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Discovery and Development of a Biological Agent to Control of *Ricania simulans* (Hemiptera: Ricaniidae)

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Abstract

Ricania simulans (Hemiptera: Ricaniidae) is a serious pest present in several countries throughout the world including Turkey, China, Japan and Taiwan. This study investigated the bacterial flora of *R. simulans*, and tested them for the insecticidal activity in order to find an effective and safe biocontrol agent for this pest. Based on conventional and modern tests, twelve bacterial isolates were identified from the microbial flora of *R. simulans*; *Pseudomonas oleovorans* (Rs1), *Pseudomonas parafulva* (Rs2, Rs3 and Rs5), *Pseudomonas sp*. (Rs4, Rs7, Rs9), *Pantoea sp.* (Rs6), *Microbacterium paraoxydans* (Rs8), *Bacillus safensis* (Rs10), *Chryseobacterium indoltheticum* (Rs11) and *Bacillus thuringiensis* (Rs12). The insecticidal activities of the bacterial isolates were determined on nymphs at five different concentrations (1.8 X 10^9 bacteria/mL, 1.8 X 10^8 bacteria/mL, 1.8 X 10^7 bacteria/mL, 1.8 X 10^6 bacteria/mL, 1.8 X 10^5 bacteria/mL), and on adults with different three concentrations (1.8 X 10^9 bacteria/mL, 1.8 X 10^8 bacteria/mL, 1.8 X 10^7 bacteria/mL) for 10 days. The highest insecticidal activity was determined with Rs4 on nymphal stage of *R. simulans* by 82% killing rate within 10 days. *Bacillus thuringiensis tenebrionis* (Xd3), *Bacillus thuringiensis kurstaki* (MnD), *Bacillus thuringiensis kurstaki* (BnBt), and *Serratia marcescens* (BnSm), which were proven effective biocontrol agents our previous studies, were also tested against this pest. BnSm showed the highest insecticidal activity with 93% killing on adult and nymph stages of *R. simulans*. These results indicate that Rs4 and BnSm isolates can be effective as potential biological control agents for the control of *R. simulans*.

Keywords: *Bacterial flora*, insecticidal activity, microbial control, *Ricania simulans*

INTRODUCTION

Ricania simulans, Ricaniidae family (Order: Hemiptera), is a polyphagous pest widely distributed in Turkey, China, Japan, and Taiwan around the world [39]. *R. simulans* is the most widespread and harmful pest in tea fields in the Black Sea Region of Turkey. This pest becomes more serious due to its increased population in Turkey since 2009. Its harm is not only limited to wild plants but also agricultural species, such as tea, bean, grapevine, cucumber, elderberry, wild blackberry, cherry laurel, fig, and kiwi in Eastern Black Sea Region [2]. Up to now, no effective control mechanism has been able to utilized against this pest except mechanical control. Fortunately, no chemical pesticide has been used to control this pest in Turkey [28]. Therefore, an effective biocontrol mechanism should be developed to keep this pest below the economic injury level (EIC).

Increasing global mobility also causes to spread harmfull and invasive pest species around the world. Integrated pest management (IPM) system aims to develop new practices to reduce this global risk and its economic consequences. These practices contain more than one control tactics to monitor pests and their natural enemies [6].

Biological pesticides, such as pheromones and microbial control agents, are known safer than traditional chemical pesticides. In addition, utilization of bacteria as microbial control agents is a well accepted approach in agricultural pest management among biological control methods. Developing an effective biocontrol agent against *R. simulans* will help to increase the yield and quality of agricultural products, especially tea, which is one of the most important cultivated plant for the region. Therefore, in this study we aimed to discover and utilize a bacterial agent or agents to use against the implied pest as a safe microbial pesticide. For this purpose, the culturable bacterial flora of the pest was isolated and their insecticidal activities were studied.

MATERIAL and METHODS

Collection of insects

Adults and nymphs of *R. simulans* were collected from Trabzon, Turkey during three months period, June, July, and August of 2013. Collected nymphs and adults were brought to the laboratory and fed with vine leaf in plastic containers with ventilated lids at room temperature under 12:12 photoperiod.

Isolation of bacteria from *R. simulans*

Insect larvae and adults were examined macroscopically for infections, and the surface of healthy insects were sterilized in 70% alcohol for 1.5 to 2 mins and then washed in sterile distilled water [29]. For microbial flora isolation, three biological replicates were used, each replicate having 20 insects. Both larvae and adults were homogenized in a nutrient broth by using sterilized glass tissue grinder. The suspensions were filtered twice through two layers of cheese cloth to remove coarse insect body debris [29]. Then, the suspensions were diluted up to 10^{-8} ml and 100 µl of diluted samples were spread on nutrient agar plates. These plates were incubated at 28°C for 48 h - 96 h. Additionally, bacterial suspensions were heated at 80°C for 10 min in a hot water bath with shaking at 200 rpm to eliminate the non-spore forming bacteria [38]. Heat-treated samples were diluted and plated in the same way as described for normal bacterial flora previously [23].

Identification of bacteria from *R. simulans*

The isolates were identified with microbiological (morphologically, physiologically) and biochemical (macromolecule detection) methods, according to *Bergey's Manual of Systematic Bacteriology*, volume 1 and 2 [20]. Cell morphology and motility were observed by light microscopy of native preparation. Gram [11] and endospore

[30] staining was performed according to previous studies [5]. All bacterial isolates were identified by various tests, such as the utilization of organic compounds, spore formation, Gram staining, NaCI tolerance, growth in pH, catalase and oxidase tests [35].

API test strips were used following the manufacturer's directions (BioMerieux, France). Bacterial colonies of each isolate were diluted in 0.85% NaCI solution, then the amount of bacteria was adjusted to 1McFarland standard turbidity. 200 µL of the bacterial suspensions was transferred into each well of API test strips. The wells were filled up with mineral oil. Then, the strips were incubated for 18-24 h at 30 °C.

Sequence analysis of 16S rRNA genes

Besides these conventional laboratory methods, the isolates were also identified at genetic level by sequencing their 16S ribosomal RNA genes. Genomic DNA extraction was performed by Sambrook [31]. DNA pellets were re-suspended in 10 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and these pellets were stored at 4ºC. PCR was performed by using BioRad MyCycler Thermal Cycler (California, US). The following primer pair was used to amplify the 16S rRNA genes from each isolates genomic DNA: UNI16SR (5'- ATG GTA CCG TGT GAC GGG CGG TGT GTA-3') and UNI16SF (5'- ATT CTA GAG TTT GAT CAT GGC TCA-3') [41]. PCR reaction mixture included 200 ng template DNA, 1 µl of 10 mM of each primer, 1 µl of 10 mM of dNTP, 3 µl of 25 mM $MgCI_2$, 5 µl of 10X enzyme without $MgCI_2$ buffer, 22.5 µl sterile dH_2O and 5 units Taq DNA polymerase (Promega, Madison,USA) in total of 50 µl volume. The PCR reaction was started with an initial denaturation step for 2 min at 95 °C; and followed by 35 cycles of denaturation for 1 min at 94 °C; annealing for 1 min at 56 °C; extension for 2 min at 72 °C; and the last annealing 5 min at 72 °C. PCR products were analyzed by electrophoresis with 1% agarose gel and visualized with EtBr staining in BioDocAnalyse System (Biometra GmbH, Goettingen, Germany). Each PCR product was cloned into pGEM-T Easy Vectors (Promega, Madison, USA) and then transformed into *E. coli* JM101 strain with electroporation. Recombinant plasmid vectors, which were isolated from selected colonies, were digested with *Eco*RI and the plasmid that carried approximately 1400 bp of fragments was sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing. Sequences were analyzed from NCBI GenBank using the BLAST alignment software [3].

Cluster analyses of 16S rRNA sequences

Evolutionary relationships of isolates and closely related species were analyzed using 16S rRNA gene sequences which are approximately 1400 bp. Sequences were collected and edited with BioEdit (version 7.09) and aligned with ClustalW [15]. The reliability of the dendrograms were tested by bootstrap analysis with 1,000 replicates using MEGA 4.0 [37].

Insecticidal effects of bacterial isolates

Fresh bacterial isolate cultures were prepared for insecticidal activity assay against *R. simulans* nymphs and adults. Each bacteria was inoculated into 5 ml of nutrient broth at a cell concetration of 0.05 OD_{600} and incubated at 30 °C with 200 rpm shaking for 18 h. These overnight (O/N) cultures were taken and the cell density was adjusted to 1.89 OD_{600} (1.8 X 10^9 CFU/mL approximately). Then, bacterial samples were centrifuged at 3.000 rpm for 10 min to pellet the cells and suspended in 5 mL of sterilized phosphate buffer solution (PBS). These cell suspensions were serially diluted to 1,8 X 10^8, 1,8 X 10^7, 1,8 X 10^6 and 1,8 X 10^5 CFU/mL to be tested on nymph stage of *R. simulans*. The same procedure was applied three times (1.8 X 10^9, 1.8 X 10^8 and 1.8 X 10^7 CFU/mL) for adult stage. PBS was used as negative control.

In addition to bacterial isolates from *R. simulans*'s microbial flora, some other important biocontrol agents; *Bacillus thuringiensis tenebrionis* (Xd3), *Bacillus thuringiensis kurstaki* (MnD), *Bacillus thuringiensis kurstaki* (BnBt) and *Serratia marcescens* (BnSm), obtained from KTU Microbiology laboratory, were also tested in a same way against this pest.

Bacterial samples with preadjusted cell concentrations were sprayed on fresh mock vine leaves then nymph and adult insect stages were placed on the leaves in very well ventilated plastic containers. Bioassays were performed with ten nymphs for five different doses and using five adults for three different doses at room temperature. Mortality of the insects was examined and dead insects were removed everyday until the 10th day and all. Each experiment was performed three times under the same laboratory conditions. Bioassay results were compared with control group using Crosstabs test (SPSS for WINDOWS version 17.0).

RESULTS

After culturing the homogenates of the adults and nymphs of *Ricania simulans,* the study selected 16 bacterial for further identification based on the colony morphology. Some of the isolates, Rs5, Rs11, Rs13 and Rs16 were discarded due to the similarities to the other isolates. Twelve bacterial strains were cultured from *R. simulans* to identify. These bacterial strains were identified at both species and genus level with conventional and modern methods.

All of the isolates were detected as bacil. While isolate Rs10 and Rs12 were the color cream, the other isolates were yellow. Three bacterial strains (Rs8, Rs10 and Rs12) were Gram positive and the rest of them were Gram negative (Rs1, Rs2, Rs3, Rs4, Rs5, Rs6, Rs7, Rs9 and Rs11). Two of them (Rs10 and Rs12) formed spores. Morphological results are shown in details on Table 1.

After morphological tests, some physical and biochemical tests were performed for phenotypic characterization of bacterial strains (Table 2). Catalase activity was negative for Rs7, and oxidase activity was negative for isolates Rs1, Rs8 and Rs12. For all the other isolates, both catalase and oxidase activities were positive.

API 20 E and API 50 CHB systems were also used to characterize the bacterial strains isolatedly API 20 E test systems were used for biochemical characterization (Table 3). API 50 CHB test systems were used for Gram-positive bacteria (Table 4).

The study also sequenced approximately 1400 bp of 16S rRNA genes for each isolate to confirm isolate identification. According to all identification tests and sequencing, the study identified the isolates as *Pseudomonas oleovorans* (Rs1), *Pseudomonas parafulva* (Rs2, Rs3 and Rs5), *Pseudomonas* sp. (Rs4, Rs7 and Rs9), *Pantoea* sp. (Rs6), *Microbacterium paraoxydans* (Rs8), *Bacillus safensis* (Rs10), *Chryseobacterium indoltheticum* (Rs11) and *Bacillus thuringiensis* (Rs12). Moreover, phylogenetic

Table 1. Morphological characteristics of bacteria from *R.simulans*.

Codes	Colonyshape	Colonycolors	Cell shape	Gram (+/-)	Spore form
Rs1	Irregular	Yellow	Bacil	-	-
Rs2	Circular	Yellow	Bacil	-	-
Rs3	Circular	Yellow	Bacil	-	-
Rs4	Irregular	Yellow	Bacil	-	-
Rs5	Circular	Yellow	Bacil	-	-
Rs6	Circular	Yellow	Bacil	-	-
Rs7	Circular	Yellow	Bacil	-	-
Rs8	Circular	Yellow	Bacil	+	-
Rs9	Circular	Yellow	Bacil	-	-
Rs10	Irregular	Cream	Bacil	+	Central, +
Rs11	Circular	Yellow	Bacil	-	-
Rs12	Irregular	Cream	Bacil	+	Central, +

Table 2. Physical and biochemical tests of bacterial strains from *R.simulans*.

	NaCl Test			Temperature Test			pH Test						Biochemical Tests		
Codes	3%	5%	9%	30ºC	40ºC	50ºC	4	5	7	11	12	13	Catalase	Oxidase	Starch Hydrolysis
Rs1	+	+	-	+	+	-	-	+	+	+	-	-	+	-	-
Rs2	+	-	-	+	-	-	-	-	+	+	+	+	+	+	-
Rs3	+	-	-	+	_	-	-	-	+	+	+	+	+	+	-
Rs4	+	-	-	+	+	-	-	+	+	+	-	-	+	+	-
Rs5	+	-	-	+	-	-	-	-	+	+	+	+	+	+	-
Rs6	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-
Rs7	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-
Rs8	+	-	-	+	-	-	-	-	+	+	+	+	+	-	-
Rs9	+	-	-	+	-	-	-	-	+	+	+	+	Z+	+	-
Rs10	+	+	+	+	+	+	-	+	+	+	+	+	+	Z+	-
Rs11	+	-	-	+	-	-	-	-	+	-	-	-	+	+	-
Rs12	+	+	-	+	+	-	-	+	+	+	+	+	+	-	+

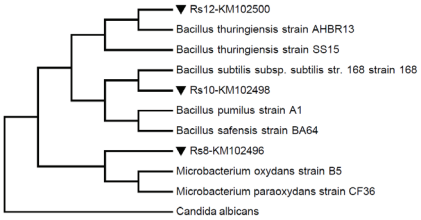

Figure 1. Maximum Likelihood Tree of Gram-positive bacterial isolates from *R. simulans* and their closely related bacterial species. Bootstrap values ≥70% was labeled. The scale on the bottom of the dendrogram showed the degree of dissimilarity.

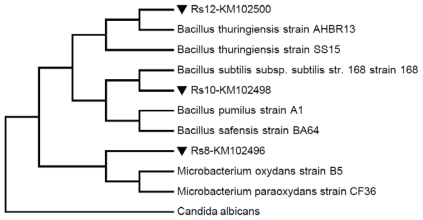

Figure 2. Maximum Likelihood Tree of negative Gram-bacterial isolates from *R. simulans* and their closely related bacterial species. Bootstrap values ≥70% was labeled. The scale on the bottom of the dendrogram showed the degree of dissimilarity.

Table 3. The results of API 20 E panel test system of bacteria from *R.simulans*.

Tests	Rs1	Rs2	Rs3	Rs4	Rs5	Rs6	Rs7	Rs8	Rs9	Rs10	Rs11	Rs12
ONPG	+	+	-	-	-	+	-	+	-	+	-	-
ADH	+	+	+	-	+	+	-	-	+	-	+	+
LDC	-	-	+	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-
CIT	+	+	+	+	+	-	+	+	+	+	+	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-	-
TDA	+	+	-	+	-	-	+	+	-	+	+	+
IND	-	-	-	-	-	-	-	-	-	-	+	-
VP	+	+	+	+	-	+	-	-	+	+	+	-
GEL	-	+	-	+	+	+	+	+	+	+	+	+
GLU	-	-	-	-	+	+	-	+	-	+	-	+
MAN	-	-	-	-	-	+	-	+	-	+	-	-
INO	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	+	-	-	-	-	-	-
SAC	-	-	-	-	-	+	-	+	-	+	-	+
MEL	-	-	-	+	-	+	+	-	+	-	-	-
AMY	-	-	-	-	-	+	-	+	-	+	-	+
ARA	+	-	-	+	-	+	+	+	+	+	-	-
OX	-	+	+	+	+	+	+	-	+	-	+	-
NO_2	-	+	+	-	+	+	-	-	+	-	-	-
N_2	+	-	-	-	-	-	+	-	-	+	+	+
MOB	-	-	+	-	-	-	-	-	-	-	-	-
McC	+	+	+	+	+	+	+	+	+	+	+	+
OF-O	+	+	+	+	+	+	+	+	+	+	+	+
OF-F	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. The results of API 50 CHB panel test system of bacteria from *R. simulans*.

Tests	Rs8	Rs10	Rs12
GLY	+	+	+
ERY	-	-	-
DARA	-	-	-
LARA	+	+	-
RIB	+	+	+
DXYL	+	+	-
LXYL	-	-	-
ADO	-	-	-
MDX	-	-	-
GAL	+	+	-
GLU	+	+	+
FRU	+	+	+
MNE	+	+	-
SBE	-	+	-
RHA	-	-	-
DUL	-	-	-
INO	-	-	-
MAN	+	+	-
SOR	-	-	-
MDM	-	+	-
MDG	-	+	-
NAG	+	+	+
AMY	+	+	-
ARB	+	+	+
ESC	+	+	+
SAL	+	+	-
CEL	+	+	+
MAL	+	+	+
LAC	-	+	-
MEL	-	-	-
SAC	+	+	+
TRE	+	+	+
INU	-	-	-
MLZ	-	-	-
RAF	-	-	-
AMD	-	-	-
GLYG	+	-	+
XLT	-	-	-
GEN	-	+	-
TUR	+	+	-
LYX	-	-	-
TAG	-	+	-
DFUC	-	-	-
LFUC	-	-	-
DARL	-	-	-
LARL	-	-	-
GNT	-	-	-
2KG	-	-	-
5KG	-	-	-

analysis of 16S rRNA genes also supported this identification (Figure 1-2).

The study also tested the insecticidal activity of the bacterial isolates, which were obtained from *R. simulans* against the nymphs and adults of the pest as a possible biocontrol agent. All of them produced different mortality values in comparison to each other and the control group ($P < 0.05$).

The highest nimphal mortalities were obtained from Rs4 with 82% within 10 days 12:12 photoperiods in laboratory conditions (Figure 3). Adult mortalities were obtained from Rs10 with 80% within 10 days 12:12 photoperiods in laboratory conditions (Figure 4).

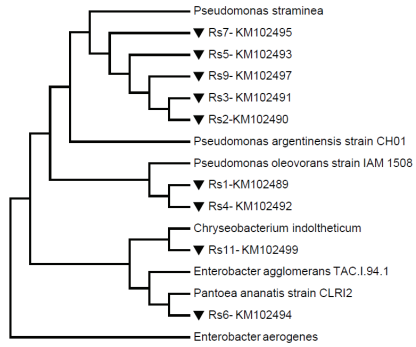

Figure 3. Insecticidal activity of bacterial isolates against nymphal-stage of *R. simulans*.

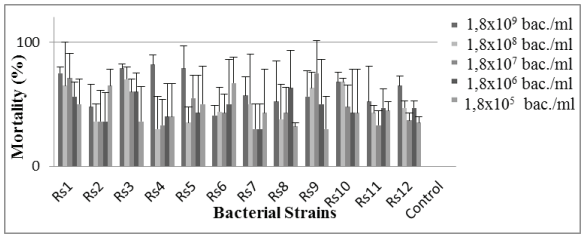

Figure 4. Insecticidal activity of bacterial isolates against adult stage of *R. simulans*.

The study also tested *Bacillus thuringiensis tenebrionis* Xd3, *Bacillus thuringiensis kurstaki* MnD, *Bacillus thuringiensis kurstaki* BnBt and *Serratia marcescens* BnSm both on nymphal stage and adult stage of *R. simulans* (Figure 5-6). The highest insecticidal activity obtained from BnSm strain was 75% in nymphal stage and 93% in adult stage.

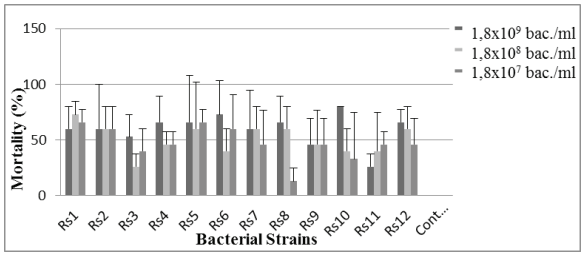

Figure 5. Insecticidal activity against nymphal stage of *R. simulans* of *B. thuringiensis* (Xd3, MnD, BnBt) and *S. marcescens* (BnSm) isolates.

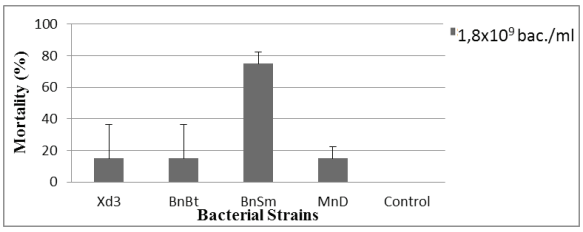

Figure 6. Insecticidal activity against adult stage of *R. simulans* of *B. thuringiensis* (Xd3, MnD and BnBt) and *S. marcescens* (BnSm) isolates.

DISCUSSION

Biological control is very important to reduce the damage caused by chemical drugs widely used in the agriculture. Biological control is also important to sustain tea production and to control pest population without using chemical pesticides in Turkey. *Ricania simulans* (Hemiptera: Ricaniidae) is a seriously harmful pest whose population has been increasing significantly in the Eastern Black Sea Region of Turkey, especially in tea fields [1]. Although *R. simulans* is widespread in China, Japan, Taiwan and Turkey, there is no effective strategy to control this harmful pest [39].

Bacterial pathogens have been tested and used effectively in pest control for a long time and some of them have been improved as bio-pesticides and are still used in pest management systems [10,35].

Although a number of pests have been investigated for bacterial flora and tested for safe biological control agent [35], there is no study on bacterial flora of *R. simulans* regarding the biology of insect and potential biological control agents. However, there has recently been an increasing interest in finding more pathogenic and safer bacterial isolate against hazardous insects. Hence, this study aimed to isolate effective and safer bacterial control agents from *R. simulans* and characterize these bacterial isolates in detail.

In a previous study, a fungal (*Lecanicillium muscarium*) sample was used as control agent against *R. simulans* in Turkey [14]. However, only 74.76% killing of this pest was achieved and fungi requires more specific conditions to grow in nature as well as their slow growth compared to bacteria. In this study, 12 bacteria were isolated from this insect and tested as potential biological control agents against this insect for the first time.

Rs1 isolate shared 99% 16S rRNA gene sequence homology with *Pseudomonas oleovorans.* Rs1 was separated from other Pseudomonas species with the help of biochemical tests [16]. *P. oleovorans* was a Gram-negative, methylotrophic bacterium that was a source of rubredoxin. It was first isolated in water-oil emulsions used as lubricants and cooling agents for cutting metals. Based on 16S rRNA analysis, *P. oleovorans* was placed in the *P. aeruginosa* group [4]. It was significant that this bacteria was found to have antiviral activity against plant viral diseases (Tobamovirus, Potyvirus, Tenuivirus, Cucumovirus and Begomovirus). *P. oleovorans* was isolated from an insect for the first time in this study. Rs1 isolate demonstrated 75 and 73% insecticidal activity on nymph and adult stages, respectively within 10

days at 12:12 photoperiods in laboratory conditions.

Rs2, Rs3 and Rs5 isolates were classified as *Pseudomonas parafulva*. 16S rRNA results, biochemical and physical characteristics were taken into account in the classification [17]. *P. parafulva* is a Gram-negative environmental bacterium [40] originally isolated from rice and commonly associated with rice plants, grains and paddy fields [24]. *P. parafulva* was isolated from an insect for the first time in this study. Mortalities were obtained from Rs2, Rs3 and Rs5 with 65%, 79% and 79% on nymphal stage and with 60%, 53% and 66% on adult stage, respectively within 10 days at 12:12 photoperiods in laboratory conditions.

Rs4, Rs7 and Rs9 were determined as *Pseudomonas* sp. at genus level. Sixteen species of the genus *Pseudomonas* have been found in insects to date. Isolation of *P. aeruginosa* and *P. fluorescens* from insects has frequently been reported in literature [34]. A number of *Pseudomonas* species are important insect pathogens, i.e. *P. aeruginosa* [26], *P. fluorescens* [34] and *P. chlororaphis* [9]. Although most of the *Pseudomonas* isolates appear insignificant as an insect pathogen [32], the *Pseudomonas* isolate (Rs4) in this study was highly pathogenic against *R. simulans.* Insecticidal activities were obtained from Rs4, Rs7 and Rs9 with 82%, 57% and 75% on nymphal stage and with 66%, 60% and 46% on adult stage, respectively within 10 days.

According to bioassay results, *Pseudomonas* sp. (Rs4) showed the highest insecticidal activity on *R. simulans*. The highest insecticidal activity occurred with 82% for Rs4 isolate on nymphal stage of *R. simulans* at a dose of 1.8 X 10^9 CFU/ml.

Rs6 isolate was classified as *Pantoea* sp. The genus *Pantoea* includes several species that are generally associated with plants, either as epiphytes or as pathogens, and some species can cause disease in humans. *Pantoea agglomerans*, the *Pantoea* species most commonly isolated from humans, is widely distributed in nature and has been isolated from numerous ecological niches, including plants, water, soil, humans, and animals [12]. Therefore, this bacterial isolate cannot be used as biological control agent. This isolate has 67 and 73% insecticidal activity on nymphal and adult stage of *R. simulans.*

Rs8 was classified as *Microbacterium paraoxydans* according to 16S rRNA results, biochemical and physical characteristics. *M. paraoxydans* is distinguished from other species wherefore glucose testing, L arabinose and H_2S test positive [18], *M. oxydans* has been isolated especially from clinical specimens and has also been isolated from an insect species before [33]. This isolate showed 63 and 66% insecticidal activities on nymphal and adult stage of *R. simulans,* respectively.

16S rRNA sequence analysis showed Rs10 similarity with *Bacillus safensis* by 99% identities. Biochemical and physical tests are easier to distinguish from other species. The presence of the species has also been reported in desert soil, sweet meat whey, root tubers, and rhizosphere [21]. *B. safensis* was isolated from an insect for the first time in this study. Rs10 showed 68% mortality on nymphal stage and 80% mortality on adult stage within ten days. Therefore, this bacterial isolate can be used as biological control agent.

According to the 16S rRNA sequence analysis, Rs11 was similar to *Chryseobacterium indoltheticum* by 99%. Rs11 is distinguished from other species wherefore indole and urea tests positive [27]. *C. indoltheticum* was isolated from an insect for the first time in this study. Rs11 showed 52% mortality on nymphal stage and 46% mortality on adult stage within 10 days 12:12 photoperiods in laboratory conditions.

Rs12 was 99% similar to *Bacillus thuringiensis* according to the result of 16S rRNA sequence analysis. These data were supported by conventional tests and the API microbial identification system. *B. thuringiensis* as control agent is widely applied to pests [22]. *B. thuringiensis* does not create toxic effects on humans and beneficial organisms [36] . *B. thuringiensis* forming toxic effects in insects is a soil bacterium [7]. In addition, some researchers emphasize that it doesn't create resistance to insects as other insecticides do [13]. The existing *Bacillus thuringiensis* among bacteria isolated from *R. simulans* has made it possible to test the insecticidal activity against *R. simulans*. According to bioassay results, the insecticidal activity occurred with 65 and 66% for *B. thuringiensis* (Rs12) isolate on nymphal and adult stages of *R. simulans* at a dose of 1.8 X 10^9 CFU/ml. Therefore, this bacterial isolate can be used as biological control agent.

Likewise, *Bacillus thuringiensis tenebrionis* (Xd3), *Bacillus thuringiensis kurstaki* (MnD), *Bacillus thuringiensis kurstaki* (BnBt) obtained from the KTU microbiology laboratory showed insecticidal activity with 15, 15 and 15% on nymphal stage of *R. simulans,* respectively and with 13, 40 and 20% on adult stage of *R. simulans,* respectively.

Moreover, *S. marcescens* had highest effect in insecticidal activity trials performed with four bacteria obtained from the KTU microbiology laboratory. The activity was 93% on adult stage of *R. simulans*. Insecticidal activity occurred with 75% for *S. marcescens* isolate on nymphal stage of *R. simulans. S. marcescens* cultures, chitinases and chitinase genes are very important biological control agents [8]. *S. marcescens*is very effective bacteria in the degradation of chitin [25]. *Serratia* species have often been associated with insect diseases and this genus contains insect pathogenic strains which are usually considered to be opportunistic or facultative pathogens, as they are often virulent to insects when present in the digestive tract but are lethal upon entering insect hemocoel following injury or stress [19]. *S. marcescens* causes disease in plants and in a wide range of invertebrate and vertebrate hosts. Additionally, there was no report of death in the control groups in trial to confirm the detection of insecticidal activity due to the tested bacteria. In light of these results, the integrated biological control in the region is also advisable to fight mechanics.

In conclusion, this is the first study to determine the bacterial flora and bacterial control agent of *R. simulans.* The study isolated and characterized twelve bacteria from *R. simulans* and tested their effectiveness on it. Some of the isolates appear to be significant candidates in the biological control of this pest. In particular, *Pseudomonas* sp., *B. safensis* and *B. thuringiensis* are the most promising isolates for the microbial control of *R. simulans*.

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