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Exploring entomopathogenic nematodes for the management of *Lobesia botrana* (Lepidoptera: Tortricidae) in vineyards: Fine-tuning of application, target area, and timing

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ABSTRACT

Lobesia botrana (Lepidoptera: Tortricidae) is a crucial grapevine pest worldwide. Expanding the available biocontrol agents can provide new management strategies compatible with organic viticulture. Entomopathogenic nematode (EPN) potential as a biocontrol agent was demonstrated against various developmental stages of *L. botrana*. For its field application, we hypothesised that by selecting the best combination of EPN-adjuvant and identifying the best area/timing for their application, we will secure their effective implementation against *L. botrana*. The aim of this study was to determine the best ecological scenarios for their use against *L. botrana*. We investigate three EPN species naturally occurring in Riojan vineyards: *Steinernema feltiae*, *S. carpocapsae*, and *Heterorhabditis bacteriophora*. We evaluated (i) EPN viability, infectivity, and adherence on leaves combined with adjuvants (Multi-Us, Maximix, Dash HC, Nu-Film-17, and Adrex), (ii) EPN biocontrol against *L. botrana* larva on leaves and grapes and damage reduction, and (iii) EPN efficacy against *L. botrana* at 22°, 15°, and 10 °C. Overall, all the adjuvants were compatible with the EPN, except Adrex with *Heterorhabditis bacteriophora*. Compared with the no application, EPNs (steinernematids) alone or with Maximix increased *L. botrana* L3 mortality on grapes and leaves, thereby reducing the damage. Pupal mortality caused by steinernematids EPN (alone or with Maximix) decreased with temperature, from ~60% at 22 °C to <30% at 10 °C. Overall, steinernematids EPN (alone or with Maximix) applied against *L. botrana* on leaves, grapes, or trunks reduced the damage and increased insect mortality. This study suggests the best-case scenario for the application of EPNs is at sunset during late spring/early autumn at mid-temperatures (22 °C-15 °C). Further field validation is necessary for their full implementation.

1. Introduction

The insect *Lobesia botrana* (Lepidoptera: Tortricidae) is one of the key pests in vineyards in Europe, introduced in Argentina and Chile in the last decades (Ioriatti et al., 2011; Varela et al., 2013), and currently eradicated in California (USA) (Gilligan et al., 2011; Simmons et al., 2021). Due to its rapid spread and potential threat, it is classified as a quarantine pest in Mexico, Canada and China (EPPO Data Base, 2023). It is a typical multivoltine species, with 2–4 generations per year, depending on the latitude and prevailing climatic conditions, which is mainly temperate (Martín-Vertedor et al., 2010; Benelli et al., 2023a). The first larval generation of the season usually attacks inflorescences, while the later generations cause damage to the fruits. The life cycle is repeated until the end of the season when it stays in the overwintering stage as a pupa. Pupation occurs in the grapevine trunk, fallen leaves, or in a suitable shelter nearby. The damage can be (i) direct: caused by larval feeding on the inflorescence or fruits, and (ii) indirect: when larval feeding wounds become infected with fungi such as the gray mold, *Botrytis cinerea* (Helotiales: Sclerotiniaceae), causing bunch rot, which substantially degrades wine quality (Roehrich and Boller, 1991; Fermaud and Le Menn, 1992). Global warming exacerbates their damage, which expands the areas suitable for their development and increases

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the number of generations per year (Reis et al., 2021).

Although L. *botrana* primary prefers the grapevine, *Vitis vinifera* (Vitales: Vitaceae), as a host, it can also attack other plant species, both wild and cultivated, from more than 25 botanical families (Maher and Thiéry 2006; Lucchi and Santini 2011; Benelli et al., 2023b). Due to its ability to adapt to climate changes and its polyphagous potential (Ioriatti, et al., 2011; Kocsis and Hufnagel, 2011; Benelli et al., 2023a), this pest can establish in any vinicultural geographic region that is introduced. Grapevine is a widely cultivated plant across arid and semi-arid ecosystems and its production is associated with relevant socioeconomic and cultural sectors worldwide, which provide a broader range of services and benefits to people and nature than the simple production of grapes and wine (Santos et al., 2020). Agricultural intensification accelerates the loss of soil biodiversity in vineyards (Veresoglou et al., 2015). Currently, the vineyard is still one of the most intensive management crops and erosion-prone land systems (Nicholls et al., 2008; Karimi et al., 2020). Overall, grapevine management is widely focused on conventional production practices, such as the use of agrochemical and soil tillage practices, which are responsible for negative environmental impact, including soil and water pollution (Pose-Juan et al., 2015; Herrero-Hernández et al., 2017), and loss of biodiversity (Karimi et al., 2020). In this sense, *L. botrana* is one example of successful management using sustainable and non-toxic means (Benelli et al., 2023b). Strategies based on prevention and monitoring are crucial for early detection of the pest. In addition, the employ of the cultural control, sterile insect release, and pheromone-mediated control strategies such as pheromone-based mating disruption are often used for their management (Benelli et al., 2023b). While these practices are commonly adopted, certain limitations need to be considered. These limitations encompass a minimum effective area of 0.5 ha, which can sometimes be fragmented among various landowners. Additionally, ensuring the optimal timing for placing the diffuser in the field (during the first adult flight) and the use of plastic products in the field is not advisable (Vicente-Díez et al., 2021a; Benelli et al., 2023b). Finally, biological control agents have been explored, and several predators, parasitoids, and entomopathogens such as *Beauveria bassiana* (Hypocreales: Clavicipitaceae) and *Bacillus thuringiensis* (Bacillales: Bacillaceae) can control various developmental stages of this pest (Vicente-Díez, et al., 2021a; Benelli et al., 2023b). However, still, the new paradigm of the European Green Deal, within the Farm-to-fork strategy, aims at reducing the use of synthetic chemical pesticides by half by 2030 (European Commission, 2020). Furthermore, incorporating novel biotools that align with organic production practices, such as employing biocontrol agents, will facilitate the transition of new areas to organic farming systems. This transition will aid in achieving the targeted expansion of agricultural land to 25% by 2030 (European Commission, 2020). Consequently, equipping the agricultural landscape with effective tools to handle biotic challenges, such as the use of biological control agents, holds great promise for the future of agriculture.

Entomopathogenic nematodes (EPNs) are well-known biocontrol agents appointed for numerous pests and target crops (Lacey et al., 2015). The infective juvenile (IJ) is the life stage naturally inhabiting the soil, including that of the vineyard (Blanco-Pérez et al., 2020, 2022a, 2022b), responsible for its resilience against both abiotic and biotic constraints. The IJs are in charge of searching for a suitable host, penetrating, and killing the insect, with the help of a symbiotically associated bacteria (Stock, 2015). Then, EPNs reproduce in the cadaver, which is in turn protected by the bacteria releasing numerous chemical compounds that repel and avoid the scavenger activity of other organisms (Gulcu et al., 2012; Blanco-Pérez et al. 2017). Finally, once the food is depleted, a new IJs-bacteria complex is developed and those emerge en masse from the carcass in search of a new host. Overall, EPN research in vineyard agroecosystems has been neglected, probably due to their main biotic threats being focused on the aboveground areas of the crop (Campos-Herrera et al., 2021). Infective juveniles display this natural behavior in the soil, where nematodes can have a water film to move within the particles and then, locate the various soil-dwelling stage of insects (Perry et al., 2012; Griffin, 2015). However, recent advances in the formulation and application system allows the use of EPNs against target aerial pests (Shapiro-Ilan and Dolinski, 2015). The use of various adjuvants has been proposed to enhance the movement in the aerial parts of the plants while, at the same time, protecting the IJs from extreme temperatures, UV exposure, and fast desiccation (Lacey and Georgis, 2012; Shapiro-Ilan and Dolinski, 2015; Ramakrishnan et al., 2022). With the exception of the field study of EPN application to fight against *Vitacea polistimorfis* (Lepidoptera: Sessidae) (Williams et al., 2010), the potential biocontrol of EPN against aerial vineyard insect pests or threatening disease vectors has been mainly addressed only as a proof of concept under laboratory conditions, targeting *Planococcus ficus* (Hemiptera: Pseudococcidae), *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), and *Philaenus spumarius* (Hemiptera: Aphrophoridae) (Vieux and Malan, 2015; Steyn et al., 2021; Vicente-Díez et al., 2021b). Recently, Vicente-Díez et al. (2021a) probed the EPN ability to kill various developmental stages of *L. botrana* under laboratory conditions and settled the basis for their application in the field. For a quick advance for their implementation in the field, the selection of adjuvants authorized for use in the vineyard to be combined with the EPN is a smart strategy (Campos-Herrera et al., 2021). The screening of their compatibility in laboratory and greenhouse approaches allows the selection of the best EPN-adjuvant mix (Platt et al., 2019a). In addition, investigating the differential ability of the EPN to kill the insect exposed to various plant organs, such as the fruit (grapes), the leaves, and the trunk, will provide critical information on the suitability of application. In addition, one strategy can be targeting the overwintering pupal stage of *L. botrana,* which often hides in the trunk and fallen leaves in late summer-early autumn. To determine the potential of EPNs for controlling this stage at mild-low temperatures, evaluating EPN pathogenicity under various environmental temperatures is crucial (Grewal et al., 2006). We hypostatized that by screening various adjuvants authorized for use in vineyards, we will find a suitable EPN-adjuvant combination that will enhance the control of *L. botrana* in various plant areas (grape, leave and trunk) and will be successful at low temperatures to control the overwintering pupal stage. The aim of this study was to investigate the compatibility of various adjuvants of variable chemical nature with the EPN species naturally occurring in vineyards (*Steinernema feltiae*, *S. carpocapsae*, and *Heterorhabditis bacteriophora*), which are also available as commercial products, to determine the best ecological scenarios for their use against *L. botrana*. Herein, we investigated (i) viability, infectivity, and adherence on leaves of EPN-adjuvant mix with five products (alone or combined in specific cases), (ii) protection capability (mortality of *L. botrana* and reduction of its damage) on leaves, and grapes, and (iii) EPN activity against *L. botrana* at low temperatures as a proxy of the overwintering period in trunk bark.

2. Material and methods

2.1. Nematodes, insects, and plants

Four EPNs were evaluated: *Steinernemal feltiae* 107, *S. feltiae* Koppert, *S. carpocapsae* All, and *Heterorhabditis bacteriophora* VM-21 (Table 1). All nematodes were reared under laboratory conditions using the last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) as the host. Once the IJs emerged from the cadavers, we harvested them in tap water and stored them until use at 14 °C in darkness. Nematodes were used within 20 days of their emergence.

The insect *G. mellonella* was used for nematode rearing and also as an experimental host in specific experiments. This insect was reared at the Instituto de Ciencias de la Vid y del Vino (ICVV, Logroño, La Rioja, Spain) under controlled conditions at 28 °C, 10% RH, and without photoperiod. For the neonate larvae, we used commercial bee pollen as food, while the rest of the larval stages were fed on an artificial diet described by Vicente-Díez et al. (2021a). The adults (n = 30–40) were

Table 1
Description of the entomopathogenic nematodes tested in this study.

Entomopathogenic nematode species	Population	ITS-GenBank Accession number	Origin
Steinernema feltiae	107	MW480131	R. Blanco-Pérez (ICVV, Spain)
Steinernema feltiae	Koppert	–	Commercial, Koppert
Steinernema carpocapsae	All	MW574913	D.I. Shapiro-Ilan (USDA-ARS, SEA, USA)
Heterorhabditis bacteriophora	VM-21	MW480135	R. Blanco-Pérez (ICVV, Spain)

kept in individual containers lined with wax paper to facilitate egg-laying. They were also provided with a sugar solution (1:10, w/v) as a supplement. The insect L. *botrana* was also reared at ICVV with an artificial diet in controlled growth chambers at 22 °C, 60% RH, and 16:8 light: darkness photoperiod. We followed the protocols and diet described by Vicente-Díez et al. (2021a). In all the experiments, larvae or pupae of the same cohort were used.

The plant species *V. vinifera* was used in all the studies. Stems with more than four extended leaves and old vine trunks were retrieved from an organic vineyard located in Logroño (La Rioja, Spain, 42° 29′ 14″ N, 2° 30′ 7″ W), where no pesticide treatment was applied. The stems were retrieved from the field 24 h before the experiments and maintained in 50 mL Falcon tubes filled with tap water. For the trunk studies, the old vines were maintained at room temperature in the darkness. For the grape studies, organic commercial grape clusters were obtained from a regional market.

2.2. Selection of adjuvants

The study investigated the compatibility of five adjuvants authorized for use in vineyards in Spain with EPNs (Table 2). All products were employed immediately upon delivery from the company to ensure their freshness and optimal properties. Then, each adjuvant was prepared by diluting it to a final volume of 0.5 l, which corresponds to twice the highest recommended concentration used in the field. This diluted adjuvant was then combined in a 1:1 ratio (volume-to-volume) with the adjusted nematode concentration. Consequently, the nematodes were exposed to the highest field-recommended concentration. Before each experiment, a fresh mixture of the adjuvant and nematode concentration was prepared.

2.3. Selection of the combination adjuvant with entomopathogenic nematodes: viability, infectivity, and adherence to leaves

A sequential experiment explored the viability and infectivity of the EPNs upon exposure to the highest recommended concentration of each of the adjuvants. For the viability test, we prepared a concentration of 20 IJs/100 μl per EPN species/population by volumetric adjustment. This concentration was reduced to half when combined with the adjuvants. For preparing the adjuvant-EPN mix, we combined 8 ml of the adjuvant (prepared at double concentration) (Table 2) with 8 ml of the nematode suspension and mixed for 1 min to ensure complete homogenization. In the first screening, for each of the four EPN species/populations, we investigated the following treatments: Control (water), Multi-Us, Maximix, Dash-HC, NuFilm 17, and Adrex. In a second study, based on the results and the approach by Platt et al. (2019a), we selected the species *S. feltiae* 107 and *S. carpocapsae* All for the evaluation of the treatments: Control (water), Multi-Us, Maximix, and the combination of Multi-Us + Maximix. Overall, for the viability studies, the experimental unit was a 30 mm. diam. Petri dishes filled with 1.5 ml of the corresponding mixture (n = 3 per treatment). In each dish, there were ~75 IJs in total. We incubated the plates covered with their corresponding lid at 22 °C, 60% RH at darkness, preventing any evaporation. Then, we evaluated viability after 4 h and 24 h post-exposure. By using a stereomicroscope, all nematodes in each plate were counted. Then, dead nematodes were registered, considering those that do not move after being touched three times with a nematological needle. The experiments were performed with all the treatments independently for each of the EPN species/population, all performed two independent trials with new nematodes and material preparation.

The subsequent infectivity study was prepared with the mixture of the EPN + adjuvant/control after 24 h exposure stored at 22 °C, 60% RH, and darkness. For this evaluation, 55 mm diam. Petri dishes filled with filter paper Whatman no. 1 at both sides (up and down) and moisten with tap water with 250 μl on each side were prepared (n = 5 per treatment). Thereafter, 250 μl of the mixtures described in the viability study were then applied to each filter paper (250 μl up and 250 μl down). The final number applied per dish was ~50 IJs. Five last instar *G. mellonella* were included in each plate, closing with a scotch tape to avoid any unexpected escape. All the plates were placed in a container with dampened paper. We conducted daily checks to ensure that the humidity remained at an optimal level. In addition to the mixtures with nematodes, we included an additional treatment only with tap water to ensure the viability of the insects. Larval mortality was evaluated after two and five days post-exposure. Finally, in addition, following the same experimental design as described above, we evaluated the application of the adjuvant alone (not combined with EPN) to verify the possible effect on the insects. The experiments were performed with all the treatments independently for each of the EPN species/population, all performed two times with new nematodes, insects, and material preparation.

For the adherence to leaves study, we used stems with more than four leaves of *V. vinifera* cv. Tempranillo. Each stem was vertically placed in a container with sterile pot soil (autoclaved for 2 h and oven dried for 3 day at 70 °C). Each of the EPN species/population suspensions was prepared to the concentration of 2000 IJs/ml (Platt et al., 2019a). The concentration of each adjuvant was prepared following the description above. Then, we mixed 80 ml of adjuvant with 80 ml of the nematode to reach the highest recommended concentration of each of the adjuvants and a final concentration of the nematodes of 1000 IJs/ml. After mixing for 1 min, each suspension was included in a hand vortex-type sprayer that produced droplets. The system was tested to release 2 ml of volume for each application. In all the cases, before application to the leaves, we sprayed 20 times while moving the suspension to ensure the pass of the nematodes throughout the system. Thereafter, we sprayed 15 times on each of the stems with four leaves per treatment (n = 3), moving the mixture frequently to avoid decantation. This volume was carefully calculated to ensure that all the leaves would eventually be saturated, resulting in complete coverage with the nematode suspension. We applied the treatments from a distance of 50 cm away from the stem. The first study evaluated the four EPN species/populations and five adjuvants, while the second study focused on the nematodes *S. feltiae* 107

Table 2
Adjuvants tested for compatibility with entomopathogenic nematodes, all certified for use in vineyards in Spain.

Commercial product	Supplier	Active ingredient	Concentration prepared	Recommended field application
Multi-Us	ASCENZA	Montana wax 20% (p/v)	20 ml/l	10 ml/l
Maximix	Agrichem	Polymenteno 96% (p/v)	1.8 ml/l	0.9 ml/l
Dash-HC	BASF	Methyl oleate 34.8% (p/v)	8 ml/l	4 ml/l
Nu-Film 17	Agrichem	Dimenteno 96% (p/v)	4.8 ml/l	2.4 ml/l
Adrex	ASCENZA	Alkyl polyglycol 20% (p/v)	12 ml/l	6 ml/l

and *S. carpocapsae* All with two adjuvants and a mix (see description above). The study was performed independently for each of the EPN species/populations with the corresponding adjuvant/control treatments. In each trial, the plants were arranged into a randomized block design in a growth chamber at 22 °C, 60–70% RH, and 16:8 light: darkness photoperiod. The total number of nematodes on leaves was evaluated immediately after application (Time 0), and after 3 h post-application (Time 3). In each sampling time, one leaf per plant was detached. From each leaf, we retrieved three leaf discs of 15 mm diam, for accounting ~5.3 cm^2 surface equally to all. The three pieces were placed into a 30 mm diam. plastic Petri dish containing 1.5 ml of tap water. Leaf discs were rinsed with tap water and finally removed for the final count. Following the procedure described above for the viability study, the total number of nematodes and the mortality were evaluated for all plates. The experiments were performed with all the treatments independently for each of the EPN species/population, with two trials per species/population, each with new nematodes, plants, and material preparation.

2.4. Efficacy of the combination of adjuvant and entomopathogenic nematodes against Lobesia botrana: impact in the biocontrol and protection of leave and grape damage

Prior to the experiments, the *L. botrana* L3 cohorts were separated from the rearing containers 16 h before experiments, and placed in new boxes for starvation. In both studies (leave and grape), we investigated the nematodes *S. feltiae* 107 and *S. carpocapsae* All, and the adjuvant Maximix. The nematode was adjusted to the concentration of 2000 IJs/ml, as described above; the adjuvant was also prepared with double the highest recommended concentration for field application (Table 2). Then, 80 ml of nematode and 80 ml of the adjuvant were combined and mixed by stirring for 1 min before passing the material to the manual sprayer, as described above. For the evaluation of biocontrol and protective action in leaves, we used *V. vinifera* cv. Tempranillo stems with four leaves recovered from the organic vineyard 24 h before the experiment and maintained as described above. Then, for the experiment, each stem was placed in a 50 ml Falcon tube filled with 25 ml tap water to maintain freshness (n = 3 per treatment). For each nematode species, the treatments investigated were (i) the controls dry (no application), (ii) water (no nematode), (iii) Maximix (no nematode), and the treatment (iv) EPN + Maximix, and (v) the EPN + water. Before the application, 20 sprays were performed to ensure the application of the nematodes. Then, each of the treatments was applied on three stem + leave systems by spraying 10 times (approximately 20 ml treatment suspension). Immediately after, five starved L3 *L. botrana* larvae were located in one selected leaf previously identified for the image data process. Larvae were confined using a mesh cloth bag. Each experiment was maintained at 22 °C, 60% RH and 16 l:8D for 24 h. Then, leaf damage was evaluated by image analysis using the "Digimizer" application. We took a picture before and after the damage (24 h), and we estimated the total area and the damaged area (in pixels), expressing the damage as a percentage of the total leaf area. For the larval mortality, after 24 h we placed the larvae in 55 mm diam. plastic Petri dishes with one moistened filter paper and a piece of food (artificial diet, as described in Vicente-Díez et al., 2023a). We revised larval mortality after two and five days. The experiments were performed individually with all the treatments for each of the EPN species, all performed two times with new nematodes, insects, plants, and material preparation.

For the evaluation of biocontrol and protective action in grapes, we used commercial *V. vinifera* grape clusters. We carefully separate the berries, cutting from the peduncle of each fruit, avoiding unintended injury. All the grapes were disinfected by using a solution of 3% NaClO and three rinsing in distilled water. The grapes were placed on tissue paper and air-dried for their subsequent use. The experimental unit was a grape included in a modified 50 ml Falcon tube with a mesh layer around the 40 ml mark, filled with 15 ml tap water to maintain moisture (n = 8 per treatment) following the design by Vicente-Díez et al. (2023b) (Fig. 1). For each nematode species, the treatments were (i) the controls dry (no application), (ii) water (no nematode), (iii) Maximix (no nematode), and the treatments (iv) EPN + Maximix, and (v) the EPN + water. All the fruit were individually weight prior to the application of treatments. Before the application, 20 sprays were performed to ensure the application of the nematodes. Then, we applied one spray per fruit (approximately, 2 ml treatment suspension). Thereafter, three starved L3 *L. botrana* larvae were placed on each fruit, and the Falcon tube was closed with the lid (Fig. 1).

The experiments were maintained at 22 °C, 60% RH, and 16:8 light: darkness photoperiod. Damage on each fruit was estimated by weighing the fruit individually after 48 h, calculating the percentage of weight loss with the initial value pre-experiment. The larvae were retrieved after 48 h, confined in a 55 mm diam. plastic Petri dish with moistened filter paper and a piece of diet. The mortality was revised after two and six days post-exposure. Individual experiments were performed with all the treatments for each of the EPN species, all performed two times with new nematodes, insects, plants, and material preparation.

2.5. Evaluation of entomopathogenic nematode activity against Lobesia botrana at various temperatures as a proxy for targetting at various season times

First, we evaluated the effect of the EPNs *S. feltiae* 107 and *S. carpocapsae* All exposure to 10° and 15 °C on the control capability against L3, L5, and pupal stage following the procedures described by Vicente-Díez et al. (2021a). Briefly, we employed 55 mm diam. Petri dish filled with 1 filter paper Whatman no. 1 (n = 5 per treatment). We applied 10 IJs/cm^2 in a final volume of 400 μl, while in the control treatment, we only added tap water. Then, we included five L3 and L5 instar per plate (a total of 25 larvae per treatment in each trial). The experiments were incubated at 10 °C with a 10:14 light: darkness photoperiod and 15 °C with a 12:12 light: darkness photoperiod, all with 60% RH. This range of temperature/light was selected as a proxy for later summer (September) and early autumn (end-October) in La Rioja (Spain) (SIAR, 2023). Mortality was assessed daily for four days. For the pupae, we used two 24-wells (Corning, New York, NY, USA) per treatment, employing 12 interleaved wells per tray (n = 24 pupae per treatment). We added 1 g of sterilized sand (pure sand, Vale do Lobo, Loulé, Portugal; autoclaved for 2 h and oven-dried at 70 °C for three days). Thereafter, we included one *L. botrana* pupa (without sexual dimorphism characterization) and we inoculated 50 IJs per well in a final volume of 200 μl. Control treatment received only the same amount of water. Pupal mortality was revised for a week.

A second study used the trunk and pupae as a proxy for the transition to the overwintering period. We investigated the nematodes *S. feltiae* 107 and *S. carpocapsae* All, alone or mixed with the adjuvant Maximix. The nematodes were adjusted to the concentration of 2000 IJs/ml, the adjuvant prepared double the highest recommended concentration to

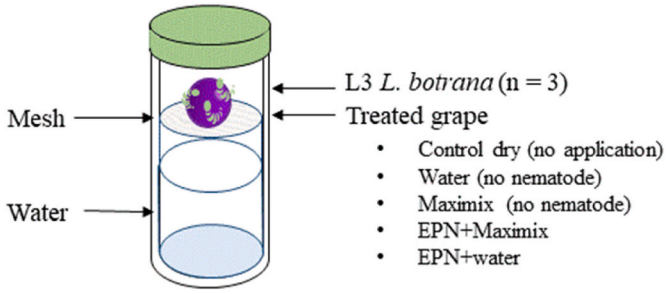

Fig. 1. Diagram of the experimental unit for the evaluation of biocontrol and protective action in grapes, following the design described by Vicente-Díez et al. (2023b).

field application (Table 2), and the final mixture was performed as described in section 2.3. In a plastic container (90 mm diam. X 45 mm high), we confined 6 g of trunk material to ensure the coverage of the surface. Then, we included ten pupae of the same cohort widespread in the container. For each nematode species, the treatments (n = 3) were (i) the controls dry (no application), (ii) water (no nematode), (iii) Maximix (no nematode), and the treatments (iv) EPN + Maximix, and (v) the EPN + water. The materials/insects/nematodes were acclimatized to the incubation conditions 1 h prior experiment. The experiments were maintained at 60% RH and specific temperature and photoperiod (light: darkness, L:D): (i) 22 °C and 16L:8D, (ii) 15 °C and 12L:12D, and (iii) 10 °C and 10L:14D. Pupal mortality was assessed after six days. Individual experiments were performed with all the treatments for each of the EPN species, all performed two times with new nematodes, insects, plants, and material preparation.

2.6. Statistical analysis

First, we ensured that the trials of the same experiment followed the same pattern of normality and homoscedasticity, so these could be combined. Thereafter, one-way ANOVA focused on the effect on the variables for each EPN species/population at different times, developmental stages, or temperatures, depending on the experiment, with Tukey posthoc test to assess statistically significant differences among treatments ($P < 0.05$) (SPSS 27.0, SPSS Statistics, SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean.

3. Results

3.1. Selection of the combination adjuvant with entomopathogenic nematodes: viability, infectivity, and adherence

In the assessment of the five adjuvants and four EPN species/populations, it was observed that, for the most part, the viability of EPNs remained unaffected within 4 h (see Appendix Fig. S1) and 24 h (Fig. 2, and Appendix Table S1). The only exceptions were noted in the case of the nematode *H. bacteriophora* when combined with Adrex after 4 h (Fig. S1A), as well as Nufilm-17 and Adrex after 24 h (Fig. 2A), where the immobility surpassed 90%.

The larval mortality after two days post-exposure was reduced for certain EPN species and adjuvants, compared with their corresponding control treatment (Fig. 2B, and Table S1). Specifically, *S. feltiae* 107 infectivity was reduced to 30% when combined with Dash-HC, while the population *S. feltiae* Koppert was affected by Nufilm-17, with 56% mortality (Fig. 2B). Only when the nematode *H. bacteriophora* VM-21 was combined with the adjuvant Dash-HC, the larval mortality was

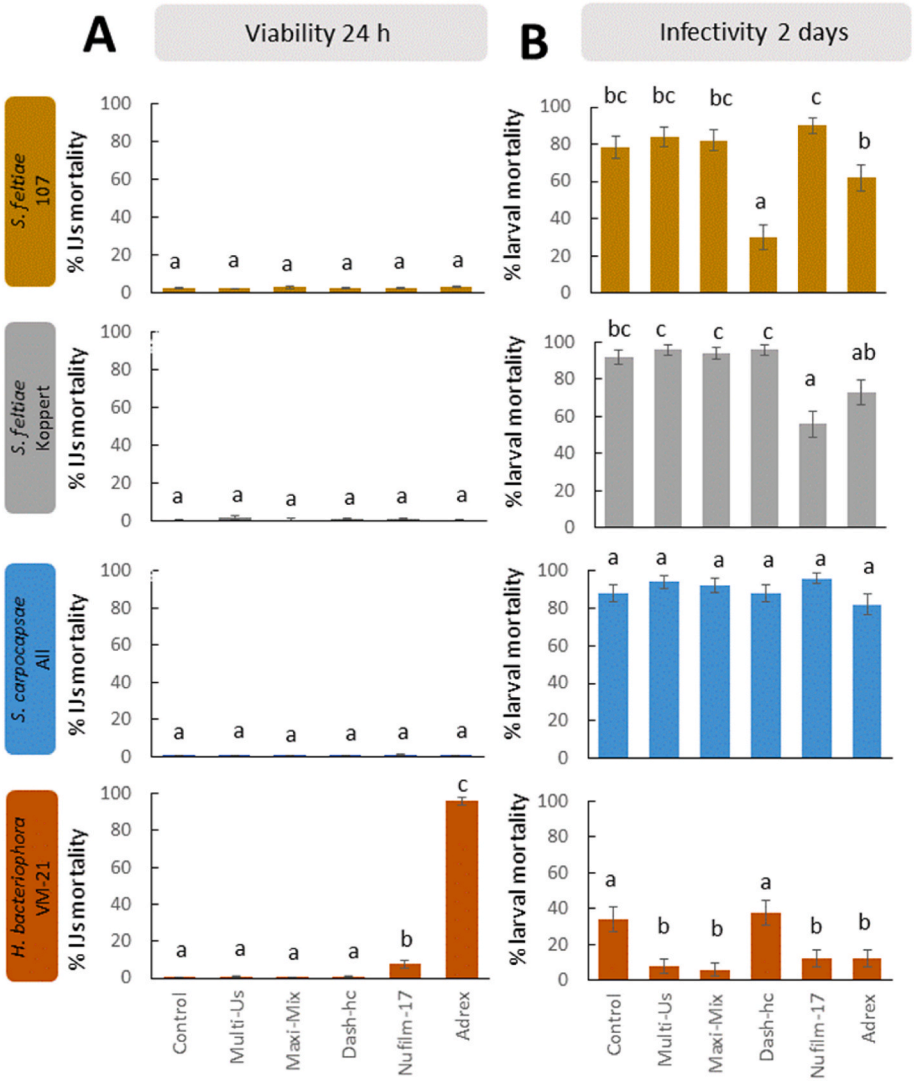

Fig. 2. Compatibility of five adjuvants with four entomopathogenic nematodes (EPNs). **A.** Viability after 24 h of exposure. **B.** Infectivity after two days post-inoculation. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison tests ($P < 0.05$).

not negatively affected (Fig. 2B). The infectivity after five days post-exposure was not altered by the combination with most of the adjuvants, with the only exception of *H. bacteriophora* combined with Adrex (Fig. S1B), which reduced to 60% larval mortality (Table S1).

In the subsequent evaluation of the adherence of four EPN species/populations to grapevine leaves when combined with five adjuvants, compared with their respective control treatment with just water, the total number of IJs present on the surface and its mortality was not affected by the adjuvant after 0 h (Appendix Fig. S2, and Table S1). After 3 h, only the mortality of *S. carpocapsae* All and *H. bacteriophora* VM-21 was reduced when combined with the adjuvant Adrex (Fig. 3B, and Table S1).

In the second study that evaluated the combination of Multi-Us, Maximix, and their mixture, the viability and infectivity were not improved by any of the treatments at any of the timings when compared with the corresponding control treatment (Fig. 4, Appendix Fig. S3, and Table S2). Similarly, overall, compared with the control treatment, the adherence and mortality of the IJs were not increased by the use of the adjuvants (Fig. 4, Fig. S3, and Table S2). Overall, the combination with Maximix showed a trend to increase the total adherence after 3 h for both nematodes tested, and hence, this adjuvant was retained for further studies.

3.2. *Efficacy of the combination of adjuvant and entomopathogenic nematodes against Lobesia botrana: impact in the biocontrol and protection of leave and grape damage*

In the study of biocontrol potential on leaves, the treatment that consistently increased the larval mortality compared with the three controls (nothing –dry-, water, and Maximix) for both nematodes species was the combination of the EPN with the adjuvant (Fig. 5, Appendix Fig. S4 and Table S3). However, the damage inferred in the leaves was only significantly reduced in the application of nematodes in both EPN species when combined with water and in combination with Maximix for *S. carpocapsae* All, with respect to the control with no application (dry treatment) (Fig. 5).

In the study of biocontrol potential in grapes, the L3 *L. botrana* mortality of both treatments with nematodes were significantly higher than any of the controls for the two nematode species (Fig. 5, Fig. S4, and Table S3). Also, the damage caused in the grapes was significantly reduced in both treatments with nematodes (EPN + water and EPN + Maximix) and both species compared with the control without any application (Fig. 5, and Fig. S4).

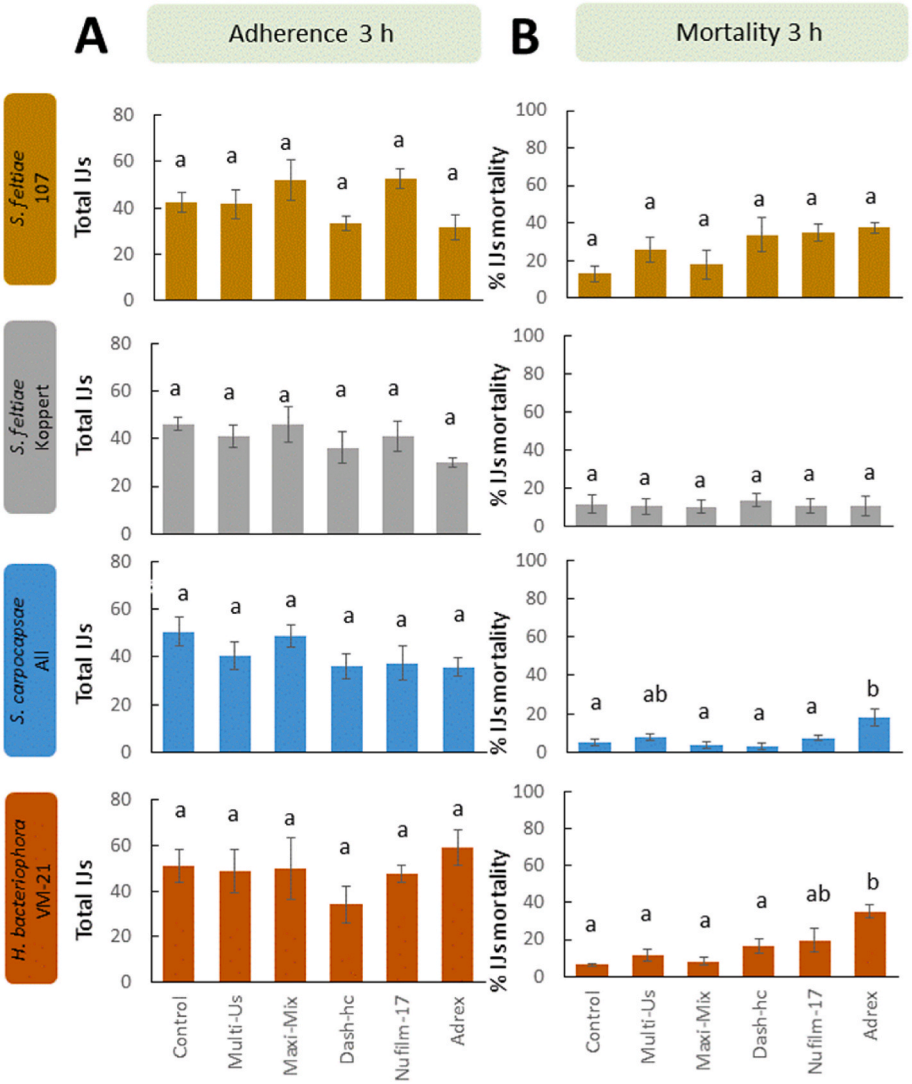

Fig. 3. Performance of four entomopathogenic nematodes after spaying on grapevine leaves combined with five adjuvants. **A.** The total number of infective juveniles (IJs) present on the leave after 3 h post-application. **B.** Percentage of IJs dead present in the leave after 3 h post-application. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison tests ($P < 0.05$).

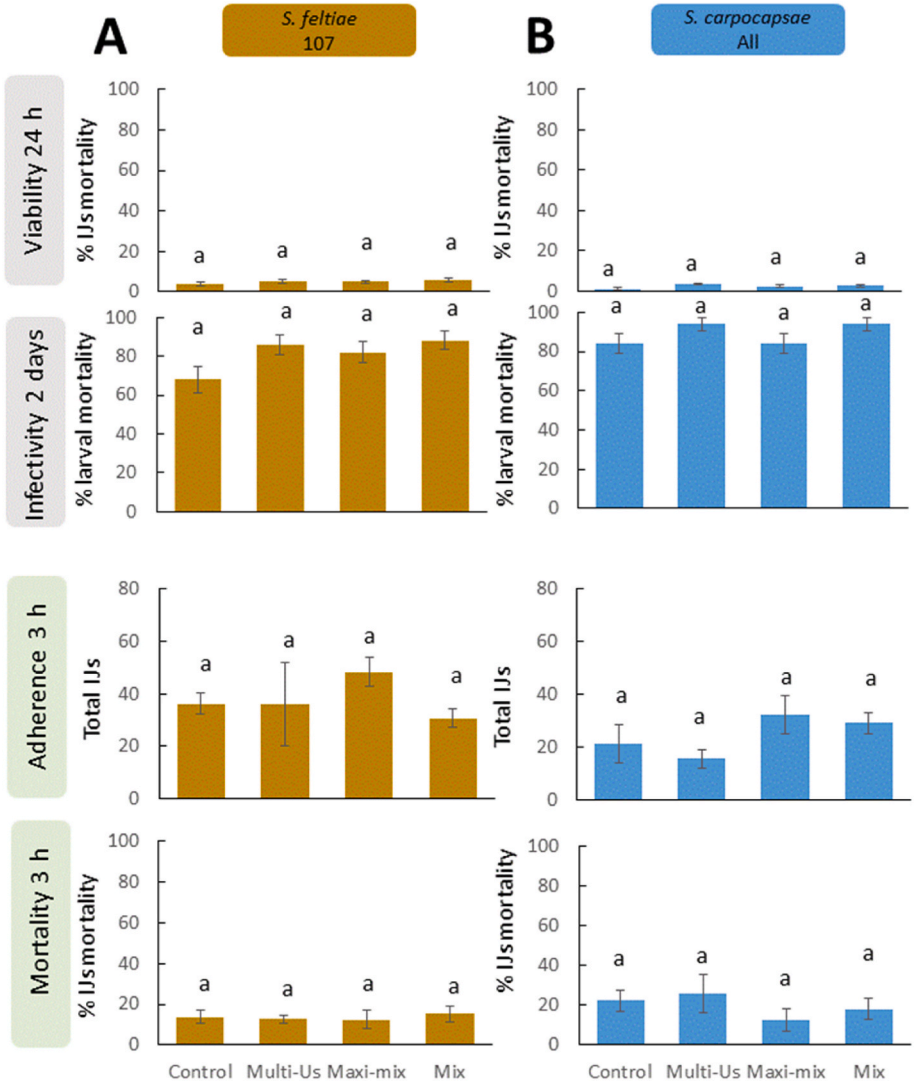

Fig. 4. Compatibility of two adjuvants and this mixture was measured as viability after 24 h of exposure and infectivity after two days post-inoculation, and performance in the leave (adherence and IJs mortality) after 3 h exposure. **A.** Values for *Steinernema feltiae* 107. **B.** Values for *Steinernema carpocapsae* All. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison tests ($P < 0.05$).

3.3. Evaluation of entomopathogenic nematode activity against Lobesia botrana at various temperatures as a proxy for targetting at various season times

Larval mortality was significantly higher than control at 10° and 15 °C for both EPN species and target stages (L3 and L5) (Fig. 6, Appendix Table S4), although the overall mortality decreased from ~80% at 15 °C to less than 50% most of the cases at 10 °C. In the control of the pupae, *S. feltiae* 107 killed 79% at 15 °C, but only 15% at 10 °C, but in both cases, significantly different from the controls (Fig. 6A, Table S4). However, *S. carpocapsae* All only reduced its pupal mortality to 19% at 15 °C, not being statistically significant at 10 °C (Fig. 6B, Table S4).

In the study using trunk and pupae as a proxy for the transition to the overwintering period, the application of EPN, irrespectively alone or with the adjuvant, caused significantly higher pupal mortality at all temperatures (22°, 15°, and 10 °C) (Fig. 7, Appendix Table S5). In all the cases, in the lower temperature, the overall mortality percentage was lower, with 22 °C reaching >50% mortality and <30% at 15° and 10 °C.

4. Discussion

Before EPNs are combined with new adjuvants for their application to the surface of a target crop, IJs should be tested for compatibility (Baur et al., 1997; Beck et al., 2013; Platt et al., 2019a). Previous studies have investigated various products with different actions (i.e. surfactants, anti-desiccants) and of different nature (polymeric materials, sprayable gels, mineral oils) (Baur et al., 1997; Schroer et al., 2005; Navaneethan et al., 2010; Shapiro-Ilan et al., 2010; Beck et al., 2014; Portman et al., 2016; Platt et al., 2019a, 2019b; Castruita-Esparza et al., 2020; Ramakrishnan et al., 2022). These products often increase the EPN aerial survival, but the final performance in the leaves or other aboveground parts tested might not be superior to the controls without adjuvants (Baur et al., 1997; Shapiro-Ilan et al., 2010; Portman et al., 2016). This was the case in this study. Although, overall, most of the commercial adjuvants authorized for use in vineyards were compatible with three of the EPN species potentially present in the vineyards (Blanco-Pérez et al., 2022a), the further use on the leaves, grapes, and trunk was not significantly superior to the application of EPNs alone. Specifically, the viability and infectivity of the Steinernematid species—*S. feltiae* (both populations, 107 and Koppert) and *S. carpocapsae*—remained largely unaffected. These nematodes exhibited a high degree of compatibility with all the adjuvants that were subjected to testing. However, *H. bacteriophora* VM-21 was negatively affected by the combination of Adrex and after 24 h with Nufilm-17.

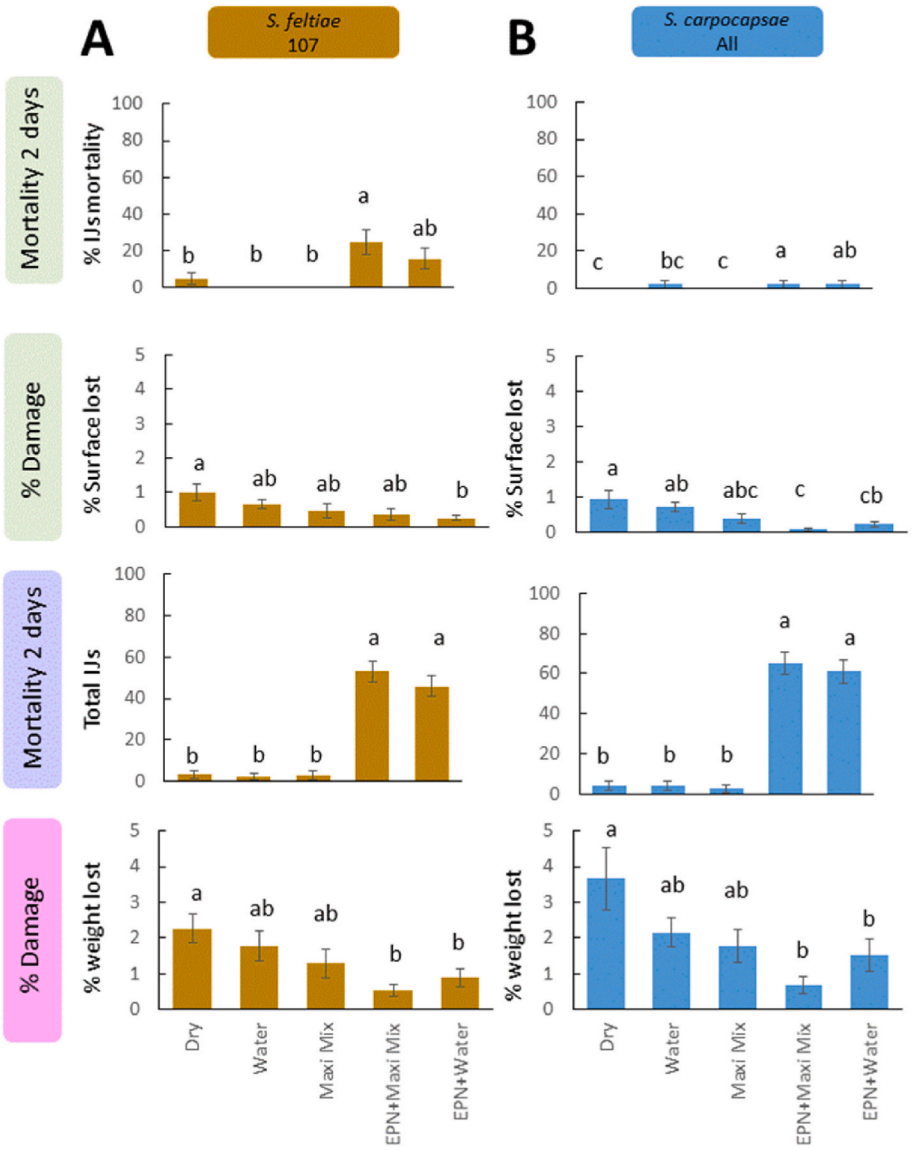

Fig. 5. Evaluation of the control ability and damage reduction on leave and grapes after two days post-application. **A.** Values for *Steinernema feltiae* 107. **B.** Values for *Steinernema carpocapsae* All. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison tests ($P < 0.05$).

Because *H. bacteriophora* showed less overall compatibility with the adjuvants, and the steinernematids showed superior performance in laboratory studies against *L. botrana* (Vicente-Díez et al., 2021a), all subsequent studies focused only on *S. feltiae* 107 and *S. carpocapsae* All, species that often are tested for aerial application for the general good toleration to desiccation and extreme temperatures (Beck et al., 2014; Glazer, 2015; Shapiro-Ilan et al., 2016; Ramakrishnan et al., 2022).

In a second attempt for increasing the leave adherence while not compromising the viability or infectivity, we combined the nematodes *S. feltiae* 107 and *S. carpocapsae* All with Maximix and Multi-Us, following the same approach of Platt et al. (2019a, 2019b). However, this combination did not result in an overall enhancement of the IJs leave adherence as observed by Platt et al. (2019a, 2019b) with the combination of the adjuvants Zeba + Nufilm-P. Having noted certain trends, including a subtle increase in adherence after 3 h and reduced IJ mortality in both nematode species, we proceeded to explore the potential positive impact of utilizing the Maximix adjuvant on EPN performance in different grapevine part (leave, grape, and trunk). This prompted us to maintain the treatment involving the Maximix adjuvant for subsequent investigations.

The use of EPNs against feeding L3 larvae on leaves and grapes significantly contributed to reducing damage. This evidence supports the use of EPNs against this pest in the most voracious moments, minimizing the L3 impact and their attack on grapes. Indeed, the IJ's ability to move in search of the larvae in the wounds and tunnels of perforation created by the larvae in the grapes is an advantage over other biocontrol agents available for managing larvae of *L. botrana* such as *B. thuringiensis* and *Metarhizium* spp. (Ioriatti et al., 2011; Sammaritano et al., 2018; Benelli et al., 2023b) that will only arrest where applied with minimal movement capability. In fact, the values of L3 control in grapes after six days were similar to those observed by Vicente-Díez et al. (2021a) in Petri dishes and filter paper for *S. carpocapsae*, reaching ~80%. However, the control ability of L3 in grapes by *S. feltiae* 107 resulted superior to in filter paper, with values > 60% in grapes compared with ~30% in filter paper (Vicente-Díez et al., 2021a). This result highlights the relevance of testing various ecological scenarios and target areas of application to unravel the best ecological settings for the potential use of the EPN in the field. When the use of the adjuvant combined with the EPN was considered in leaf and grape application, overall, no significant benefit was observed. However, a consistent trend on a beneficial effect

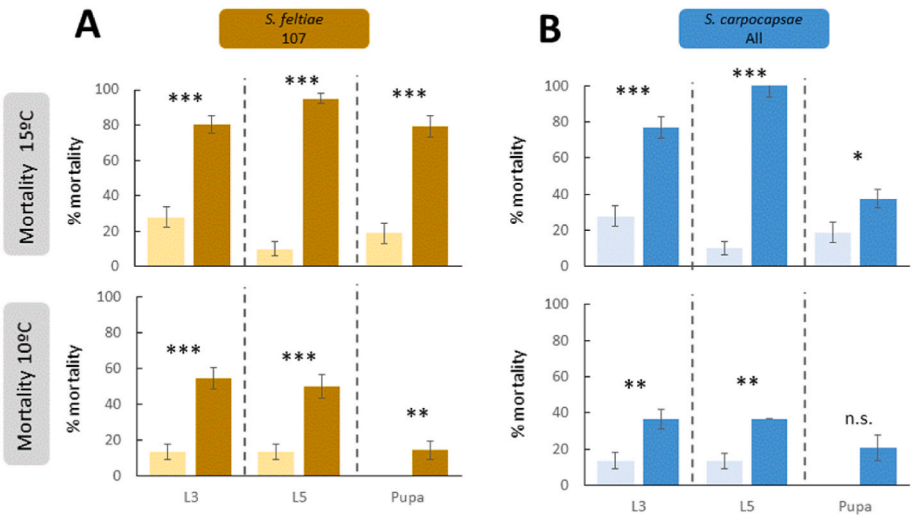

Fig. 6. Mortality of *Lobesia botrana* 3rd and 5th larval instar (L3 and L5, respectively) and pupae when exposed to entomopathogenic nematodes (EPNs) at 15° and 10 °C. **A.** Values for *Steinernema feltiae* 107. **B.** Values for *Steinernema carpocapsae* All. For each insect developmental stage, controls are light-colored bars, and treatment with nematodes are dark-colored bars. Asterisk indicates significant differences at ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n. s., not significant.

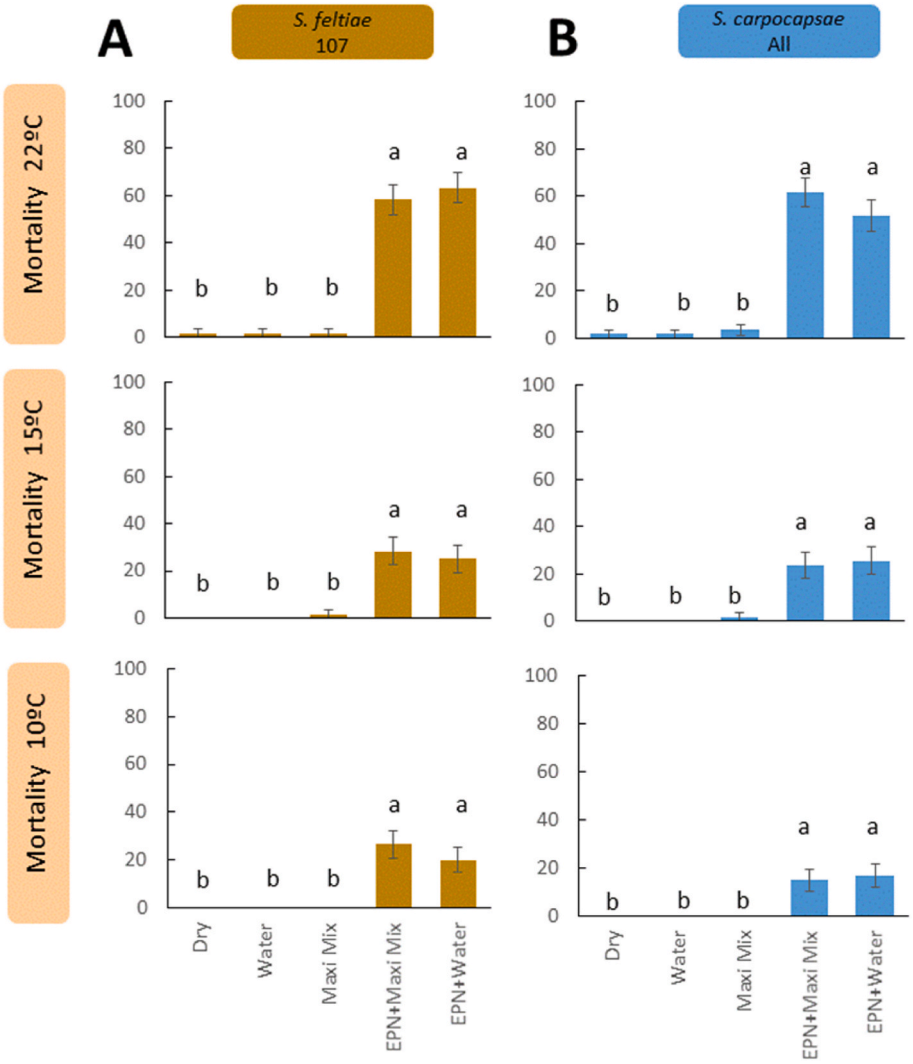

Fig. 7. Pupal mortality after entomopathogenic nematode exposure at 22°, 15°, and 10°. **A.** Values for *Steinernema feltiae* 107. **B.** Values for *Steinernema carpocapsae* All. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison tests ($P < 0.05$).

for both EPN species was observed after two days, which was slightly improved after five-six days due to the presence of EPN in combination with the adjuvant Maximix, with slightly better results on the reduction of damage and higher mortality in the treatment with this adjuvant.

As expected, reducing the temperature from the 22 °C tested by Vicente-Díez et al. (2021a) to 15° and 10 °C evaluated herein modulated the overall EPN ability to kill L3, L5, and pupae of *L. botrana,* but with differences between EPN species. For *S. feltiae,* IJs exposed at 15 °C were able to control >80% of the larvae and pupae, while at 10 °C, only ~50% of the larvae and <20% of the pupae. In contrast. *S. carpocapsae* maintained similar values of control for larvae at 15 °C compared with those observed at 22 °C by Vicente-Díez et al. (2021a). However, at 10 °C, the killing ability of *S. carpocapsae* All decreased below 50% in both larval stages (L3 and L5), and in pupae, mortality was not significantly different from in the control. Similarly, the evaluation of pupae control over trunk bark as a proxy for managing overwintering stages confirmed the suitability of the EPNs at various temperatures, following the same pattern observed herein and by Vicente-Díez et al. (2021a) using filter paper or 24-well plates as the experimental arena. This evidence is in agreement with the thermal niche breadths for infection described by Grewal et al. (2006), where *S. feltiae* was 8–30 °C, while for *S. carpocapsae* 10-32 °C. Hence, our study at 10 °C was within the limit for *S. carpocapsae* infection, and out of the range for its reproduction, described from 20 to 30 °C, while *S. feltiae* was still able to reproduce from 10 to 25 °C. Then, both nematode species would be suitable for L3, L5, and overwintering pupae management in later spring and early autumn, but *S. feltiae* would be better suited for lower temperatures.

In conclusion, we showed that EPNs resulted excellent biocontrol agents against *L. botrana* on leaves, grapes, and trunks, virtually, at any place where this pest can be present in the grapevine and at any developmental stages. Considering the expansion forecast due to overall Earth warming and global trade (Reis et al., 2021; Benelli et al., 2023b), providing new alternatives for their management is crucial to ensure their reduction and damage. Also, because these nematodes are also capable of controlling other aerial pests and disease vectors associated with the vineyards (Vieux and Malan, 2015; Platt et al., 2019a; Steyn et al., 2021; Vicente-Díez et al., 2021a, 2021b), their use can target various pests at once. Still, further investigation is required to ensure that the EPN aerial application is not detrimental to the presence of beneficial arthropods that can act as predators or parasitoids (Campos-Herrera et al., 2021; Incedayi et al., 2021). Similarly, compatibility with agrochemicals used for managing other biotic threats in the vineyards, such as *Plasmopara viticola* (Peronosporales: Peronosporaceae), *Erysiphe necator* (Erysiphales: Erysiphaceae), and *Botrytis cinerea* (Helotiales: Sclerotiniaceae), responsible for most of the phytosanitary treatments applied in vineyards (Pertot et al., 2017), is desirable to ensure their viability after field application. Furthermore, considering the potential application near grape harvest, there is a need for studies examining the EPN's impact on the quality of the resulting must. Finally, the use of the EPNs to protect vineyards is nowadays experiencing an expansion with the employ of by-products derived from their symbiotic bacteria (Eroglu et al., 2019; Vicente-Díez et al., 2023a, 2023b). However, it is important to note that only the short-term application of EPNs is feasible. In contrast to the necessary scaling-up process for by-products derived from their bacteria, EPNs are readily available as commercial products. Future research conducted in productive grapevine fields will further establish EPN as a valuable tool for growers adopting integrated and organic management practices.

Authors' contributions

Conceptualization, RCH; Methodology RCH, MMGT, IVD, EC, MP, EV, and RC; Analysis RCH, MMGT, IGV, and EC; Investigation, and Data Curation: RCH; Resources, RCH; Writing—Original Draft Preparation and Visualization, RCH; Writing—Review and Editing, RCH, MMGT, IVD, EC, MP, EV, and RC; Funding, Acquisition and Administration, RCH. All authors have read and agreed to the published version of the manuscript.

Archive of data

The data presented in this study will be archived in https://digital.csic.es/, to ensure that we compile with the FAIR mandate and accessibility to any researcher.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106392.

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