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RESEARCH ARTICLE

The Investigation of the Biological Control of *Icerya purchasi* Maskell, 1878 (Hemiptera: Margarodidae) with Entomopathogenic Fungi and Bacteria

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ABSTRACT

Cottony cushion scale *Icerya purchasi* Maskell, 1878 (Hemiptera: Margarodidae) is an important pest that inhibits the plant growth and development by sap sucking of the plants, and causes sooty mold in more than 200 plant species, especially in citrus plantation. The present study investigated the biological control of the nymphs and adult *I. purchasi*, which densely populates the mimosa plants (*Acacia dealbata*) in Artvin, Turkey. For this purpose, one fungal isolate [*Beauveria bassiana* (ET 10)] and eight bacterial strains [*Brevibacillus brevis* (CP-1), *Bacillus thuringiensis* subsp. *kenyae (*FDP-8, FDP-42), *B. thuringiensis (*FDP-1), *B. sphaericus* (FD-49), *B. pumilus* (TV-67C), *Pseudomonas fluorescens* (RK-1773) and *B. atrophaeus* (RK-1774)] were assessed against the nymphs and adult of *I. purchasi* under controlled conditions. Fungal and bacterial suspensions were sprayed onto 20 nymphs and 20 adults of *I. purchasi* in plastic boxes. The death rates of the nymphs and adults were recorded. The *B. bassiana* (ET 10) caused a death up to 100% and 80% in nymphs and adults, respectively. Moreover, *P. flourescens* (RK 1773) caused 90.5% death of nymphs and *B. thuringiensis* subsp. *kenyae* (FDP-42) presented 88.5% death to the nymphs of *I. purchase*. However, the use of the bacterial strains was not much successful against the adults, as compared to the nymphs.

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Introduction

Icerya purchasi Maskell, 1878 (Hemiptera: Margarodidae) is a cosmopolitan plant pest native to Australia that causes harm to more than 200 plant species including especially citrus fruits, other subtropical fruits, ornamental plants and weeds (Kollar et al., 2016). It was first identified in New Zealand and spread to the other regions of the world through global trade and continued to spread to the northern regions due to global warming. In Turkey, it is spread along the entire coastline and passages. Different from other *Icerya* species, its tolerance to climatic factors allows its residence in Northern Europe (Salisbury and Booth, 2004). Due to the damage caused by the species, the offshoots and branches of forest trees and ornamental plants dry and the trees and plants lose their leaves. Furthermore, its inhibition of photosynthesis by causing sooty mold in leaves is another important damage caused by the pest. The uncontrolled infestation of cottony cushion scale has a severe effect on the pomiculture and horticulture industries and the endemic fauna of small islands.

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Moreover, the damage it causes to plants leads to the extinction of the hosts of other species from the Lepidoptera order that feed on these plants and their natural enemies are also negatively affected by this issue (Hoddle, 2011). Organophosphates and petroleum oils are used to control the pest, and although buprofezin is effective on young nymphs, it fails to affect the adult pests. On the other hand, predator, *Rodolia cardinalis*, had shown considerable potential to control the population of cottony cushion scale (*Anonymous, 2008, 2015)*.

However, the activity of natural enemies diminishes due to blind use of broad-spectrum insecticides by farmers that have adverse effects on the environment (Carruthers and Hural, 1990; Inglis et al., 2001).

The inclusion of effective biopesticides that do not have the risk of resistance and any toxic effects on the environment and human health especially in areas where the activity of its natural enemies is low is of great importance for the control of the pest whose chemical control is not recommended. Many entomopathogens such as *Bacillus thuringiensis, Beauveria bassiana* and *Metarhizium anisopliae* can be mass produced, formulated, and applied to pest populations in a manner analogous to chemical pesticides, i.e. as nonpersistent remedial treatments that are released inundatively (Bhattarai et al., 2016).

This study investigated the insecticidal effects of eight bacterial strains and one fungal isolate against *I. purchasi*. The review of the literature has not revealed any survey on the control of *I. purchasi* by using fungal and bacterial microorganisms.

Materials and Methods

Host Plant, Pest, Bioagent Fungal Isolate and Bacteria Strains

The materials of the study consist of the mimosa (*Acacia dealbata* Willd. var. *dealbata* (Link)) plants naturally infected with *I. purchasi* and adult *I. purchasi* and its nymphs in the Artvin Çoruh University Seyitler Campus in Artvin, Turkey (Figure 1).

Figure 1. *Acacia dealbata* infected with *Icerya purchasi* adults

Table 1. Identification and similarity indices of the bacterial strains and fungal isolate used in the study

Isolate and Strains Number	Fungal Isolate				
	İsolated from	**ITS**	**S***	**AN****	**Literature**
ET 10	*Sphenoptera antiqua*	*Beauveria bassiana*	0.99	GB|KY806126|	Tozlu et al., 2017
	Bacterial Strains				
	Isolated from	**MIS Identification Results**	**S**	**HR*****	**Literature**
CP-1	*Ricania simulans*	*Brevibacillus brevis*	0.65	-	Göktürk et al., 2018
TV-67C	Rasberry	*Bacillus pumilus*	0.63	-	Erman et al., 2010
FDP-1	*Malacosoma neustria*	*Bacillus thrungiensis*	0.64	-	Göktürk et al., 2018
FDP-8	*Hypera postica*	*Bacillus thuringiensis* subsp. *kenyae*	0.45	-	Tozlu et al., 2011
FDP-42	*Apion* spp.	*Bacillus thuringiensis* subsp. *kenyae*	0.47	-	Tozlu et al., 2011
FD-49	*Culex* sp.	*Bacillus sphaericus*	0.71	-	Dadaşoğlu, 2013
RK-1773	*Pseudaulacaspis pentagona*	*Pseudomonas fluorescens*	0.59	-	In this study
RK-1774	*Pseudaulacaspis pentagona*	*Bacillus atrophaeus*	0.63	-	In this study

*S: Similarity; **AN; Accession number (GenBank); ***HR: Hypersensitivity –: Negative Reaction

The bacterial strains and fungal biocontrol isolate the insecticidal effects of which were investigated in the study were obtained from the Ataturk University Faculty of Agriculture Plant Protection Department Culture Collection. Previous studies have determined the effectiveness of a portion of these agents on different plant pests and pathogens (Göktürk et al., 2018; Erman et al., 2010; Tozlu et al., 2011, Dadaşoğlu, 2013), while others were first tested in this study (Table 1). The bacterial biocontrol strains cultured in the Nutrient Agar (NA; Difco) medium were kept in 30% glycerol-containing Nutrient Broth (NB; Difco) at -80 ºC, while the fungal isolate was kept in slant Potato Dextrose Agar (PDA, Difco) at 4 ºC in the Atatürk University Faculty of Agriculture Plant Protection Department Culture Collection.

Identification of the Bacterial Species by MIS

Preparation and analysis of FAME 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) (Miller and Berger, 1985; Roy, 1988). FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25mx0.2mmx0.33µm) with cross-linked 5% phenylmethyl silicone. FAME profiles of each bacterial strain were identified by comparing the commercial databases (TSBA 40) with the MIS software package. The identity of bacterial strains was revealed by computer comparison of FAME profiles of the unknown test strains with those in the library. For MIS, Biolog, ELISA, hypersensitivity, pathogenicity and biochemical tests *E. amylovora* strain PD-761 was used as a positive control.

Hypersensitivity to Tobacco Test of the Bacterial Biocontrol Agents (HR)

The fresh leaves of the tobacco, *Nicotiana tabacum* L. var. Samsun were grown in pots were used in the hypersensitivity to tobacco test. The suspensions (10^8 cells/mL) that were prepared, using the bacterial cultures grown in the NA medium for 24h-48h, were injected between two adjacent trachea and the leaves were inspected for signs of symptoms. Those that didn't show symptoms in tobacco leaves were regarded as unfavorable, while those that show symptoms in tobacco leaves were regarded as positive (Klement, 1964).

Preparation of the Bioagent Fungal Isolate and Bacterial Strains

The bacteria inoculated onto NA media were incubated at 28 °C for 48h and, then, transferred to NB media and incubated in a horizontal shaker. Then, the concentration of the bacterial suspension was adjusted to $1x10^8$ cfu/mL. For this purpose, the absorbance of the suspension was spectrophotometrically adjusted to 0.1 at 600 nm.

The potential bioagent fungus was cultured in PDA for about 15 days to allow spore formation. Then, sterile water was poured into the Petri dishes containing the PDA in which spore-forming fungus was grown. The spores were transferred into the baker with a glass pipette to make the suspension stirred homogeneously with a micropipette up to clear suspension. Finally, the spore concentration was adjusted to $1x10^6$ conidia/mL with a hemocytometer.

Testing the Fungal Isolate and Bacteria Strains Against the Pest Under Controlled Conditions

The effectiveness of the bacterial strains and fungal isolate against *I. purchasi* was tested under controlled conditions. The mimosa branches naturally infected with *I. purchasi,* were brought to the Plant Clinical Laboratory of the Atatürk University, Faculty of Agriculture, Plant Protection Department. The branches were cut into small pieces, with 20 insects on each piece and placed in plastic boxes (19x12.5x7 cm). All suspensions were separately sprayed onto the branches. After the application, the plastic boxes were kept at 25 ºC and 80% humidity and a photoperiod of 12h:12h (light:darkness). Only the NB medium in which bacteria were grown was applied to the control group. The study was carried out in accordance with the randomized block design in three repetitions.

The number of dead insects was recorded daily and the death rates in percentages (%) was calculated using the formula below

$$Death\ rate\ (\%) = 100\ x\ \frac{Number\ of\ dead\ insects}{Total\ number\ of\ dead\ insects} \quad (1)$$

The potential bioagent bacterial strains and fungal isolate were re-isolated from the dead insects and the Koch's postulates were fulfilled.

Analysis of the Results

Arcsine transformation was applied to the data and, then, one-way variance analysis was applied and the differences between the mean values were compared using the LS Means Student test at a significance level of $P<0.01$. The data analysis was carried out using the JMP IN statistical software (SAS Institute, Cary, NC, 0% PC version).

Results and Discussion

Table 2 shows the insecticidal effects of the bacterial strains and fungal isolate on the nymphs and adults of *I. purchasi*.

The results revealed the bioagent fungal isolate and bacterial strains had varying levels of insecticidal effect on the nymphs (49.50±27.79% and 100±0.00%) and adult pests (0.00±0.00%-80.00±37.75%) (Table 2). The highest death rate was determined in the nymphs. A death rate of 100% was obtained in the application in which the ET 10 isolate of *B. bassiana* was applied to the nymphs, followed by the application of *P. fluorescens* that was isolated from *Pseudaulacaspis pentagona* (mulberry scale) (RK 1773) (90.50%). The FDP-42 (*B. thuringiensis* subsp. *kenyae*) (88.50%) and FDP-8 (*B. thuringiensis* subsp. *kenyae*) (82.00%) applications were in different groups, while the CP-1 (*B. brevis*) (58.00%), FDP-1 (*B. thuringiensis*) (58.00%), TV-67C (*B. pumilus*) (55.00%), RK 1774 (*B. atrophaeus*) (54.50%) and FD-49 (*B. sphaericus*) (49.50%) applications were in the same group and yielded different results from the control group (0.00%).

In the applications to adult pests, the highest death rate was obtained with the ET 10 isolate of *B. bassiana* (80.00%), as was the case in the nymphs, followed by the TV-67C (20.00%) strain of *B. pumilus* and FDP-1 (18.06%) strain of *B. thuringiensis*. RK 1774 (*B. atrophaeus*) (7.22%), RK 1773 (*P. fluorescens*) (6.94%), FDP-8 (*B. thuringiensis* subsp. *kenyae*) (5.56%), FDP-42 (*B. thuringiensis* subsp. *kenyae*) (5.28%), FDP-49 (*B. sphaericus*) (1.11%) were in the same group, while no deaths were observed in the CP-1 (*B. brevis*) and control applications (0.00%) (Table 2).

Table 2. Efficacy of some entomopathogen bacterial strains and fungal isolate on *Icerya purchasi*

Treatment	Percentage death ratio (%)*	
	Nymph	**Adult**
ET 10 (*Beauveria bassiana*)	100.00±0.00 A	80.00±37.75 A
RK 1773 (*Pseudomonas fluorescens*)	90.50±9,65 AB	6.94±6.67 C
FDP-42 (*Bacillus thuringiensis* subsp. *kenyae*)	88.50±6.25 B	5.28±4.99 C
FDP-8 (*Bacillus thuringiensis* subsp. *kenyae*)	82.00±17.73 B	5.56±8.20 C
CP-1 (*Brevibacillus brevis*)	58.00±38.07 C	0.00±0.00 C
FDP-1 (*Bacillus thrungiensis*)	58.00±37.85 C	18.06±12.30 B
TV-67C (*Bacillus pumilus*)	55.00±18.44 C	20.00±15.05 B
RK 1774 (*Bacillus atrophaeus*)	54.50±16.83 C	7.22±7.71 C
FD-49 (*Bacillus sphaericus*)	49.50±27.79 C	1.11±2.30 C
Control	0.00±0.00 D	0.00±0.00 C
CV	**24.28**	**78.19**
LSD	**11.15**	**7.42**

*Mean values in the same column by the same letter are not significantly different to the test of LS Means Differences Student's ($p<0.01$)

Figure 2 shows the effects of the ET 10 isolate, which was determined to be the most effective application, on the nymphs and adult of *I. purchasi* and the effects of RK 1773, FDP-42 and FDP-8 on the nymphs.

Entomopathogens are among important factors suppressing pest populations. Various pesticides are commonly used worldwide in the biological control of pests in greenhouse products, ornamental plants, stored products, forest products and products from vegetable and fruit gardens (Lacey et al., 2001).

There are many studies on *B. bassiana* (Kumar and Suktana, 2017), which is frequently included in Integrated Pest Management (IPM) programs due to its environmental-friendly structure, biological retention and ability to kill pests at various developmental periods in their life cycles (Diehl-Fleig, 1986; Adane et al., 1996, Loureiro and Monteiro, 2005; Marannino et al., 2006, Sabour et al., 2007; Castilho, 2010; Zibae et al., 2013, Tangtrakulwanich et al., 2014; Swiergiel et al., 2015). The studies have revealed that the fungal infection begins with the attachment of the spore cuticle. The fungus penetrates through the thin regions of the cuticula or mouthparts and the host become dead by feeding the inoculated food. The death of the host is caused due to the production of fungal toxins e.g. beauvericin, bassianin, bassianolide, beauverolide, tenellin, oosporein, oxalic acid, bassiacridine, cyclosporin A and hydrophilic chitinase (Yıldız, 2015). Today, there are many commercial products of *B. bassiana* and they are effectively used against different insect pests. The results of the present study about the effectiveness of *B. bassiana* represented significant potential against the nymphs and adult of *I. purchasi* and the ET 10 isolate caused a 100% death rate in the nymphs and 80% death rate in adults.

Facultative aerobic and spore-forming bacteria that have crystal proteins were commonly used for eco-friendly biological control of pests (Katı, 2008). Insecticidal crystal proteins cause a death against numerous insect pests and vector pests (Jackson et al., 2000; Katı, 2008; Azizoğlu et al., 2012). Crystal proteins are taken from the water during feeding, dissolves in the intestines of the pest, a high-alkali environment, and the toxin activated by protease goes through the cell wall after binding to specific parts of middle intestinal cells and perforates the cell wall. This disturbs the ion balance of intestinal epithelial cells and kills the pest (Nielson-LeRoux et al., 2001; Smith et al., 2005; El-Bendary, 2006).

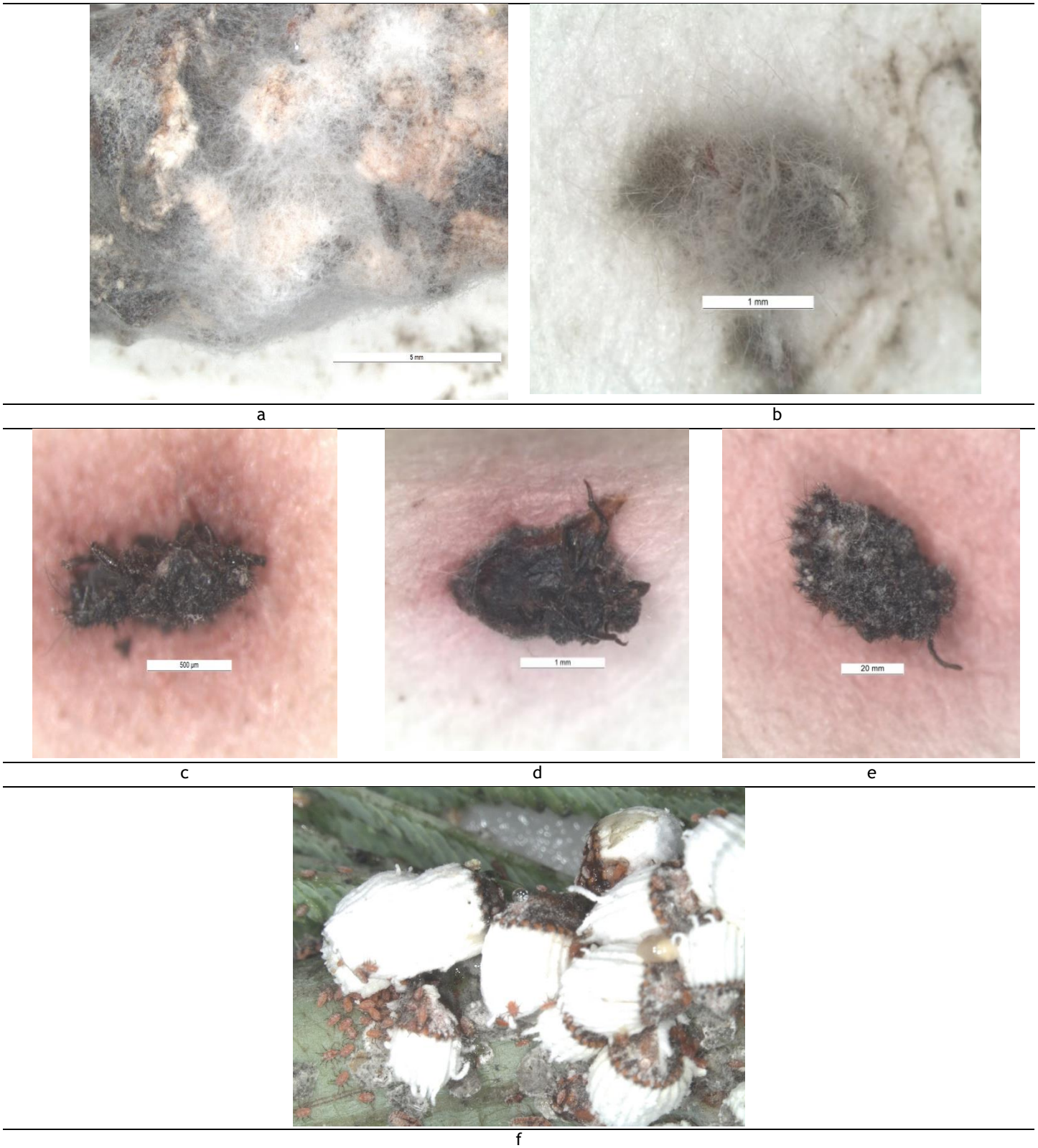
Among these bacteria, the most important group is the *Bacillus* species (Gray et al., 2001; Alper et al., 2013) and the most investigated bacteria from the *Bacillus* genus is *B. thuringiensis*, which constitutes 2% of world insecticide market (Bravo et al., 2007) and known as a pesticide with a lower risk than chemicals (Ertürk and Yaman, 2019). The bacteria have many varieties and each variety could kill a specific insect and produce a different toxin. Accordingly, in this study, *B. thuringiensis* subsp. *kenyae* was the most effective bacterial agent against the nymphs while *B. thuringiensis* was on adults. However, Hajaij et al. (2005) reported that there were significant decreases in the number of spores immediately after the application and bacteria spores could not reproduce during the dead stages of the pest in areas where *B. thuringiensis* was applied and emphasized that the *B. thuringiensis* applications should be periodically repeated.

In the study, *P. fluorescens* was the another bacterial species that was effective on the nymphs. Suganthi et al. (2017) reported that the MP-13 isolate of *P. fluorescens* resulted in a death rate of 100% in tea mosquito bug (*Helopeltis* spp. (Hemiptera: Miridae)) under *in vitro* conditions and recorded that *P. fluorescens* affected the pest by enzymatically hydrolyzing the chitin in the exoskeleton of the insect. Moreover, the researchers also reported that the enzyme affected the digestion of the insect and caused death by directly inhibiting the growth and development of the insect.

Another important species used in the biological control of pests is *B. sphaericus*. In general, after the application of *B. sphaericus* to the pests, the feeding of the insect stepped, the insect activity decreased within two hours and insect was paralyzed after around six hours. Furthermore, the reproduction of the *B. sphaericus* spores on dead larvae has been reported to be important in the control of pests (Boonserm et al., 2006). In this study, the FD-49 strain of *B. sphaericus* whose insecticidal effect on *I. purchasi* was investigated caused a death rate of 49.40% in the nymphs and

1.11% in the adults and, thus, was less effective compared with other bioagents.

In conclusion, the fungal bioagent *B. bassiana* ET 10 isolate was found to be effective on the nymphs and adult *I. purchasi* under controlled conditions. In addition, *P. fluorescens* RK 1773 and *B. thuringiensis* subsp. *kenyae* FDP-42 were effective on the nymphs and *B. pumilus* TV-67C and the FDP-1 strain of *B thuringiensis* were effective on the adults.



a b c d e f

Figure 2. ET 10 on *Icerya purchasi* adults (a), ET 10 (b), RK 1773 (c), FDP-42 (d), FDP-8 (e) on *Icerya purchasi* nymphs and control (f)

Conclusion

The study emphasizes the importance of the inclusion of biopesticides that do not have the risk of resistance and any toxic effects on the environment and human health and are useful in the areas where the effectiveness of the natural enemy is low into the control systems. The effectiveness of the bioagents used in the study may vary under the field conditions at different temperatures and humidity levels. Thus, carrying out field studies involving the bacteria strains and fungus isolate that are effective on the pest in the future is of great importance.

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