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Full Length Research Paper

Studies on bacterial flora and biological control agent of *Cydia pomonella* L. (Lepidoptera: Tortricidae)

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In the present study, in order to find a more effective and safe biological control agent against *Cydia pomonella*, we investigated the bacterial flora and tested them for insecticidal effects on this insect. According to morphological, physiological and biochemical tests, bacterial flora were identified as *Proteus rettgeri* (Cp1), *Eschericia coli* (Cp2), *Pseudomonas stutzeri* (Cp3), *Pseudomonas aeroginosa* (Cp4), *Bacillus laterosporus* (Cp5), *Micrococcus sp.* (Cp6), *Proteus vulgaris* (Cp7) *and Deinococcus sp.* (Cp8). Insecticidal effects of bacterial isolates were performed on larvae *C. pomonella.* The highest insecticidal effect determined was 65 % by *Bacillus laterosporus* within eight days. The insecticidal effects of the other isolates (Cp1, Cp2, Cp3, Cp4, Cp6, Cp7 and Cp8) we determined as 22, 19, 25, 60, 20, 57 and 17% within the same period, respectively. No mortality was detected on control groups.

Key words: *Cydia pomonella*, bacterial flora, biological control.

INTRODUCTION

The codling moth (*Cydia pomonella* L. Lepidoptera: Tortricidae) is an important cosmopolitan pest of apple other fruits in Turkey (The Ministry of Agriculture of Turkey, 1995). This pest damages the apple leaves during spring and summer. It causes approximately 100-60% economic damage particularly on apple production per year in Turkey (The Ministry of Agriculture of Turkey, 1995). Up till now, chemical substances (such as Carbaryl 85 (WP), Etrimfos 520 g/l (EC), Phosalone, 350 g/l E.C., Phosalone 30 (WP) and Triflumuro 25 W.P.) have been utilized to control this pest (The Ministry of Agriculture of Turkey, 1995). However, recent concern about the apple effect of chemical pesticides on the environment has encouraged scientists to consider finding more effective and safer control agents. In the search for safer and more lasting methods, researchers 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. 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 and Frachon, 1997; Sezen et al., 1999; Yaman et al., 2000a,b).

Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling *C. pomonella* Surprisingly, despite their mass occurrence and wide distribution, very little is known about the bacterial pathogens limiting their population. In addition, nothing is known about what its bacterial 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.

MATERIALS AND METHODS

Collection of insects

Larvae of *Cydia pomonella* were collected from the vicinity of Amasya, Kayseri, Ordu, Trabzon and Gümüşhane in Turkey from

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Table 1. The morphological characteristics of bacterial isolates.

Isolate Number	Cp1	Cp2	Cp3	Cp4	Cp5	Cp6	Cp7	Cp8
Color and Shape of colonies	Cream rough concentric	White, round, concentric	Cream, round, concentric	Cream, round, concentric	Cream rough, concentric	Light Cream smooth, rough	Cream rough, concentric	Yellows mooth, round
Gram Stain	-	-	-	-	+	+	-	+
Shape of Bacteria	Bacillus	Bacillus	Bacillus	Bacillus	Bacilli	Micrococci	Bacillus	Bacillus
Spore Stain	-	-	-	-	+	-	-	ND[2]
Spore Shape	ND	ND	ND	ND	Terminal	ND	ND	ND
Spore Form	ND	ND	ND	ND	Ellipsoid	ND	ND	ND
Length (µm)	1.3-2.6	1.7-2.1	1.4-1.6	1.4-1.7	2.2-3.1	0.9	1.4-2.2	ND
Wideness (µm)	0.5-0.7	0.7-0.9	0.7-0.9	0.7-1.0	0.6-0.8	ND	0.5-0.9	1.5-2.5
Turbidity When Grown in NB[1]	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Clear
Motility	+	+	+	+	+	-	+	-
Anaerobic Growth	+	+	+	+	+	+	+	+

[1]NB: Nutrient broth.
[2]ND: No data.

June to July 2000. Insects were collected from leaves of apple trees.

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 30ºC 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, 3, 5, 7 and 10% NaCl) and heat (grown at 28, 30, 37, 41 and 50ºC). We also determined whether bacterial isolates produce various enzymes and products by plating on media including different special features (Palleroni, 1986; Sneath, 1986; Thiery and Frachon, 1997).

Isolation of spore-forming bacteria

After suspension was prepared as explained above, it was heated at 80ºC 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 30ºC 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 third-fourth instar larvae of *C. pomonella* were used for the insecticidal assay of bacterial isolates. Isolates were incubated for 18 h (72 h for *Bacillus* to sporulation) at 30ºC in nutrient broth. After incubation, 5 mL of culture was centrifuged at 3000 rpm for 10 min (Ben-Dov et al., 1995). The pellet was resuspended in 5 mL of sterilized PBS. The density of cells was set at 1.89 at OD_{600} and used for bioassays (Moar et al., 1995). Specific diet cultures prepared for *C. pomonella* (20 g. corn flour, 15 g. bran, 25 g yeast, 5 g ascorbic acid 2 g and benzoic acid are mixed in 850 mL sterilized water). It was used in bioassays as diet. The diet was contaminated with prepared bacterial isolates and was placed into individual glass containers (80 mm in diameter). Ten healthy third-fourth instar larvae were placed into each container and larvae received diet contamined with bacterial suspensions for 24 h. and then fresh diet every 24 h. Containers were kept at 26 ± 2ºC and 60% (relative humidity, RH) on a 12:12 h photoperiod (Lipa et al., 1994). The mortality of insects was recorded every 24 h and all dead insects were removed from containers. Because mortality was not notice after eight days post infection, bioassays were finished at 8th day, and living insects were obliterated. Data were evaluated by using Abbott's formula (Abbott, 1925).

RESULTS

We selected and characterized six bacterial isolates from *C. pomonella* by color and shape of colonies, spore formation and nutritional features (Table 1). Cp1, Cp5 and Cp7 colonies were cream, concentric and Cp3 and Cp4 colonies were cream, concentric and Cp2 colony was white, concentric Cp6 colony was light cream, smooth and Cp8 colony was yellow, round. While the shape of three colonies (Cp2, Cp3 and Cp4) was round, Cp1, Cp5 and Cp7 were rough, and the others (Cp6 and

Table 2. Physiological and biochemical characteristics of the bacterial isolates.

Isolate Number	Cp1	Cp2	Cp3	Cp4	Cp5	Cp6	Cp7	Cp8
Nitrate reduction	+	+	-	+	+	-	+	+
Catalase test	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
Indole test	-	-	+	-	-	-	+	ND
Metyhl red test	+	-	+	-	-	-	+	+
Voges proskauer test	+	-	+	-	-	-	-	+
H_2S production	-	-	+	+	-	-	+	+
Hydrolysis of gelatine	-	-	W	+	-	+	+	-
Hydrolysis of urea	-	-	+	-	+	-	+	+
Citrate utilisation	-	W	+	+	-	-	+	+
Propionate utilisation	-	W	+	+	-	-	+	+
Growth with lysozyme	ND	ND	ND	ND	+	-	ND	ND
Utilisation of L-arginine	-	+	-	+	ND	ND	-	-
Kligler iron agar	W	+	+	+	-	ND	+	+
Starch test	ND	ND	+	-	-	-	-	-
Glucose fermentation	+	+	+	+	-	+	+	+
Arabinose fermentation	ND	+	ND	-	-	-	ND	ND
Xylose fermentation	ND	W	ND	-	-	W	ND	ND
Mannitol fermentation	+	+	+	+	+	+	+	+
Lactose fermantation	ND	+	ND	W	ND	ND	-	+
Sucrose fermentation	ND	+	ND	+	ND	ND	+	+
Growth in 2% NaCl	+	+	+	+	ND	+	+	+
Growth in 3% NaCl	+	+	+	+	+	+	+	+
Growth in 5% NaCl	W	+	+	+	+	+	ND	+
Growth in 7% NaCl	-	+	W	-	W	+	ND	+
Growth in 10% NaCl	-	-	W	-	-	-	ND	W
Growth at 28ºC	+	+	+	+	+	+	+	+
Growth at 30ºC	+	+	+	+	+	+	+	+
Growth at 37ºC	+	+	+	+	+	+	+	+
Growth at 41ºC	-	W	+	+	-	+	-	-
Growth at 50ºC	-	-	-	-	-	-	-	-
Optimum pH 5.7 Growth	+	-	-	+	+	-	ND	W
Growth at pH>7 MVRPbroth	ND	W	+	+	-	ND	ND	ND
Growth at pH<6 MVRP broth	ND	-	W	+	+	ND	ND	W
Optimum Growth (ºC)	37	37	35	37	37	35	32	37

[1]W: Weak growth
[2]ND: No data.

Cp8) were smooth. Cp6 and Cp8 isolates were micrococci, Cp5 isolate is Bacilli and the other isolates were Bacilli, turbid and motile. Three isolates (Cp5, Cp6 and Cp8) were Gram positive and the others (Cp1, Cp2, Cp3, Cp4 and Cp7) were Gram negative (Table 1). Enrichments and purification procedures carried out with larvae of *C. pomonella* allowed the isolation of one spore forming isolate (Cp5). The shapes of the spore (Cp5) were ellipsoid. Form of spore was terminal and central in Cp5.

Physiological and biochemical characteristics of isolates are indicated in Table 2. The optimal growth of the isolates was at pH 6-7 and 32ºC. The insecticidal effects of the isolates on *C. pomonella* larvae are shown in Figure 1. The highest insecticidal effect determined was 65% by *Bacillus laterosporus* within eight days. The insecticidal effects of the other isolates (Cp1, Cp2, Cp3, Cp4, Cp6, Cp7 and Cp8) were determined as 22, 19, 25, 60, 20, 57 and 17%, respectively, within the same period. No mortality was determined on control groups.

DISCUSSION

This is the first study on the bacterial isolation and biological control agent of *C. pomonella* a common pest

of apple. We isolated and determined eight bacterial isolates from this pest. According to preliminary observations and tests, we determined that bacterial flora of *C. pomonella* consists of *Proteus rettgeri* (Cp1), *Eschericia coli* (Cp2), *Pseudomonas stutzeri* (Cp3), *Pseudomonas aeroginosa* (Cp4), *Bacillus laterosporus* (Cp5*), Micrococcus* sp. (Cp6), *Proteus vulgaris* (Cp7) and *Deinococcus* sp. (Cp8). Hundreds of bacterial species have been associated with insects (Deacon 1983). It is known that many bacteria which can be isolated from insects belongs to genera *Bacillus*, *Enterobacter, Streptococcus* and *Pseudomonas* (Tana-da and Kaya, 1993; Bucher, 1981). In all bioassays, the highest insecticidal infectivity determined on *C. pomonella* larvaes was 65% with by *B. laterosporus* (Cp5). The result of bioassays indicated that all isolates (spore-forming and non-spore-forming) are pathogenic at different ratios on the pests (Figure 1).

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). Yaman and Demirbağ (2000a) isolated *B. thuringiensis* from *Hyphantria cunea* and indicated that it has approximately insecticidal effect on *Gypsonoma dealbana*, *Euproctis chrysorrhoea*, *Melolontha melolontha* and *Agelastica alni*. Esters (1996) indicated that effects of each *B. thuringiensis* strains are different on different insect species, because there is some evidence of different enzymes released. 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 be established themselves in the haemocoel if they have enough time to pass through the wall and enter susceptible cells.

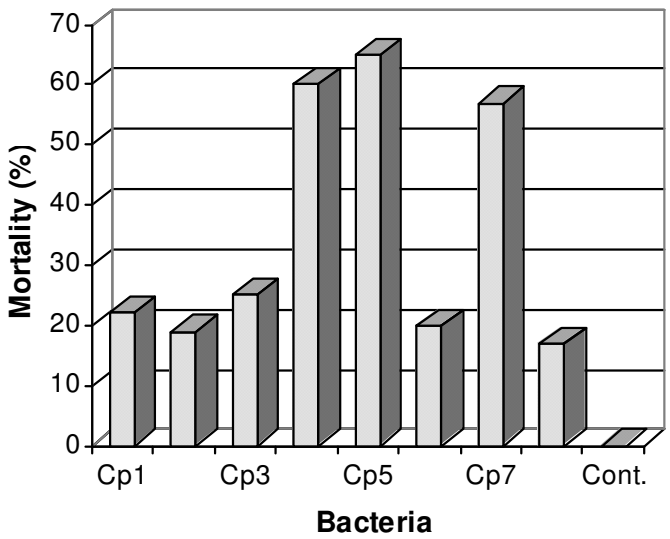

Figure 1. The insecticidal effects of bacterial isolates from *C. pomonella* on larvaes of this pest within eigth days. Cp1, *Proteus rettgeri*; Cp2, *Escherichia coli*; Cp3, *Pseudomonas stutzeri*; Cp4, *Pseudomonas aeroginosa*; Cp5, *Bacillus laterosporus*; Cp6, *Micrococcus* sp.; Cp7, *Proteus vulgaris*; Cp8, *Deinococus* Sp. and Control.

This is a very important result for the biological control for *C. pomonella,* because so far nothing has been known about its natural bacterial enemies. *Pseudomonas aeroginosa, Bacillus laterosporus* and *Proteus vulgaris* can be used as biological control agent for *C. pomonella.* However, further research will be directed to improve the insecticidal effect of this biological control agent for this pest using recombinant techniques.

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