Investigations on Bacteria as a Potential Biological Control Agent of Summer Chafer, *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae)

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Studying the bacteria of hazardous insects allows the opportunity to find potentially better biological control agents. Therefore, in this study, bacteria from summer chafer (*Amphimallon solstitiale* L., Coleoptera: Scarabaeidae) we isolated and identified the insecticidal effects of bacteria isolated from *A. solstitiale* and *Melolontha melolontha* L. (common cockchafer, Coleoptera: Scarabaeidae) and the mixtures of these bacterial isolates were investigated on *A. solstitiale* larvae. Crystals from *Bacillus* sp. isolated from *M. melolontha* were also purified, and tested against the second and third-stage larvae of *A. solstitiale*. The bacterial isolates of *A. solstitiale* were identified as *Pseudomonas* sp., *Pseudomonas* sp., *Bacillus cereus* and *Micrococcus luteus*, based on their morphology, spore formation, nutritional features, and physiological and biochemical characteristics. The insecticidal effects of the bacterial isolates determined on the larvae of *A. solstitiale* were 90% with *B. cereus* isolated from *A. solstitiale*, and 75% with *B. cereus*, *B. sphaericus* and *B. thuringiensis* isolated from *M. melolontha* within ten days. The highest insecticidal effects of the mixed infections on the larvae of *A. solstitiale* were 100% both with *B. cereus*+*B. sphaericus* and with *B. cereus*+*B. thuringiensis*. In the crystal protein bioassays, the highest insecticidal effect was 65% with crystals of *B. thuringiensis* and *B. sphaericus* isolated from *M. melolontha* within seven days. Finally, our results showed that the mixed infections could be utilized as microbial control agents, as they have a 100% insecticidal effect on the larvae of *A. solstitiale*.

Key words: *A. solstitiale*, biological control, insecticidal activity, summer chafer

Summer chafer is a serious pest towards the hazelnuts cultivated in the Black Sea Region of Turkey (Allen, 1995; T. C. Tarım ve Köyişleri Bakanlığı, 1995; Goffau, 1996). Endosulphan, at 350 g/l, and 25% chlorpyrifos are major pesticides utilized for the control of this pest. However, these chemicals are no longer recommended for agricultural use. Increasing interest in the development of environmentally safe pest control methods has inspired our study of potential microbial agents for the control of *A. solstitiale*. An important way to investigate microbial control agent of any noxious insect is to search for entomopathogens of the target or closely related pests. Until now, there has been no study investigating bacterial flora as microbial control agents for use against this insect. This is the first study on the isolation and characterization of the bacteria of *A. solstitiale* and for the determination of the insecticidal potential of these isolates on *A. solstitiale*. In addition, bacteria isolated from *Melolontha melolontha* were also tested for their insecticidal effect on *A. solstitiale*. *M. melolontha* lives in the same environment and

causes the same damage as *A. solsititale*. Mixtures of these insect-originated bacteria and bacterial crystals were also prepared and tested against *A. solsititale*.

Materials and Methods

Collection of Insects
Larvae of the summer chafer were collected within the vicinity of Trabzon, Turkey, on a daily basis. The collected insects were taken to the laboratory in bottles with perforated covers to permit airflow.

Quantitative Analysis and Isolation of Bacteria from Larvae
The living larvae were surface sterilized with 70% alcohol. The suspensions obtained from larvae and adult bodies were separately mixed 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 plated on nutrient agar and incubated at 30°C for 24 h. After incubation, the total number of bacteria in each larva was determined. Isolates were determined based on their color and morphology of the colonies. Pure cultures of the bacterial colonies were pre-

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pared, which were identified by various taxonomical tests.

Identification of Bacterial Isolates

The identification procedure for the isolated bacteria was performed 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, optimum pH, optimum temperature, catalase test, oxidase test and gelatine hydrolysis, were performed for all isolates.

Infectivity Test of Bacterial Isolates

Bacterial isolates were incubated in nutrient broth medium at 30°C for 18 h. After incubation, the density was adjusted to 1.89 at OD_{600} (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 sterilized PBS and used in bioassays. In the mixture infection tests, 0.5 ml of the suspensions of two bacterial isolates having a 70% or higher insecticidal effect on the larvae of *A. solstitiale* were mixed and used in bioassays. The mixtures included *B. cereus* (from *A. solstitiale*, As3) and *B. sphaericus* (from *M. melolontha*, Mm5); *B. cereus* (As3) and *B. thuringiensis* (Mm2); *B. cereus* (Mm7) and *B. sphaericus* (Mm5); *B. cereus* (Mm7) and *B. thuringiensis* (Mm2); *B. sphaericus* (Mm5) and *B. thuringiensis* (Mm2). Bacterial isolates of *M. melolontha* were isolated and identified in a similar study (Unpublished data).

Isolation and Infectivity Test of Crystal Proteins from Bacillus sp.

After incubation for 3-5 days at 30°C, the bacteria were collected and lysed. The mixture of spores and crystals was suspended in 0.5 M NaCl and centrifuged at 13,800 g for 5 min. The pellet was resuspended in 1% SDS and 0.001% β-mercaptoethanol, boiled for 10 min., and then centrifuged at 13,800 g for 10 min. (Sivripoulou *et al.*, 2000). The supernatant, which contained mainly crystal proteins, was removed, measured by spectrophotometry (OD_{595}), and used for an infectivity test at a concentration of 100 μg/ml.

General Conditions for Infectivity Test

A diet was prepared from roots of *Corylus* sp. and collected field soil. The sterilized diet was placed into individual glass containers (80 mm in diameter), each containing a single bacterial culture. Bacterial isolates and crystal proteins prepared in PBS were applied individually to the surface of the diet. Second and third stage larvae, 10 each, were placed on the diet in the containers, and kept at 26±2 °C, 60% RH on a 12:12 h photo regime, with the diet changed after eating (Lipa *et al.*, 1994). The mortalities of larvae were recorded every 24 h, with all dead larvae removed from the containers. Infectivity tests were carried out, with the positive and negative controls. All bio-

assays were repeated three times on different occasions. At least 30 larvae were assayed for each infectivity test. Data were evaluated using Abbott's formula (Abbott, 1925).

Results

The number of bacteria in the larvae was determined by counting the number of colonies on the plates that had been inoculated with the diluted bacterial suspensions. The total number of bacteria was found to be $3.6 \pm 0.35 \times 10^6$ cfu/larva (n=20) in *A. solstitiale*.

Only four different isolates were found among the total bacteria. All bacteria were identical with one of these four isolates. Therefore, four isolates were finally selected, and characterized for the morphology, spore formation, nutritional features, and physiological and biochemical characteristics (Table 1). Most of the isolates were creamy in color, but isolate As4 was yellow, on the agar plates. Colonies of isolate As3 became filamentous after one day of incubation on the nutrient agar plate at 30°C.

Enrichment and purification procedures were carried out for the larvae of *A. solstitiale*, which allowed the isolation of three non-spore forming bacteria. The morphological characteristics of the bacteria are shown in Table 1. All of the isolates were rod-shaped, with the exception of isolate As4, which occurred singly, with a length of approximately 1.2 - 7.6 μm. Two isolates (As3 and As4) were gram positive, but the rest were gram negative. Motility was observed in all isolates, with the exception of As4.

The physiological and biochemical characteristics of the isolates are reported in Table 1. All of the isolates were determined as being positive in the catalase test. All isolates, with the exception of As3, were found to be negative in the oxidase test. All isolates were found to be positive for gelatin hydrolysis, were tolerant to different concentrations of NaCl: 2, 5, 7 and 12% and grew optimally in nutrient broth medium where the pH values were between 6 and 8 and were facultatively anaerobic. Growth was observed for all isolates in the presence of lysozyme, with the exception of As4.

Isolates 1 and 2, which were non-spore forming bacteria, were identified as *Pseudomonas* sp. Sixteen species of the genus *Pseudomonas* are found in insects. Isolates 1 and 2 showed some features of *P. fluorescens, P. aeruginosa* and *P. chlororaphis*; therefore, these bacteria were identified as *Pseudomonas* sp. Since they showed no significant insecticidal effect, further detailed studies were not performed.

Isolate 3 was rod-shaped, motile, Gram-positive, 0.8 to 1×3.8 to 7.6 μm, catalase positive, spore-forming, a facultative anaerobe, produced acid from glucose, reduced nitrate, had grey-yellow colonies, was dry and formed filamentous colonies on nutrient agar. Morphological, phys-

Table 1. The morphological, physiological and biochemical characteristics of the bacterial isolates from *A. solstitiale*

| Tests/Isolate Numbers	As1	As2	As3	As4
Colony color	cream	cream	cream	yellow
Colony margin	smooth	smooth	filamentous	smooth
Colony shape	round	round	filamentous	round
Shape of bacteria	bacillus	bacillus	bacillus	coccus
Gram stain	-	-	+	+
Spore stain	ND	ND	+	-
Spore shape	ND	ND	Ellipsoid	ND
Spore form	ND	ND	Central	ND
Diameter (μm)	ND	ND	ND	0.8 - 1.2
Length (μm)	1.2 - 3.4	1.3 - 3.5	3.8 - 7.6	ND
Wideness (μm)	0.6 - 1	0.5 - 1	0.8 - 1	ND
Motility	+	+	+	-
Nitrate reduction	+	-	+	-
Hydrolysis of starch	-	-	+	-
Oxidase	+	+	-	+
Hydrolysis of urea	-	W+	+	+
Propionate utilization	+	-	ND	ND
Methyl red test	-	+	+	-
Voges proskauer test	+	-	+	-
Growth in 12% NaCl	+	-	-	+
Growth with lysozyme	+	+	+	-
Growth at 4°C	+	+	ND	ND
Growth at 40°C	ND	ND	ND	-
Growth at 41°C	-	-	ND	ND
Hydrolysis of tween 80	+	-	+	-
Tyrosinase production	ND	ND	+	ND
Hydrolysis of casein	ND	ND	+	ND
Egg-yolk lecithinase	ND	ND	+	ND
Growth at pH>7 MVRP	ND	ND	+	ND
Growth at pH<6 MVRP	ND	ND	+	ND
Glucose fermentation	+	+	+	-
Arabinose fermentation	+	-	-	-
Xylose fermentation	+	-	-	-
Mannitol fermentation	W+	+	-	W+
Sucrose fermentation	+	-	+	+
Optimum pH	6 - 8	6 - 8	6 - 8	6 - 8
Optimum Growth (°C)	30	30	30	30

ND: No Data
W: Weak Growth

iological and biochemical tests showed this bacterium was *Bacillus cereus*.

Isolate 4 was non-motile, nonspore-forming, Gram-positive, spherical 0.8-1.2 μm in diameter occurred in tetrads, catalase positive, produced yellowish pigment that was insoluble in water and had smooth round colonies on nutrient agar. Morphological, physiological and biochemical tests showed that isolate 4 was *Micrococcus luteus*.

A number of bioassays were carried out using larvae of *A. solstitiale* as the test insect that had been fed with isolated bacteria and crystal proteins. The insecticidal effects of the bacteria isolated from *A. solstitiale* and *M. melolontha*, the mixed infections and crystal proteins on the larvae of *A. solstitiale* are shown in Figs. 1 and 2. The pathogenic effects of all agents were assumed the results of cytotoxicity.

Of the bioassays used for the bacterial isolates, the highest insecticidal effect of the bacteria on the larvae of *A. solstitiale* was 90%, which occurred with As4 within ten days of utilizing the bacteria isolated from *A. solstitiale,* and 75%, which occurred with Mm2, Mm5 and Mm7 within ten days of utilizing the bacteria isolated from *M. melolontha* (Fig. 1).

The insecticidal effects of the mixed infections were 100, 100, 85, 90 and 85% for As3+Mm5, As3+Mm2, Mm7+Mm5, Mm7+Mm2 and Mm5+Mm2 within eight days, respectively. The crystal proteins of *Bacillu*s spp. had insecticidal effects on *A. solstitiale* larvae. The highest insecticidal effect was 65%, with the crystal proteins of *B. thuringiensis* (Mm2) and *B. sphaericus* (Mm5) isolated from *M. melolontha* within seven days. Conversely, the crystal protein of the reference strain, *B. thuringiensis*
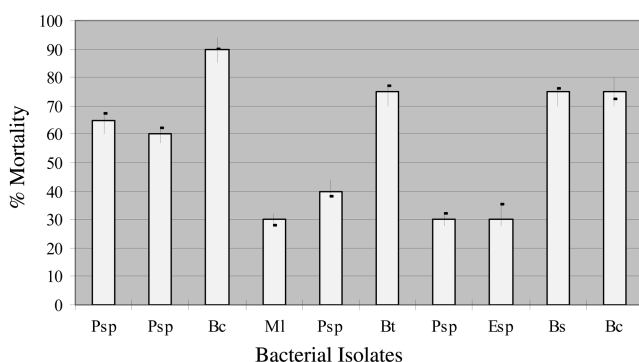
Fig. 1. The results of the insecticidal effects of bacterial isolates on *A. solstitiale* larvae. The first 4 bacterial isolates from *A. solstitiale* are, Psp: *Pseudomonas* sp.; Psp: *Pseudomonas* sp.; Bc: *B. cereus* and Ml: *M. luteus*; The others from *M. melolontha* are, Psp: *Pseudomonas* sp.; Bt: *B. thuringiensis* ; Psp: *Pseudomonas* sp.; Esp: *Enterobacter* sp.; Bs: *B. sphaericus* and Bc: *B. cereus*. Vertical bars represent the standard deviation.
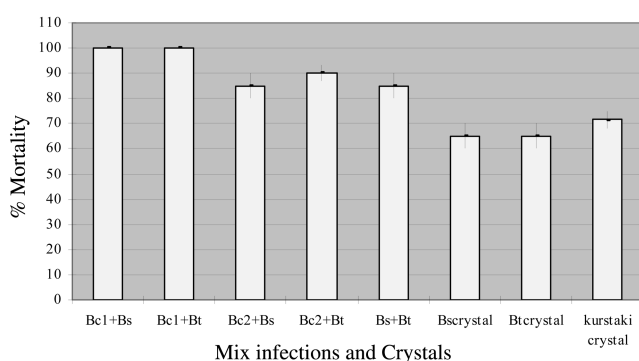
Fig. 2. The results of the insecticidal effects of the mixed infection and crystals on *A. solstitiale* larvae. Bc[1]+Bs: *B. cereus* + *B. sphaericus*; Bc[1]+Bt: *B. cereus* + *B. thuringiensis*; Bs+Bt: *B. sphaericus* + *B. thuringiensis*; Bscrystal: *B. sphaericus crystal*; Btcrystal: *B. thuringiensis crystal*; kurstakicrystal: *B. thuringiensis* subsp. *kurstaki* HD-1. Vertical bars represent the standard deviation.
B. cereus[1] : from *A. solstitiale*
B. cereus[2] : from *M. melolontha*

subsp. *kurstaki* HD-1, showed a mortality percentage of 72% for *A. solstitiale* (Fig. 2). The result of the negative control result for the insecticidal effects of bacterial isolates was 7%, but those for the mixed infection and crystals were 5%.

Discussion

There has recently been increasing interest in the discovery of more effective and safer biological control agents against hazardous insects. To date, no study has been conducted on the determination of bacterial isolates as potential biological control agents of *A. solstitiale*.

In the present study, four bacterial isolates were verified from *A. solstitiale*. Based on morphological, nutritional, physiological and biochemical tests, the isolates from the larvae of *A. solstitiale* were identified as *Pseudomonas* sp. (As1), *Pseudomonas* sp. (As2), *Bacillus cereus* (As3) and *Micrococcus luteus* (As4) (Sneath, 1986; Thiery and Frachon, 1997). These bacteria have been commonly isolated from several insects (Lipa and Wiland, 1972; Sezen and Demirbağ, 1999; Broderick *et al.*, 2000).

The larvae were shown to have different signs before death following ten days of infection with the bacterial isolates; generally, the larvae were sluggish and showed a loss of appetite. In addition to the other major symptoms, infection decreased the larval survival. The average larval life span of healthy individuals at $26 \pm 2°C$ was 30 ± 2 days, whereas the average life span of infected larvae under the same conditions was 10 ± 3 days.

In the bioassays, the highest insecticidal effect of the bacterial isolates of *A. solstitiale* on the larvae of *A. solstitiale* was 90%, which was shown with *B. cereus* (As3) within ten days. Conversely, the highest insecticidal effect of the bacterial isolates of *M. melolontha* on *A. solstitiale* larvae was 75%, which were observed with *B. thuringiensis* (Mm2), *B. sphaericus* (Mm5) and *B. cereus* (Mm7) within ten days.

Most of the time, bacterial pathogens show higher insecticidal effect against their isolated host compared to that against other insects. A similar result was found in a similar study (Demir *et al.*, 2002); namely, a strain of *B. sphaericus* isolated from *Anoplus roboris* Sufr. (Coleoptera: Curculionidae) showed a 67% insecticidal effect toward its adults. In addition, *B. cereus* isolated from *A. solstitiale* showed a 90% insecticidal effect on the larvae of *A. solstitiale*. *B. cereus* is also a common bacterial pathogen. Until now, many *B. cereus* strains have been isolated from insects (Lipa and Wiland, 1972; Broderick *et al.*, 2000). Glare *et al.* (1993) determined that *Bacillus popillia* Type A1 isolated from *A. solstitiale* had a high infectivity on *A. solstitiale* population, but not on *M. hippocastani*.

Although the insecticidal effect of bacteria against non isolated insects is generally very low, in this study, the effect of bacterial isolates of *M. melolontha* on *A. solsti-*

tiale larvae was found to be significantly high. Katı *et al.* (2005) also recorded a mortality of 76% for *Malacosoma neustria* using *B. thuringiensis* (MnD) isolated from *M. neustria*. Conversely, MnD showed no insecticidal activity against *Agelastica alni*. This shows that *B. thuringiensis* strains isolated from coleopteran have higher insecticidal effects on the larvae belonging to coleopteran than those belonging to Lepidoptera. As discussed by Burgerjon and Martouret (1971), many species of larvae exhibit no toxemia due to *B. thuringiensis* because of the alkaline pH in their gut.

B. thuringiensis is now the most widely used biologically produced pest control agent. Worldwide sales of *B. thuringiensis* were $90 million, representing about 2% of the total global insecticide market. Rowe *et al.* (1987) reported an annual worldwide distribution for *B. thuringiensis* of 2.3×10^6 kg. As of early 1998, there were nearly 200 registered *B. thuringiensis* products in the United States. While the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favor the future development of *B. thuringiensis*. The cry proteins studied thus far are not pathogenic to mammals, birds, amphibians or reptiles, but are very specific to groups of insects and invertebrate pests against which they have activity. Cry-based pesticides generally have low developmental and registration costs (Crickmore *et al.* 1998).

In this study, two *Pseudomonas* and one *Micrococcus* species were identified and their insecticidal effect on *A. solstitiale* larvae determined. Previous studies have also shown that the isolation of these bacterial species from various insects (Lipa and Wiland, 1972; Sezen and Demirbağ, 1999; Sezen *et al.* 2004), but the insecticidal effect of these bacteria was not high. Sezen and Demirbağ (1999) determined that *P. fluorescens* isolated from *B. nucum* had 20% insecticidal activity towards *B. nucum* larvae, and determined that *Micrococcus luteus* was pathogenic towards *B. nucum* larvae (30%). Until now, various bacteria belonging to *Micrococcaceae* have been isolated and identified from different insect (Lipa and Wiland, 1972; Sezen and Demirbağ, 1999).

When bacterial isolates having a 70% or more insecticidal effect were administered to *A. solstitiale* larvae together, their individual insecticidal effect was increased, but the time to mortality was decreased. Our results showed that the mixtures of *B. cereus* and *B. sphaericus* or *B. cereus* and *B. thuringiensis* caused 100% mortality within eight days. Broderick *et al.* (2000) determined that the addition of a *B. cereus* UW85 culture significantly increased the mortality of gypsy moth larvae when administered with the constant addition of the *B. thuringiensis* subsp., *kurstaki*. In another study, Wirth *et al.* (2000) observed that the Cyt1A toxin of *B. thuringiensis* and *B. sphaericus* had a very important insecticidal effect against

the resistant mosquito population.

In crystal protein bioassays, the insecticidal effects of *B. thuringiensis* and *B. sphaericus* crystals from *M. melolontha* were 65%. Lopez-Meza and Ibarra (1996) recorded that parasporal crystals of *B. thuringiensis* isolated from living larvae of *Anopheles pseudopunctipennis* in Mexico have no infectivity against four species of caterpillar, three species of mosquito and two species of beetle. In contrast, crystal protein of the reference strain, *B. thuringiensis* subsp. *kurstaki* HD-1, showed a mortality percentage of 72% for *A. solstitiale*.

Consequently, *B. cereus*, *B. sphaericus* and *B. thuringiensis,* in particular, were determined to be used as biological control agents against *A. solstitiale* larvae. However, the mixture of these bacteria was found to confer better infectivity. Future studies will be conducted with the aim of finding a biological control agent against these hazardous insects, using these agents or other newly improved pesticides. The present study has contributed significantly to the literature on bacterial isolates and the biological control of summer chafer.

Acknowledgements

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