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Study of the bacterial flora as a biological control agent of *Agelastica alni* L. (Coleoptera: Chrysomelidae)

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The alder leaf beetle (*Agelastica alni* L., Coleoptera: Chrysomelidae) is one of the sources of damage to hazelnut and alder trees throughout the world. Chemical substances have been utilized to control this pest. In the present study, in order to find a more effective and safe biological control agent against *A. alni*, we investigated the bacterial flora of the alder leaf beetles that were collected from the vicinity of Trabzon, Turkey, during 2000–2002, and tested them for insecticidal activities on it. It was determined that the number of total bacteria is $9.8 \times 10^4 \pm 0.121$ bacteria/larva and $1.31 \times 10^5 \pm 0.310$ bacteria/adult. Based on morphological, physiological and biochemical tests, bacterial flora were identified as *Enterobacter agglomerans* (Aa1), *Listeria* sp. (Aa2), *Pseudomonas chlororaphis* (Aa3) and *Pseudomonas fluorescens* (Aa4). The highest insecticidal effect determined on both the larvae and adults of *A. alni* were 70% and 56% with Aa4 within seven days, respectively. The insecticidal activities of the other isolates (Aa1, Aa2 and Aa3) were determined as 65%, 48% and 37% on the larvae, and 50%, 34% and 30% on the adults of *A. alni*, respectively.

Key words: alder leaf beetle, *Agelastica alni*, bacterial flora, insecticidal activity, biological control.

Introduction

The alder leaf beetle, *Agelastica alni* (Coleoptera: Chrysomelidae) is among the most serious pests of hazelnut (Suchy, 1988; Baur et al., 1991; Urban, 1999) and alder trees in the Black Sea Region of Turkey. This pest damages the hazelnut and alder leaves during spring and summer. Up to now, according to the *Technical guide of agricultural control* (issued by the Ministry of Agriculture, Turkey, 1995), chemical substances such as Carbaryl 5%, Methiocarb 50% and Malathion 200 g/L have been utilized to control this pest. However, recent concern about the hazardous effect of chemical pesticides on the environment have encouraged scientists to consider finding more effective and safe control agents. In the search for safer and more lasting methods, scientists 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 ani-

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mals 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 biological control of insects (Thiery & Frachon, 1997; Sezen & Demirba, 1999; Yaman et al., 1999; Sezen et al., 2001, Demir et al., 2002).

Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling the alder leaf beetle, *Agelastica alni*. 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 alder leaf beetle have been neglected. Although there are a few studies on biology and control of *A. alni*, there is no study on investigation of bacterial flora as biological control agent.

In this study we isolated and identified bacteria from the alder leaf beetle and determined the insecticidal effects of these isolates against the larvae and adults of the alder leaf beetle as possible biological control agents.

Material and methods

Collection of insects

Adults and larvae of the alder leaf beetle were collected from the vicinity of Trabzon, Turkey, during the period of 2000–2002. While adults were collected weekly from the beginning of March, larvae were collected daily from the beginning of May. 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 suspensions, which were 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 & Thomas, 1978). The suspension was diluted to 10^{-8} (Christine & Ted, 1992). Each suspension (100 μL) was plated on nutrient agar. Plates were incubated at 30 °C for 24 hours. After incubation, the total number of bacteria in larvae and adults was determined.

Isolation of bacteria

Living larvae and adults of field collected *A. alni* 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" (Krieg & Holt, 1986; Sneath et al., 1986). Tests, such as utilization of organic compounds, spore formation, NaCl tolerance, optimum pH and optimum temperature, were performed for all isolates.

General conditions of bioassays

A diet was prepared from fresh leaves of *Corylus* 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 hr. After incubation, the density was measured as 1.89 at OD_{600}(Ben-Dov et al., 1995; Moar et al., 1995). Five mL of this culture was centrifuged at 3.000 rpm for 10 min. The pellet was resuspended in 5 mL of sterilised PBS. Ten third-stadium larvae and adults were placed on the diet in containers and kept at 26 ± 2 °C and 60% RH on a 12:12 hr photoregime (Mitchell & Smith, 1985). The mortalities of larvae and adults were recorded every 24 hr, and all dead larvae and adults were removed from containers. At least 30 adults and 60 larvae were assayed for each bacterial isolate (1, 2, 3 and 4). The data were evaluated using Abbott's formula (Abbott, 1925).

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 number of bacteria was found to be $9.8 \times 10^4 \pm 0.121$ bacteria/larva ($n = 20$) and $1.31 \times 10^5 \pm 0.310$ bacteria/adult ($n = 10$).

Isolation of bacteria

A total of four isolates were finally selected and characterised for morphology, spore formation, nutritional features, and physiological and biochemical characteristics (Tabs 1, 2). Table 3 shows the frequency of isolation of bacterial isolates. On

Table 1. The morphological characteristics of bacteria isolated from *Agelastica alni*.

Isolate number	Aa1	Aa2	Aa3	Aa4
Colour and shape of colonies	Cream, smooth, irregularly, round	Cream, filamentous, round	Cream, smooth, round	Cream, smooth, round
Turbidity[a]	Turbid	Turbid	Turbid	Turbid
Shape of bacteria	Bacillar	Bacillar	Bacillar	Bacillar
Gram Stain	–	+	–	–
Length (μm)	0.95–2.85	1.05–2.85	2.85–5.7	1.42–2.85
Width (μm)	0.76–0.95	0.57–0.95	0.95–1.04	0.66–0.85
Motility	+	+	+	+

[a] When grown in Nutrient Broth.

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

Isolate No.	Aa1	Aa2	Aa3	Aa4
Nitrate reduction	–	–	+	+
Catalase test	+	+	+	+
Starch test	–	–	+	+
Oxidase test	–	+	+	+
Hydrolysis of gelatine	–	–	+	+
Hydrolysis of urea	+	–	–	–
Citrate utilisation	+	+	–	+
Propionate utilisation	+	+	–	–
Indole test	–	–	–	–
Methyl red test	–	–	+	+
Voges-Proskauer test	–	–	+	+
Growth in 2% NaCl	+	+	+	+
Growth in 5% NaCl	+	+	+	+
Growth in 7% NaCl	W+	+	–	+
Growth in 12% NaCl	W+	–	ND	ND
Growth with lysozyme present	+	+	W+	+
Growth at 37 °C	+	+	+	+
Using of L-arginine	–	–	+	+
Hydrolysis of Tween-80	+	–	+	+
Tyrosinase production	ND	+	ND	ND
Hydrolysis of phenyl alanine	ND	–	ND	ND
Glucose fermentation	+	–	+	+
Arabinose fermentation	–	–	–	–
Xylose fermentation	–	–	–	–
Sucrose fermentation	–	+	–	–
Mannitol fermentation	–	–	–	+
Lactose fermentation	–	–	–	–
Optimum pH	6–8	6–8	6–8	6–8
Optimum growth (°C)	30	30	30	30
Growth at 4 °C	ND	ND	–	+
Growth at 41 °C	ND	ND	+	+
Anaerobic growth	+	+	+	+

[a]ND: not determined; W: weak growth.

agar plates, all isolates were cream. The colonies of isolate Aa1, Aa3 and Aa4 were smooth and round, the colony of Aa2 was filamentous after one day of incubation on Nutrient Agar plate at 30 °C.

Morphological characteristics of bacterial isolates

Enrichments and purification procedures carried out for both larvae and adults of *A. alni* allowed the isolation of four non-spore forming bacteria. The morphological characteristics of the bac-

Table 3. Bacteria isolated from *Agelastica alni* larvae and adults.

Source	Isolated bacteria	Frequency[a] (%)
Adult	*Enterobacter agglomerans*	9.09
Adult	*Listeria* sp.	18.18
Larvae and adult	*Pseudomonas chlororaphis*	36.36
Larvae and adult	*Pseudomonas fluorescens*	36.36

[a]Frequency of isolation or identification.

teria are indicated in Table 1. All of the isolates were rod-shaped, occurring singly, commonly 0.95–5.7 μm in length. One isolate (Aa2) was Gram-positive and the rest of the isolates were Gram-negative. Motility was observed in all isolates.

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 positive in isolates Aa2, Aa3 and Aa4. Gelatine hydrolysis was positive in isolates Aa3 and Aa4. All isolates tolerated different values of NaCl: 2%, 5%, 7% and 12% respectively. The isolates grew optimally in nutrient broth medium where pH values were 6 to 8. All of the isolates were facultatively anaerobic. Fermentation of many carbohydrates was determined to be poor for all isolates. Growth was observed for all isolates with lysozyme present.

Bioassays

A number of bioassays were carried out using larvae and adults of *A. alni* as the test insect, which was fed with isolated bacteria. Both the insecticidal activity of the isolates on *A. alni* larvae and adults and the comparison of the pathogenicity of these bacteria between larvae and adults of *A. alni* were determined.

Among all assays, the highest insecticidal effect determined on *A. alni* larvae was 70% with Aa4, and 56% with Aa4 on *A. alni* adults within seven days. Aa1, Aa2 and Aa3 infected both *A. alni* larvae and adults at different ratios. The insecticidal activities of these bacteria were 65, 48 and 37% on larvae, and 50, 34 and 30% on adults respectively.

Discussion

There has recently been an increasing interest in finding more effective and safe biological control agents against hazardous insects. To date, no study has been performed on the determination of bacterial isolates and the investigation of biological control agents of *A. alni*.

In the present study, we determined four bacterial isolates from *A. alni*. Based on morphological, nutritional, physiological and biochemical studies, bacterial flora of *A. alni* consists of *Enterobacter agglomerans* (Aa1), *Listeria* sp. (Aa2), *Pseudomonas chlororaphis* (Aa3) and *Pseudomonas fluorescens* (Aa4). Based on performed tests there are 73.3%, 84.6%, 90.1% and 92.3% similarity between bacterial isolates and related species in Bergey's Manual, respectively (Krieg & Holt, 1986; Sneath et al., 1986). However, more detail studies are necessary to determine the species exactly.

In the present study, it was determined that, following one week of bioassay, larvae showed different signs before death; generally, the larvae displayed sluggishness and loss of appetite. In addition to the other major symptoms, infection decreased larval survival. The average duration of larval life span for healthy individuals at 26 $\pm$ 2 ℃ was 20 $\pm$ 2 days, whereas the average life span for infected larvae at 26 $\pm$ 2 ℃ was 7 $\pm$ 3 days.

In all bioassays, the highest insecticidal effect determined on *A. alni* larvae and adults within seven days were 70% and 56% with *P. fluorescens* respectively. The results of bioassays indicate that all isolates are pathogenic at different ratio on the pest. Most of the pathogenic bacteria are found in the families of *Bacillaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Streptococcaceae* and *Micrococcaceae* (Tanada & Kaya, 1993). *P. fluorescens* was isolated from many insects and is recognized as facultative insect pathogen (Krieg, 1961). Lipa & Wiland (1972) isolated 5 strains of *P. fluorescens* var. *septicus* from dead and alive larvae of *Agrotis segetum* (Schiff), and determined the insecticidal effects of 5 strains of *P. fluorescens* on *A. segetum* larvae (about 70%). Demir et al. (2002) isolated *Enterobacter* sp. from *Anoplus roboris* (Coleoptera), a pest of hazelnut, and indi-

cated that it has an insecticidal effect on that pest. According to COPPEL & MARTINS (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 be established themselves in the haemocoel if they have enough time to pass through the wall and enter susceptible cells.

Consequently, it was determined that *P. fluorescens*, which shows the highest insecticidal effect, can be used as a biological control agent against *A. alni* larvae and adults. Future studies will be conducted with the aim of finding a better biological control agent against *A. alni* using this bacterium or other newly improved pesticides. The present study has contributed significantly to the literature on bacterial isolates and biological control of the alder leaf beetle.

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References

ABBOTT, W. S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. **18:** 265–267.

BAUR, R., BINDER, S. & BENZ, G. 1991. Nonglandular leaf trichomes as short-term inducible defense of the grey alder *Alnus incana* (L.), against the chrysomelid beetle, *Agelastica alni* L. Oecologia **87:** 219–226.

BEN-DOV, E., BOUSSIBA, S. & ZARITSKY, A. 1995. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:** 2851–2857.

CHRISTINE, L. C. & TED, R. J. 1992. Laboratory Experiments in Microbiology, Third Edition. The Benjamin/Cummings Publishing Company, Inc., California.

COPPEL, C. H. & MARTINS, J. W. 1977. Biological Insect Pest Suppression. Springer Verlag, Berlin, 314 pp.

DEMIR, İ., SEZEN, K. & DEMIRBAĞ, Z. 2002. The first study on bacterial flora and biological control agent of *Anoplus roboris* (Sufr., Coleoptera). J. Microbiol. **40:** 104–108.

KRIEG, A. 1961. Grundlagen der insektenpathologie. Dr. D. Steinkopt Verlag, Darmstadt, 304 pp.

KRIEG, N. R. & HOLT, J. G. 1986. Gram-negative aerobic rods and cocci, pp. 140–218. In: PALLERONI, N. J. (ed.) Bergey's Manual of Systematic Bacteriology, Volume 1, Williams and Wilkins, Baltimore.

LIPA, J. J. & WILAND, E. 1972. Bacteria isolated from cutworms and their infectivitiy to *Agrotis sp.* Acta Microbiol. Polonica Ser. B **4:** 127–140.

MITCHELL, F. L. & SMITH, J. W. 1985. Pathology and bioassays of the lesser cornstalk borer (*Elasmopalpus lignosellus*) entomopoxvirus. J. Invertebr. Pathol. **45:** 75–80.

MOAR, W. J., PUSZTZAI-CAREY, M. & MACK, T. P. 1995. Toxity of purified proteins and the HD-1 strain from *Bacillus thuringiensis* againts lesser cornstalk borer (Lepidoptera: Pyralidae). J. Econ. Entomol. **88:** 606–609.

POINAR, G. O. & THOMAS, G. M. 1978. Diagnostic Manual for the Identification of Insect Pathogens. Plenum Press, New York, 218 pp.

SEZEN, K. & DEMIRBA, Z. 1999. Isolation and insecticidal activity of some bacteria from the hazelnut beetle (*Balaninus nucum* L.). Appl. Entomol. Zool. **34:** 85–89.

SEZEN, K., YAMAN, M. & DEMIRBAĞ, Z. 2001. Insecticidal potential of *Serratia marcescens* Bn10. Biologia, Bratislava **56:** 333–336.

SNEATH, P. H. A., MAIR, N. S., SHARPE, M. E. & HOLT, J. G. 1986. Regular, nonsporing Gram-positive rods, pp. 1208–1260. In: KANDLER, O. & WEISS, N. (eds) Bergey's Manual of Systematic Bacteriology, Volume 2, Williams and Wilkins, Baltimore.

SUCHY, J. 1988. Note on the biology of the chrysomelid *Agelastica alni* (L.) and the predator hister helluo truqui. Zpravy Muzei Zapadoceskeho Kraje **93:** 36–37.

TANADA, Y. & KAYA, H. K. 1993. Insect pathology. Academic Press, San Diego, 666 pp.

THIERY, I. & FRACHON, E. 1997. Idendification, isolation, culture and preservation of enthomopathogenic bacteria, pp. 55–73. In: LACEY, A. L. (ed.) Manual of Techniques in Insect Pathology, Academic Press, London.

URBAN, J. 1999. Results of the study of biology and harmfulness of alder leaf beetle (*Agelastica alni* L.) (Chrysomelidae, Coleoptera). Acta Univ. Agric. Sivil. Men. Brun. **47:** 47–71.

YAMAN, M., SEZEN, K. & DEMIRBAĞ, Z. 1999. Insecticidal effects of some biological agents on the larvae of *Balaninus nucum* (Coleoptera: Curculionidae) and *Euproctis chrysorrhoea* (Lepidoptera: Lymantridae). Pak. J. Biol. Sci. **2:** 616–617.

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