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Additive and synergistic interactions of entomopathogenic fungi with *Bacillus thuringiensis* for the control of the European grapevine moth *Lobesia botrana* (Denis and Schifferrmüller) (Lepidoptera: Tortricidae)

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Abstract

Background: The European grapevine moth, *Lobesia botrana* (Denis and Schifferrmüller) (Lepidoptera: Tortricidae) is currently the most damaging pest in many viticultural regions across the Mediterranean basin and elsewhere. Its feeding activity also enhances the development of secondary infections by *Botrytis cinerea* - and other fungi - to wine grapes. The gram-positive bacterium *Bacillus thuringiensis* (Bt) has been reported to partially control larval populations of *L. botrana*, while it requires to be digested by the insect to cause infection. Entomopathogenic fungi (EPF) are possibly capable of acting synergistically with Bt to increase its efficacy against insect pests.

Results: The hypothesis of synergy or antagonism between Bt and EPF for the control of *L. botrana* was tested in two bioassays: A) Insects fed on Bt diet and subsequently some groups were sprayed by conidia of *Beauveria bassiana* or *Paecilomyces fumosoroseus*, and B) Grapes were sprayed by Bt, or *B. bassiana*, or combination of the two, and then untreated insects were placed to feed on the grapes. In both bioassays, combination treatments performed better than single treatments, indicating additive action or synergy. The Bt and *B. bassiana* combination treatment (Bt diet for 30 h and then sprayed with conidia of *B. bassiana*) resulted in 91% larval mortality while the single Bt and *B. bassiana* treatments caused 28% and 34% mortality respectively. Such results indicated synergism. Combination treatment on grapes also caused significantly higher mortality on *L. botrana* larvae, compared to single treatments. The median lethal time (LT₅₀) was estimated as 8.43 days for the single Bt treatment, 7.87 days for the single *B. bassiana* treatment and 6.3 days for the combination Bt + *B. bassiana* treatment.

Conclusions: Absence of antagonism as well as additive action or synergy were indicated by the results. Larval populations of the pest can be effectively controlled by using microbial biocontrol agents. Further research is needed to investigate the biotic and abiotic factors that affect interactions between insect hosts and entomopathogenic organisms. However, the entomopathogens used in the present study showed remarkable action and may be included parallelly in control strategies against vineyard pests.

Keywords: *Lobesia botrana*, Entomopathogenic fungi, *Bacillus thuringiensis*, Biological control, *Vitis vinifera*

Background

The European grapevine moth, *Lobesia botrana* (Denis and Schifferrmüller) (Lepidoptera: Tortricidae) is the most damaging vineyard pest in South and Central Europe,

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while recently it has been reported to cause significant damages in important viticulture regions of Chile, Argentina and California (López-Plantey et al. 2019). It mainly damages grape berries and flowers (Moreau et al. 2010), while larval stage affects the level of damage (Delbac and Thiery 2015). The direct losses that *L. botrana* are often followed by secondary infections by *Botrytis cinerea* and several more fungi (Delbac et al. 2010). Direct infestations by *L. botrana* and damages caused by its subsequent fungal infections, have been reported to substantially affect the quality and quantity of the grapes, as well as the quality and chemical properties of the wines produced from those grapes (Ioriatti et al. 2011).

Chemical control has significant environmental, economic and public health impact in addition to the resistance that *L. botrana* develops to specific insecticidal chemical compounds (Durmuşoğlu et al. 2015). Common control strategies against the pest include the mating disruption technique (Ioriatti et al. 2011) and the use of egg parasitoids (Vogelweith et al. 2014). Alternative control approaches such as the exposure of the insect to Gamma irradiation (Mansour and Al-Attar 2014) and the use of nanotechnology (Benelli et al. 2020) have been recently tested.

Regarding microbial bio-control agents, *Bacillus thuringiensis* (*Bt*) has been proven to control larval populations of *L. botrana* (Ruiz de Escudero et al. 2007). Many *Bt* strains produce crystal proteins, called δ -endotoxins, which have insecticidal action (Schünemann et al. 2014). The Cry toxin can be extracted and used as a bio-insecticide. Such insecticides have already been used in viticulture for controlling the European grapevine moth and other vineyard pests. Toxicity caused by *Bt* is considered as insect specific. This selective action of most *Bt* formulations, results in effective control of the populations of *L. botrana* without affecting the populations of its parasitoids and predatory insects and mites (Tzanakakis and Katsoyiannis 2003). However, the efficacy of *Bt* on pests, depends on multiple biotic and abiotic factors such as environments conditions (Moustafa et al. 2018). The physiology of *L. botrana* also depends substantially on environmental factors (Gutierrez et al. 2018; Reineke and Selim 2019). Furthermore, *Bt* needs to be digested by the insect to cause infection (Guo et al. 2019).

Entomopathogenic fungi (EPF) have been reported to present additional efficacy against insect pests when applied in combination with *Bt*-based products. Although there is no information on the use of such combination treatments on *L. botrana*, results showing additive effects or synergistic interactions between EPF and *Bt* on the control of various pests, have been previously published. Interactions between *B. bassiana* and Cry1Ac (a toxic crystal protein produced by *Bt*) were observed by

Ma et al. (2008) on laboratory bioassays for the control of *Ostrinia furnacalis*. Moreover, combined effects of *B. bassiana* and bio-insecticides based on *Bt* were investigated in field populations of Colorado potato beetle larvae (Wraight and Ramos 2005). Synergistic interactions of *B. bassiana* and *Bt* on control of the Colorado Potato Beetle were also reported by Costa et al. (2001), after applying sublethal stress with the CryIII δ -endotoxin of *Bt* and subsequent exposure to *B. bassiana*.

In the present study, an attempt was made to investigate whether *Bt* can act synergistically with EPF for effective control of *L. botrana*.

Methods

Insects

Second and 3rd larval instars of *L. botrana* were used in the following bioassays. Insects were reared, under controlled conditions, on a semisynthetic diet (Standard *L. botrana* diet) consisting of the following ingredients: distilled water 1.4 l, corn flour 225 g, wheat germs 58 g, agar 32 g, brewing yeast 22 g, sorbic acid 8 g, Nipagin 4 g, Benzoic acid 4 g.

Larvae were initially collected, along with infested grapes, from a vineyard (*V. vinifera* cv. Savatiano) in the area of Spata (Attica - Greece). Then, infested grapes were placed in cages until first adult insects emerged. Adult female and male insects were placed on plastic cups, covered with filter paper on the top, and the females laid their eggs on the walls of the cups. Then, the cups were half-filled with artificial diet which was previously cut into cubes of about 1 cm length. When the eggs hatched, young larvae fed on diet until they pupated. After the emergence, all adult males and females were placed into plastic cups using a glass tube. The insects were left to lay their eggs for 3–4 days and then removed and killed. Adult insects fed on a solution of 5% honey dissolved into distilled water, which was provided to the insects via a cotton disc placed on the bottom of the cups. The colony of *L. botrana* was maintained at a temperature of $24 \pm 1^\circ \text{C}$ and $60 \pm 10\%$ relative humidity. The photoperiod was 16:8 h (light: dark) and the luminosity 500 lx photophase. Insects used in both bioassays remained under starvation for 24 h before the initiation of the experiments.

Bacillus thuringiensis

Bacillus thuringiensis var. *kurstaki* (Dipel ES, 17,600 International Units (IU)/mg, Abbott, North Chicago, Illinois) was used at both following bioassays. Dipel ES contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B endotoxins of *Bt* along with supplementary components which improve its performance as a microbial insecticide. For the In vitro test, 4 ml of Dipel ES were

dissolved in 1.4 l of distilled water, which replaced the total amount of water in an individual block of semi-synthetic diet (1650 g). This process resulted in a concentration of ~42,700 IU per g of diet. For the latter bioassay, a concentration of 70,400 IU/ml was made by adding 2 ml of Dipel ES into 500 ml of sterile distilled water.

Fungal preparations

The EPF isolates *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01, as well as a commercial bio-insecticide based on *B. bassiana* strain GHA (BotaniGard® ES), were used at the following bioassays. The two non-commercialised fungi were initially isolated using the “*Galleria* Bait Method” (Zimmermann 1986). Upon initiation of the experiments, these isolates were sub-cultured and grown on 9 cm Ø Petri dishes with half-strength Sabouraud Dextrose Agar (SDA), at 25° C for 20 days. Spore suspensions of 1×10^8 conidia/ml were made for each isolate, by scraping conidia from the surface of the plates using a microscope slide. Then, a sterile liquid solution of distilled water with 0.1% Tween 80 was poured into the plates to remove, disperse and integrate the conidia. The containing conidia liquid suspension was stirred using a magnetic stirrer and filtered twice using a sterile nylon membrane. The dose of 1×10^8 conidia/ml was selected for all fungal treatments due to the highest efficacy levels that presented in previous bioassays. This concentration has been considered as optimum, especially for single concentration experiments (Galindo-Velasco et al. 2015). In the case of the commercial bio-pesticide (BotaniGard® ES), a spore suspension was adjusted to 1×10^8 conidia/ml by dissolving 6.15 ml of product into 500 ml of sterile distilled water. All spore concentrations were measured, under microscope (400×), using a standard (improved Neubauer) haemocytometer. When needed, extra distilled water was added to adjust the dilution until reaching the 1×10^8 conidia/ml concentration.

Spore viability was calculated by counting the percentage of germinated conidia, which were spread on half-strength SDA plates, under a microscope (400×). *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01 presented conidial viability of 99 and 98.3%, respectively while *B. bassiana* GHA (BotaniGard® ES) showed 96.7% spore viability. A concentration of 1×10^5 conidia/ml was made for each isolate, using the method described above; 100 µl of the suspension was added on each plate using a micropipette and then spread using a microscope slide. After sealing, the plates were incubated at 25° C for 18 h. Three sets of 100 conidia were measured for each isolate. All conidia with visible germ tubes of any length were counted as viable.

Mortality of *L. botrana* larvae after exposure to *Bt* diet and subsequent fungal treatment

The 2nd instar *L. botrana* larvae were used in the present bioassay. For treatments involving *Bt* consumption, insects were exposed and fed on a diet, which contained *Bt* var. *kurstaki* (Dipel ES). Control larvae, as well as insects that were prepared for the single fungal treatments, were fed on standard *L. botrana* diet (described above). *Bt* diet was made exactly as the standard, except the addition of 4 ml of the product (Dipel ES) in 1.4 l of distilled water, which produces approximately 1650 g of diet (42,700 IU/g). Insects were fed on either diet in groups of 10 (5 groups for each treatment), inside sterile Petri dishes for 30 h.

Subsequently, fungal treatments were applied by spraying the insects, using Mist trigger micro-sprayers “Hozelock 4120 Spray 0.5 l”, inside sterile Petri dishes with filter paper covering the bottom of the plates. Ten larvae were placed into each dish. Insects were sprayed until the entire area of the paper was wet but not saturated. Single and combination *B. bassiana* and *P. fumosoroseus* treatments were sprayed with the respective suspensions (1×10^8 conidia/ml of sterile distilled water with 0.1% Tween 80). Control and single *Bt* insects were sprayed with a solution of sterile distilled water with 0.1% Tween 80.

Therefore, six (6) groups of differently treated insects were evaluated:

1. *Control*: Larvae fed on standard *L. botrana* diet for 30 h and then, sprayed with sterile distilled water.
2. *Bt*: Larvae fed on *Bt* diet for 30 h and then, sprayed with sterile distilled water.
3. *B. bassiana*: Larvae fed on standard *L. botrana* diet for 30 h and then, sprayed with a spore suspension of 1×10^8 conidia *B. bassiana*/ml.
4. *P. fumosoroseus*: Larvae fed on standard *L. botrana* diet for 30 h and then, sprayed with a spore suspension of 1×10^8 conidia *P. fumosoroseus*/ml.
5. *Bt*+*B. bassiana*: Larvae fed on *Bt* diet for 30 h and then, sprayed with a spore suspension of 1×10^8 conidia *B. bassiana*/ml.
6. *Bt*+*P. fumosoroseus*: Larvae fed on *Bt* diet for 30 h and then, sprayed with a spore suspension of 1×10^8 conidia *P. fumosoroseus*/ml.

Post treatment, larvae were provided with a standard *L. botrana* diet inside Petri dishes (10 larvae per dish). Four cubes of about one cubic centimetre each were added on each plate. Then, plates were sealed and placed into incubators at 25 °C, 60 ± 1%RH and 14:10 (L:D) photoperiod (Sanyo - MLR-351H). Each

treatment consisted of 50 replicates (5 dishes \times 10 larvae each) and the entire experiment was repeated twice.

Mortality measurements took place 3 and 6 days after the spraying treatments. Any larva which either remained still after contact with the pincers, or was rotten or disintegrated, or presented symptoms of mycosis was recorded as dead. After each mortality measurement, all dead insects were removed from the plates.

Combined effect of *Bt* and *B. bassiana* for the control of *L. botrana* on grapes

Young 3rd instar *L. botrana* larvae were exposed to grapes that were previously sprayed by either *B. bassiana* strain GHA (BotaniGard® ES), or *Bt* var. *kurstaki* (Dipel-ES) or a combination of both entomopathogens. Control insects were exposed to grapes, sprayed with sterile distilled water. The *B. bassiana* suspension was adjusted to 1×10^8 conidia/ml of sterile distilled water and 0.5 l of such suspension was made. The *Bt* treatment was made by dissolving 2 ml of Dipel ES into 500 ml of sterile distilled water, resulting in a final concentration of 70,400 IU/ml. Equal parts of both liquids (250 ml) were mixed to prepare the combination treatment.

Ripe grapes of *Vitis vinifera* cv. Sauvignon Blanc were used in the bioassay (11.8–14.1 Baumé degrees at 20° C). Small grape clusters or parts of clusters (6–15 berries each) were totally covered by the spraying liquid. Then, grapes were rested to dry for 2 h. Subsequently, grape clusters were placed into plastic jars (9.5 cm Ø and 14 cm height). The lids of the jars were pierced multiple times so air was allowed in the jars. A piece of wet filter paper was placed to cover the bottom of each jar. Grapes of the respective treatment (one small cluster) as well as 10 *L. botrana* larvae were placed into each jar. All jars were incubated at 25° C, $60 \pm 1\%$ RH and 12:12 h (L:D) photoperiod for 48 h and larvae were observed to feed on the grapes. Then, insects from each jar were transferred into a sterile Petri dish, using entomological pincers. Standard *L. botrana* artificial diet was added into the dishes and insects were incubated under the same conditions as above for 5 more days. Twenty replicates of 10 insects were made for each treatment and the experiment was repeated twice. Mortality measurements were conducted 3, 5 and 7 days after the initial placement of the insects into the jars. Any larva that was rotten, covered by mycelium, or remained still was counted as dead. Dead larvae were removed from the plates during each measurement.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted to compare different treatments in terms of larval mortality (SPSS, 2008). In both bioassays, Levene's test was used to estimate if variances were assumed homogenous

or not. According to those estimations, respective Post-Hoc tests were used to indicate significant differences between particular treatments (Hilton and Armstrong 2006). In the latter bioassay, Probit analysis was used to estimate the median lethal time (LT_{50}) (Reddy et al. 2016). The synergism factor (SF) was calculated by dividing the predicted theoretical mortality value for each treatment by the observed value. According to Tabashnik (1992), an SF ratio equal to 1 was additive, lower than 1 was antagonistic, and a ratio over 1 was synergistic.

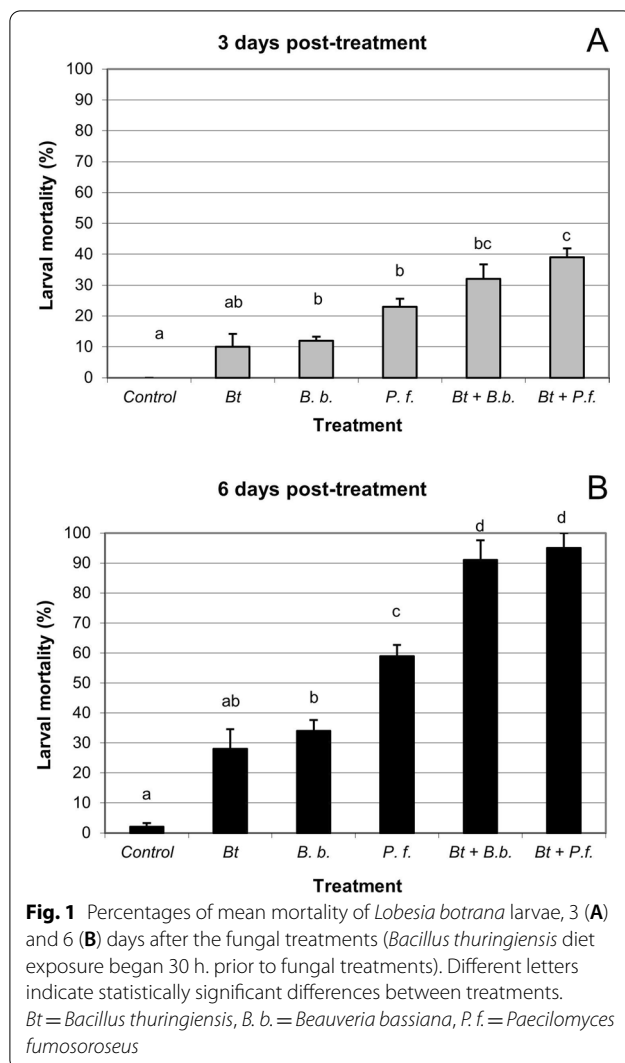
Results

Mortality of *L. botrana* larvae after exposure to *Bt* diet and subsequent fungal treatment

One-way analysis of variance (ANOVA) showed that even 3 days after the fungal treatments, different applications resulted in significant differences in terms of larval mortality of *L. botrana* ($P=0.0018$, $F=23.25$, $df=5$, 24) (Fig. 1A). Significant differences - among treatments - in mortality of *L. botrana* larvae were also detected in the final measurement that took place 6 days after the fungal treatment ($P<0.001$, $F=95.12$, $df=5$, 24) (Fig. 1B). In both cases, Levene's statistic indicated that variances were not homogenous and therefore Games Howell Post-hoc test was used to determine significant differences among treatments. Control insects presented no mortality in the first measurement (3 days post-treatment), while minimum levels of mortality (2%) were observed 6 days post-treatment (Fig. 1). The highest mortality levels of *L. botrana* larvae were observed when insects were treated with the combination of *Bt* diet and subsequent treatment with *B. bassiana* (91%) and *P. fumosoroseus* (95%). Those results indicated that combination treatments differed significantly from single treatments causing remarkably high mortality levels. The combination of *Bt* diet and subsequent *B. bassiana* treatment showed a synergistic action, while additive effects were observed in the case of *Bt* + *P. fumosoroseus*. About 35% of insects sprayed with fungal suspensions presented symptoms of external mycosis 11 days post-treatment.

Combined effect of *Bt* and *B. bassiana* for the control on *L. botrana* on grapes

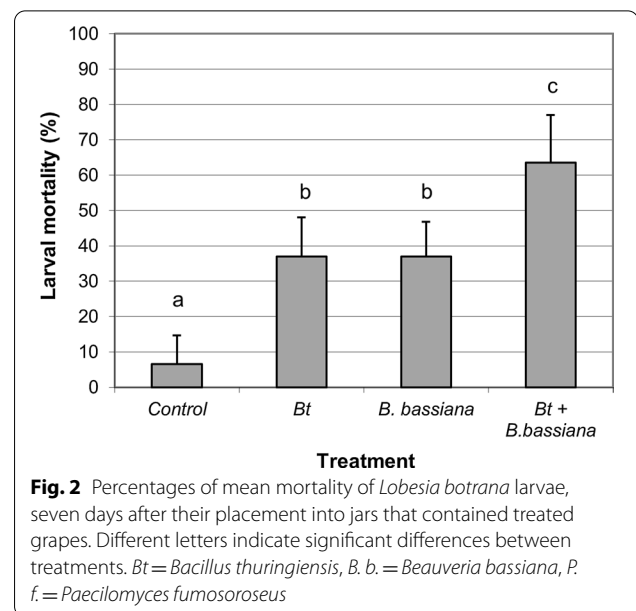
There were statistically significant differences among treatments 7 days post placement of the insects into the jars that contained treated grapes ($P<0.001$, $F=84.62$, $df=3$, 76). Single *Bt* and *B. bassiana* treatments resulted in equal larval mortality (37%). Mortality in the combination treatment was significantly higher than single treatments, while all treatments differed significantly from control (Fig. 2). Tukey HSD test was used to determine differences between treatments due to Levene's test which proved homogeneity within variances ($P=0.201$).



Measurements taken 3 and 5 days post-treatment presented low levels of mortality in all treatments and therefore, results were neither taken into consideration nor statistically analysed. According to Probit analysis, the LT_{50} (median lethal time 50%) for the single *Bt* treatment was 8.43, for the single *B. bassiana* treatment was 7.87 and for the combination *Bt* + *B. bassiana* treatment was 6.3 (days), at standard concentrations of 1×10^8 conidia/ml (*B. bassiana*) and 70,400 IU/ml (*Bt*). Additive effects were observed (when combining *Bt* and *B. bassiana*), but in this case, no synergy was suggested.

Discussion

Results published in previous assays indicated efficacy of *Bt* on the control of *L. botrana*. Lethal effects were observed under field and laboratory conditions by Roditakis (1986), while Ifoulis and Savopoulou-Soul-tani (2004) tested various *Bt* formulations at laboratory



bioassays. Ruiz de Escudero et al. (2007) also reported the efficiency of *Bt* toxins on the control of *L. botrana*. Analogous Research was extended to several more lepidopteran Pests (Ruiz de Escudero et al. 2014). Furthermore, selective action (*Bt* controls *L. botrana* without harming its predators and parasitoids) has been indicated (Tzanakakis and Katsoyiannos 2003). However, in a recent study, *Bt* showed to affect host-searching behaviour and reproductive activity of the parasitoid *Palm-istichus elaeis* reducing its immature production and survival (Rolim et al. 2020). Those results indicate low compatibility between bio-insecticides based on *Bacillus* sp. and a group of wasp parasitoids. In another study, mortality levels of *L. botrana* after being treated by 5 commercial formulations of *Bt*, (*B. thuringiensis* subsp. *kurstaki*/subsp. *aizawai*), Dipel (*Bt* subsp. *kurstaki*), Bactospeine (*Bt* subsp. *kurstaki*), Xentari (*Bt* subsp. *aizawai*) and BMP (*Bt* encapsulated δ -entotoxin), were recorded by Anagnou and Kontodimas (2003) under laboratory conditions. All products (which were added on artificial diet) caused high larval mortality. Results derived from both bioassays of the present study indicated that *Bt* did not inhibit the action of EPF against *L. botrana*. In contrast, combination treatments resulted in higher mortality levels of *L. botrana* than the single treatments presenting additive action or even synergism.

Synergistic action between *Bt* and EPF has been previously reported by Costa et al. (2001) who investigated mortality of the Colorado potato beetle (*Leptinotarsa decemlineata*) after sub-lethal stress caused by *Bt* and subsequent exposure to *B. bassiana*. Synergy was indicated particularly when insects were exposed to the

highest concentration of *B. bassiana*. In other studies, Wraight and Ramos (2005) found that combined treatments produced a statistically significant (6–35%) greater reduction in larval populations than would have been predicted to have with *Bt* and *B. bassiana* when applied separately against the Colorado potato beetle. Moreover, high mortality levels (near 70%) were caused by the combination of *Bt* with *B. bassiana* to *Ostrinia nubilalis* larvae (Lewis et al. 1996). Also, Ma et al. (2008) presented results that indicated the additive effect of *Bt* and *B. bassiana* on mortality of *Ostrinia furnacalis* in most cases, except for the combination of Cry1Ac (0.2 µg/g) and *B. bassiana* (1.8×10^6 conidia/ml) that showed antagonism. EPF have also presented enhanced efficacy when applied in combination treatments not only with other entomopathogens but also with synthetic insecticides (Furlong and Groden 2001). Regarding their co-existence with plant extracts, EPF have shown both inhibited action (Mann and Davis 2020) and positive interaction as Neem seed cake improved pathogenicity of the fungus *Metarhizium anisopliae* against the Black Vine Weevil (Shah et al. 2008).

Conclusions

Both *Bt* and EPF have showed remarkable potential as bio-control agents of many lepidopteran and coleopteran pests. Research is needed to investigate the biotic and abiotic factors that affect interactions between different entomopathogens in the control of a specific target pest. The additive and synergistic action between *Bt* and EPF - concluded from the present assay - indicated that both entomopathogens can be applied in combination treatments for the control of *L. botrana* and can be included simultaneously in Integrated Pest Management (IPM) programs designed for vineyards.

Abbreviations

Bt: *Bacillus thuringiensis*; EPF: Entomopathogenic fungi; LT₅₀: Median lethal time; IU: International units; SDA: Sabouraud Dextrose Agar; RH: Relative humidity; IPM: Integrated Pest Management; SF: Synergism factor.

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Authors' contributions

EB conducted the bioassays, while EB and EK designed the experiments, carried out the statistical analysis of data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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