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ORIGINAL PAPER

Biological Control of Pine Sawfly (*Diprion pini* L.) and Molecular Characterisation of Effective Strains

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Abstract

Diprion pini is one of the most important pests in forest trees around the world. Also the pest was determined in pine forests around the Turkey, causing outbreaks in some regions. Therefore, this study aimed to develop bacterial biopesticides, which can be used to control Diprion pini remarkably and at the same time which do not threaten the environment and human health. Bacterial strains which were effective against different pests in some studies were used. Their insecticidal effects were tested on larvae of Diprion pini in vitro on petri dishes. Seven days after applications, some of the tested strains showed more or less insecticidal activity against this pest. The highest activity was obtained for strains FD-17 and FD-1causing 66.7-80% mortality, respectively. The activities of the other bacterial applications were not statistically different from that of the negative control. In conclusion, strains FD-17 and FD-1 are good candidates for use as biological control agents against this economically important pest. These effective strains were characterized to the species level by 16S rDNA sequence analysis. According to the results, both strains have been identified as Bacillus atrophaeus.

Keywords: *Bacillus, Biocontrol, Biopesticide, 16S rDNA sequence analysis, pine sawfly*

1. Introduction

The common pine sawfly (*Diprion pini*) is one of the most serious pine pests in the forests. *Pinus sylvestris,* a species within *Genus of Pinus* is among the hosts of *Diprion pini* (1, 2, 3, 4). It has been determined that the larvae are fed gregariously, rendering the shoots nude, with only main veins of needles remaining (5). Pine sawfly can cause serious economic damages in pine trees in many region of Turkey, mainly in the provinces of Afyon, Amasya, Ankara, Artvin, Antalya, Bolu, Çanakkale, Edirne, Elazığ, Erzurum, Eskişehir, Giresun, Gümüşhane, Isparta, İstanbul, Kahramanmaraş, Kastamonu, Konya, Mersin, Muğla, Sakarya, Sinop, Uşak, and Zonguldak (6). The chemical drugs are commonly used against this pest (7). But the problems of insecticide resistance as well as the environmental and consumer health hazards associated with insecticide residues in plant materials 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 several studies related to the biocontrol of pests using bacterial biological agents, but few studies have been carried out on *Diprion pini* (8, 9, 10, 11). Molecular methods are considered as study material, which are carbonhydrates, lipids, protein and genetic material (DNA and RNA) (12). Thus, these methods are used the identification and characterization of microorganisms by using one or more of these macromolecules. Advances in molecular biology techniques such as fatty acid methyl ester (FAME), genomic fingerprinting, and 16S rDNA sequencing have provided an excellent opportunity for identification and characterization of microorganisms at species and subspecies levels (13). Thus, if the goal is to identify an unknown organism on the basis of no a priori knowledge, FAME and the 16S rRNA gene sequencing are an excellent and extensively used choice. The aim of this study is to investigate the insecticidal effect of bacterial strains isolated from different insect species in the Eastern Anatolia region of Turkey, on *Diprion pini* and also, to make the molecular characterisation of bacterial strains determined to be effective.

2. Materials and Methods

2.1. Isolation and cultivation of the bacterial strains

A total of 7 bacterial strains were tested for their insecticidal activities against *Diprion pini* invitroon Petri plates. They were isolated from larvae of *Yponomeutida evonymella*, *Malacosoma neustria* and *Culex* sp. collected from the Eastern 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 at the Department of Plant Protection, Faculty of Agriculture, Atatürk University(14).

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

Identification of the tested bacterial strains were 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 (15).

2.3. 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 GP2 plate, bacterial strains were streaked on BUG agar plates. Eachwell of Biolog GP2 micro-titer plates was inoculated with 125 µl of the Gram-positive bacterial suspension, 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 micro plate 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/ GP601 KID software(16). Carbon source utilization rates of the strains were estimated as percentages.

2.4. 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 (sdH2O) was used as a negative control(14).

2.5. 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 (sdH2O) to a final concentration of 10^8 cfu/ml with a turbidimeter. The needles of pine 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 leaves, were released in each Petri plate. The plates were sealed with parafilm and transferred to desiccators containing 40 ml sdH2O. When plates were removed from the desiccators, the mortality was recorded after 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.

2.6. Genomic DNA extraction of bacteria

Total genomic DNA was extracted from 24-h cultures grown on NA using a commercial extraction kit as suggested by the QIAcube robotic platform (QIAGEN).

2.7. PCR amplification of 16S rDNA

The 16S rDNA PCR was performed according to the schedule below (17).

PCR methods	Primers	Master mix (for a sample)		PCR programme	
16S rDNA PCR: *Bacillus* sp.	**EUB-F:**GCACAAGCGGTGGAGCATGTGG **EUB-R:**GCCCGGGAACGTATTC-ACCG	10 x PCR buffer	5 µl	**1.** Denaturation a	95 ºC, 7 min
		dNTP	1 µl	**2.** Denaturation b	94 ºC, 1 min
		Primers	0.3'er µl	**3.** Annealing	56 ºC, 1 min
		Taq DNA polimerase (5U)	0.5 µl	**4.** Extension	72 ºC, 3 min
		sdH$_2$O	39.9 µl	**5.** Cycle (2, 3, 4)	20 repeat
		Template DNA (50 ng/µl)	3 µl	**6.** Extention	72ºC,10 min

2.8. Sequencing analysis

Following PCR amplification and cloning of the 16S rDNA genes of our strains, the 16S rDNA gene sequences were determined sequencer by using the Miseq Illumina (Intergen, ANKARA). The nucleotide sequence analysis of the 16 S rDNA of the isolates was done at NCBI server using BLAST (www.ncbi.ncm.gob/blast) by aligning the partial sequences with the 16S rDNA gene sequences of recognized species of the genus Bacillus obtained from the GenBank database. The 16S rDNA gene sequences of the species most closely related to our strains were retrieved from the database.

2.9. Data analysis

In order to determine significant differences in toxicity among the insecticidal activities, analysis of variance (ANOVA) was carried out using the SPSS 18.0 statistical software package. The results showed significant differences at the $P<0.01$ level.

3. Results

FD-48, FD-49, FD-50 and FD51 bacterial strains isolated from *Culex* sp., FD-16 and FD-17 strains isolated from larvae of *Yponomeutida evonymella* and FD-1 strain isolated from larvae of *Malacosoma neustria*are shown in Table 1. Also, the results of hypersensitivity test of all the strains on tobacco leaves, which were negative are shown in Table 1. According to the MIS and BIOLOG identification results of tested bacterial strains, *Bacillus sphaericus* GC subgroup D/ *Bacillus sphaericus* (strain FD-48), *Bacillus sphaericus* GC subgroup D/ *Bacillus sphaericus* (strain FD-49),*Bacillus thuringiensis* var. *kurstakii*/*Bacillus thuringiensis* (strain FD-50) and *Bacillus thuringiensis* var. *kurstakii*/*Bacillus thuringiensis* (strain FD-51), were identified as *Bacillus thuringiensis* var. *kurstakii*/*Bacillus thuringiensis* (strain FD-16) and *Bacillusatrophaeus*/*Bacillus licheniformis* (strain FD-17), identified as *Brevibacillus brevis*/ *Bacillus subtilis* (strain FD-1)are shown in Table 2.

The insecticidal effects of bacterial strains on *Diprion pini* are shown in Table 3. Some of these strains are toxic and have a significant insecticidal effect on *Diprion pini*. According to the results of ANOVA, the insecticidal effect was significant ($P< 0.01$) (Table 3). On the first day, the high mortality rate (53.3%) was observed from positive control (1% Kingbo). No mortality were observed with negative control (TSB) and all of the bacterial strains. On the 4th day, the highest mortality rates were observed from positive control (1% Kingbo) and the lowest mortality rates were observed negative control. Also, the FD-16 strain caused mortality but the mortality rate of FD-16 were not different statistically from the negative control. The mortality rates of *Bacillus atrophaeus* strain FD-17 and *Brevibacillus brevis* (FD-1) ranged from 33.3 to 63.3% on the fourth day. Their insecticidal activities were different from the negative control. On the 4th day, the mortality level of *Bacillus atrophaeus* strain FD-17 in the bacterial applications had the highest rate. But, its activity was lower than that of positive control (1% Kingbo). On the 7th day, two of the bacterial applications showed insecticidal activity ranged from 66.7 to 80% and mortality rate of *Bacillus atrophaeus* strain FD-17 was statistically indifferent from that of positive control (1% Kingbo). Also on the fourth day and the seventh day strains of FD-48, FD-49, FD-50, FD-51 and FD-16 insecticidal activities on *Diprion pini* were not statistically different from the negative control. As a result of, the highest mortality rates were observed from *Bacillus atrophaeus* strain FD-17 and *Brevibacillus brevis* strain FD-1. The insecticidal activities of these strains were significantly different from the negative control but *Bacillus atrophaeus* strain FD-17 were not different from the positive control(1% Kingbo).

Profiles of fatty acid methyl esters (FAMEs) of effective strains are presented in Table 4. According to the obtained results, the fatty acids in the strains are almost the same and low differences were determined among all of them. The 15;0 anteiso fatty acid which is found to be in large amounts in both strains, i.e. 55.9% in FD-17 strain and 50.61% in FD-1. FD-17 and FD-1 strains'17;0 antesio, 15;0 iso and 16;0 iso fatty acids containing 17.45%-13.17%, 8.88%-17.53% and 6.17%-3.66%, respectively. Additionally, these two strains contained some of other fatty acids in different but closer proportions.

The results of carbon utilization of effective strains are presented in Table 5. According to these results, FD-17 strain utilized from 21 and FD-1 from 19 of 95 different carbon resources. In both strains, 13 different carbon resources are used, which are Dextrin, D-Fructose, α-D-Glucose, Maltotriose, 3-Methyl-Glucose, β-Methyl-D-Glucoside, Palatinose, Salicin, Sucrose, D-Trehalose, Turanose, Methyl-Pyruvate and Pyruvic Acid.

As a result of 16S rDNA sequence analysis the strains were identified to the species level and results were compared with the data in the gene bank blast analysis. FD-1 and FD-17 strains have been identified as *Bacillus atrophaeus*, a similarity value of 99% was determined.

Table 1. Bacterial strains isolated from pests and the results of the hypersensitivity test (HR)

Strain no	Isolated from hosts	HR
FD-1	Larvae of *Malacosoma neustria*	-
FD-16		-
FD-17	Larvae of *Yponomeutida evonymella*	-
FD-48		-
FD-49		-
FD-50	*Culex* sp.	-
FD-51		-

-: Hypersensitivity test result was negative on tobacco plant.

Table 2. MIS and BIOLOG identification results of bacterial strains

Strain no	MIS results	SI%	BIOLOG results	SI%
FD-1	*Brevibacillus brevis*	62	*Bacillus subtilis*	34
FD-16	*Bacillus thuringiensis* var. *Kurstakii*	80	*Bacillus thuringiensis*	40
FD-17	*Bacillus atrophaeus*	45	*Bacillus licheniformis*	34
FD-48	*Bacillus sphaericus* GC subgroup D	59	*Bacillus sphaericus*	53
FD-49	*Bacillus sphaericus* GC subgroup D	68	*Bacillus sphaericus*	55
FD-50	*Bacillus thuringiensis* var. *kurstakii*	42	*Bacillus thuringiensis*	46
FD-51	*Bacillus thuringiensis* var. *kurstakii*	37	*Bacillus thuringiensis*	42

SI: similarity index

Table 3. The mortality rate of *Diprion pini* after bacterial application measured after 1, 4 and 7 days under laboratory conditions.

Applications of bacteria	Mortality rate					
	First day		Fourth day		Seventh day	
FD-1 *Brevibacillus brevis*	0.0	a	33.3	b	66.7	b
FD-16 *Bacillus thuringiensis* var. *kurstakii*	0.0	a	0.33	a	1.0	a
FD-17 *Bacillus atrophaeus*	0.0	a	63.3	c	80.0	bc
FD-48 *Bacillus sphaericus* GC subgroup D	0.0	a	0.0	a	0.0	a
FD-49 *Bacillus sphaericus* GC subgroup D	0.0	a	0.0	a	0.0	a
FD-50 *Bacillus thuringiensis* var. *kurstakii*	0.0	a	0.0	a	0.0	a
FD-51 *Bacillus thuringiensis* var. *kurstakii*	0.0	a	0.0	a	0.0	a
Control (+) Kingbo %1	53,3	b	100.0	d	10.0	c
Control (-)TSB	0.0	a	0.0	a	0.0	a

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

Table 4. Profiles of fatty acid methyl esters (FAMEs) of effective strains

Fatty acids	FD-1	FD-17
14:0 iso	1,58	2,27
15:0 iso	17,53	8,88
15:0 anteiso	50,61	55,9
16:00	2,21	3,93
16:0 iso	3,66	6,17
16:1 w7c alcohol	1,03	0,73
16:1 w11c	1,04	0,75
17: 0 iso	4,26	3,32
17:0 anteiso	13,17	17,45
18:00	0,46	0,6
Summed future	2,16	0
Others	2,29	0

***Table 5.* Profiles of BIOLOG of effective strains**

Strains	Wells	1	2	3	4	5	6	7	8	9	10	11	12
FD-1	A	0	-	-	+	-	-	-	-	-	-	-	-
FD-17		0	-	+	+	-	-	-	-	-	+	-	-
FD-1	B	-	-	+	-	+	-	-	-	-	-	+	-
FD-17		-	-	-	+	+	-	-	-	-	-	+	-
FD-1	C	-	-	-	+	-	+	-	-	-	-	+	-
FD-17		-	-	-	+	-	-	-	-	-	-	+	-
FD-1	D	+	-	+	+	-	-	-	+	-	-	-	+
FD-17		+	-	+	-	-	-	+	+	-	-	-	+
FD-1	E	-	+	+	-	-	-	-	-	-	-	-	-
FD-17		-	+	+	-	-	-	-	-	-	-	-	-
FD-1	F	-	-	-	-	+	+	-	-	+	-	-	-
FD-17		-	-	-	-	-	+	-	-	+	-	-	-
FD-1	G	-	-	-	-	-	-	-	-	-	-	-	-
FD-17		-	-	-	-	-	-	-	-	-	-	+	+
FD-1	H	-	-	-	-	-	-	-	-	-	-	-	-
FD-17		+	+	+	+	+	-	-	-	-	-	-	+

*BIOLOG GP2 MicroPlate: **A1:** water, **A2**: α-cyclodextrin, **A3**: β-Cyclodextrin**A4**: Dextrin **A5**: glycogen **A6**: Inulin **A7**: Mannan, **A8**: Tween 40, **A9**: Tween 80, **A10**: N-acetyl-D-glucosamine, **A1**1: N-acetyl-D-Mannosamine, **A12**: Amygdalin, **B1**: L-Arabinose **B2**: D-arabitol, **B3**: Arbutin **B4**:D-Cellobiose, **B5**: D-Fructose, **B6**: L-Fucose, **B7**: D-galactose, **B8**: D-galacturonic acid, **B9**: Gentiobiose, **B10:** D-gluconic acid, **B11**: α-D-Glucose**,** **B12**: m-Inositol, **C1**: α-D-Lactose **C2**: Lactulose, **C3**: Maltose **C4**: Maltotriose **C5**: D-Mannitol **C6**: D-Mannose, **C7**: D-Melezitose **C8**: D-Melibiose, **C9**: α-Methyl D-Galactoside, **C10**: β-Methyl-D-Galactoside, **C11**: 3-Methyl-Glucose, **C12**: α-Methyl-D-Glucoside, **D1**: β-Methyl-D-Glucoside, **D2**: **α-Methyl-D-Mannoside**, **D3**: Palatinose, **D4**: D-Psicose, **D5**: D-Raffinose, **D6**: D-Rhamnose, **D7:** D-Ribose **D8:** Salicin, **D9:** Sedoheptulosan , **D10:** D-Sorbitol, **D11:** Stachyose, **D12:** Sucrose, **E1:** D-Tagatose, **E2:** D-Trehalose, **E3:** Turanose, **E4:** Xylitol, **E5:** D-Xylose, **E6:** Acetic AcidD, **E7**: α-Hydroxy Butyric Acid, **E8**: β-Hydroxy Butyric Acid, **E9**: γ-Hydroxy Butyric Acid, **E10:**p-Hydroxy Phenyl Acetic Acid, **E11** α-Keto Glutaric Acid, **E12**: α-Keto Valeric Acid, **F1:** Lactamide, **F2:**D-Lactic Acid Methyl Ester, **F3:** L-Lactic Acid, **F4:** D-Malic Acid, **F5:** L-Malic Acid, **F6:** Methyl-Pyruvate, **F7:** Mono-methyl Succinate, **F8:** Propionic Acid, **F9:** Pyruvic Acid, **F10:** Succunamic Acid, **F11:** Succinic Acid, **F12:**N-Acetyl L-Glutamic Acid, **G1:** L-Alalinamide, **G2:** D-Alanine , **G3:** L-Alaline, **G4:** L-Alanyl-glycine, **G5:** L-Asparagine, **G6:** L-Glutamic-Acid, **G7:** Glycyl- LGlutamic Acid, **G8:** L-Pyroglutamic Acid, **G9:** L-Serine, **G10:** Putrescine, **G11:** 2,3-Butanediol, **G12:** Glycerol, **H1:** Adenosine, **H2:**2'-Deoxy Adenosine, **H3:** Inosine, **H4:** Thymidine, **H5:** Uridine, **H6:**Adenosine-5'- Monophosphate, **H7:**Thymidine-5'-Monophosphate, **H8:**Uridine-5'-Monophosphate, **H9:**Fructose-6- Phosphate, **H10:**Glucose-1-Phosphate, **H11:** Glucose-6-Phosphate, **H12:**D-L-α-Glycerol Phosphate

4. Discussion and Conclusion

In this study, 7 bacterial strains in total were identified as *Bacillus* and *Brevibacillus* species on the basis of FAME analysis and carbon source utilization profiles by using MIS and BIOLOG. When the MIS identification results of the tested bacterial strains were compared with the BIOLOG identification results, MIS results of all strains were confirmed by BIOLOG at the species level and only strain FD-1 was different from others in the genus level. These results showed that FAME and BIOLOG analysis are appropriate phenotypic

methods for the discrimination of *Bacillus* strains at genus level, but not at species level. As a result, it can be concluded that FAME and BIOLOG profilings may be useful for the characterization of *Bacillus* strains at genus level. Similar findings related to the FAME and BIOLOG profiles of *Bacillus* have been reported in the literature (13,18).

Conventionally, *Bacillus* species have been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (19, 20). These are complex and labor intensive procedures. However, the scarcity of reproducible and distinguishable phenotypic characteristics for several bacterial species often makes difficult a precise identification. So far, the development of gene amplification and sequencing, especially that of the 16S rRNA gene sequences, has simplified the identification and the detection of specific bacteria (21, 22), especially those lacking distinguishable phenotypic characteristics. The 16S rRNA gene is now used as a framework for the modern classification of bacteria including those in the genus *Bacillus* (23). Therefore, 16S rDNA sequence analysis for characterisation of strains are preferred. According to these results, FD-1 and FD-17 strains have been identified as *Bacillus atrophaeus*.

The best-known example of an entomopathogenic bacterium is *Bacillus* genus (24). In this study, 7 *Bacillus* sp. strains isolated from insects have been tested for their potential to be used for the biological control of insect pests. The *Bacillus*-based biological control agents (BCAs) are among the best-known, most widely developed and safe alternatives against insect pests (25). It can be stated that 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. Use of these insecticides is relatively easy and provides an effective pest control; it is likely that they will always be a component of pest management programs. Unfortunately, insecticides have some undesirable attributes. They usually present to a degree a hazard to applicator and other people who may come in contact with them; they can leave residues that some find unacceptable; they can contaminate soil and water and affect wildlife, aquatic life, and other nontarget organisms; they can interfere with beneficial organisms, such as pollinating insects and natural enemies of pests; and insects can develop resistance to insecticides, effectively eliminating those materials as pest management options (26, 27, 28). Therefore, considering the deleterious effects of insecticides on life supporting systems, there is an urgent need to search for alternative approaches for the management of pests. The use of microorganisms biocontrol agents is one of the first choices for pest control (28, 29).

We observed that *Bacillus atrophaeus* strains FD-17 and FD-1 were the most effective strains. Generally, the insecticidal activities of tested bacterial strains on the seventh day were higher than that registered on first and fourth day. We consider that some strains need more time to adapt to the environmental conditions, and 24 or 48 h probably were not enough to produce spores. The results of experimental infections are very promising. Therefore, these strains successfully can be used on the biological control against *Diprion pini* as potential environment-friendly, biocontrol agents risk free for human health and other livings products in place of hazardous chemical pesticides.

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