosinase production	-	-	-	-
Hydrolysis of phenylalanine	-	-	ND	+
Hydrolysis of casein	-	+	ND	-
Egg-yolk lecithinase	+	-	ND	-
Growth at pH>7 MVRP broth	W+	W+	ND	+
Growth at pH<6 MVRP broth	+	+	ND	+
Glucose fermentation	-	+	-	-
Arabinose fermentation	-	+	-	-
Xylose fermentation	-	+	-	-
Mannitol fermentation	-	+	-	-
Sucrose fermentation	-	-	-	-
Optimum pH	6-8	6-8	6-8	6-8
Optimum growth °C	30	30	30	30

[1]W: Weak Growth
[2]ND: No Data

Physiological and biochemical characteristics of isolates are indicated in Table 2. While the catalase test was positive, propionate utilization, indole test, using of L-arginine, tyrosinase production, and sucrose fermentation were negative for all isolates. On the other hand, organic acid and egg-yolk lecithinase were produced by only Ar1; nitrate reduction, hydrolysis of casein, and fermentation of glucose, arabinose, xylose and mannitol were observed by only Ar2; production of H_2S was determined only in Ar3 and hydrolysis of phenylalanine was performed only in Ar4. All isolates grew in 2%, 5% and 7% NaCl, and at 28°C, 30°C and 37°C, except Ar3 grew in 12% NaCl and Ar2 grew weakly at 50°C. All Gram positive isolates grew in MRVP broth at pH>7 and pH<6. The optimal growth was at pH 6-8 and 30°C.

The insecticidal effects of the isolates on *A. roboris* adults are shown in Fig. 1. The highest insecticidal infectivity was 67% with Ar4 within eight days. The insecticidal effects of the other isolates (Ar1, Ar2 and Ar3) were determined as 33%, 47% and 47% within eight days, respectively. No mortality was determined on control groups.

Discussion

This is the first study on the bacterial isolation and biological control agent of *Anoplus roboris*, a common pest of *Coryllus* sp. and *Quercus* sp. We isolated and determined four bacterial isolates from this pest. According to preliminary observations and tests, morphology, reaction to stains, endospore formation, motility, aerobic/anaerobic growth and catalase production, secondary observations and metabolic (biochemical) tests, nitrate reduction, starch, oxidase and IMViC (indole, methyl red, Voges-Proskauer tests and citrate utilisation) tests, hydrolysis of gelatin, urea, tween 80, phenylalanine and casein, H_2S and tyro-

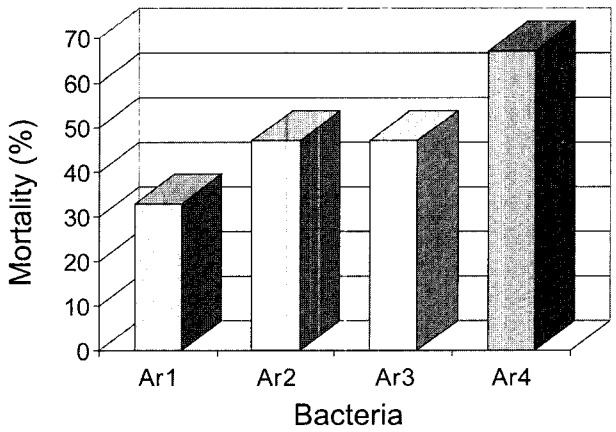

Fig. 1. The insecticidal effects of bacterial isolates from *A. roboris* on adults of this pest within eigth days. Ar1; *Bacillus circulans*, Ar2; *Bacillus polymyxa*, Ar3; *Enterobacter sp.*, Ar4; *Bacillus sphaericus*.

sinase production, egg-yolk lecithinase, fermentation, and growth tests, we determined that bacterial flora of *A. roboris* consists of *Bacillus circulans* (Ar1), *Bacillus polymyxa* (Ar2), *Enterobacter sp.* (Ar3) and *Bacillus sphaericus* (Ar4). Hundreds of bacterial species have been associated with insects (Deacon 1983). It is known that many bacteria which can be isolated from insects belong to genera *Bacillus* and *Enterobacter* (Tanada and Kaya, 1993).

In all bioassays, the highest insecticidal infectivity determined on *A. roboris* adults was 67% with *Bacillus sphaericus* (Ar4). The other isolates (Ar1, Ar2 and Ar3) had 33%, 47% and 47% insecticidal effects on this pest, respectively. The result of bioassays indicated that all isolates (spore-forming and non-spore-forming) are pathogenic at different ratios on the pests (Fig. 1). Most of the pathogenic enterobacteria are found in the families *Bacillaceae*, *Pseudomonadaceae*, *Enterobacteriaeae*, *Streptococcaceae* and *Micrococcaceae* (Tanada and Kaya, 1993). Because *Bacillus* strains are pathogens for various insects, it is by far the most important microbial pesticide genus. Different *Bacillus* species have been used as microbial control agents in agricultural areas (Flexner and Belnavis, 2000). According to Copping (1998), *Bacillus sphaericus* has been registered for mosquito control. Yaman and Demirbağ (2000) isolated *B. sphaericus* from *Pieris brassica* and indicated that it has approximately 30% insecticidal effect on *Gypsonema dealbana*, *Euproctis chrysorrhoea*, *Melolontha melolontha* and *Agelastica alni*. In addition, we also determined that it has a high insecticidal effect (67%) on *Anoplus roboris*, Coleoptera. Esters (1996) indicated that the effects of each *Bacillus* strain are different on different insect species, because there is some evidence that the enzymes of insects release different polypeptides from the proteins. Thus, further specifity may arise in this way (Deacon, 1983). According to Coppel and Mertins (1977), non-spore-forming bacterial pathogens include all of the potential pathogens for the insects. Potential pathogens do not normally multiply in the gut, but they can establish themselves in the haemocoel if they have enough time to pass through the wall and enter susceptible cells.

As a result, it was determined that all isolates are natural enemies of pests. This is a very important result to biological control for *A. roboris*, because so far nothing has been known about its natural enemies. Especially, *Bacillus sphaericus* can be used as a biological control agent for *A. roboris*. However, further research will be directed to improve the insecticidal effect of this biological control agent for this pest using recombinant techniques.

Consequently, great effort has been exerted toward identification of natural enemies to effectively suppress various pests in different types of production systems. As more information is learned and these systems become more refined, we will see even more applications of this technology used in the future.

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