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Potentials of some entomopathogens against the brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae)

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Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae), is an invasive harmful pest species due to its economic losses. Its wide host range and continuous movement make its control difficult with insecticides. Biological control has recently gained importance due to the negative aspects of chemical control. The study evaluated the biological control tools by testing the entomopathogens against the pest by 11 bacteria strains and 1 fungal isolate. *Brevibacillus*, *Bacillus*, *Pantoea*, *Vibrio*, *Pseudomonas*, and *Beauveria* were tested against the nymphs of *H. halys* under controlled conditions. All applied entomopathogens had potentials for controlling *H. halys*. Mortality rates of 75 and 100% were obtained by the bacteria strains and 76.19% by the fungus, *B. bassiana*. Successfully reaching a 100% control rate, the bacterial isolates of the *Bacillus cereus* GC subgroup B and *Pantoea agglomerans* GC subgroup were recorded to have a greater potential than the others.

Keywords: *Halyomorpha halys*, Entomopathogens, Biological control, Potentials

Background

The brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) is a polyphagous pest that has more than 300 host plants. It is originated from East Asia (Rider 2006). It was identified as a new invasive species in the USA in 2001 (Hoebeke and Carter, 2003) and then widely populated in several countries worldwide (Haye et al. 2015). *H. halys* was first detected in Turkey by Çerçi and Koçak (2017) in Istanbul. Göktürk and Tozlu (2019) reported that they identified the species in 2016 in the coasts of the Black Sea. The damage caused by *H. halys* is known by its feeding on various plant species worldwide including economically important plants (Kuhar et al. 2012 and Rice et al. 2014). The pest is known also spreading certain plant diseases (Bernon et al. 2004).

Chemical control is often the first tactic that farmers or pest managers think of such notorious pest. However, its continuous movement by easily flying from field to field makes its control with insecticides difficult (Bariselli et al. 2016). The negative effects of pesticides on the environment and human health have reached vast dimensions and brought along the absolute need to develop alternative control strategies and reduction of the use of pesticides. Biological methods have gained importance in the solution of this problem, and the need to develop biological products that can reduce the negative effects of chemicals on the environment and human health has been emphasized in every platform, leading to the idea to investigate the means of biological control. 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 non-persistent remedial treatments that are released inundatively (Bhattarai et al. 2016).

This study aimed to evaluate the potentials of some entomopathogens such as *B. thuringiensis kurstaki*, *Bacillus atrophaeus*, *Bacillus sphaericus*, *Bacillus cereus*, *Pantoea agglomerans*, *Pseudomonas flourescens*, *Vibrio*

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hollisae, and *Brevibacillus brevis* that can be recommended as the biological control of *H. halys.* The study was carried out under laboratory conditions.

Materials and methods

Collection of harmful nymphs

Nymphs of *H. halys* were collected from the hazelnut, corn, and bean in Artvin, Turkey, using sweep net and shaking various parts of the plants on a white bed sheet (3 × 3.5 m^2), while its eggs were collected using visual inspection. The hazelnut leaves with egg packs and nymphs were placed in containers and brought to the Plant Clinical Laboratory of the Plant Protection Department of Atatürk University, Faculty of Agriculture, Turkey.

Entomopathogenic bacterial and fungal isolates

Entomopathogen strains, previously tested against other pests in other studies, and were available in the Atatürk University, Faculty of Agriculture, Plant Protection Department and kept at − 80°C in the Nutrient Broth (NA; Difco) containing 15% glycerol after culture in the Nutrient Agar (NA; Difco) medium were used. As the entomopathogen fungus, *Beauveria bassiana* ET 10 fungal isolate that was available in the Mycology Laboratory of the Atatürk University, Faculty of Agriculture, Plant Protection Department and kept in tubes containing Potato Dextrose Agar (PDA; Difco) after isolation the *Sphenoptera antiqua* (Illiger) larvae, a pest of sainfoin (*Onobrychis sativa* L. (Fabacea)), was investigated (Table 1).

Identification of entomopathogenic bacteria with MIS

The extraction and analysis of the fatty acid methyl esters of the bacterial biocontrol isolates were carried out, following the standard protocol of the Microbial Identification System (MIS) (MIDI, Inc., Newark, DE) (Paisley 1995). The bacteria strains that were prepared from the fresh cultures, using a sterile platin loop, were analyzed, using the MIS device and the identification results were obtained.

Hypersensitivity to tobacco test of the bacterial biocontrol agents

The fresh leaves of the tobacco, *Nicotina tabacum* L. var. Samsun tobacco variety that 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 24–48 h, were injected between 2 adjacent trachea and the leaves were inspected for signs of symptoms. Those that did not show symptoms in tobacco leaves were regarded as negative, while those that show symptoms in tobacco leaves were regarded as positive (Klement, 1964).

Preparation of the bacterial suspensions

The 3-4-phase inoculation of the tested bacteria into the NA medium was carried out and the strains were cultured for 24 h at 30 °C to obtain fresh cultures. A single bacteria colony that was collected from the cultures, using a sterile loop under controlled conditions, was inoculated into the Erlenmeyer flasks containing 300 ml Nutrient Broth (NB) and incubated at 27 °C for 24 h in a thermostatic shaker at 250 rpm. The bacteria density in

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

Bacterial strains

Strain	Isolated from	MIS Identification results	S	HR	Reference
FD 1	*Malacosoma neustria*	*Brevibacillus brevis*	0.625	–	Tozlu et al. 2011
FD 16	*Yponomeuta evonymella*	*Bacillus thuringiensis kurstaki*	0.805	–	Dadaşoğlu et al. 2016
FD 17	*Yponomeuta evonymella*	*Bacillus atrophaeus*	0.459	–	Tozlu et al. 2011
FD 49	*Culex* sp.	*Bacillus sphaericus*	0.681	–	Dadaşoğlu et al. 2016
FD 51	*Culex* sp.	*Bacillus thuringiensis kurstaki*	0.368	–	Dadaşoğlu et al. 2016
FD 63	*Yponomeuta evonymella*	*Bacillus cereus*	0.241	–	Tozlu et al. 2011
FD 68	*Melolontha melolontha*	*Pantoea agglomerans*	0.734	–	In this study
FD 69	*Melolontha melolontha*	*Pantoea agglomerans*	0.552	–	In this study
FD 70	*Melolontha melolontha*	*Vibrio hollisae*	0.476	–	In this study
FD 71	*Melolontha melolontha*	*Pseudomonas flourescens*	0.913	–	In this study
FDP 8	*Bemisia tabaci*	*Bacillus cereus*	0.652	–	Tozlu et al. 2011

Fungal isolate

Isolate	Isolated from	ITS Identification Result	S	ITS 1 sequences	Reference
ET 10	*Sphenoptera antiqua*	*Beauveria bassiana*	0.99	GB |KY806126|	Tozlu et al. 2017

S Similarity, – negative effect, *HR* hypersensitivity

the aqueous culture was adjusted at (1×10^8 CFU/ml) by a spectrophotometry, using NB medium and transferred to sterile spray bottles.

Preparation of the fungal conidia suspension

Conidia formation was obtained by the incubation of the *B. bassiana* ET 10 isolate in the Sabourth Dextrose Agar (SDA) medium at 25 °C and 80% RH for 2–3 weeks. Then, the surface of the culture was rinsed with 0.2 ml/l Tween-80 solution (Quesada-Moraga et al. 2006) into sterile water-containing bottles to prepare the stock suspension. The conidia suspension was adjusted at 5.7×10^5 conidia/ml using hemocytometry.

Testing the entomopathogens against the pest

Fresh green beans were placed in each Petri dish (9 cm), and 6 nymphs (third and fourth nymphal instars) were counted and left in the Petri dishes and then sprayed by the bacteria and fungus suspensions. The entomopathogen-applied Petri dishes were kept under controlled conditions at 25 ± 2 °C and 65–70% RH and under a photoperiod regime of 16:8 (light: darkness). The number of dead nymphs was cumulatively recorded daily. The final evaluation of the trial was carried out and the mortality rates were determined at 264 h. Re-isolation from the nymphs that were determined to be infected according to the Koch Postulates was performed and the entomopathogenic fungus and bacteria were recovered. The sterile water containing both the sterile NB medium used in the dilution of the bacteria solutions and 0.2 ml/l tween 80 solution used in the fungus suspensions was used as the negative control in the study. The trial was carried out in four repetitions for each combination in the same day.

Potentials of some bacteria strains and the fungal isolate against the eggs of the pest

After counting the eggs in the egg packs, the packs were removed from leaves and placed in Petri dishes, placed on blotting papers, to determine the effects of 11 bacterial strains and 1 fungal isolate (Table 1) on the eggs. The (1×10^8 CFU/ml) bacteria and (5.7×10^5 conidia/ml) fungal suspensions that were previously prepared for the nymph applications were sprayed onto the egg packs including 5–37 eggs. Sterile water was applied as a control application and the number of hatched eggs was counted.

Statistical analysis

The data obtained from different trials under controlled conditions were statistically analyzed, using the JMP 5.0 program. The differences among the applications were determined according to ANOVA results and "LSMeans Differences Student's" multiple comparison test.

Results and discussion

The effects of a total of 11 bacteria strains comprising: 6 *Bacillus*, 2 *Pantoea*, 1 *Brevibacillus*, 1 *Pseudomonas*, and 1 *Vibrio* and 1 fungal isolate on the nymphs of *H. halys*, the invasive pest, were tested and the results showed that all applications yielded different results than the control group and resulted in mortality rates between 75 and 100%.

The bacterial strains yielded relatively more significant results as they were 5.0–40.0% after 24 h, 10.0–65.0% after 48 h, 29.2–76.7% after 72 h, 37.5–76.7% after 96 h, 37.5–85.8% after 120 h, 41.7–90.0% after 144 h, 41.7–95.0% after 168 h, 50.0–95.0% after 192 h, 55.0–95.0% after 216 h, 62.5–95.0% after 240 h, and 75.0–100% after 264 h (Fig. 1).

According to the percentage mortality of nymphs, the difference between applications was found to be statistically significant (F 13.59; $p < 0.01$). The mean values of the mortality rates of the nymphs and hatched eggs to each of the entomopathogen bacterial strains and fungal isolate showed that the highest nymphal mortality rates (%) were obtained by the FD 63 and FD 68 strains (100%) (Table 2). The lowest mortality rate was obtained by the sterile water and NB medium-sprayed applications. Hyphal growths from 72 h showed the status of the nymphs (third and fourth nymphal instars) after 264 h from the applications of the bacteria and fungus that yielded the best results (Fig. 2).

The effects of the applications on egg hatching were investigated in the study. The control eggs and the eggs to which the ET 10 fungal isolate of *B. bassiana* and the FD 51 and FD 16 bacterial strains of *B. thuringiensis kurstaki* were applied, hatched in 96 h, while hatching did not occur in *B. cereus* (FD 63, FDP 8), *P. agglomerans* (FD 68, FD 69), *B. brevis* (FD 1), *P. flourescens* (FD 71), *B. atropheus* (FD 17), *V. hollisae* (FD 70), and *B. sphaericus* (FD 49), even 264 h (Table 2). Figure 3 illustrates the pictures of the unhatched eggs and hatched eggs in the applications.

Although there are various studies on the use of bacteria and fungus in the biological control of pests, limited numbers of studies were found on the microbiological control of *H. halys*, comprising the studies of Gouli et al. (2011) and Kumar and Suktana (2017).

B. bassiana has a wide host range of hosts including hemipteran species (Gouli et al., 2011). Due to its environmentally friendly nature, bio-persistence and ability to kill pests at various developmental stages in their life cycle, the use of *B. bassiana* in Integrated Pest Management (IPM) programs is of great importance (Kumar and Suktana, 2017). The spores of *B. bassiana* attach to the insect's cuticle, they germinate, the hyphae penetrate the insect's body, and proliferate. The insects die after about 3–5 days and infected

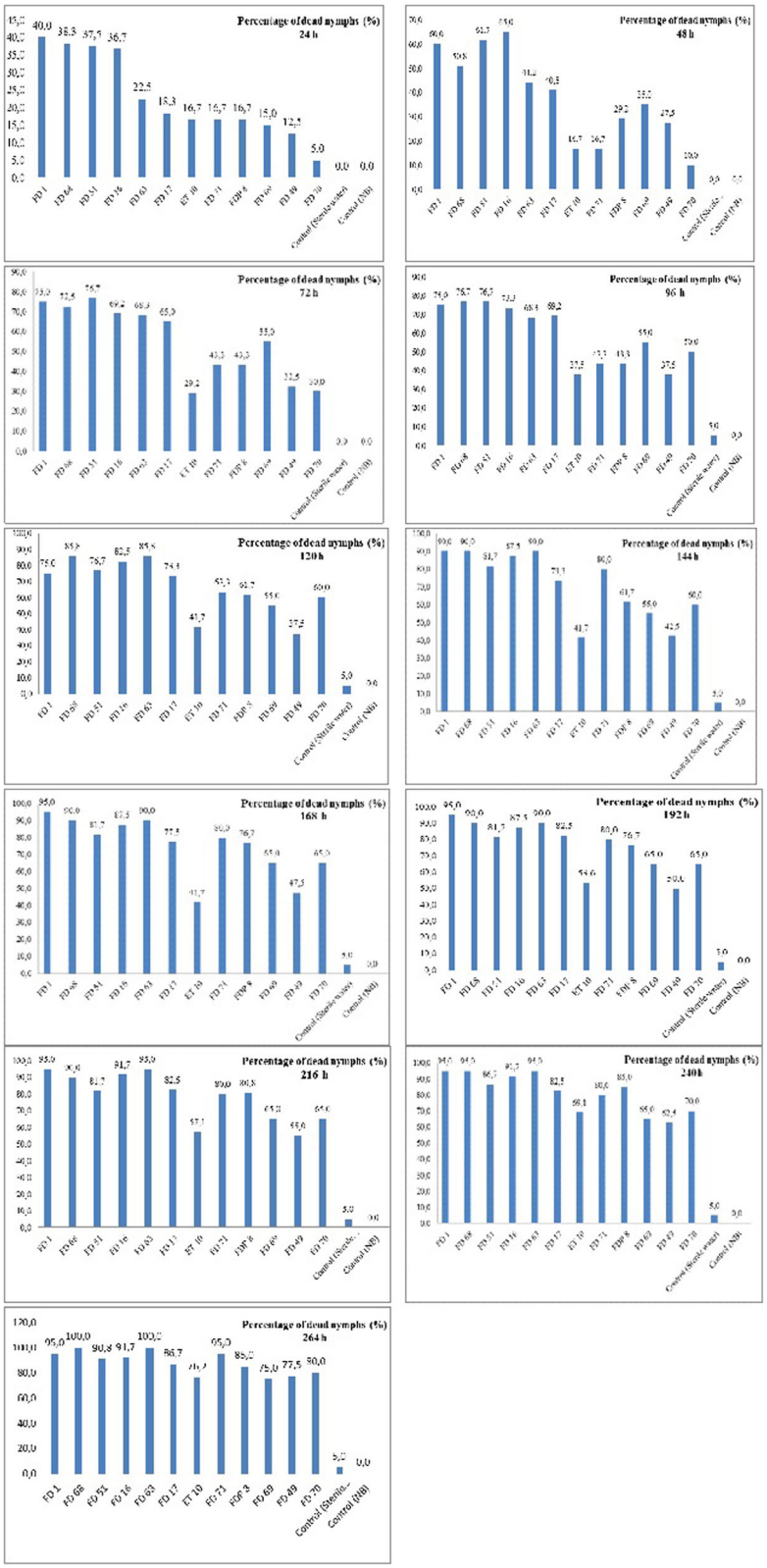

Fig. 1 Percentages of mortality of *Halyomorpha halys* nymphs in response to some entomopathogenic bacterial strains and fungal isolates by hours

Table 2 Efficacy of some entomopathogen bacterial strains and fungal isolate on nymphs of *Halyomorpha halys* under controlled conditions after 264 h

Treatment	Death nymph ratio (%)		Number of eggs in packs (number)	Number of hatched eggs
FD 63 (*Bacillus cereus*)	100.00	A	19	0
FD 68 (*Pantoea agglomerans*)	100.00	A	37	0
FD 1 (*Brevibacillus brevis*)	95.00	AB	14	0
FD 71 (*Pseudomonas flourescens*)	95.00	AB	12	0
FD 16 (*Bacillus thuringiensis kurstaki*)	91.67	AB	28	28
FD 51 (*Bacillus thuringiensis kurstaki*)	90.83	AB	5	5
FD 17 (*Bacillus atrophaeus*)	86.67	AB	11	0
FDP 8 (*Bacillus cereus*)	85.00	AB	13	0
FD 70 (*Vibrio hollisae*)	80.00	AB	14	0
FD 49 (*Bacillus sphaericus*)	77.50	B	26	0
ET 10 (*Beauveria bassiana*)	76.19	B	8	8
FD 69 (*Pantoea agglomerans*)	75.00	B	17	0
Control (Sterile water)	5.00	C	10	10
Control (NB)	0.00	C	12	12
CV	23.01			
LSD	24.87			

cadavers may serve as a source of spores for secondary spread of the fungus.

The first study on the use of entomopathogen fungus for the control of this pest was carried out in 2011 (Gouli et al. 2011). The efficacy of 2 *Metarhizium anisopliae* isolates and commercial preparations of *B. bassiana* isolates were investigated for the control of the pest. The researchers reported that using *M. anisopliae* achieved mortality rates ranged between 40 and 88% in adults *H. halys*, while it ranged between 67 and 100%, 12 days after the application of *B. bassiana*, under controlled conditions. Other researchers applied commercial preparations of 2 different formulations of *B. bassiana* concentrations (5×10^6 and 1×10^7 conidia ml^{-1}) under controlled conditions and found that the best results were obtained by using the concentration (1×10^7 conidia ml^{-1}) as a wettable powder formulation. Mortality rates ranged between 78 and 100% after 12 days of application (Parker et al. 2015). In this study, mortality rates of 76.19% in the application of *B. bassiana* were also achieved after 264 h, which agree with the results obtained in the previous study. Furthermore, ET 10 isolate (*B. bassiana*) was tested against *Syrista parreysii* larvae. The number of dead larvae and mortality rate (%) of 10^6, 10^7, and 10^8 conidial suspensions of ET 10 were 7.44, 7.56, 8.11, and 82.72%, 83.95%, and 90.12%, respectively (Tozlu et al. 2017).

Erper et al. (2016) applied 2 *Simplicillium lamellicola*, 4 *Lecanicillium muscarium*, and 1 *B. bassiana* and *Isaria fumosorosea* isolates under laboratory conditions at 25 °C to control the nymphs of *Palonema prasina*, a pentatomid species. They found that the LT_{50} values were between 3.20 and 8.48 days, while the LT_{90} values were between 9.32 and 40.30 days on day 12 of the study. In addition, they reported that all isolates, used in the study had efficacies over 83% and the highest percentage mortality (98 and 95%) were obtained by *L. musacarium* and *B. bassiana*, respectively.

Various studies have reported that the most important bacterial species that yielded successful results in the biological control of pests were *Bacillus* varieties (Alper et al. 2013). *B. thuringiensis* was reported to be successful in controlling different pests' species (*Helicoverpa armigera*, *Spodoptera litura*, *Pieris brassicae*, and *Spilarctia oblique*) (Mohan et al. 2014).

A review of the scientific literature revealed no studies testing the efficacy of entomopathogen bacteria against *H. halys*, but the effects of the bacterial strains used in this study to test their efficacy against *H. halys* were determined for different pests in previous studies. In the present study, the efficacy of two *B. cereus* strains (FD 63 (36.6%), FDP 8 (76.6%)), one *B. atrophaeus* strain (FD 17 (36.6%)) and one *B. brevis* strain (FD 1 (23.0%)) against *Bruchus dentipes* under controlled conditions were investigated. The strains were reported to control the pests in 7 days (Tozlu et al. 2011). Dadaşoğlu et al. (2016) tested the efficacies of two strains of *B. thuringiensis kurstaki* (*F* 16, F51) and one strain of *B. sphaericus* GC subgroup (*F* 49) against *Diplion pini* where the mortality rates ranged between 66.7 and 80%, respectively, under controlled conditions. The results of the present study, also showed that *B. cereus* (FD 63,

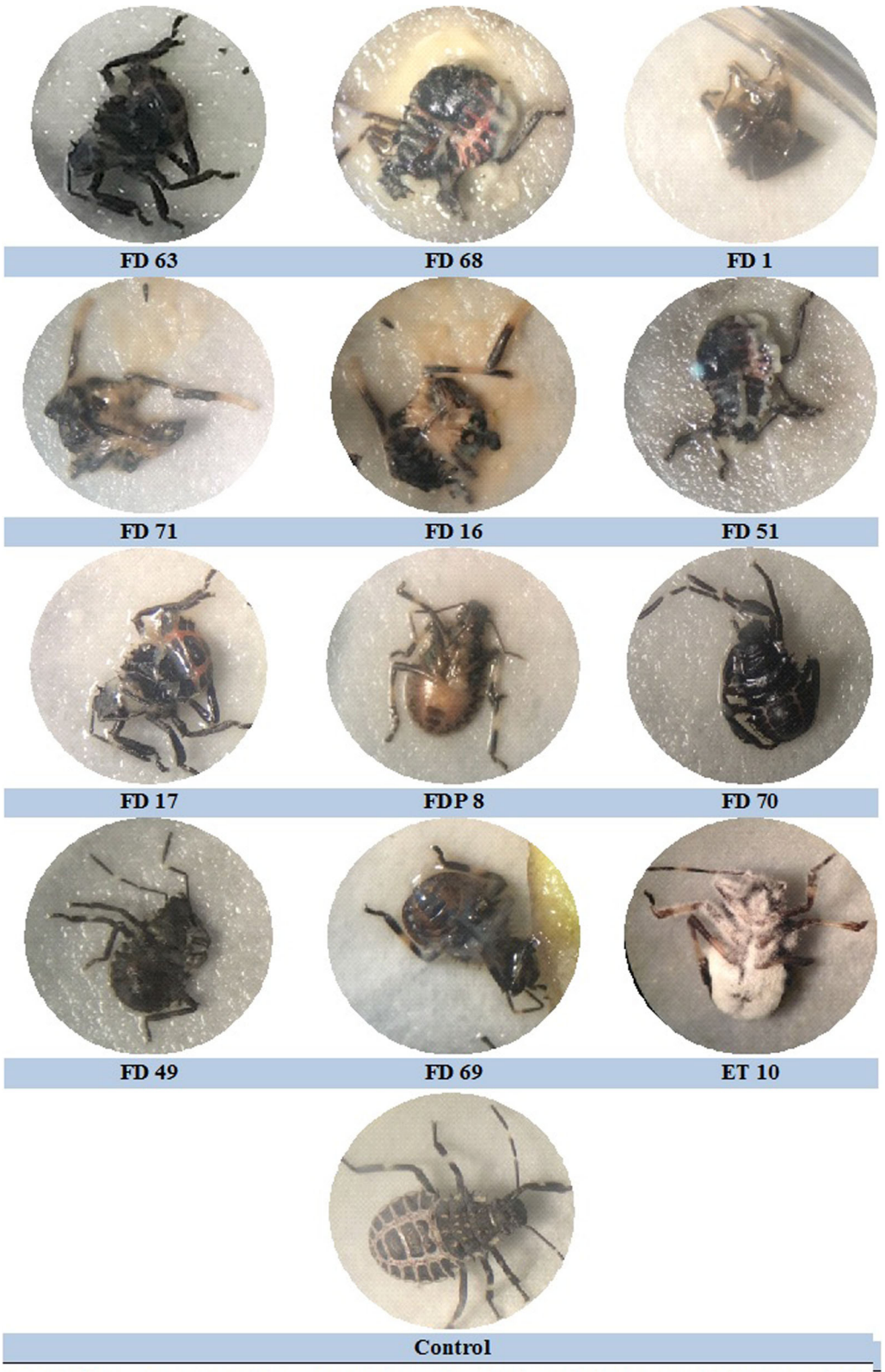

Fig. 2 Effects of the applications of bacterial strains and fungal isolate on the *Halyomorpha halys* nymphs after 264h

Fig. 3 Appearance of the unhatched and hatched eggs after 264 h

100.00%), *B. thuringiensis kurstaki* (FD 16 (91.67%), FD 51 (90.83%)), *B. atrophaeus* (FD 17, 86.67%), *B. cereus* (FDP 8, 85.00%), *B. sphaericus* (FD 49, 77.50%), and *B. brevis* (FD 1, 95.00%) strains were effective against *H. halys* nymphs.

In addition, the pathogenicity of the two *P. agglomerans* strains (FD 69 (75 %) FD 68 (100 %)) and one *V. hollisae* strain (FD 70, 80 %) against the nymphs of *H. halys* were successfully exterminated in this study. The eggs hatched in fungal isolate, *B. thuringiensis kurstakii* strains applications and control, although the eggs unhatched in other applications.

Conclusion

The study highlighted a successful usage of the environment-friendly control tool, the entomopathogens, against *H. halys* as a substitution of the chemical pesticides. Such potentials may be varied under field conditions. Therefore, the efficacy of the entomopathogens under field conditions against the different developmental stages of the pest should be tested.

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Authors' contributions
ET, IS, GT, and RK conceived and designed research. IS collected *H. halys* nymphs from Artvin, Turkey. ET, GT, and RK conducted experiments. ET studied fungal experiments. RK and FD studied bacterial experiments. ET, GT, and NT studied controlled assay. ET analyzed the data. ET, GT, and RK wrote the manuscript. NT took photos of the study. All authors read and approved the final manuscript.

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Not applicable

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Competing interests
The authors declare that they have no competing interests.

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