INSECTICIDAL EFFECT OF SOME BACTERIA ON *BRUCHUS DENTIPES* BAUDI (COLEOPTERA: BRUCHIDAE)

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ABSTRACT

In this study, a total of 10 bacterial strains (2 *Bacillus thuringiensis* subsp. *kenyae*, 2 *Bacillus cereus*, 1 *Bacillus thuringiensis* subsp. *kurstaki*, 1 *Bacillus atrophaeus*, 1 *Bacillus pumilus*, 1 *Bacillus globisporus*, 1 *Brevibacillus brevis* and 1 *Alcaligenes denitrificans* subsp. *xylosoxydans*), isolated from different insect species in the Eastern Anatolia region of Turkey, were identified on the basis of fatty acid methyl ester analysis and carbon source utilization profiles by using Microbial Identification System (MIS) and/or Biolog MicroPlate Systems (BIOLOG). Their insecticidal effects were tested on adults of *Bruchus dentipes* Baudi *in vitro* on Petri dishes. Seven days after the applications, all of the tested strains showed more or less insecticidal activity against this pest. The highest activity was obtained from *Bacillus thuringiensis* subsp. *kenyae* strain FDP8 causing 76.6% mortality, and its activity was not statistically different from that of the positive control (Kingbo). The mortality rates of the other bacterial applications were lower than that of the positive control, but not that of the negative control. In conclusion, *Bacillus thuringiensis* subsp. *kenyae* strain FDP8 is a good candidate for use as a biological control agent against this economically important pest.

KEYWORDS: *Bacillus thuringiensis*, Biocontrol, Biopesticide, Bruchid beetles, *Bruchus dentipes*

1. INTRODUCTION

In Turkey, legumes are an important component of agricultural and food systems after cereals. As with other agricultural products, pests damage and cause significant yield loss in legumes. In Turkey, it has been determined that seed beetles, pests feeding on legume seeds, cause quality and quantity loss of seeds (20%), and power of germination (90%) in the field and in storage [1-11]. *Bruchus dentipes* (Baudi) is an important pest of *Vicia faba*, *V. hyrcana*, *V. lutae* and *V. sative* [12]. This species is found in Southern and Central Europe, North Africa, Armenia, Syria, Lebanon, Israel, Afghanistan, Iraq, Iran, India and Turkey [13, 14].

There are many control methods for Bruchid beetles, including early harvesting of grain, stubbling, underwinter plowing, fumigation of grain in granaries, and insecticide treatments in fields during the period of budding and blossoming [15]. However, the negative effects of the chemicals used against pests should not be ignored. The problems of insecticide resistance as well as the environmental and consumer health hazards associated with insecticide residues in plant material have focused attention on alternative methods for controlling pests. The development of biocontrol agents may help to decrease the negative effects (i.e., residues, resistance and environmental pollution) of chemical pesticides that are commonly and extensively used for plant disease management in agriculture.

There are many studies related to the biocontrol of pests using bacterial biological agents [16-26], but there have been few studies on Bruchid beetles. One of these studies is on *Bruchus rufimanus* [27], but not *B. dentipes*.

The aim of this study is to investigate the insecticidal effect of 10 bacterial strains (for details see Summary), isolated from different insect species in the Eastern Anatolia region of Turkey, on *Bruchus dentipes*.

2. MATERIALS AND METHODS

2.1. Insect collection

B. dentipes Baudi adults were collected 2009, during the mass occurrence of this insect from the vicinity of Erzurum, Turkey.

2.2. Isolation and cultivation of the bacterial strains

A total of 10 bacterial strains were tested for their insecticidal activities against *Bruchus dentipes* Baudi *in vitro* on Petri plates. They were isolated from larvae of *Yponomeutida evonymella*, *Hypera postica*, *Malacosoma neustria* and adults of *Apion* spp. collected from the East-

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ern Anatolia region of Turkey. The bacterial cultures were grown on nutrient agar (NA) for routine use, and maintained in Luria Broth (LB) with 15% glycerol at -80 °C for long-term storage [28], at the Department of Plant Protection, Faculty of Agriculture, Atatürk University.

2.3. Identification of the bacterial strains by Microbial Identification System (MIS)

Identification of the tested bacterial strains was confirmed by using MIS systems. Preparation and analysis of FAMEs from whole cell fatty acids of bacterial strains were performed according to the method described by the manufacturer`s manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA). FAMEs were separated by gas chromatography (HP-6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m x 0.2 mm, with cross-linked 5% phenyl methyl silicone). FAME profiles of each bacterial strain were identified by comparing the commercial databases (TSBA 40) with the MIS software package [29].

2.4. Identification of the bacterial strains by Biolog Microplate System (BIOLOG)

Identification of the tested bacterial strains was confirmed by using BIOLOG systems. One or two days before the inoculation of Biolog GN2 and GP2 plates, bacterial strains were streaked on TSA or BUG agar plates. Each well of Biolog GN2 or GP2 micro-titer plates was inoculated with 125 µl of the Gram-negative or positive bacterial suspension, respectively, adjusted to the appropriate density (10^8 cfu/ml) and incubated at 27 °C for 24 and 48 h. The development of color was automatically recorded using a microplate reader with a 590-nm wavelength filter. Identification (Biolog Microlog 34.20 database) and ASCII file output of test results, applying the automatic threshold option, were performed using BIOLOG420/Databases/GN601 and GP601 KID software [30]. Carbon source utilization rates of the strains were estimated as percentages.

2.5. Hypersensitivity tests (HR)

All of the bacterial strains were tested for hypersensitivity on tobacco plants (*Nicotina tabacum* L. var. Samsun). The bacterial suspension (10^8 cfu/ml) prepared in sterile distilled water and infiltrated into the inter-costal area of the leaves of tobacco plants by using a 3-cc syringe (Becton Dickinson, Franklin Lakes, NJ, U.S.A). The inoculated plants were incubated in a completely randomized design on the greenhouse bench for 24–48 h at 20–28 °C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24–48 h after infiltration. This test was repeated, at least three times, for each strain. For HR tests, sterilized distilled water (sdH_2O) was used as a negative control.

2.6. Bioassay

Tryptic Soy Agar (TSA, Oxoid) and Tryptic Soy Broth (TSB, Oxoid) plates were used in the experiments. All bacterial isolates were incubated in TSA at 27 °C for 24 h. After incubation period, a single colony was transferred to 500-ml flasks containing TSB, and grown aerobically in the flasks on a rotating shaker (150 rpm) for 48 h at 27 °C (Merck KGaA, Germany). The bacterial suspension was then diluted in sterile distilled water (sdH_2O) to a final concentration of 10^8 cfu/ml with a turbidimeter.

Ten seeds of legumes were added to each of the Petri plates to feed the insects. The prepared solutions were transferred to sterile glass injection containers, and the suspensions were sprayed onto Petri plates. Ten insects, collected from naturally affected legume seeds, were released in each Petri plate. The plates were sealed with parafilm and transferred to desiccators containing 40 ml sdH_2O. When plates were removed from the desiccators, the mortality was recorded after 1, 4 and 7 days. The assay was designed in randomized complete blocks with three replications; 1% kingbo and sterile media TSB were used as the positive and negative control, respectively.

2.7. Data analysis

In order to determine significant differences in toxicity among the insecticidal activities, analysis of variance (ANOVA) was carried out using the SPSS 11.0 statistical software package. The results showed significant differences at the $P<0.01$ level.

3. RESULTS

The MIS and BIOLOG identification results of tested bacterial strains and their hypersensitivity test results on tobacco plants are shown in Table 1. According to the MIS/BIOLOG results, bacterial strains isolated from larvae of *Yponomeutida evonymella* were identified as *Alcaligenes denitrificans* subsp. *xylosoxydans*/*Alcaligenes faecalis* (strain FD-61), *Bacillus globisporus*/*Bacillus amyloliquefaciens* (strain FD-64), *Bacillus cereus* GC subgroup B/*Bacillus amyloliquefaciens* (strain FD-63) and *Bacillus atrophaeus*/*Bacillus licheniformis* (strain FD-17); from larvae of *Hypera postica* identified as *Bacillus thuringiensis* subsp. *kenyae*/*Bacillus thuringiensis* (strain FDP-8), *Bacillus cereus* GC subgroup B/*Bacillus cereus* (strain FDP-24) and *Bacillus pumilus* GC subgroup B/*Bacillus pumilus* (strain FDP-32); from adults of *Apion* spp. identified as *Bacillus thuringiensis* subsp. *kurstakii*/*Bacillus thuringiensis* (strain FDP-41) and *Bacillus thuringiensis* subsp. *kenyae*/*Bacillus thuringiensis* (strain FDP-42); from larvae of *Malacosoma neustria* identified as *Brevibacillus brevis*/*Bacillus subtilis* (strain FD-1). Hypersensitivity test results of all strains were negative on tobacco leaves. Carbon source utilization rates of the tested bacterial strains changed from 18.42 to 38.90% (Table 1).

The insecticidal effects of the tested bacterial strains on *Bruchus dentipes* are summarized in Table 2. The results show that these strains are toxic and have a significant insecticidal effect on *Bruchus dentipes*. According to

the results of ANOVA, the insecticidal effect was significant (P< 0.01) (Table 2).

On the first day, the high mortality rate (100%) was observed from positive control (1% Kingbo). The low mortality rates (0.0%) were observed with negative control (TSB), *Bacillus globisporus* strain FD-64 and *Bacillus pumilus* GC subgroup B strain FDP-32. The mortality rates of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8, *Bacillus thuringiensis* subsp. *kurstakii* strain FDP-41, *Bacillus thuringiensis* subsp. *kenyae* strain FDP-42 and *Bacillus cereus* GC subgroup B strain FDP-24 were changed from 26.6 to 63.3% on the first day. All of them were different from the negative control applied to TSB medium.

On the 4 th day, the highest and lowest mortality rates were observed from positive control (1% Kingbo) and negative control, respectively. All of the bacterial applications caused mortality but the mortality rate of *Bacillus cereus* GC subgroup B strain FDP-24, *Bacillus atrophaeus* strain FD-17, *Bacillus pumilus* GC subgroup B strain FDP-32, *Brevibacillus brevis* strain FD-1, *Alcaligenes denitrificans* subsp. *xylosoxydans* strain FD-61 and *Bacillus globisporus* strain FD-64 were not different from the negative control. The mortality rates of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8, *Bacillus thuringiensis* subsp. *kurstakii* strain FDP-41, *Bacillus thuringiensis* subsp. *kenyae* strain FDP-42 and *Bacillus cereus* GC subgroup B strain FD-63 were changed from 36.6 to 73.3% on the first day. Their insecticidal activities were different from the negative control. The 4th day mortality rate of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8 in the bacterial applications was found to be the highest. But, its activity was lower than that of positive control (1% Kingbo).

On the 7th day, all of the bacterial applications showed insecticidal activity changed from 10.0 to 76.6%. The 7th day mortality rate of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8 was statistically indifferent from that of positive control (1% Kingbo). *Brevibacillus brevis* strain FD-1, *Alcaligenes denitrificans* subsp. *xylosoxydans* strain FD-61 and *Bacillus globisporus* strain FD-64 caused insecticidal activities on *Bruchus dentipes* but their mortalities were not statistically different from the negative control.

TABLE 1 - MIS and BIOLOG identification results of bacterial strains, their similarity index (SI), Hypersensitivity test (HR) results and carbon source utilization rates (CSU%)

Isolated from	Strain no	MIS results	SI	BIOLOG results	SI	HR	CSU
Larvae of *Yponomeutida evonymella*							
	FD-61	*Alcaligenes denitrificans* subsp. *xylosoxydans*	55	*Alcaligenes faecalis*	74	-	33.68
	FD-64	*Bacillus globisporus*	34	*Bacillus amyloliquefaciens*	30	-	25.26
	FD-63	*Bacillus cereus* GC subgroup B	24	*Bacillus amyloliquefaciens*	17	-	25.26
	FD-17	*Bacillus atrophaeus*	45	*Bacillus licheniformis*	34	-	26.31
Larvae of *Hypera postica*							
	FDP-8	*Bacillus thuringiensis* subsp. *kenyae*	32	*Bacillus thuringiensis*	57	-	38.90
	FDP-24	*Bacillus cereus* GC subgroup B	38	*Bacillus cereus*	72	-	27.36
	FDP-32	*Bacillus pumilus* GC subgroup B	45	*Bacillus pumilus*	31	-	33.68
Adults of Apion spp							
	FDP-41	*Bacillus thuringiensis* subsp. *kurstakii*	55	*Bacillus thuringiensis*	44	-	24.21
	FDP-42	*Bacillus thuringiensis* subsp. *kenyae*	35	*Bacillus thuringiensis*	72	-	18.42
Larvae of *Malacosoma neustria*							
	FD-1	*Brevibacillus brevis*	62	*Bacillus subtilis*	34	-	17.89

-: Hypersensitivity test result was negative on tobacco plant.

TABLE 2 - The mortality rate of *Bruchus dentipes with* bacterial application measured after 1, 4 and 7 days under laboratory conditions.

Applications	Mortality rate					
	First day		Fourth day		Seventh day	
Bacillus thuringiensis subsp. *kenyae* strain FDP-8	63.3	d	73.3	e	76.6	de
Bacillus thuringiensis subsp. *kurstakii* strain FDP-41	43.3	c	43.3	cd	63.3	cd
Bacillus thuringiensis subsp. *kenyae* strain FDP-42	50.0	c	56.6	de	56.6	cd
Bacillus cereus GC subgroup B strain FDP-24	26.6	b	26.6	abc	40.0	bc
Bacillus cereus GC subgroup B strain FD-63	13.3	a	36.6	bcd	36.6	bc
Bacillus atrophaeus strain FD-17	3.3	a	6.6	a	36.6	bc
Bacillus pumilus GC subgroup B strain FDP-32	0.0	a	26.7	abc	33.3	bc
Brevibacillus brevis strain FD-1	3.3	a	16.6	ab	23.3	ab
Alcaligenes denitrificans subsp. *xylosoxydans* strain FD-61	10.0	a	16.6	ab	16.6	ab
Bacillus globisporus strain FD-64	0.0	a	10.0	a	10.0	ab
Positive control (1% Kingbo)	100.0	e	100.0	f	100.0	e
Negative control (TSB)	0.0	a	0.0	a	0.0	a

* Values followed by different days differ statistically significant (P<0.01).

The highest mortality rate was observed from *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8. The insecticidal activities of the rest of bacterial applications were significantly different from the negative but not positive control (1% Kingbo). On 1st, 4th and 7th day, the mortality rates of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8 were the highest in the bacterial applications but its activity was lower than that of the positive control.

4. DISCUSSION AND CONCLUSION

In this study, a total of 10 bacterial strains were identified as *Bacillus*, *Brevibacillus* and/or *Alcaligenes* species on the basis of FAME analysis and carbon source utilization profiles by using MIS and BIOLOG. Their insecticidal effects were tested on adults of *Bruchus dentipes.* When the MIS identification results of the tested bacterial strains were compared with the BIOLOG identification results, MIS results of all strains, except strain FD-1, were confirmed by BIOLOG in the genus level but not species level for some strains (FD-17, FD-61, FD-63 and FD-64). The results show that MIS and BIOLOG alone are accurately enough to serve as a primary method for identifying entomopathogenic *Bacillus* species, at least in genus level.

The 10 bacterial species identified are known as insect pathogenic bacteria for the possible use to control insect pests. The development of natural or biological insecticides will help to reduce the negative effects of synthetic insecticides, such as residue formation, resistance development and environmental pollution. The best-known example of an entomopathogenic bacterium is *Bacillus thuringiensis* which produces parasporal protein crystals in association with spore formation [31-36]. These protein toxins lyse the insect after ingestion [37]. The bacteria *Bacillus thuringiensis* produce an entomopathogenic protein, D-endotoxin, which yields crystals toxic to insects belonging to the classes Lepidoptera, Diptera, and Coleoptera. In addition to D-endotoxin, they are capable of synthesizing other secondary metabolites, namely, exotoxins of various nature, antibiotics, hemolysins, proteases, and phospholipases [38]. Therefore, natural and genetically engineered strains of *Bacillus thuringiensis* have been used commercially to control pests of many representatives of the orders of Lepidoptera, Diptera, and Coleoptera [31-34]. DiPel®PRO DF of *Bacillus thuringiensis* subsp. *kurstaki* is used against *Plodia interpunctella* larvae (Lepidoptera, Pyralidae) [39].

Studies on bacterial pathogens of Bruchids are scarce as compared with other pests. Comparing our isolates with bacteria recorded from adults of *Bruchus dentipes*, none of them were isolated earlier from these insects. In a paper published in 2007 by Sabour et al. [27], activities of two kinds of commercially prepared fungi, *Beauveria bassiana* (Biovar) and *Metarhaizum anisopilae* (Bionaz), as well as fungal isolate *Verticillium lecanii*, and bacterial strain *B. thuringiensis* (Bt) were tested with regard to *Bruchus rufimanus* adult beetles. Both *Beauveria bassiana* and *Metarhaizum anisopliae* gave the highest protection to broad bean against *Bruchus rufimanus* infestation up to 90 days. The highest percentage of infestations recorded had been 77% [27].

In this study, we observed that *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8, *Bacillus thuringiensis* subsp. *kurstakii* strain FDP-41 and *Bacillus thuringiensis* subsp. *kenyae* strain FDP-42 were the most effective strains of all tested bacterial strains. For the 7th day, the mortality rate of *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8 was recorded to be 76.6%. This was the highest effectiveness in the bacterial applications and statistically indifferent from that of the positive control (1% Kingbo). On the 7th day, the mortality rates in *Bacillus cereus* GC subgroup B strain FDP-24 and *Bacillus cereus* GC subgroup B strain FD-63 applications were observed as 40.0 and 36.6%, respectively. These mortality rates were different from the negative l but not the positive control.

On the first day, *Bacillus pumilus* strain FDP-32 was not effective. But, the mortality rates of this strain on the fourth and seventh day were recorded to be 26.7 and 33.3%, respectively. In a previous study, it was reported that *Bacillus pumilus* causes of bacterial spotting on immature peaches [36]. However, the hypersensitivity test results (HR) of the strain FDP-32 used in this study were negative. Generally, the insecticidal activities of tested bacterial strains on the seventh day were higher than on first and fourth day. We think that some strains need time to adapt to the environmental conditions, and 24 or 48 h probably were not enough to produce spores for the *Bacillus* species. The results of experimental infections are very promising but all results were provided under laboratory conditions. Some treatments of these isolates should be carried out under storage condition.

Carbon source utilization rates of the tested bacterial strains changed from 8.42 to 38.90%. *Bacillus thuringiensis* subsp. *kenyae* strain FDP-8 had the highest percentage of carbon source utilization. This is very important for potential entomopatogenic bacteria to adapt easily to the environmental conditions.

In conclusion, our results showed that many strains of *Bacillus thuringiensis* (FDP-8, FDP-41 and FDP-42) have insecticidal activity against *Bruchus dentipes*. These strains may have the potential to be used as biocontrol agents, but further study is necessary for determining the mechanism of insecticidal activity of these strains.

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