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# Investigation of some enthomopathogens as biocontrol agents of *Tinocallis* (*Sappocallis*) *saltans* (Nevsky, 1929) (Hemiptera: Aphididae)

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**Abstract:** In Turkey, *Tinocallis (Sappocallis) saltans* (Nevsky) on *Ulmus glabra* Hudson was detected for the first time, and it was determined that it caused a considerable amount of damage. The study was conducted for the purpose of determining the usability of enthomopathogens in the control against it, while avoiding the negative effects of chemicals on the environment and human health. A total of 12 bacteria strains were tested for control efficacy and verified: *Brevibacillus brevis* (2), *Bacillus thuringiensis* (1), *Bacillus thuringiensis* subsp. *kenyae*(2), *Bacillus thuringiensis* subsp. *kurstakii* (2), *Bacillus subtilis* (1), *Pseudomonas chlororaphis* (1), *Bacillus sphaericus* GC subgroup D (1), *Pseudomonas fluorescens* biotype C (1), *Bacillus atrophaeus* (1), and 1 fungus isolate (*Beauveria bassiana*). The trials were carried out in 2 different forms: under controlled conditions and under field conditions. Data showed that the mortality rates were between 12.84% and100% in controlled condition and between 7.72% and 31.79% in field condition over the 72 h period. *B. thuringiensis* subsp. *kurstakii* (FDP-41), *B. subtilis* (EK-7), and *B. thuringiensis* subsp. *kenyae* (FDP-42) were the most effective bacterial strains against the aphids in controlled conditions. The percentage of mortality related to these applications was 100% in 48 h in controlled condition. However, the effectiveness of *B. thuringiensis* subsp. *kurstakii* (FDP-41) was 31.79% in 72 h in field conditions. On the other hand, fungal isolate *B. bassiana* (ET 10) suppressed the harmful population significantly in controlled conditions (86.64%); however, it was less effective in field conditions (13.04%). As a result, it was concluded in the present study that these bacteria strains may be used successfully as bioagents in the biological control against *T. saltans*.

**Key words:** *Bacillus* spp., *Beauveria bassiana*, biological control, *Tinocallis (Sappocallis) saltans*

## 1. Introduction

The Aphididae (Hemiptera) family, which has a total of 570 species in Turkey (Görür et al., 2020), causes worldwide destruction in crops, especially in fruits, vegetables, vine, and ornamental plants and forest plants, either directly by sucking the sap of the plants or indirectly by carrying plant viral diseases. The Ulmaceae family is included as hosts of these harmful aphids and is represented by more than 100 species of *Ulmus* L. and *Zelkova* Spach. (Yaltırık, 1993). *Ulmus* has a total of 4 species in Turkey. The species that is called *Ulmus glabra* Hudson because its shell does not crack is one of them. Blackman and Eastop (2019)[1] list about 75 species of aphids as feeding on elms worldwide; 22 of these species are fed on *Ulmus glabra*. It was observed that *Tinocallis saltans* had had a population at high density on this tree for the past several years and caused damage. *Tinocallis* Matsumura, 1919 is a small group of aphids that is mainly associated with the Ulmaceae and contains 8 known species (Richards, 1967).

There are studies reporting that *T. saltans* causes significant damage on *Ulmus* species. Khamraev and Davenport (2004) reported that *T. saltans* was a very common and important aphid, and added that it had negative effects on photosynthesis because it fed on the sap of the leaves, which were left small; and aside from this, the aphid reallocates sugary substances over leaf surfaces, polluting them and the area under the tree canopy. Because of feeding elm aphids on the leaves, the leaves swell and fold down along the leaf edge. Elm aphids secrete copious amounts of honey extract as their nutrition and proliferation increase. This sledgehammer is particularly uncomfortable because it covers over pavements, cars, and other objects under trees (Anonymous, 2017).

Khamraev and Davenport (2004) reported that lambda-cyhalothrin 55 s.c (0.05%) bifenthrin 10% s.c (%0.1), and tau-fluvalinate 2U 25 0.1% s.c were effective insecticides used to control this species. Similarly, Anonymous (2017) reported that leaves can be pruned in the cultural control

[1] Blackman RL, Eastop VF (2019). Aphids on the world's plants. An online Identification and Information Guide [online] Website http://www.aphidsonworldsplants.info [accessed 03 May 2019].

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of elm aphids. It is also stated that insecticidal soap, diazinon, or malathion can be applied in the chemical control of aphids (Anonymous, 2017). The negative effects of pesticides on the environment and human health have led researchers to develop alternative control strategies and a reduction in pesticide use. Biological methods have gained importance over time to resolve this situation, and it has been emphasized in every platform that biological products must be developed to reduce the negative effects of the pesticide chemicals on the environment and human health. Researchers have focused on studies that investigate the methods that may be used in the biologically-based control against harmful species under field conditions and/or controlled conditions.

In this study, the purpose was to determine the efficacy of some bacterial isolates and a fungal isolate for control of *T. saltans*, tested under controlled and field conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and fungal isolate used in this study

A total of 12 bacterial strains and 1 fungal isolate used in this study were tested for their entomopathogenic activities against pests in controlled and field conditions. Ten of the bacterial strains (CP-1, FDP-1, FDP-8, FDP-41, FDP-42, FD-1, EK-7, NEM-28, FD-49, BAB-410) and the fungi (ET 10) were isolated in our previous studies (Tozlu et al., 2011; Dadaşoğlu et al., 2013; Tozlu et al., 2016a; Göktürk et al., 2018; Tozlu et al., 2016b). They were obtained from the culture collection unit in the Department of Plant Protection, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. The bacterial strains were kept at –86 °C in stock growth media containing 30% glycerol and Loria Broth (LB), and fungi were maintained on Potato Dextrose Agar (PDA) slant cultures at 4 °C in the refrigerator until use.

### 2.2. Fungal isolate

ET 10 was obtained from *Sphenoptera antiqua* Illiger (Buprestidae: Coleoptera) larvae and identified as *Beauveria bassiana,* according to molecular identification in the previous study (Tozlu et al., 2017). The genome of the pure isolate was identified using PCR method with primer specific for identification of *B. bassiana*. According to the molecular identification test results, ET 10 fungal isolate was identified as *Beauveriabassiana* (GB |KC753398.1|) at 0.99 similarity index (Tozlu et al., 2017).

### 2.3. Host plants and insects

Elm trees (*Ulmus glabra*) were used as the host plant. Elm trees were naturally infested with *T. saltans* as harmful insects in this study. *U. glabra* was identified by Dr. Hilal Turgut, a specialist in ornamental plants, and *T. saltans* was identified by Dr. Işıl Özdemir, a specialist in Aphididae in Turkey.

### 2.4. Identification of the bacteria by MIS

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

### 2.5. Hypersensitivity test of bacteria

The bacterial strains were tested on tobacco plants (*Nicotina tabacum* L. var. Samsun) for their hypersensitivity activity, as described by Klement et al. (1964).

### 2.6. Preparation of bacterial suspensions

All bacterial isolates were incubated in Tryptic Soy Agar (TSA, Oxoid) at 27 °C for 24 h. After the incubation period, a single colony was transferred to 500 mL flasks containing Tryptic Soy Broth (TSB, Oxoid), and grown aerobically in the flasks on a rotating shaker (150 rpm) for 48 h at 27 °C (Merck KGaA, Darmstadt, Germany). The bacterial suspension was then diluted in sterile distilled water (sdH2O) to a final concentration of $1 \times 10^8$cfu mL-1 with a turbidimeter.

### 2.7. Preparation of conidial suspensions

*B. bassiana* (ET 10) was cultured on Sabouraud Dextrose Agar (Merck KGaA) with 1% yeast extract (SDAY) plates in several petri dishes (9 cm in diameter) and was grown for 2–3 weeks at 25 ± 1 °C under a 16 h/8 h (light/dark) photoperiod and 80% ± 10% RH for fungal growth and conidial production (Tozlu et al., 2017). A suspension of inoculum was prepared by scraping conidia from the cultures into an aqueous solution of 0.02% Tween 80 (Quesada-Moraga et al., 2006). The surface of a 14-day-old culture was gently scratched with an inoculation needle and transferred to vials containing 5 mL sterile Tween-80 solution (0.1% v/v). The concentration of conidia in stock suspensions was determined by direct count using a hemocytometer. $1 \times 10^6$ (conidia/mL) conidial suspensions (Quesada-Moraga et al., 2006) were prepared in sterile distilled water containing Tween 80 and vortexed for 3 min to produce a homogenous suspension for the bioassay.

### 2.8. Controlled condition assays

The efficacy of the bacterial strains and fungal isolate against *T. saltans* was tested using elm tree leaves under controlled condition [25 °C, in 75%–80% relative humidity (RH) in a photoperiod of 16 h light and 8 h dark (Vu et al., 2007)]. The elm tree leaves naturally infested with *T. saltans* were brought to a laboratory of the Department of Plant Protection, Faculty of Agriculture, Atatürk University. The leaf area of the leaves was measured, and the number of *T. saltans* was recorded to determine the density per leaf. Almost all aphid samples were in the nymph stage.

Each leaf infested with *T. saltans* was placed in a polyethylene-lined plastic box (19 × 12.5 × 7 cm) and $10^8$ cfu/mL bacterial suspensions, or $1 \times 10^6$ conidia/mL fungal suspension was sprayed on the infested leaf (Figure

1a). Positive and negative control leaves including *T. saltans* were sprayed with only TSB and Malathion. The experimental design included 3 replications, and each replicate contained 1 leaf. The dead individuals were counted on the leaves, after 24, 48, and 72 h.

**2.9. Field assays**

Shoots were selected from different parts of the elm tree that was infested with *T. saltans*. Each shoot had 9 leaves. $10^8$ cfu/mL bacteria suspension or $1 \times 10^6$ conidia/mL fungal suspension was applied to these leaves, and only TSB and Malathion were sprayed in the positive and negative control application. These leaves were then placed in a 23 × 25 cm tulle cage (Figure 1b).

24 h after the first application, 3 of the 9 leaves were selected randomly and brought to the laboratory. The leaf area was measured, and the living and dead aphid counts were recorded using a microscope. This process was repeated at the 48th and 72nd h by taking the remaining leaves.

**2.10. Data analysis**

The mortality rate of *T. saltans* was analyzed by JMP, 5.0, and separation of means was performed with LSMeans Students tests at $P < 0.01$ probability level.

**3. Results and discussion**

Identification results and similarity indexes (SIM) of bacterial strains and fungal isolate and HR test results of the bacteria are given in Table. According to these results, HR result of all of the bacteria was negative.

In controlled conditions, the visible mortality rates after 24, 48, and 72 h of the applications are given in Figure 2. The counting of the dead aphids was cumulative. According to the results obtained, the highest mortality rate after 24 h was 100% in aphids to which FDP-42 strains were applied; followed by the EK-7 (97.98% mortality) and FDP-41 (93.09% mortality) strains, respectively. After 48 h, 100% death rate was observed in the EK-7 and FDP-41 applications. After 72 h, the mortality rate was 93.70% in RK-1774, and 90.34% in RK-1773. High mortality rates were observed in all microbial treatments and Malathion application; however, no individuals died in the control application. In the fungal isolate ET 10 application, the mortality rates were 26.81% after 24 h, 77.20% after 48 h, and 86.64% after 72 h (Figure 2).

The mortality rates that were observed in field conditions after 24, 48, and 72 h of the application are given in Figure 3. The mortality rates varied between 4.16% and 13.40% at 24 h, 10.70% and 25.79% at 48 h, and between 7.72% and 31.79% at 72 h in field conditions. ET 10 mortality rates at 24, 48, and 72 h were 12.40%, 11.73%, and 13.04% in field conditions, respectively (Figure 3).

*T. saltans* in the most effective bacterial and fungal isolates treatment at the end of a 3-day follow-up period is given in Figure 4.

It was reported that *T. saltans* caused damage on *Ulmus pumilla* in North America (Halbertand Pike, 1990), Spain (Núñez-Pérez et al., 1991), Italy (Patti and Barbagallo, 1998), East Europe (Romania, Hungary), and Mid and East Asia (Eastern Iran, Urals, Kazakhstan, Uzbekistan, Tajikistan, Pakistan, Siberia, Afghanistan, Korea, and China); and on *Zelkova serrata* in Hungary (Ripka, 1998).

However, since pests can have resistance to chemical agents, new control measures are sought for pests such as aphids (Devonshire, 1989). For this reason, the importance of studies that are intended to develop new methods of using biological agents that may be alternatives in controlling pests is increasing. Biopesticides do not have negative effects on humans and the environment, predatory insects and do not leave toxic residue on foods (Tozlu et al., 2019). They may resolve the above mentioned problems permanently and are cost-effective in the long run. They provide high protection with low costs, are effective only on the target pests, do not cause endurance problem on the target pests, and may sustain their existence even in lowly populated hosts. This is why they are preferred in the control (Öncüer, 1991).


**Figure 1.** Applications of leaves in laboratory (a) and field (b).

**Table.** The identification results of bacterial strains and fungal isolate and hypersensitivity test results of the bacteria used in this study.

| Strain No. | Isolated from | Microbial identification results of bacteria | SIM | HR | Reference |
|---|---|---|---|---|---|
| CP-1 | *Ricaniz simulans* (Hem.: Ricaniidae) | *Brevibacillus brevis* | 0.65 | - | Gokturk et al., 2018 |
| FDP-1 | *Malacosoma neustria* (Lep.:Lasiocampidae) | *Bacillus thuringiensis* | 0.64 | - | Gokturk et al., 2018 |
| FDP-8 | *Hypera postica* (Col.: Curculionidae) | *Bacillus thuringiensis* subsp. *kenyae* | 0.45 | - | Tozlu et al., 2011 |
| FDP-41 | *Apion* spp. (Col.: Brentidae) | *Bacillus thuringiensis* subsp. *kurstakii* | 0.57 | - | Tozlu et al., 2011 |
| FDP-42 | *Apion* spp.(Col.: Brentidae) | *Bacillus thuringiensis* subsp. *kenyae* | 0.47 | - | Tozlu et al., 2011 |
| FD-1 | *Malacosoma neustria* (Lep.:Lasiocampidae) | *Brevibacillus brevis* | 0.65 | - | Tozlu et al., 2011 |
| EK-7 | *Rosa canina* (Rosales: Rosaceae) | *Bacillus subtilis* | 0.65 | - | Tozlu et al., 2016 |
| NEM-28 | *Ricanizsimulans* (Hem.: Ricaniidae) | *Pseudomonas chlororaphis* | 0.40 | - | Gokturk et al., 2018 |
| FD-49 | *Culex* sp. (Dip.: Culicidae) | *Bacillus sphaericus* GC subgroup D | 0.71 | - | Dadasoglu et al., 2013 |
| BAB-410 | *Ricaniz simulans* (Hem.: Ricaniidae) | *Bacillus thuringiensis* subsp. *kurstakii* | 0.62 | - | Gokturk et al., 2018 |
| RK-1773 | *Pseudaulacaspis pentagona* | *Pseudomonas fluorescens* | 0.59 | - | In this study |
| RK-1774 | *Pseudaulacaspis pentagona* | *Bacillus atrophaeus* | 0.60 | - | In this study |
| Strain No. | Isolated from | ITS identification results of fungi | | | |
| ET 10 | *Sphenoptera antiqua* Illiger (Col.: Buprestidae) | *Beauveria bassiana* | 0.99 | | Tozlu et al., 2017 |

SIM: Similarity index, -: Negative reaction, HR: Hypersensitivity test

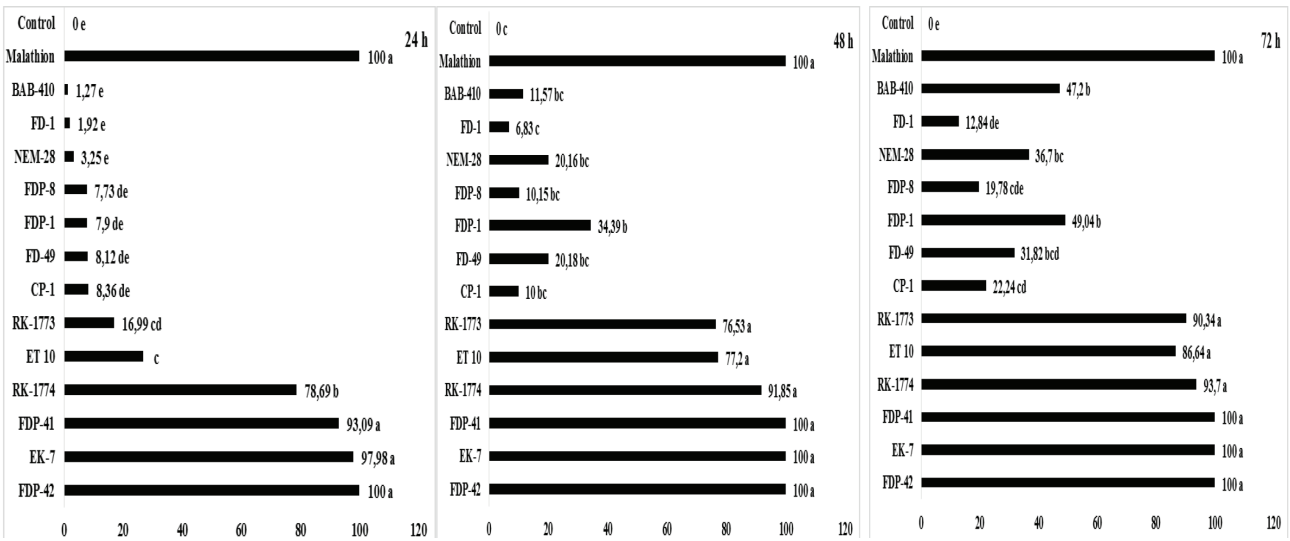


**Figure 2.** The mortality rates (%) from the bioagent fungus and bacteria that were tested against *Tinocallis saltans* under controlled conditions.

The use of *Bacillus* species in a successful manner in the biological control against pests has been reported in various studies conducted previously (Gray et al., 2001; Alper et al., 2013), and it was reported that especially *B. thuringiensis* (BT) constituted 2% of the insecticidal market (Bravo et al., 2011). It is stated that the sales of entomopathogens (bacteria, viruses, and fungi etc.) in Europe in 2010 were approximately 42 million euros, and the majority of them (58%) were obtained from *B. thuringiensis* (Glare et al., 2012). BT products are used to target lepidoptera or diptera larvae, and a few coleopterans (van Lenteren et al., 2018). It is reported BT is better in terms of efficacy against aphids (van Lenteren et al., 2018), such as *Macrosiphum euphorbiae* (Walters and English, 1995) and *Acyrthosiphon pisum* (Porcar et al., 2009) in some investigations. However, there is not any report about the biological control of *T. saltans* with *Bacillus*.

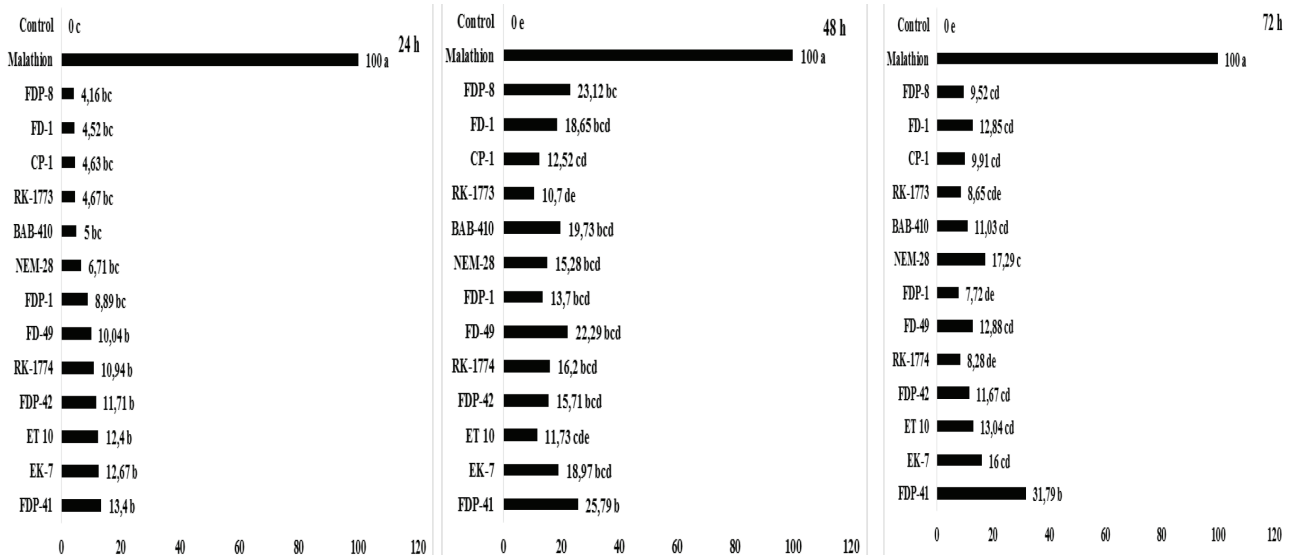


**Figure 3.** The mortality rates (%) obtained from the bioagent fungus and bacteria tested against *Tinocallis saltans* under field conditions.

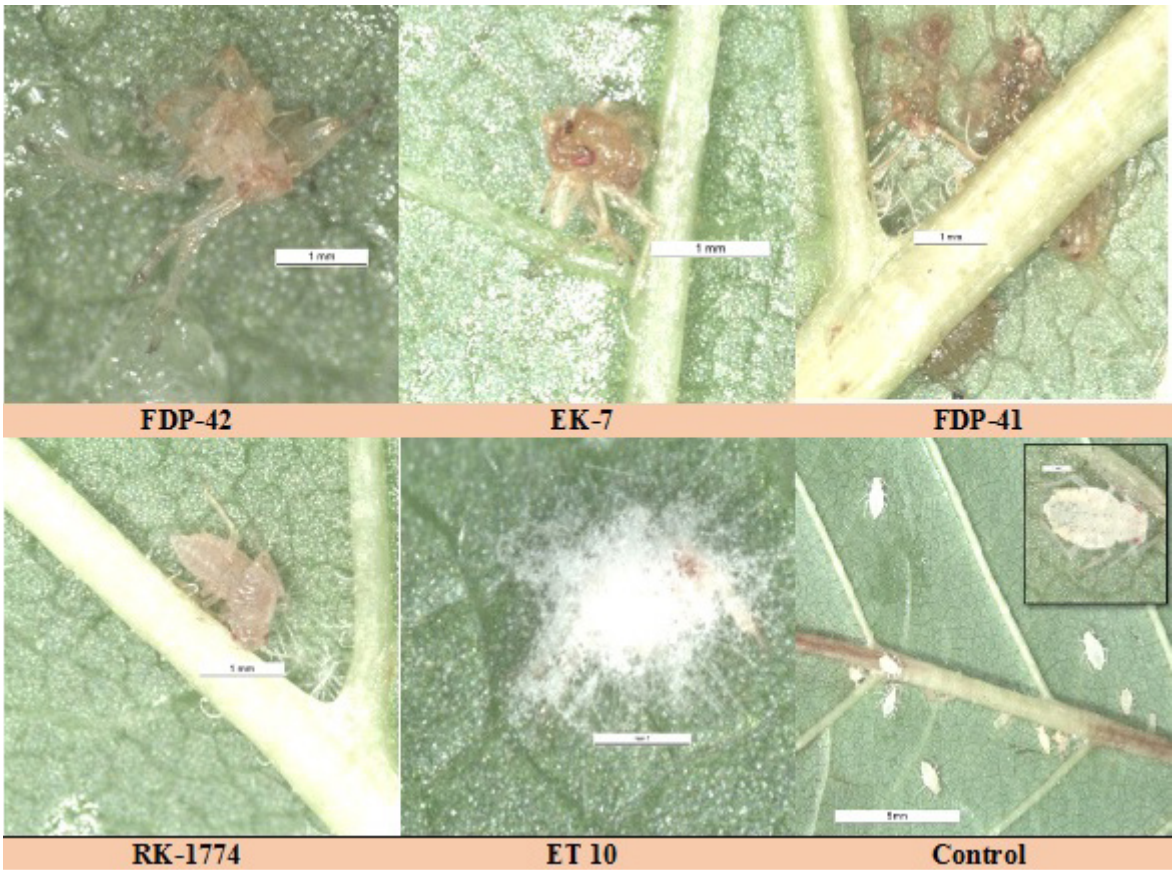


**Figure 4.** Appearance of the *Tinocallis saltans* individuals following bacterial and fungal isolates treatments.

The *Bacillus* species were also tested in this study, and the mortality rates that were determined in controlled conditions varied under field conditions that had different temperature and moisture. It is likely that the reason for this is the adaptation of the biological control agents under different conditions. In controlled conditions, the

*B. thuringiensis* subsp. *kenyae* (FDP-42), and *B. atrophaeus* (RK-1774) were used against *T. saltans*, and *B. subtilis* (EK-7) and *B. thuringiensis* subsp. *kurstakii* (FDP-41) gave the most effective results both in controlled and in field conditions.

*Bacillus* species is a well-known biocontrol agent that has been used for many years for pest control in agriculture and disease-related insect vectors (Veliz et al., 2017). In our previous studies, some bacterial strains and fungal isolate used in this study were reported to be successful in controlling different pest species and/or fungal pathogens. For example, *B. brevis* FD-1 showed significant insecticidal effect on *Culex pipiens* larvae but not *B. thuringiensis* FDP-41 and *B. thuringiensis* FDP-42. Mortality rate was recorded as 100% at 72h in *B. brevis* FD-1 application (Dadaşoğlu et al., 2013). We also have other studies revealing *B. brevis* strains FD-1 has important insecticidal activity against pests, including different genus. *B. brevis* FD-1 was also tested against *Caliroa cerasi* L. (Dadasoglu et al., 2014), *Diprionpini* L. (Dadasoglu et al., 2016), and *Ricania simulans* (Göktürk et al., 2018). Its insecticidal activities were obtained as 86%, 66%, and 27%, respectively. In another study, *B. thuringiensis* FDP-41, *B. thuringiensis* FDP-42, and *B. subtilis* EK-7 isolates were also tested against *R. simulans*, and the mortality rates of these applications were determined as 25%, 42%, and 25, respectively (Gokturk et al., 2018). Yet in another study, it was reported that *B. thuringiensis* FDP-41 and *B. thuringiensis* FDP-42 strains could be used as biocontrol agent against *Bruchus dentipes* (Tozlu et al., 2011).

As a result of the antibacterial, antifungal, insecticidal, or nematicidal activities of microbial chitinases, the cell walls of many pests and pathogens weaken and deteriorate (Edreva, 2005). Several chitinolytic organisms, such as *Bacillus* sp, *Pseudomonas* sp., and *Streptomyces* sp., have been shown to be potential biological control agents of various fungal pathogens and pests (Bélanger, 2001). Jabeen et al. (2018) reported that chitinolytic bacteria degraded chitin content of termites can be used as a biocontrol agent for this pest. In this study, the mortality rate of *T. saltans* was 100% after 48 h and 16% after 72 h in *B. subtilis* EK-7 strain application in controlled condition and field condition, respectively. Our previous study showed this strain has also remarkable antifungal activity against *Penicillium digitatum*, and chitinase and glucanase enzyme activity of this strain was positive (Mohammadi et al., 2017). Percentage of mortality employing chitinases to control the pests proved that chitinolytic *B. subtilis* EK-7 is an effective biological control agent for some insect pests. Upon degradation of chitin by a number of organisms, severe damage and even death may occur in pathogens and pests whose external surfaces contain this polymer (Veliz et al., 2017). Some studies also report a synergistic effect between *B. thuringiensis* endotoxins and chitinases (Kramer and Muthukrishnan, 1997). In some studies, chitins have been shown to affect insect growth. Both the feeding speed and body weight of the larvae in contact with chitinases decrease, and as a result, death can be observed (Veliz et al., 2017).

In our previous study, *B. brevis* strains FD-1 bacterial strain and *B. bassiana* ET 10 fungal isolate were tested against the nymphs of *Halyomorpha halys* (Stal, 1855) (Hemiptera: Pentatomidae). Mortality rates of 95% and 76% were obtained by FD-1 and ET-10, respectively (Tozlu et al., 2019). The effectiveness of bioagents in controlled conditions was higher than their activities in field conditions in this study. For example, while *B. thuringiensis* FDP-41 is 100% effective in controlled conditions, its effectiveness in field conditions has dropped to 31%. The effectiveness of bioagents in controlled conditions cannot be expected to be the same in field conditions. The dosage of bioagent bacteria or fungus used against pests is also very important. In another previous study of ours, *B. bassiana* ET 10 isolate was tested against *Syrista parreysii* larvae. The mortality rate (%) of $10^6$, $10^7$, and $10^8$ conidial suspensions of ET 10 were 82%, 83%, and 90%, respectively (Tozlu et al., 2017). Therefore, their effectiveness can be further increased by increasing the usage dose of the bioagents used.

Entomopathogenic fungi also provide a sustainable solution with their cosmopolitan existence and a rich variety in integrated pesticide management (IPM) programs. Due to their environmentally-friendly nature and biopermanency, these fungi are ideal in killing pests in various growth stages of their lives (Kumar and Sultana, 2017). Entomopathogenic fungi that attack insects are important agents for biocontrol and play an important role in promoting integrated pest management (Cooke, 1977). They have been produced as biopesticides since the 1970s and are preferred against aphids because aphids are sensitive to natural fungal epizootics (Milner, 1997). However, it is difficult to mass-produce and formulate entomophthorales, which are the most successful against aphids in nature (Leite et al., 2003). Thus, efforts to produce the environmentally-friendly species have increased recently (*Beauveria, Isaria, Metarhizium,* and *Lecanicillium*). Up until now, a variety of strains of entomopathogenic fungi, such as *Lecanicillium* sp. (Jackson et al., 1985; Jung et al., 2006; Steenberg and Humber, 1999; Kim, 2004), *B. bassiana* (Quesada-Moraga et al., 2006), *M. anisopliae* (Shia and Feng, 2004; Wright et al., 2004), *Isaria* (Shia and Feng 2004), and *Nomuraea rileyi* (Devi et al., 2003) have been used for the management of aphids and many other pests. Hesketh et al. (2008), Shan and Feng (2010), and Tesfaye and Seyoum (2010) demonstrated

high (>75%) mortality of adult *M. persicae* or *A. gossypii* exposed to various *Beauveria* and *Metarhizium* isolates. *B. bassiana*, which is one of the environmentally-friendly entomopathogenswith a wide host range (Khachatourians, 1986; Leathers and Gupta, 1993; Quintela and McCoy, 1998; Zibae et al., 2013), caused death against *T. saltans* at a rate of 86.64% in controlled conditions, and 13.04% in field conditions in this study.

## 4. Conclusion

As a result, the controlled condition assays display more promising results, compared to the field assays. *B. thuringiensis* subsp. *kenyae* FDP-42, *B. subtilis* EK-7, *B. thuringiensis* subsp. *kurstakii* FDP-41, and *B. atrophaeus* RK-1774 strains originally isolated from Turkey were effective under controlled condition assays. Among these, FDP-42 strains were also effective in field assays. Considering the negative effect of insecticides, it was concluded that the FDP-42 producing chitinase could be an ideal candidate for environment-friendly control. Moreover, aphid has a complex life cycle, and thus when considering the effects of these strains as a biocontrol agent against aphid species, more integrated studies should be carried out.

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