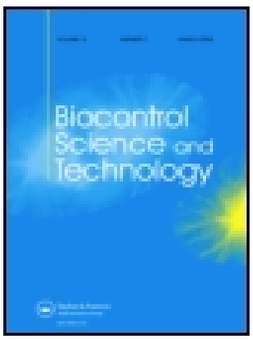

Biocontrol Science and Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/cbst20

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To cite this article: Yara El Khoury , Elise Noujeim , Josip Ravlić , Monica Oreste , Rocco Addante , Nabil Nemer & Eustachio Tarasco (2020): The effect of entomopathogenic nematodes and fungi against four xylophagous pests, Biocontrol Science and Technology, DOI: 10.1080/09583157.2020.1781059

To link to this article: https://doi.org/10.1080/09583157.2020.1781059

Published online: 17 Jun 2020.

BIOCONTROL SCIENCE AND TECHNOLOGY
https://doi.org/10.1080/09583157.2020.1781059

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RESEARCH ARTICLE

The effect of entomopathogenic nematodes and fungi against four xylophagous pests

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ABSTRACT

The effects of entomopathogenic nematodes EPN (Steinernematidae and Heterorhabditidae) and fungi EPF (*Beauveria bassiana*) strains were evaluated in laboratory assays against larvae of four xylophagous pests: the Asparagus moth *Parahypopta caestrum*, the European goat moth *Cossus cossus*, the pine longhorn *Arhopalus syriacus* and the black Buprestid *Capnodis tenebrionis*. Due to their biology and ethology, these insects may be included in the category of pests residing in cryptic habitats. The control of these species is considered difficult, due to the inability of chemical pesticides to penetrate the cryptic habitats and reach the targets. The pathogenicity of the entomopathogenic nematodes and fungi was tested *in vitro* against the pests. Two experimental models were considered and aimed to imitate the natural environment of the pests, in Petri dishes filled with plant material and inside wood galleries respectively. Main results showed that the majority of the tested strains of nematodes and fungi affected the insects' survival rate. *Steinernema feltiae* and *B. bassiana* caused the highest percentage of larval mortality (80–100%). Considering the lack of effective chemical control means, the microbial control of the xylophagous pests by EPN and EPF reveals promising perspectives. Nematodes and fungi are able to penetrate the cryptic habitats because they are living organisms and may be horizontally transmitted by infected hosts. The distribution of EPF as preventive control method and the injection of EPN suspensions to reach and infect the larvae inside the wood galleries can be a combined sustainable control system.

ARTICLE HISTORY
Received 28 April 2020
Accepted 8 June 2020

KEYWORDS
Xylophagous insects; Entomopathogenic fungi; Entomopathogenic nematodes; Biological control

Introduction

Biological control agents such as entomopathogenic fungi (EPF) and nematodes (EPN), could be promising alternatives to chemical pesticides and have emerged as effective biological control agents for soil-borne insect pests (Campos-Herrera et al., 2015; Grewal et al., 2005; Lozano-Tovar et al., 2013). However, the potential of EPF and EPN as

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biocontrol agents against xylophagous insects has not been studied enough. Xylophagous or wood-boring insects reside in cryptic habitats, in the xylem or under the bark of the plant. Thus, they are difficult to control by chemical insecticides. Among xylophagous insects in the Mediterranean, *Parahypopta caestrum* (Hübner) (Lepidoptera, Cossidae), *Cossus cossus* (L.) (Lepidoptera, Cossidae), *Capnodis tenebrionis* (L.) (Coleoptera, Buprestidae) and *Arhopalus syriacus* (Reitter) (Coleoptera, Cerambycidae) are highly destructive cryptic pests.

Firstly, *P. caestrum* is considered a major destructive pest of *Asparagus* spp. in Italy. The larvae are born in the soil and start boring mines into the roots and the shoots, leading to the total destruction of the crop in 2–3 year (Pollini, 1989). The goat moth *C. cossus* is a xylophagous pest of fruit and forest trees. The larvae attack the bark and the xylem of the tree by boring large galleries, reducing thus the growth and the vigour of the tree, and causing limbs and branches to fall (Gumus et al., 2015). The Mediterranean flat-headed peachborer *C. tenebrionis* is a destructive pest of cultivated Rosaceae fruit trees. As referred by its name it is present in many Mediterranean countries, for example in southern Italy heavy attacks were reported on stone fruit trees. *Capnodis tenebrionis* usually attacks stressed or declining trees but also healthy plants. Eggs are laid during the summer on the trunk base and in the vicinity of it, either on superficial roots or freely in the soil (Marannino & de Lillo, 2007). Lastly, *A. syriacus* is native of the Eastern Mediterranean region, but also found in Italy and Greece (Brown, 1968; Martelli, 1952). It develops in pine trees mainly. This wood borer has induced the mortality of numerous trees in Salento (Apulia region) southern Italy and Lebanon in the Middle East. The larvae feed first under the bark and they bore later into the sapwood of dying trees. Adults of this nocturnal species can be found on the host plants at night or in bark crevices and under loose bark (Webb & Eldridge, 1997).

The control of pests residing in cryptic habitats such as xylophagous insects is challenging as they are hiding where chemicals cannot reach (Gumus et al., 2015). In addition, the use of chemicals in forest habitats is prohibited which makes even more difficult the management of cryptic living insects in forests. Due to the absence of effective treatment methods, phytosanitary control measures of cutting and incineration of infested trees are considered (Gumus et al., 2015). Alternatively, microbial control agents could be considered, as entomopathogenic nematodes (EPN) and fungi (EPF), because they may be able to penetrate into cryptic habitats and to be horizontally transmitted within the pest populations (Ashtari et al., 2011; Gumus et al., 2015; Kreutz et al., 2004; Marannino et al., 2007). Therefore, the aim of the present work is to test the pathogenicity of native entomopathogenic fungi (EPF) and nematodes (EPN) against four different xylophagous insects (*P. caestrum*, *C. cossus*, *A. syriacus* and *C. tenebrionis*), and to evaluate their potential use against larval stages as a substitute to chemical insecticides.

Materials and methods

Insects

Four xylophagous insects were collected from their natural habitat, reared in the laboratory until use in the bioassays.

The third instar larvae of *P. caestrum* feeding on Asparagus plantations were collected from Ortanova (Foggia province, Apulia). Collected larvae were reared in the laboratory of entomology at the University of Bari. The larvae were feeding on *Asparagus officinalis* (L.) plant under controlled conditions (20–22°C, 50–60% R.H., 16:8 L:D photoperiod).

Larvae of *C. cossus* were obtained from infested cherry trees in Apulia (Bari Province). The bark was peeled off using a small axe, and collected larvae were placed in plastic containers under room temperature (20–22°C) until use in the experiment.

For obtaining *C. tenebrionis* larvae, newly emerged adults were collected in autumn from infested stone fruit orchards (Apulia, Bari and Taranto Provinces), to be reared under laboratory conditions (28–30°C, 40–50% R.H., 16:8 L:D photoperiod). As a food source, fresh apricot twigs were given to the adults regularly (De Lillo, 1998). The dishes filled with sifted sand were used for the adults to lay eggs (Garrido et al., 1987) and only one-day-old neonate larvae were included in the experiments.

To collect *A. syriacus* larvae, the bark of infested pine trees (Qsaybeh, Lebanon) was peeled off with a small axe. Collected larvae were reared in plastic containers with pine tree bark and kept at room temperature (20–22°C) until their use in the experiments.

Nematodes and fungal isolates

Bioassays were carried out with isolates of EPN belonging to *Steinernema feltiae* Filipjev (CZ19, CO1, OT9, OT15, TC5, GR1, MF1, GF16, ALG18, LIB132); *S. affine* (Bovien) (CZ7), *S. arenarium* (Artyukhovsky) (C31), *S. ichnusae* (Tarasco, Mracek, Nguyen & Triggiani) (SAR2), *S. carpocapsae* (Weiser) (MR7), *S. apuliae* (Triggiani, Mracek & Reid) (CS3, LE13) and *Heterorhabditis bacteriophora* (Poinar) (LU1, D1) (Rhabditida: Steinernematidae and Heterorhabditidae). EPN were collected using the 'Galleria baiting technique' (Bedding & Akhurst, 1975) during soil surveys in different habitats in Lebanon and Italy (Noujeim et al., 2016; Tarasco et al., 2015). To obtain fresh infective juveniles (IJs), *Galleria mellonella* (Lepidoptera, Pyralidae) larvae were placed in Petri dishes (100 × 10 mm) lined with two filter papers and exposed to IJs at the concentrations of 2.000 IJs/1.5 ml tap water at 22°C (Tarasco et al., 2015). Dead last-instar larvae were put on modified White traps (White, 1927); juveniles emerging from *G. mellonella* cadavers were collected and used in the bioassay. Isolates of *Beauveria bassiana* (Bals.) (OF13, RF1, ALB55, CG2) obtained from soils of different Italian ecosystems, within previous studies (Tarasco & Triggiani, 1997; Tarasco & Triggiani, 2007) were used. The conidial suspension was prepared by growing the *B. bassiana* strains in Petri dishes on 2% malt extract agar and incubated at 25°C in the dark for 15 days. Conidia of *B. bassiana* were harvested directly from fungal cultures by pouring sterile distilled water with the addition of 0.002% Tween 80 in the Petri dish and scarping the sporulating colonies. The suspension was filtered through cheesecloth to eliminate mycelial fragments. The concentration of the conidial suspension was counted and adjusted using a Neubauer Haemacytometer.

Infectivity bioassays of entomopathogenic nematodes and fungi against xylophagous larvae

The pathogenicity of EPN and *B. bassiana* strains was tested under laboratory conditions against four xylophagous pests *P. caestrum*, *C. cossus*, *A. syriacus* and *C. tenebrionis*.

Bioassays against *P. caestrum*, *C. Cossus*, and *A. syriacus* were conducted in glass Petri dishes (95 × 32 mm) filled with bark and frass from the same environment of the larvae collected (Figure 1). For each xylophagous pest, five larval individuals were treated with 5 ml of *B. bassiana* conidial suspension (2×10^5conidia/ml) or nematodes strains (100 and 200 IJs/larva) and a single larva was considered a replicate. EPN or EPF solutions were applied homogenously onto the surface of the Petri dish, and the larva was covered with the adequate plant material (Figure 1). As control, sterile distilled water with 0.002% Tween 80 was considered for EPF bioassays while only tap water was used as a control in EPN bioassays. Five toten replicates were considered for each treatment against each insect species depending on the availability of the larvae.

For performing the EPF bioassay against *C. tenebrionis*, the previous experimental design was used. As for the EPN bioassay against *C. tenebrionis*, another experimental model was considered (Figure 2). Reared larvae were reintroduced into the wood galleries to imitate their natural habitat. Then, the suspensions of EPN strains (200 IJs/individual) diluted in 10 ml of tap water were injected into each gallery using a micropipette. The galleries' holes were covered by a mesh fabric.

For both experimental designs, the final larval mortality was recorded 5 days after treatment (DAT). Afterwards, the dead larvae were collected, rinsed in tap water and dissected after 48 h to confirm the infection of nematodes. Also, the mortality induced by *B. bassiana* was confirmed by culturing the dead larva on malt extract agar media. The sporulation of the dead larva, if occurred, indicated that the entomopathogenic fungus was the cause of the mortality.

Statistical analysis

Statistical analyses and plots were performed using the software IBM SPSS-Statistics 22. The results data were analysed using a general linear model procedure (ANOVA – analysis of variance) and significant differences among means were separated by HSD Tukey's test. The minimum level of significance was taken as $P < 0.05$.

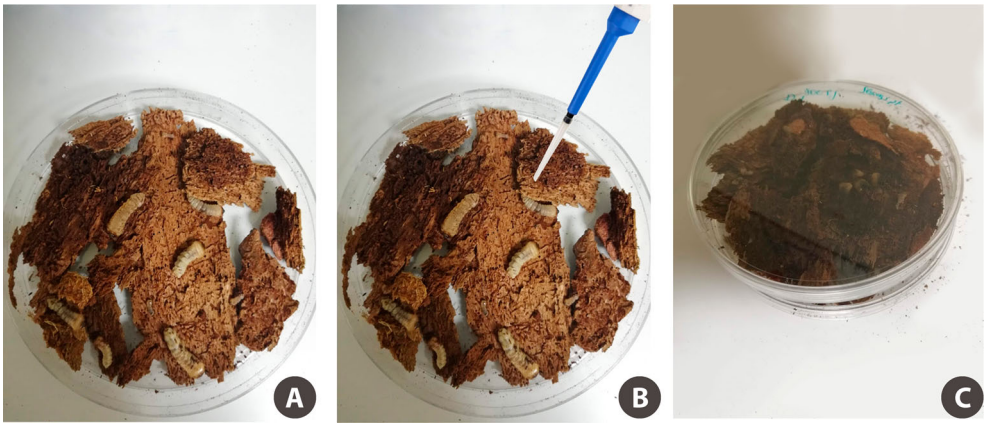

Figure 1. Experimental design of EPN or EPF on the larvae in Petri dishes. A. Plant material (here pine bark) and larvae in a Petri dish; B. Application of the microbial solution using a micropipette; C. Larvae covered with pine bark before incubation.

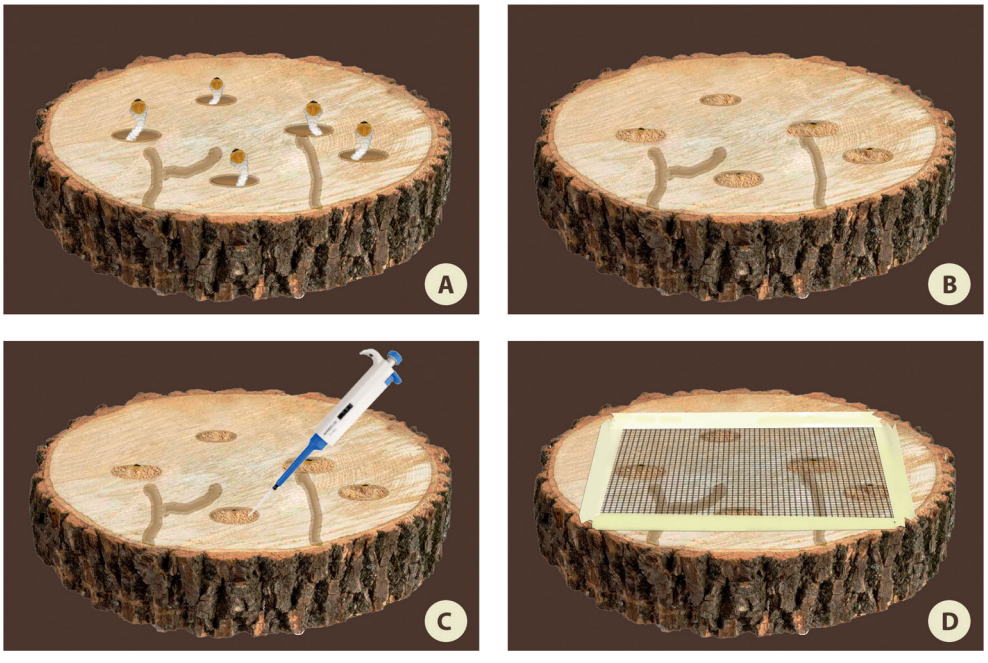

Figure 2. Experimental design of EPN on *C. tenebrionis* in wood trunks. A. Larvae introduced into the galleries; B. Galleries filled with wood frass; C. Treatment application into the gallery with a micropipette; D. Galleries' holes covered by mesh fabric.

Results

The bioassays showed that all the entomopathogenic nematodes and fungi strains affected the survival rate of *P. caestrum*, *C. cossus*, *C. tenebrionis* and *A. syriacus*. No nematode emergence or fungi growth was recorded in the control treatments.

Parahypopta caestrum

Results of the analysis of variance revealed that both the EPN ($F = 72.46$; $df = 10$; $P < 0.0001$) and EPF ($F = 178.57$; $df = 3$; $P<0.0001$) strains affected the larval mortality of *P. caestrum* (Figure 3 and Figure 4). Concerning the type of microorganism, entomopathogenic fungi induced higher mortality rate than the entomopathogenic nematodes. The majority of nematodes and fungal strains were pathogenic to *P. caestrum* larvae. Though, *S. affine* CZ7 and *H. bacteriophora* LU1 were the least efficient (Figure 3). *S. feltiae* MF1 and *B. bassiana* OF13 showed the best performances (killing the 97% and 94% of *P. caestrum* larvae, respectively). *B. bassiana* RF1 (90%) and *S. feltiae* GF16 (87%) and *B. bassiana* ALB55 (87%) scored also high mortality rates (Figure 4). *S. arenarium* caused 40% larval mortality.

Cossus cossus

In the experiments targeting *C. cossus*, results of the analysis of variance showed that both the EPN ($F = 17.77$; $df = 15$; $P < 0.0001$) and EPF ($F = 12.23$; $df = 5$; $P = 0.000$) strains

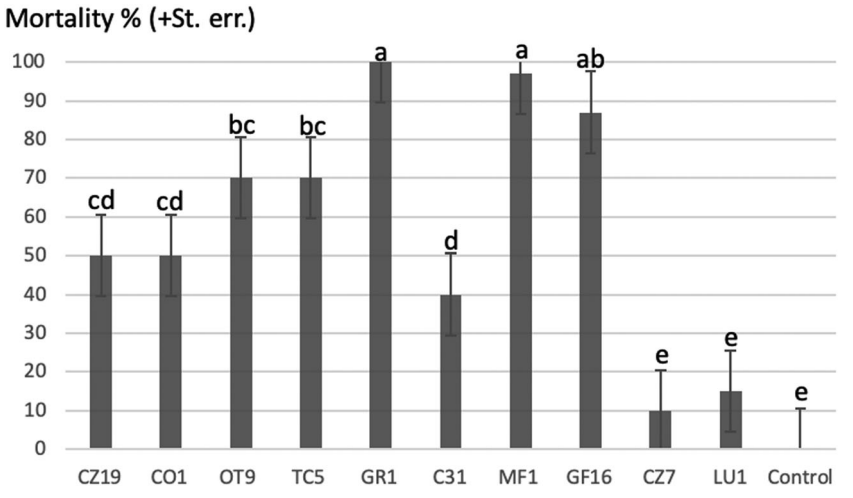

Figure 3. Mean mortality (%) of *P. caestrum* instar larvae after exposure to different EPN strains. *S. feltiae*: CZ19, CO1, OT9, TC5, GR1, MF1, GF16; *S. arenarium*: C31; *Steinernema affine:* CZ7; *H. bacteriophora*: LU1. Different letters above bars indicate significant differences at $P < 0.05$.

affected significantly the larval mortality. All the nematode and fungi strains were able to kill the larvae of *C. cossus*, but mortality rates differed significantly (Figure 5 and Figure 6). *Steinernema feltiae* (CZ19, GR1, ALG18) caused the highest mortality rates (80–100%). Nonetheless, *H. bacteriophora* LU1 and the remaining *S. feltiae* strains were the least effective. As for *B. bassiana*, the strains (OF13, RF1, CGD2) caused 90% of mortality.

Capnodis tenebrionis

Results of the analysis of variance revealed that both the EPN ($F = 166$; $df = 3$; $P < 0.0001$) and EPF ($F = 36.57$; $df = 4$; $P < 0.0001$) strains affected significantly the larval mortality of *C. tenebrionis* (Figure 7 and Figure 8). Both strains of *S. feltiae* (GR1 and OT15) showed high efficiency against the larvae of *C. tenebrionis*, causing 80% and 100% mortality, respectively. However, no mortality was recorded by *H. bacteriophora*. As for the entomopathogenic fungi, two *B. bassiana* strains (GC2 and OF13) recorded different results respectively 76% and 26% (Figure 8).

Arhopalus syriacus

In the last experiment targeting *A. syriacus*, results of the analysis of variance revealed the EPN treatments ($F = 72.33$; $df = 2$; $P < 0.0001$) affected significantly the larval mortality (Figure 9). *S. feltiae* LIB132 succeeded to kill 67% of *A. syriacus* larvae, and lower mortality rate (53%) was recorded by *H. bacteriophora* D1 (Figure 9).

Discussion and conclusion

In the present study, we assess the pathogenicity of EPN and EPF against *P. caestrum*, *C. cossus*, *C. tenebrionis* and the pathogenicity of EPN against *A. syriacus*. The studied

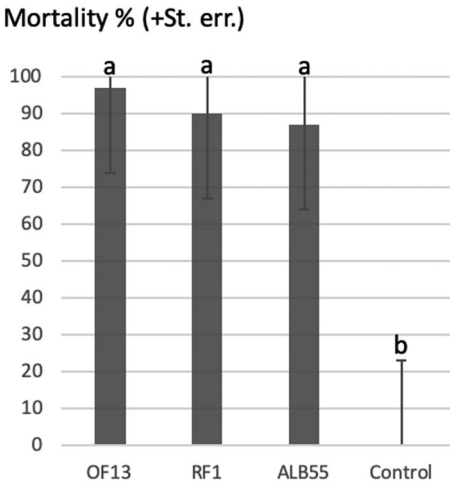

Figure 4. Mean mortality (%) of *P. caestrum* instar larvae after exposure to different EPF (*B. bassiana*) strains. Different letters above bars indicate significant differences at $P < 0.05$.

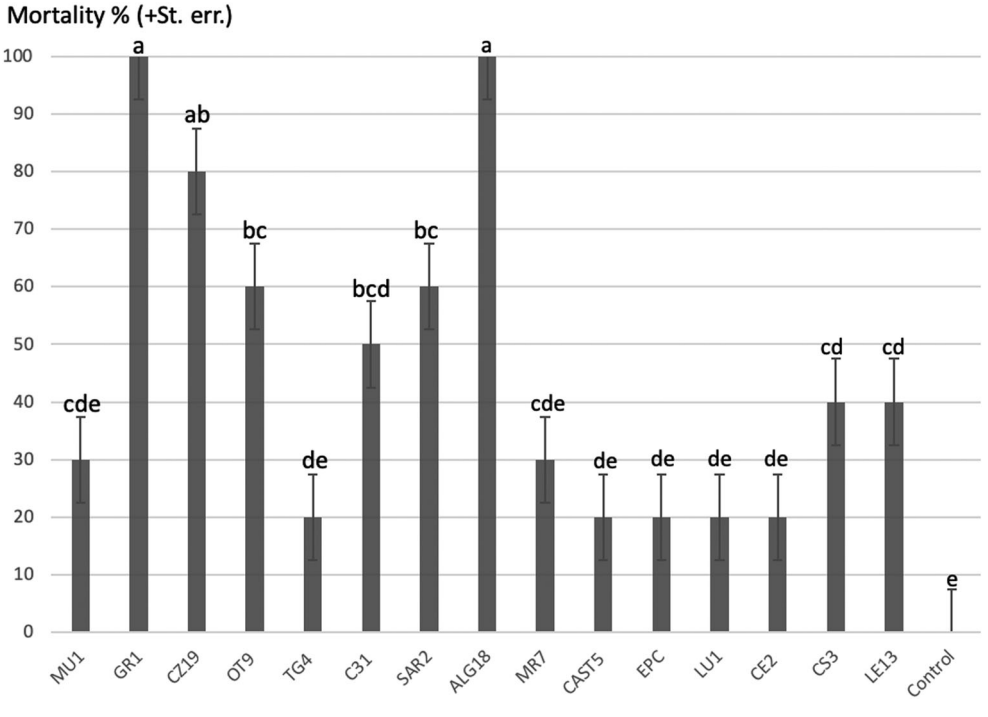

Figure 5. Mean mortality (%) of *C. cossus* instar larvae after exposure to different EPN strains. *S. feltiae*: MU1, GR1, CZ19, OT9, TG4, ALG18, CAST5, EPC, CE2; *S. carpocapsae*: MR7; *Steinernema a.*: C31; *S. ichnusae:* SAR2; *S. apulia*: CS3, LE13; *H. bacteriophora*: LU1. Different letters above bars indicate significant differences at $P < 0.05$.

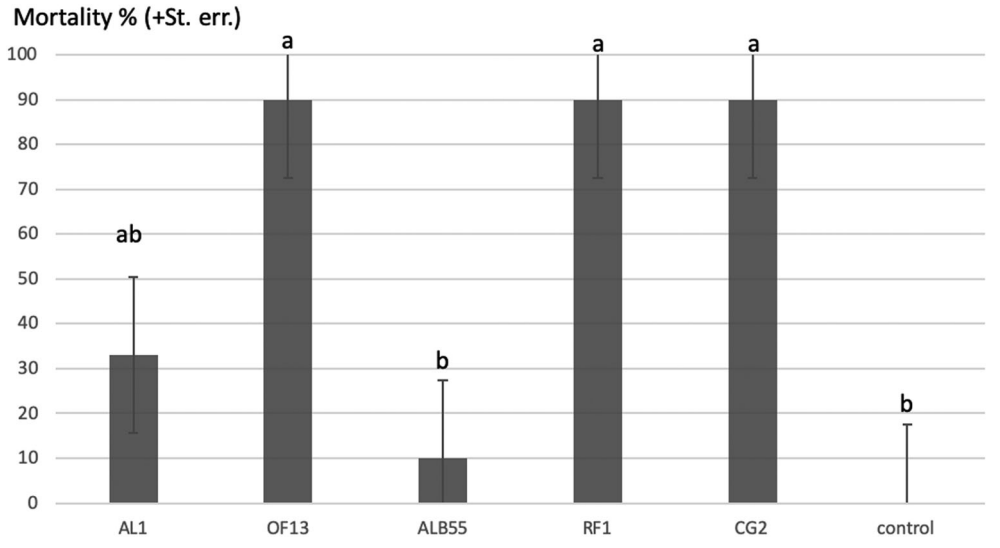

Figure 6. Mean mortality (%) of *C. cossus* instar larvae after exposure to different EPF (*B. bassiana*) strains. Different letters above bars indicate significant differences at $P < 0.05$.

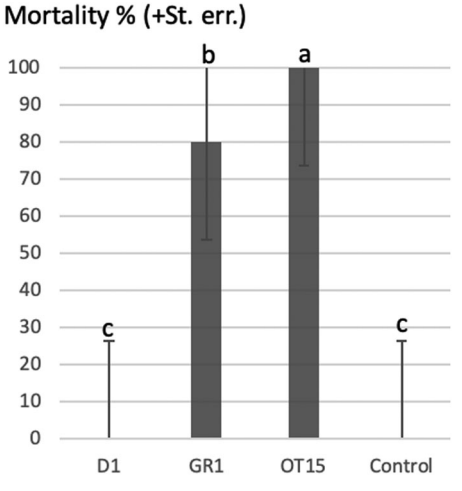

Figure 7. Mean mortality (%) of *C. tenebrionis* instar larvae after exposure to different EPN strains. *S. feltiae*: GR1, OT15; *H. bacteriophora*: D1. Different letters above bars indicate significant differences at $P < 0.05$.

insects may be included in the category of pests inhabiting cryptic habitats, as insects that bore into the plant tissue (wood-boring insects) or under the bark (bark beetles) or in the soil (wireworms). These pests, being residents in cryptic habitats, are harsh to be reached and to be controlled by chemical means (Gumus et al., 2015). As a result, the remaining option to manage those pests and to reduce infestations are sanitary measures considering removal and destruction of the infested or the injured plants (Gumus et al., 2015; Langstrom et al., 2004); hence, the necessity to find alternative control method that could

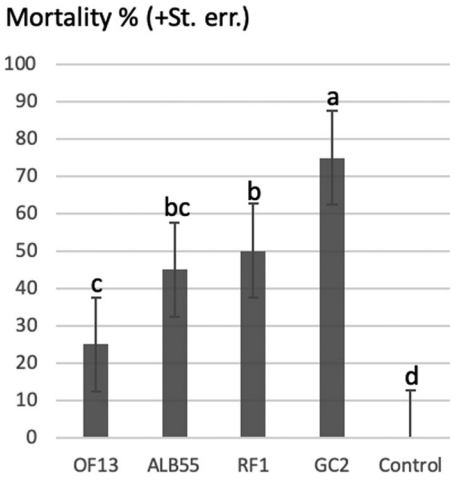

Figure 8. Mean mortality (%) of *C. tenebrionis* instar larvae after exposure to different EPF (*B. bassiana*) strains. Different letters above bars indicate significant differences at $P < 0.05$.

possibly save the plants. Nematodes and fungi are living microorganisms; thus, they are able to reach the cryptic habitats and may be horizontally transmitted by the infected hosts. The pathogenicity of EPN and EPF against pests residing into cryptic habitats was the subject of several studies (Ashtari et al., 2011; Gumus et al., 2015; Kreutz et al., 2004; Martinez de Altube et al., 2008; Şahin & Gözel, 2019; Yiğit et al., 2018; Yuksel & Canhilal, 2018).

Our results showed that EPN and EPF were effective against *P. caestrum* larvae under laboratory conditions. Mortality rates varied according to the species and strains. *S. feltiae* and *B. bassiana* showed the best performances, by killing on average 90% of the *P. caestrum* larvae in laboratory assays. Considering the efficacy of EPN, our results

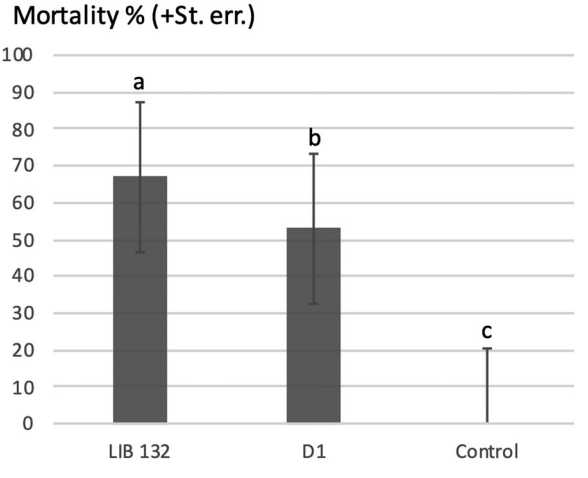

Figure 9. Mean mortality (%) of *A. syriacus* instar larvae after exposure to different EPN strains. *S. feltiae*: LIB 132; *H. bacteriophora*: D1. Different letters above bars indicate significant differences at $P < 0.05$.

partially agreed with those of Salpiggidis et al. (2008), who found that both *S. feltiae* and *H. bacteriophora* provided high larval mortality of *P. caestrum* (70–90%) in laboratory assays, although *S. feltiae* had a quicker effect. Our results are in alignment with the results obtained by Tarasco et al. (2016) where *B. bassiana* caused a high mortality rate (96%) of *P. caestrum* larvae.

As for *C. cossus*, Gumus et al. (2015) showed that live insect hosts that were pre-infected with EPN and released in the habitat, induced a high mortality (86%) of *C. cossus* larvae infesting chestnut logs. However, in the bioassays performed in Petri dishes, our results showed a higher pathogenicity (80-100% versus 4% Mortality rate) of the tested EPN and EPF strains against *C. cossus* larvae. Since the strains used are different, we hypothesised that this could be due to the higher virulence of the strains selected in our study.

The results of our study clearly demonstrate that *B. bassiana* is pathogenic against the larvae of *C. tenebrionis*, both strains used were virulent and the mortality ranged between 26% and 76%. Similar efficacies were obtained by Marannino et al. (2006) evaluating the efficacy of four *Metarizium anisopliae* (Metschn.) and *B. bassiana* isolates against neonate larvae of *C. tenebrionis*. Higher mortality against the larvae of *C. tenebrionis* (100%) was recorded in a recent study by applying the *B. bassiana* at the concentration of 10^6 spores/cm^2 (Yiğit et al., 2018). This higher mortality could be due to the higher conidial concentration used in this study, and the soil environment where the treatment was applied could have maintained a higher moisture content thus the viability of spores was well preserved. On another note, the considered strain of *B. bassiana* could be more virulent than the strains used in our study. As for entomopathogenic nematodes, the efficacy of *S. feltiae* against larvae of *C. tenebrionis* in semi-field conditions obtained in this experiment (80% and 100% mortality) are in alignment with different studies. Morton and García-del-Pino (2008) tested the efficacy of *S. feltiae* against *C. tenebrionis* in pot experiments where it killed 88.2% of the larvae. In a recent study, Şahin and Gözel (2019) evaluated the pathogenicity of four entomopathogenic nematodes. In accordance with our results, *S. feltiae* was able to kill 92.5% of the larvae in infested potted apricot 5th DAT. In the same study, *H. bacteriophora* caused 75% mortality 5th DAT. Similarly, another study reported 98.9% mortality of endophytic larvae of *C. tenebrionis* by *H. bacteriophora* (Marannino et al., 2003). However, in our study, *H. bacteriophora* (D1) caused no mortality to *C. tenebrionis* larvae residing inside the galleries. This might be due to the lower concentration of IJs applied, and a probable difference between the virulence of the strains could have resulted in different results. The same strain (D1) caused 53% mortality to *A. syriacus* larvae in Petri dishes. A potential factor that has negatively impacted the nematodes pathogenicity could be the compacted environment (wood frass inside galleries) where the EPN were applied. As compacting might diminish the ability of the EPN to move toward their host (Kapranas et al., 2017). To our knowledge, no data are available about the pathogenicity of EPN against *A. syriacus*, thus our preliminary positive results are considered the first report.

Despite that our results showed that EPN and EPF are effective separately against the studied xylophagous insects, the combination of the bioagents should not be dismissed in field applications. As EPF could persist for long periods in some environments (Scheepmaker & Butt, 2010) and the mobile EPN have the ability to move inside cryptic habitats, the application of both bioagents simultaneously could constitute a greater promising control measurement. Nevertheless, their effectiveness as bio-agents against the tested

insects needs to be confirmed in the field, and various parameters remain to be explored. On the first hand, environmental conditions where the microbials are applied constitute the main limiting factors of their success. Thus, soil or bark humidity should be adequate while applying EPN and EPF. On the other hand, the timing of the application is another key factor for the success of the treatment. For instance, as suggested by Tarasco et al. (2016), the optimal period for the use of EPF and EPN against *P. caestrum* in the field could be directly following the egg hatching, in order to target the susceptible young larvae on their way to infest the roots. On another note, the choice of the most virulent strain is crucial for the success of the infestation, as different strains of the same microbial species could have varied pathogenicity level.

Considering the high destructiveness of the four xylophagous insects studied, and the difficulty to access to xylophagous insects, major attention from the research institutions should be given to the control of these pests, focusing on the improvement of the most suitable microbial control agents and their application methods as a substitute to chemical pesticides.

Acknowledgements

Research supported by the University of Bari 'Aldo Moro' funds. Also, the authors would like to acknowledge the National Council for Scientific Research Lebanon CNRS-Lebanon for granting a doctoral fellowship to Yara El Khoury.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Research was supported by the University of Bari 'Aldo Moro' funds.

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