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Prospects of Entomopathogenic Bacteria and Fungi for Biological Control of *Ricania simulans* (Walker 1851) (Hemiptera: Ricaniidae)

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ABSTRACT

Ricania simulans causes harm in almost all plants that grow along the Eastern Black Sea coast. The chemicals used to control this pest are prohibited in this region due to tea cultivation. For this reason, new strategies are needed to control this pest. With the awareness on the negative effects of the chemicals used in the control against pests and with the increasing awareness on environmental issues, alternative methods were sought in the past; and in this context, studies were conducted to find new methods in which fungi and bacteria were used in the biological control against pests. Totally 10 bacterial strains including 2 strains of *Brevibacillus brevis* (CP-1, FD-1), 1 strain of *Bacillus thuringiensis* (FDP-1), 2 strains of *Bacillus thuringiensis* subsp. *kenyae* (FDP-8, FDP-42), 2 strains of *Bacillus thuringiensis* subsp. *kurstakii* (FDP-41, BAB-410), 1 strain of *Bacillus subtilis* (EK-7), 1 strain of *Pseudomonas chlororaphis* (NEM-28) and 1 strain of *Bacillus sphaericus* GC sub-group D (FD-49) and additionally 1 *Beauveria bassiana* (ET 10) fungus isolate were examined for their insecticidal activities in this study. The studied bio agents were tested by spraying on *R. simulans* nymphs and adults. *B. thuringiensis* subsp. *kenyae*, *B. brevis* and *B. sphaericus* GC subgroup D were the most effective on nymphs, whereas *B. thuringiensis* subsp. *kurstakii*, *P. chlororaphi,* and *B. brevis* were the most effective on adults. Under controlled conditions, mortality rate varied between 19.58%-42.08% in nymph applications, and between 6%-18% in adult applications.

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TG, ET and RK designed the study, each authors contributed equally to the study.

Key words
Bacteria, *Beauveria bassiana*, Biological control, Entomopathogens, *Ricania simulans*.

INTRODUCTION

Ricania simulans (Walker, 1851) (Hemiptera: Ricaniidae) is an important polyphagous pest that causes harm by sucking sap from hosts. Females lay their eggs under the skin of fresh sprouts and thin branches, causing death in these tissues over time (Gokturk and Aksu, 2014; Ak *et al*., 2015). The most common option for control against the *R. simulans* is chemical pest control. However, the region where this pest is common is reserved for tea cultivation, and also use of chemical pest control is prohibited in this region.

For this reason, recent efforts have focused on developing environmentally safe, long lasting and effective bio control methods for the management of pests. One method of biological pest control is the utilization of microbial agents for control against pests. Among bacterial bio insecticides, especially the spore-forming species belonging to the genus *Bacillus*, such as *Bacillus popilliae*, *Bacillus thuringiensis* and *Bacillus sphaericus* are commonly used in biological pest control. *B. thuringiensis* has been shown to be effective against some insects under the order Dipteran and Coleopteran, and particularly the harmful Lepidoptera larvae (Lacey *et al*., 2001).

Another group that is used in biological pest control is the fungi, and they are known to be entomopathogens against over 700 species belonging to 90 genera (Khachatourians and Sohail, 2008). Most of the entomopathogenic fungi are classified under the divisions of Ascomycota and Zygomycota. They have been effective in controlling many species of insects belonging to the genera *Metarhizium, Beauveria, Trichoderma, Verticillium, Nomuraea, Entomophtora* and *Neozygites* (Desphande, 1999). Among these, *Beauveria* isolates, and particularly *Beauveria bassiana* have been reported to be used in biological pest control against many harmful insect species, and their commercial preparations have been developed and introduced to the market (Inglis *et al*., 2001; Wraight *et al*., 2001; Vestergaad *et al*., 2003; Copping, 2004) and have minimum risk against natural enemies (Huang *et al*., 2012).

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The present study was planned and conducted with the aim of determining and identifying entomopathogens effectively be used in biological control against *R. simulans* which continue to spread rapidly along the Eastern Black Sea coast and lead to economic losses.

MATERIALS AND METHODS

Harmful insects, bacterial strains, fungal isolates and host plant

Different biological stages of *R. simulans* were obtained from the tea cultivation fields and kiwi gardens in Artvin and Rize provinces of Turkey. In this study, we obtained 100 bacterial and 100 fungal strains from adult and nymph stages of dead and diseased *R. simulans*. After 10 bacterial strains and 10 fungal isolates with different characteristics were chosen according to colony morphology and colour. Among these, only 4 bacterial strains were found to be effective, and the remaining 6 bacterial strains and 10 fungal isolates were grouped as other bacteria and fungi. In addition, 1 strain of *Brevibacillus brevis* (FD-1), 1 strain of *Bacillus subtilis* (EK-7), 2 strains of *B. thuringiensis* subsp. *kenyae* (FDP-8, FDP-42), 1 strain of *B. thuringiensis* subsp. *kurstakii* (FDP-41) and 1 strain of *B. sphaericus* GC subgroup D (FD-49) isolated from various diseased or dead insects in previous studies (Tozlu *et al*., 2011, 2016; Dadasoglu *et al*., 2013) and defined in the MIS and BIOLOG system based on fatty acid methyl esters and *B. bassiana* (ET 10) isolated from *Sphenoptera antiqua* (Illiger, 1803) larvae that are harmful to the trefoil plant (*Onobrychis sativa* L. (Fabacea)) were used in this study. Stock cultures of the bacterial strains and fungi isolate were kept in Atatürk University Faculty of Agriculture Plant Clinical Laboratory.

Tomato seedlings (*Solanum lycopersicum* L.) obtained from Artvin were used as host plants in order to determine the efficacy of bio agents studied in the laboratory.

Isolation of bacterial and fungal isolates

The diseased and dead nymphs and adults of *R. simulans* were subjected to surface sterilization by treating with 95% ethyl alcohol for 5 minutes. Then, cuticles of the nymphs were cut with a sterile scissor, and a drop of hemolymph was obtained and diluted in sterile water for inoculation (Thiery and Frachon, 1997). Adults were homogenized by pulverizing in a sterile mortar with sterile saline solution and serial dilutions were obtained from this homogenate. The dilutions prepared from the nymphs and adults were inoculated on Nutrient Agar (NA) and Tripticase Soy Agar (TSA) for bacterial growth and Water Agar (WA) and Potato Dextrose Agar (PDA) for fungal growth. The growth media were incubated at 30 °C, and at the end of 24-72 h. The colonies were purified according to different characteristics by transferring new growth media (Sezen and Demirbag, 2007). Each microorganism was given a separate code number, and information regarding the isolation conditions (location, altitude, insect form, date, *etc*.) were noted. Until the time of identification, the samples were kept at -86 °C in stock growth media containing 30% glycerol and Loria Broth (LB) for bacterial strains and in slant agar at +4 °C for fungal isolates.

Identification of the bacteria by MIS and BIOLOG

The identity of all bacterial strains used in this study was confirmed according to fatty acid methyl esters (FAME) analysis by using Sherlock Microbial Identification System (Microbial ID, Newark, DE, USA) (Sasser, 1990).

The identity of the bacteria was confirmed according to BIOLOG systems. Two days before the inoculation of Biolog GP2 plates (Biolog), bacterial strains were streaked on Biolog Universal Growth Agar+25% Maltose agar plates. Each well of Biolog GP2 microtiter plates was inoculated with 125 µl of the bacterial suspension adjusted to the appropriate density (10^8 cfu/ml), and incubated at 27°C for 24 and 48 h. Colour development was automatically recorded using a microplate reader with a 590 nm wavelength filter. Identification results and similarity index of the bacteria was performed using BIOLOG420/Databases/GP601 (Holmes *et al*.,. 1994).

Hypersensitivity tests (HR) of the bacteria

The potential biocontrol bacterial strains were tested for hypersensitivity on tobacco plants (*Nicotina tabacum* L. var. Samsun) as described by Klement *et al*. (1964). The bacterial suspension (10^8 cfu/ml) prepared in sterile distilled water and infiltrated into inter costal area of the leaves of tobacco plants by using a 3-cc syringe without needle (Becton Dickinson, Franklin Lakes, NJ, USA). 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. Sterilized distilled water (sdH_2O) was used as a negative control.

Insecticidal effect of the bacteria under controlled conditions

Bacteria were grown in 50 ml flasks containing 20 mL of TSB medium on a rotary shaker at 27°C for 24 h. Absorbance of the bacterial suspensions was measured spectrophotometrically at 600 nm and appropriately diluted

to $1x10^8$ CFU/ml in sd.H_2O.

B. bassiana ET 10 isolate was cultured on SDA with 1% yeast extract (SDAY) plates in several Petri dishes (9 cm in dia-meter), and were grown at 25 ± 1°C under a 16 h/8 h (light/dark) photoperiod and 60 ± 5% RH for fungal growth and conidial production. Surface of a 14-day-old culture was gently scratched with inoculation needle and transferred to vials containing 5 ml sterile Tween-80 solution (0.1% v/v). The concentration of conidia in stock suspensions were determined by direct count using hemocytometer. The conidial suspensions (10^6 conidia/ml) was prepared for the bioassay (Quesada *et al*., 2006).

Tomato seedlings were placed on pots, and the nymphs and/or adults collected 12-24 h ago were placed on each pot (20 on each) under cheesecloth, the prepared suspensions were sprayed over them. Plants were controlled daily and the number of dead adults and/or nymphs were noted. At the end of the experiment, number of dead adults and nymphs were used in the following formula to calculate the percent mortality rate:

$$\text{Death rate } (\%) = 100 \times \frac{\text{Count of dead nymphs or adults}}{\text{Total count of nymphs or adults}}$$

Pathogens from infected nymphs and adults were re-isolated according to Koch postulates; pathogenicity tests were repeated and their results were recorded. Sterile NB growth media used for diluting bacterial suspensions was used as a negative control and plant-origin pesticide Neemazal ® T/S containing the active substance Azadirachtin A, and pesticide Laser containing the active substance Spinosad were used as positive controls, these agents were previously tested against *R.simulans* and yielded successful results.

Analysis of results

All data in the present study were processed by JUMP 5.0 and the means were separated by LS Means Students tests. The statistical analyses of percentage values were performed by using transformed values.

RESULTS

MIS and BIOLOG identification results and HR test results of 10 bacterial strains obtained from *R. simulans* were given Table I. According to BIOLOG identification results, CP-1 as *Bacillus subtilis*, FDP-1 and BAB-410 as *Bacillus thuringiensis*, NEM-28 as *Pseudomonas* sp. studied in this study as first were also confirmed by MIS identification results of the bacteria. Their similarity indexes in BIOLOG were changed from 45 to 76. None of them showed hypersensitivity positive reaction on tobacco plants.

The insecticidal activities of treatments tested against *R. simulans* nymphs and adults on tomato plants were given (Figs. 1, 2, 3, 4). According to these results, all the bacteria and fungi showed more or less insecticidal activity against pest in controlled conditions.

Table I.- MIS and BIOLOG identification results and HR (hypersensitivity) test results of the bacterial strains used in this study.

Strain	Pest (isolated from)	MIS identification results	SIM	BIOLOG identification results	SIM	Reference
CP-1	*Ricania simulans*	*Brevibacillus brevis*	0.65	*Bacillus subtilis*	45	This study
FDP-1	*Malacosoma neustria*	*Bacillus thuringiensis*	0.64	*Bacillus thuringiensis*	56	This study
FDP-8	*Hypera postica*	*Bacillus thuringiensis* subsp. *kenyae*	0.45	*Bacillus thuringiensis*	59	Tozlu *et al.* (2011)
FDP-41	*Apion* spp.	*Bacillus thuringiensis* subsp. *kurstakii*	0.57	*Bacillus thuringiensis*	45	Tozlu *et al.* (2011)
FDP-42	*Apion* spp.	*Bacillus thuringiensis* subsp. *kenyae*	0.47	*Bacillus thuringiensis*	75	Tozlu *et al.* (2011)
FD-1	*Malacosoma neustria*	*Brevibacillus brevis*	0.65	*Bacillus subtilis*	45	Tozlu *et al.* (2011)
EK-7	*Rosehip*	*Bacillus subtilis*	0.65	*Bacillus subtilis*	76	Tozlu *et al*. (2017)
NEM-28	*Ricania simulans*	*Pseudomonas chlororaphis*	0.40	*Pseudomonas* sp.	57	This study
FD-49	*Culex* sp.	*Bacillus sphaericus* GC subgroup D	0.71	*Bacillus sphaericus*	58	Dadasoglu (2013)
BAB-410	*Ricania simulans*	*Bacillus thuringiensis* subsp. *kurstakii*	0.62	*Bacillus thuringiensis*	57	This study
X	*Ricania simulans*	Not done	-	Not done	-	This study

SIM, similarity ındex; -, negative reaction; X, other tested bacterial strains and fungal isolates.

Their insecticidal effects changed 19.58 and 100 (Fig. 1) on nymphs. The highest mortality rate values were observed plant-origin pesticide containing the active substance Azadirachtin A and the pesticide containing the active substance Spinosad (100). FDP 42 (42.08), CP 1 (40.41), FD-49 (37.91), FD-1 (27.08), EK-7 (25.83), FDP-41 (25.83), NEM-28 (24.16), FDP-1 (21.25), ET 10 (entomopathogen fungi *B. bassiana*) (20), FDP-8 (20) and BAB-410 (19.58) followed these applications against nymphs. Six bacterial strains and 10 fungal isolates isolated from *R. simulans* nymphs and adults collected from the field were in the same group with the control. They were shown in Figure 1 as other bacteria and fungi tested. The lowest mortality rates were observed with control (0.41), other bacteria (0) and fungi tested (0) (Fig. 1).

The nymph mortality rates according to days during the 8 days follow-up period were given in Figure 2. All *R. simulans* nymphs died in Azadirachtin A and Spinosad applications on first day. The nymphs started to die in FDP 42, CP-1, FD 1, EK 7, FDP-41 and FDP-1 applications on second days and in FD-49, NEM-28, and ET 10 applications on thirds day (Fig. 2).

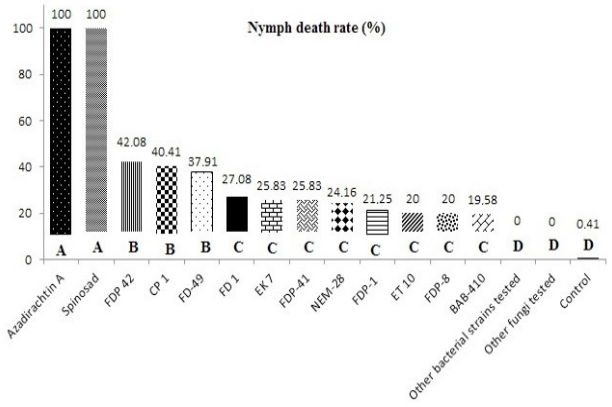

Fig. 1. Effectiveness of potential biological control agent bacterial strains and fungal isolates against *Ricania simulans* nymphs.

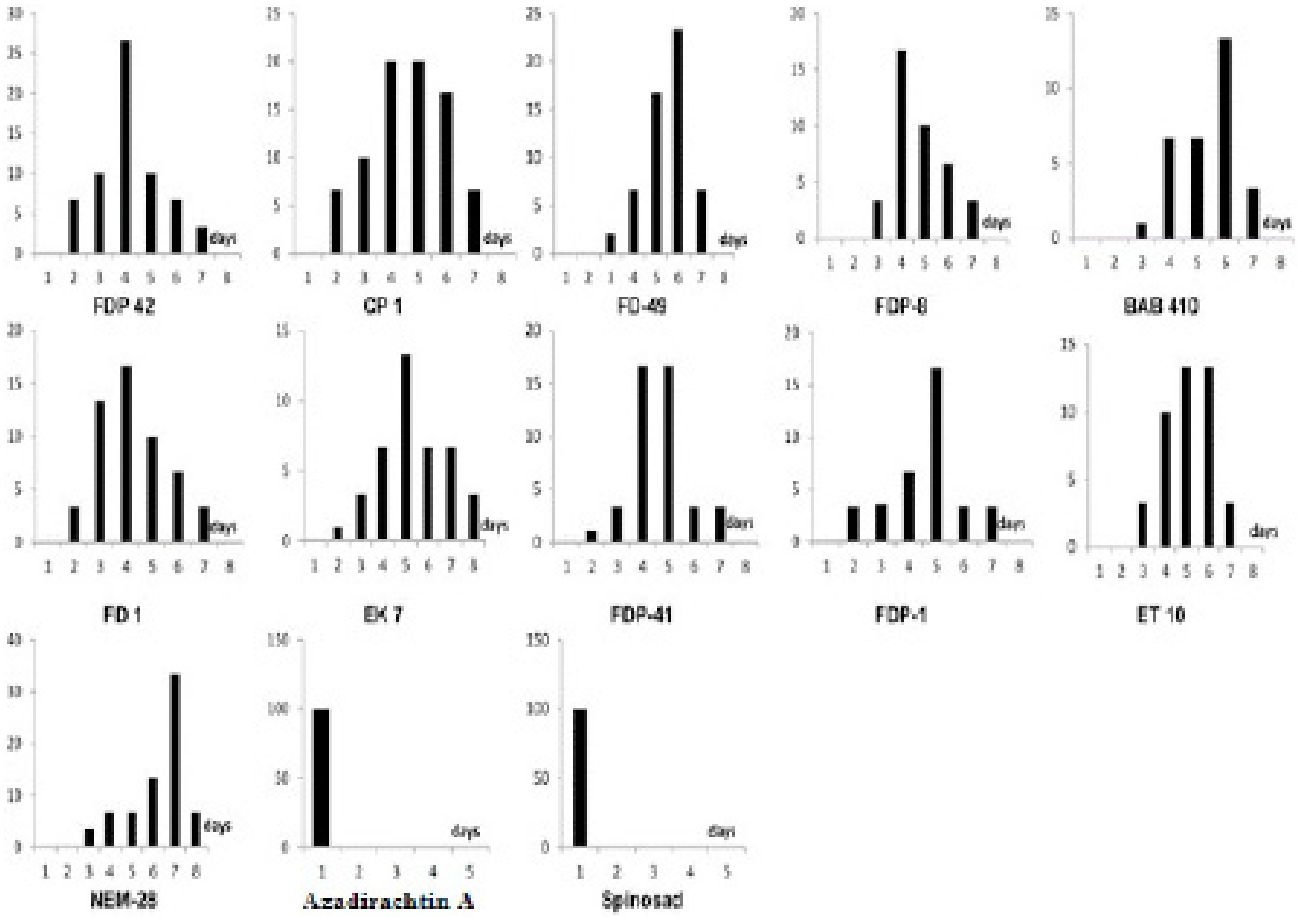

Fig. 2. Nymphs' death rates with respect to the days at which counts were made.

Insecticidal effects of bacterial and fungal isolates changed 11.33 and 100 (Fig. 3) on adults. The highest mortality rate values were observed Azadirachtin A and Spinosad (100) as applications against nymphs. FDP 1 (18.00), NEM-28 (17.33), FD-1 (17.33), BAB-410 (16.67), FDP-8 (16.67), FDP-42 (14.67), FD-49 (14.00), CP-1 (14.00), FDP-1 (13.33), EK-7 (11.33) AND ET 10 (6) followed these applications against adults. Other bacterial strains and other fungi tested in the same group with the control (Fig. 3). The lowest mortality rates were observed with control, other bacteria and fungi tested (0) (Fig. 3).

According to days during the 5 days follow-up period, the adult's mortality rates were given in Figure 4. All *R. simulans* adults died in Azadirachtin A and Spinosad applications on first day. The nymphs started to die in FDP 42, CP-1, FD 1, EK 7, FDP-41 and FDP-1 applications on second days and in FD-49, NEM-28, and ET 10 applications on thirds day (Fig. 4).

Figure 4 showed mortality rates according to days during a follow-up period of 5 days. All adults were died in Azadirachtin A, Spinosad in first day. The adults started to die in FDP-41, NEM-28, FDP-8 and BAB-410 applications in first day, in FDP 42, CP-1, FD-49, FD 1, EK 7 and FDP-in second days and in ET10 applications in third days.

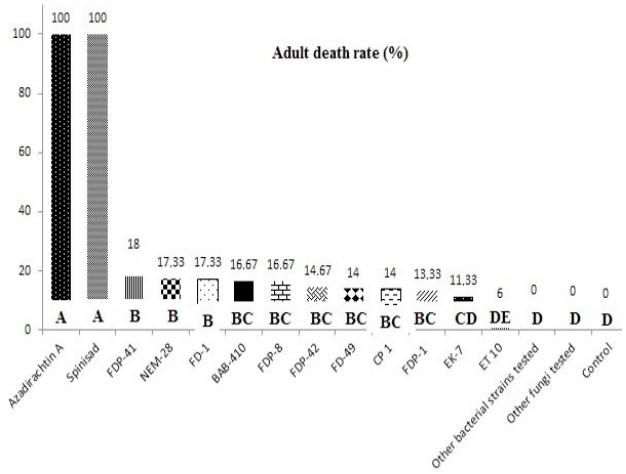

Fig. 3. Effectiveness of potential biological control agent bacterial strains and fungal isolates against *Ricania simulans* adults.

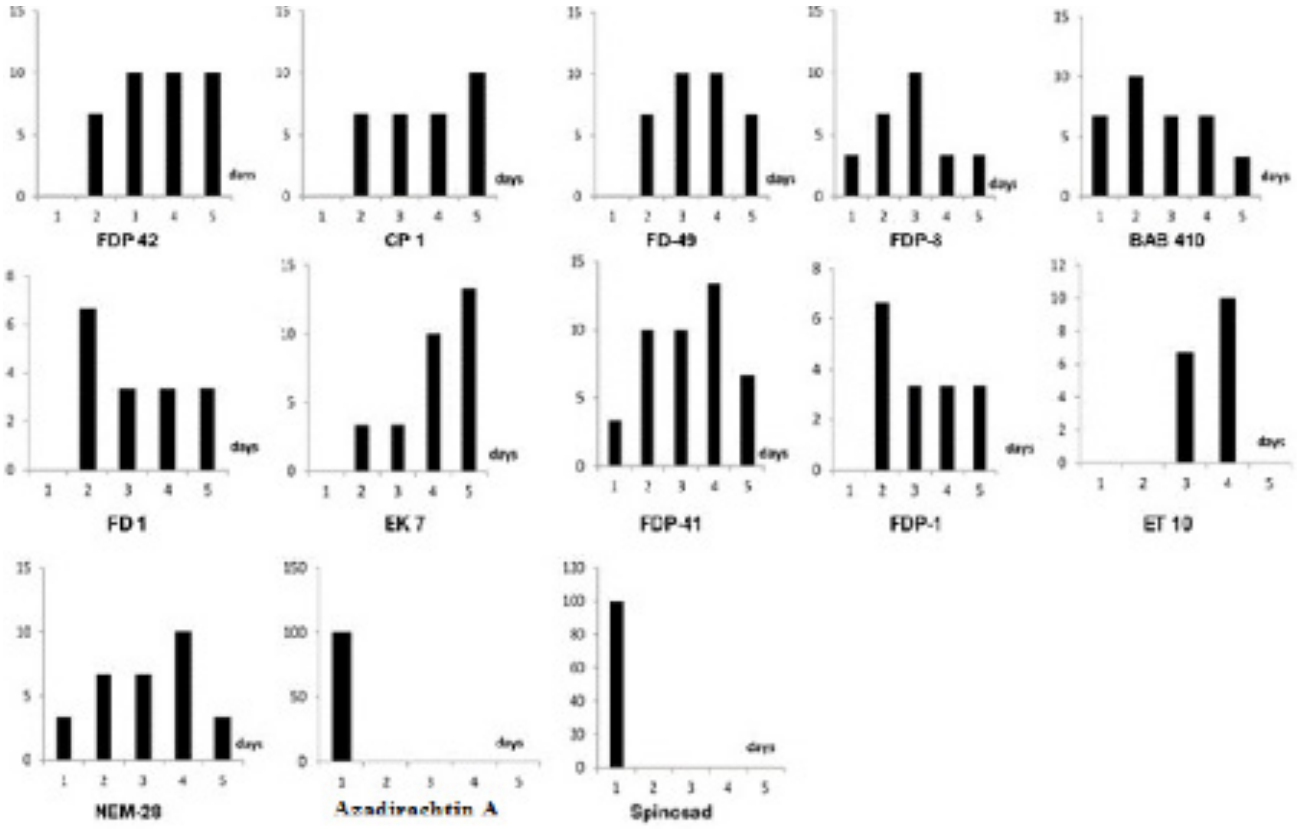

Fig. 4. Adult death rates with respect to the days at which counts were made.

DISCUSSION

There are many studies related to utilization of entomopathogenic bacteria and fungi in biological pest control (Inglis *et al*., 2001; Wraight *et al*., 2001; Lacey *et al*., 2001; Vestergaad *et al*., 2003; Copping, 2004; Aslantas *et al*., 2008; Khachatourians and Sohail, 2008; Ak *et al*., 2013, 2014). However, studies that investigate biological pesticides against the *R. simulans* are very limited.

One study that was conducted on tea leaves in laboratory and kiwi plant in field conditions tested 6 isolates of *Lecanicillium muscarium* (Petch) at a dose of 10^7 conidia/ml against nymphs of the pest, and found 50.95-74.76% mortality in 2.34-3.90 days, respectively; and a dose of 10^7 conidia/ml was effective against nymphs and adults in field conditions in 4.18-6.49 days, respectively. The authors concluded that *L. muscarium* could be an alternative and environment-friendly pest control agent against *R. simulans* (Guclu *et al*., 2010). Ak *et al*. (2014) applied *Conidiobolus coronatus,* another entomopathogenic fungus against this species, in both laboratory and field conditions, and they reported 100% success.

Sixteen bacterial strains from this species were isolated and 9 of them were identified as species and 7 of them were identified as genus in the other study. Among these, *Pseudomonas* sp. (Rs4 isolate) showed highest insecticidal activity at the nymph stage with 82% efficacy, while *B. thuringiensis* (Rs16 isolate) showed the highest insecticidal activity at the adult stage with 86% efficacy (Alev, 2014).

In this study, totally of 9 bacterial strains belonging to *Bacillus* genera, 1 bacterial strain belonging to Pseudomonas genera and 1 fungal isolates *Beauveria bassiana* were tested for insecticidal activities against *R. simulans* nymphs and adults. All of the tested bacterial strains and fungal isolate showed more or less insecticidal activity against the pest in controlled conditions. The most effective bacteria were *B. thuringiensis* FDP-42, *B. subtilis* CP-1 and *B. sphaericus* FDP 49 strains against nymphs and *B. thuringiensis* FDP-41, *Pseudomonas* sp. NEM-28 and *B. subtilis* FD-1 strains in adults. ET 10 against both nymphs and adults were different from control. Mortality rates were between 19.58-42.08% in nymphs, and between 6-18% in adults.

Studies conducted by different researchers have previously shown that *Bacillus* species can be successfully used in biological pest control (Gray *et al*., 2001; Alper *et al*., 2013), and particularly *B. thuringiensis* was reported to have approximately 2% share of the insecticide market (Bravo *et al*., 2007). *B. bassiana*, whose efficacy was tested in the present study, is one of the most important entomopathogens used in biological pest control (Zibaee *et al*., 2013). As an environment-friendly entomopathogen, the fungus *B. bassiana* has a wide host range and is commercially available (Keyhani, 2015). Although Al-Mazra'awi and Shipp (2006), reported that *B. bassiana* was given successful results in biological pest control against the Hemiptera species. In this study, we found that *B. bassiana* was more effective compared to control, while it showed less efficacy when compared to the studied bacterial strains.

Our results indicated that, the applications were more effective against nymphs than adults. These effects may be varied in field treatments. In the future, it is important that this study should be conducted in controlled conditions. We plan to prepare a commercial preparation after making a good carrier consisting of organic material with long shelf life for the most effective bacterial strain. In the midst of these obstacles, the bacterial strains *B. thuringiensis*, *B. subtilis* and *B. sphaericus* can be commercialized for management of *R. simulans* in both agricultural and horticultural crops.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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