Ak *Egyptian Journal of Biological Pest Control* (2019) 29:12
https://doi.org/10.1186/s41938-019-0115-y

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Efficacy of entomopathogenic fungi against the stored-grain pests, *Sitophilus granarius* L. and *S. oryzae* L. (Coleoptera: Curculionidae)

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Abstract

Efficacy of the five native entomopathogenic fungi (EPFs), *Beauveria bassiana*, *Isaria fumosorosea*, *Lecanicillium muscarium*, *Metarhizium anisopliae*, and *Simplicillium lamellicola*, against adults of the stored-grain insect pests, *Sitophilus granarius* and *Sitophilus oryzae* (Coleoptera: Curculionidae), was evaluated under laboratory conditions at two different temperatures. Conidial suspensions (1×10^8 conidia ml^{-1}) of the fungi were directly applied to both the pests in Petri dishes (2 ml per dish), using a Potter spray tower. All the dishes were incubated both at 20 and 25 °C in 16 h light/8 h dark and in 75 ± 5% relative humidity (RH). Dead individuals were counted daily, following treatments, for 7 days. Lethal time values (LT_{50} and LT_{90}) for EPFs were calculated. The results demonstrated that the mortality rates varied according to both the temperature and the tested EPFs. The highest effect among EPFs at (1×10^8 conidia ml^{-1}) concentration on *S. granarius* at 20 °C at the end of day 7 was showed by *I. fumosorosea* (92.69%) and *M. anisopliae* (90.35%), followed by the other EPFs. Similarly, *M. anisopliae* and *I. fumosorosea* were the most effective ones with 90.48 and 84.21% mortality rates, respectively, at 25 °C. However, while *M. anisopliae* (85.68%) showed the highest effect among all the EPFs applied on *S. oryzae* at 20 °C, *B. bassiana* with a mortality rate of 93.66% was the most effective one at 25 °C. LT_{50} values for *I. fumosorosea* and *M. anisopliae* were 2.75 and 2.88/days, respectively, and LT_{90} values were 4.17 and 4.47/days, respectively, at 20 °C for *S. oryzae*. However, LT_{50} values for *M. anisopliae* on *S. granarius* in both temperatures were the lowest. This study indicated that *M. anisopliae* and *I. fumosorosea* had a significant potential as a biological control agent against *S. granarius* and *S. oryzae*. Further studies are necessary to evaluate the efficacy of the isolate on the pests under storage conditions.

Keywords: Entomopathogenic fungi, *Sitophilus granaries*, *Sitophilus oryzae*, Biological control

Background

Sitophilus weevils, including *Sitophilus oryzae* (rice weevil), *S. zeamais* (maize weevil), and *S. granarius* (granary weevil) (Coleoptera: Curculionidae), are well-known stored-grain insect pests in Turkey and many other countries in the world (Bağcı et al. 2014). These weevils have a nearly cosmopolitan distribution, occurring throughout all warm and tropical parts of the world (Hong et al. 2018). Generally, because of the storage-grain pests infestation, it has been estimated that during storage, 10–25% of the grain crops are damaged yearly worldwide. Damages caused by the insects not only contain the direct feeding harm resulting in loss of weight, but they also seriously decrease nutrients, lowering seeds germination rate, reducing quality, and lowering their marketing value due to the mass of waste, webbing, and insect cadavers (Abdel-Raheem et al. 2015).

Stored-grain protection against the pests is currently based on the use of synthetic insecticides and fumigants (Arthur 1996). As a result, these have caused problems including insecticide resistance along with contamination of many food products with chemical residues and

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consumer demand for pesticide-free grain. Thus, there is a growing interest in using biological control agents against the pests as an alternative (Wakil et al. 2015).

Entomopathogenic fungi (EPFs) are common natural enemies of arthropods worldwide, attracting attention as a potential biological control agent. There are more than 700 species of EPFs (Sandhu et al. 2012; Erper et al. 2016). Fungal entomopathogens such as *Beauveria bassiana*, *B. brongniartii*, *Isaria farinosa*, *I. fumosorosea*, *Lecanicillium* spp., and *Metarhizium anisopliae* play an important role in the regulation of insect populations (Zimmermann 2008; Gurulingappa et al. 2011). Also, since they exist in nature, EPFs have low environmental impact and are generally considered environmentally safe agents with low mammalian toxicity (Rumbos and Athanassiou 2017).

The using of EPFs for the control of the insect pests in stored-grain products is one of the most promising alternative control methods (Moore et al. 2000). Especially, the species *B. bassiana* and *M. anisopliae* have a wide host range and have been tested against most of the major stored-grain pests (Batta 2018; Rumbos and Athanassiou 2017).

Temperature plays a significant role on the effectiveness of EFPs, especially high temperatures affect negatively conidial viability and germination (Rumbos and Athanassiou 2017). For example, *B. bassiana* was found to be more effective against *R. dominica*, *S. oryzae* at 26 °C than at 30 °C (Vassilakos et al., 2006), and *S. granarius* (Athanassiou and Steenberg 2007) in stored wheat. Similarly, Michalaki et al. (2007) found that *Isaria fumosorosea* was more effective at 20 °C than at 25 °C. In another study, *I. fumosorosea* was effective against *Tribolium confusum* and *Ephestia kuehniella*, but its effectiveness was highly dependent on the target species and life stage, exposure interval, and temperature (Michalaki et al. 2007).

The aim of this study was to evaluate the efficacy of five EPFs isolates, belonging to *I. fumosorosea*, *Simplicillium lamellicola*, *B. bassiana*, *M. anisopliae*, and *L. muscarium*, against the storage-grain pests, *S. granarius* and *S. oryzae*, at two different temperatures under laboratory conditions.

Materials and methods

Fungi cultures

Five respect isolates (TR-01, TR-07, TR-78-3, TR-106, and TR-217) of the entomopathogenic fungi (EPFs), *I. fumosorosea*, *Simplicillium lamellicola*, *B. bassiana*, *M. anisopliae*, and *L. muscarium*, were used in the present study. They were isolated from different infected hosts in hazelnuts orchards in the Black Sea region of Turkey (Erper et al. 2016; Kushiyev et al. 2018). The single-spore cultures of *B. bassiana* (TR-217 isolate), *I. fumosorosea* (TR-78-3 isolate), *L. muscarium* (TR-07 isolate), *M. anisopliae* (TR-106 isolate), and *S. lamellicola* (TR-01 isolate) were stored at 4 °C on Sabouraud dextrose agar (SDA; Merck Ltd., Darmstadt, Germany) slants and deposited in the fungal culture collection of the Mycology Laboratory at the Ondokuz Mayis University, Faculty of Agriculture's Department of Plant Protection in Samsun, Turkey.

Insect cultures

Adults of *S. granarius* and *S. oryzae* were used. Adult insects were obtained from stock cultures in the Black Sea Agricultural Research Institute (Samsun-Turkey). Insects in cultures were grown at 25 ± 2 °C, 65 ± 3% RH in 16-h light/8-h dark conditions and fed on sterile wheat grains. Adults from cultures were collected by an oral aspirator and 1-day-old adults were used in the study.

Inoculum of EPF

The five isolates of EPFs were incubated on potato dextrose agar (PDA; Merck Ltd., Darmstadt, Germany) at 25 ± 1 °C for 10–14 days. Conidia were harvested by sterile distilled water, containing 0.02% Tween 20. Then, conidia suspensions were filtered through four layers of sterile cheesecloth to remove mycelium, and conidia were counted under an Olympus CX-31 compound microscope (Olympus America Inc., Lake Success, NY), using a Neubauer hemocytometer to calibrate a suspension of 1×10^8 conidia ml^{-1} of each isolate (Erper et al. 2016).

Conidial germination assessment

The viability of conidia of the five isolates belonging to *B. bassiana*, *I. fumosorosea*, *L. muscarium*, *M. anisopliae*, and *S. lamellicola* was determined. A conidial suspension (200 μl) of each isolate at (1×10^4 conidia ml^{-1}) obtained by dilution was sprayed onto Petri plates (9-cm dia.), containing PDA (Merck Ltd., Darmstadt, Germany). These plates were incubated at 25 ± 1 °C. After 24 h of incubation, the percentage of germinated conidia was counted, using an Olympus CX-31 compound microscope at × 400 magnification. Conidia were regarded as germinated, when they produced a germ tube, at least half of the conidial length. The germination ratios for each isolate were calculated after examining a minimum of 200 conidia from each of the three replicate plates (Saruhan et al. 2015).

Experimental design

Ten *S. granarius* and *S. oryzae* adults were released in each Petri plate (9-cm dia.), containing 10 pieces of sterilized wheat grain. Bottoms of plate cups were covered by a filter paper moisturized with sufficiently sterile distilled water. Conidial suspension (1×10^8 conidia ml^{-1}) of each EPF (TR-217, TR-78-3, TR-07, TR-106, and TR-01) was applied to the *S. granarius* and *S. oryzae* adults (2 ml per plate), using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). Control Petri plates were treated by sterile distilled water (2 ml), containing 0.02% Tween 20. All the

plates were loosely covered by a Parafilm to prevent their escape and incubated both at 20 ± 1 and at 25 ± 1 °C in 16 h light/8 h dark and in 75 ± 5% RH, using the Memmert incubator (Model ICP 110; Germany). The spray tower was cleaned by 70% ethanol and sterile distilled water after each application of the fungus suspension.

Dead adults were counted, using a Leica EZ4 stereo dissecting scope at × 40–70 magnification. They were removed daily from the plates and immediately surface disinfected by dipping it in 1% sodium hypochlorite (NaOCl) for 3 min and in 70% ethanol for 3 min. Then, the dead insects (belonging to *S. granarius* and *S. oryzae*) were washed three times in sterile distilled water and placed in 75 ± 5% RH, using the Memmert incubator. Mortality rates were confirmed by examining of hyphae on the cadavers under Leica EZ4 stereomicroscope, 10 days after placing the dead insects (Kocaçevik et al. 2016). The bioassay was performed by using a completely randomized experimental design with five replicates. Each replicate consisted of 10 1-day-old adults of the pests and placed in a Petri plate (9-cm dia.), and the experiment was conducted once (Saruhan et al. 2015).

Statistical analysis

The mortality rate was observed at 7 days, following each application. Dead individuals were counted under a stereoscopic microscope and the mortality rate was calculated. Data was corrected by Abbott's formula (Abbott 1925). Fifty percent lethal time (LT_{50}) and 90% lethal time (LT_{90}) were determined, using the probit analysis by SPSS (ver. 21) program. The effects on mortality rates of the *S. granarius* and *S. oryzae* were analyzed, using the two-way analysis of variance (ANOVA), followed by a comparison of means, using the Tukey HSD test (SPSS) ($P < 0.05$).

Results and discussion

The efficacy of the five different EPFs, *I. fumosorosea*, *Simplicillium lamellicola*, *B. bassiana*, *M. anisopliae*, and *L. muscarium*, against adults of storage-grain pests *S. granarius* and *S. oryzae* at two different temperatures (20–25 °C) under laboratory conditions was evaluated.

Among the EFPs, *I. fumosorosea* (92.69%) and *M. anisopliae* (90.35%) recorded the highest effects on *S. granarius* at 20 °C at the end of day 7, followed by *B. bassiana* (72.91%), *S. lamellicola* (62.02%), and *L. muscarium* (33.91%). The same isolates were tested at 25 °C, where the highest effect, recorded at this temperature, was by *M. anisopliae* (90.48%), followed by *I. fumosorosea* (84.21%), *S. lamellicola* (59.26%), *B. bassiana* (56.14%), and *L. muscarium* (22.81%). The effects of these isolates on *S. granarius* at different temperatures were similar but slightly low at 25 °C (Figs. 1 and 2). Sheeba et al. (2001) applied *B. bassiana* against *S. oryzae* and recorded (86.2%) the mortality rate in adults after day 25. In another study, Khashaveh et al. (2011) tested the commercial product of *B. bassiana* against *S. granarius*, *Oryzaephilus surinamensis*, and *Tribolium castaneum* at 24 ± 2 °C recording 88.33, 78.31, and 64.99% mortality, respectively. Among these three pests, *S. granarius* was reported to be the most sensitive.

Among the isolates applied on *S. oryzae* at 20 °C, *M. anisopliae* showed the highest effect (85.68%), followed by *I. fumosorosea* (63.32%), *S. lamellicola* (48.06%), *L. muscarium* (45.10%), and *B. bassiana* (40.74%). For the same pest at 25 °C, *B. bassiana* had the highest effect (93.66%) at this temperature, followed by *M. anisopliae* (90.40%), *I. fumosorosea* (58.02%), *L. muscarium* (56.86%), and *S. lamellicola* (54.74%). With the rise of temperature, the effect of isolates against *S. oryzae* was increased (Figs. 3 and 4).

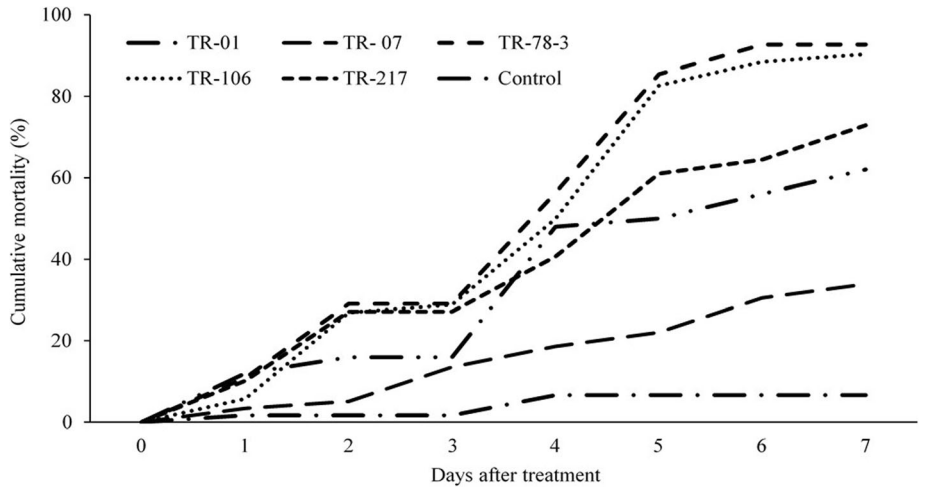

Fig. 1 Mortality rate of *Sitophilus oryzae* treated with entomopathogenic fungi at 20 °C

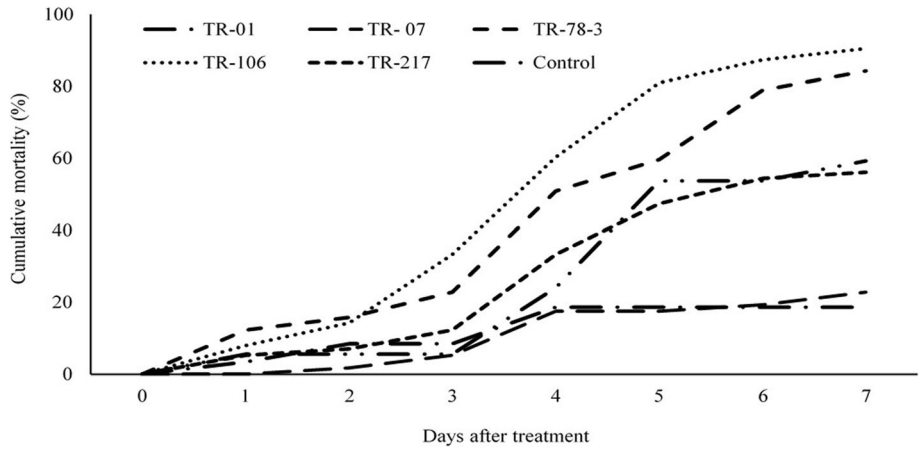

Fig. 2 Mortality rate of *Sitophilus oryzae* treated with entomopathogenic fungi at 25 °C

Temperature plays a significant role for the effectiveness of EPFs. It is widely accepted that high temperatures affect negatively the conidial viability and germination (Rumbos and Athanassiou 2017). Generally, different fungal species have different temperature requirements. For instance, regarding several strains of *B. bassiana*, the optimum temperature for conidial germination and vegetative growth is around 25 °C (Rumbos and Athanassiou 2017), while *I. fumosorosea* was more effective at 20 °C than at 25 °C (Michalaki et al. 2007). In the present study, the efficacy of the isolate of *I. fumosorosea* was the highest (92.69%) on *S. granarius* at 20 °C, while it showed a lower effect (84.21%) on the pest at 25 °C. Similarly, the isolate of *B. bassiana* had the highest effect (93.66%) at 25 °C, while it showed the lowest effect (40.74%) on the *S. oryzae* at 20 °C. In contrast, *B. bassiana* was found to be more effective (72.91%) at 20 °C than (56.14%) at 25 °C against *S. granarius*. Similarly, Tefera and Pringle (2003) found that among different isolates of *B. bassiana*, germination, radial growth, and sporulation of all isolates were retarded at 15 and 35 °C, while the optimum temperature of different isolates of *B. bassiana* was between 20 and 30 °C (Tefera and Pringle 2003). Also, the pathogenicity and virulence of *B. bassiana* isolates vary remarkably among the host species and the life stage of the target pest. In the present study, the isolate of *B. bassiana* was more effective on *S. granarius* than on *S. oryzae*. This finding is also in line with Kassa et al. (2002) who tested 11 isolates of *B. bassiana* against adults of *S. zeamais* and *Prostephanus truncatus* (larger grain borer) (Coleoptera: Bostrychidae), and as a result, determined that *P. truncatus* was more susceptible to the *B. bassiana* than *S. zeamais*.

The LT_{50} values of the *I. fumosorosea* and *M. anisopliae* isolates (2.75 and 2.88 days, respectively), used against *S. oryzae* at 20 °C at a concentration of 1×10^8 conidia/ml, showed that they were the most effective ones and they were statistically different from the other

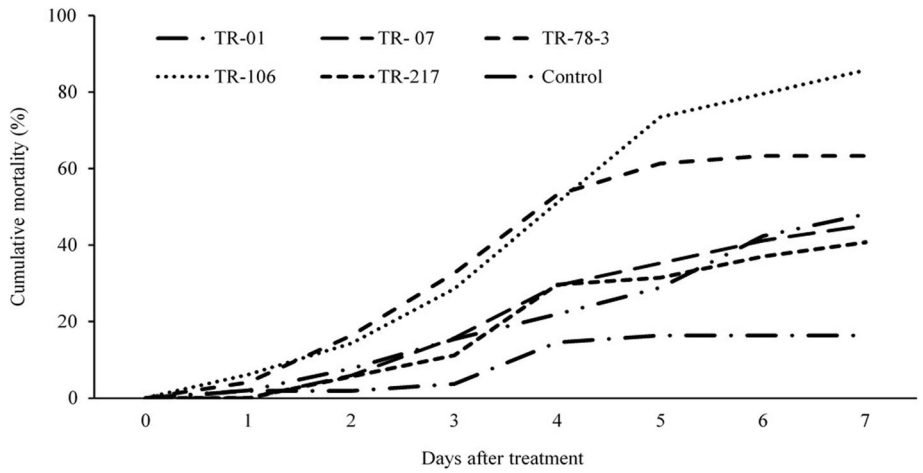

Fig. 3 Mortality rate of *Sitophilus granarius* treated with entomopathogenic fungi at 20 °C

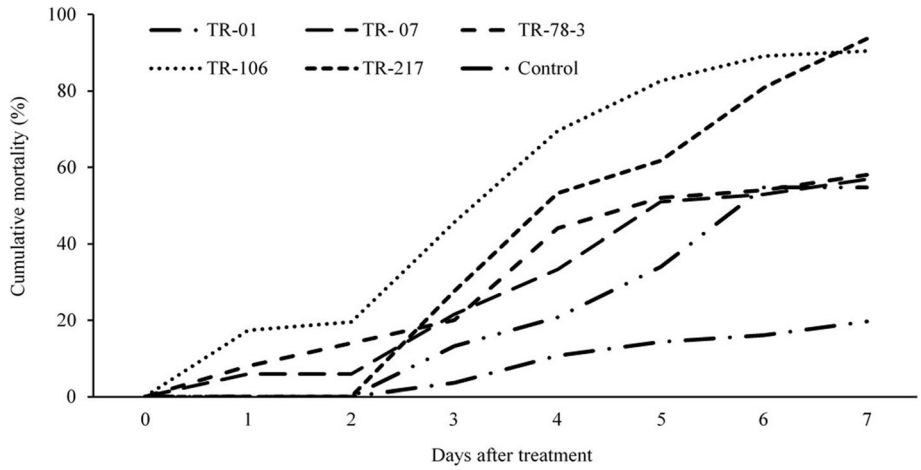

Fig. 4 Mortality rate of *Sitophilus granarius* treated with entomopathogenic fungi at 25 °C

used isolates. Correspondent LT_{50} values of *L. muscarium*, *S. lamellicola*, and *B. bassiana* at the same concentration and at 20 °C were 4.78, 4.87, and 5.14 days, respectively. The LT_{90} values of the EPFs used against *S. oryzae* at 20 °C had a similar trend to those of LT_{50}. Considering the LT_{90} values, the most effective EPFs were *I. fumosorosea* and *M. anisopliae* (4.17 and 4.47 days), respectively, although the mortality period in adults was lengthen out. These EPFs were determined as *L. muscarium*, *S. lamellicola*, *B. bassiana* (7.67, 7.84, 8.26 days), respectively. The most effective isolate at LT_{50} at 25 °C was *M. anisopliae* (2.20 days), followed by *B. bassiana* (3.17 days), *I fumosorosea* (3.34 days), *L. muscarium* (3.73 days) and *S. lamellicola* (4.57 days). Similarly, LT_{90} values of *M. anisopliae* and *B. bassiana* were 3.82, 3.94 days, respectively, followed by *I. fumosorosea* with 5.62 days. The lowest effect was recorded for *L. muscarium* and *S. lamellicola* (6.42 and 5.92 days, respectively) (Table 1). When the temperature sensitiveness of LT_{50} values of the used isolates was analyzed, *L. muscarium* and *S. lamellicola* isolates were found statistically in the same group ($P < 0.05$). *M. anisopliae*, *I. fumosorosea*, and *B. bassiana* were different from these two isolates. In a study, the LT_{50} value was determined as 3.52 days after AAU D (*Metarhizium*) application against *S. oryzae* at a dose of 1×10^8 conidia ml^{-1}, and (96.6%) the mortality rate was determined at the end of day 10. In the same study, the LT_{50} value of DLCO 141 (*Beauveria*) was reported as 6.53 days and the mortality rate was 70.0%, and the LT_{50} value of DLCO 26 (*Metarhizium*) was 6.21 days and the mortality rate was 60.0% (Kassaye 2011). In this study, the effect of isolates used against *S. oryzae* was similar to those of the isolates that were used against the same pest species.

According to the results of the isolates of EPFs against *S. granarius*, there was insignificant difference between the results obtained at 20 and 25 °C ($P < 0.05$) (Table 2). In the present study, LT_{50} values of the five isolates of EPFs, used against *S. granarius* at 20 °C, were evaluated; *S. lamellicola*, *B. bassiana*, *I. fumosorosea*, and *M.*

Table 1 Lethal time (LT_{50} and LT_{90}) for *Sitophilus oryzae* treated with the tested entomopathogenic fungal isolates at 20 and 25 °C

Isolates***	Temperatures					
	20 °C			25 °C		
	LT_{50} (95% confidence limit)	LT_{90} (95% confidence limit)	χ^2	LT_{50} (95% confidence limit)	LT_{90} (95% confidence limit)	χ^2
TR-01	4.87 (4.56–5.23) a* A**	7.84 (7.25–8.63) a B	3.17	4.57 (4.16–4.98) a A	6.42 (5.87–7.27) a B	4.57
TR-07	4.78 (4.22–5.43) a A	7.67 (6.75–9.26) a B	12.79	3.73 (3.47–3.98) b A	5.96 (5.59–6.42) a B	8.17
TR-78-3	2.75 (2.54–2.97) b A	4.17 (3.84–4.63) b B	1.30	3.34 (3.08–3.60) b A	5.62 (5.25–6.08) a B	9.37
TR-106	2.88 (2.68–3.13) b A	4.47 (4.09–5.02) b B	3.32	2.20 (-----) c A	3.82 (-----) b B	11.87
TR-217	5.14 (4.47–6.04) a A	8.26 (7.08–10.59) a B	16.28	3.17 (3.02–3.32) b A	3.94 (3.74–4.22) b B	3.67
F	17.642	11.627		34.481	24.935	
P	0.000	0.001		0.000	0.000	

*The same small letters within columns indicates no significant differences between means
**The same capital letters within rows indicates no significant differences between means
***TR-01 (*Simplicillium lamellicola*), TR-07 (*Lecanicillium muscarium*), TR-78-3 (*Isaria fumosorosea*), TR-106 (*Metarhizium anisopliae*), and TR-217 (*Beauveria bassiana*)

Table 2 Lethal time (LT_{50} and LT_{90}) for *Sitophilus granarius* treated with the tested entomopathogenic fungal isolates at 20 and 25 °C

Isolates***	Temperatures					
	20 °C			25 °C		
	LT_{50} (95% confidence limit)	LT_{90} (95% confidence limit)	χ^2	LT_{50} (95% confidence limit)	LT_{90} (95% confidence limit)	χ^2
TR-01	3.21 (2.45–3.99) ab* A**	5.50 (4.57–7.45) a B	20.64	4.01 (3.29–4.78) b A	5.90 (5.07–7.60) b B	32.61
TR-07	6.03 (5.54–6.67) a A	10.09 (9.02–11.70) a B	4.88	7.26 (6.13–9.96) a A	11.39 (9.09–17.83) a A	14.35
TR-78-3	2.43 (1.27–4.05) b A	4.13 (3.07–10.40) a A	16.92	2.78 (1.96–3.72) b A	4.52 (3.62–7.08) b A	21.05
TR-106	2.71 (1.93–3.80) b A	4.47 (3.51–7.92) a A	10.19	2.82 (2.52–3.26) b A	4.54 (3.93–5.60) b B	2.96
TR-217	2.95 (1.95–5.42) a b A	5.35 (3.88–14.57) a A	11.03	3.91 (3.66–4.17) b A	6.11 (5.74–6.58 b B	8.66
F	4.310	1.092		11.514	6.234	
P	0.028	0.411		0.001	0.009	

*The same small letters within columns indicates no significant differences between means
**The same capital letters within rows indicates no significant differences between means
***TR-01 (*Simplicillium lamellicola*), TR-07 (*Lecanicillium muscarium*), TR-78-3 (*Isaria fumosorosea*), TR-106 (*Metarhizium anisopliae*), and TR-217 (*Beauveria bassiana*)

anisopliae were found in the same group, while *L. muscarium* was in a different group. LT_{50} values of the isolates, used against *S. granarius*, were 3.21 days (*S. lamellicola*), 2.95 days (*B. bassiana*), 2.43 days (*I. fumosorosea*), and 2.71 days (*M. anisopliae*), while *L. muscarium* was determined as (6.03 days). LT_{90} values of EPF applied against *S. granarius* at 20 °C were found to be at the same group statistically (Table 2). The LT_{50} results of the isolates used against *S. granarius* at 25 °C showed a similar trend to those obtained at 20 °C. In terms of LT_{50} values, *I. fumosorosea* (2.78 days), *M. anisopliae* (2.82 days), *B. bassiana* (3.91 days), and *S. lamellicola* (4.01 days) were found to be at the same group statistically, whereas *L. muscarium* (7.26 days) isolate was found to be low in effect and in a different group statistically. Additionally, in terms of LT_{90}, it was determined that *L. muscarium* had a low effect (11.39 days) and it was in a different group than other isolates statistically, and LT_{90} values of the other four isolates ranged 4.52 to 6.11 days and were at the same group statistically (Table 2) ($P < 0.05$).

Conclusion

In conclusion, the five different EPFs evaluated in this study showed that they were effective against *S. oryzae* and *S. granarius*, and may be considered as alternatives to chemical control. In addition, *M. anisopliae* and *I. fumosorosea* showed about 90% efficacy against both pests at the end of the day 7. Thus, they are promising biocontrol agents in terms of practical application according to the results obtained from similar studies. Further studies are necessary to evaluate the efficacy of the isolate on the pests under storage conditions.

Acknowledgments
I am grateful to Ismail Erper and Islam Saruhan (Ondokuz Mayıs University, Faculty of Agriculture-Turkey) for help in collecting, reproducing, and applying the isolates.

Funding
No funding

Availability of data and materials
All data are available at the end of the article and the materials used in this work are of high quality and grade.

Authors' contributions
KA designed the study, supervised the work, wrote the manuscript, and carried out the experiments. The author read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The author declares that there are no competing interests.

Publisher's Note

Received: 13 November 2018 Accepted: 15 February 2019
Published online: 05 March 2019

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