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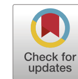

The deterrent ability of *Xenorhabdus nematophila* and *Photorhabdus laumondii* compounds as a potential novel tool for *Lobesia botrana* (Lepidoptera: Tortricidae) management

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A R T I C L E I N F O

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A B S T R A C T

The grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae), is a critical pest for vineyards and causes significant economic losses in wine-growing areas worldwide. Identifying and developing novel semiochemical cues (e.g. volatile bacterial compounds) which modify the ovipositional and trophic behaviour of *L. botrana* in vineyard fields could be a novel control alternative in viticulture. *Xenorhabdus* spp. and *Photorhabdus* spp. are becoming one of the best-studied bacterial species due to their potential interest in producing toxins and deterrent factors. In this study, we investigated the effect of the deterrent compounds produced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* on the ovipositional moth behaviour and the larval feeding preference of *L. botrana*. Along with the in-vitro bioassays performed, we screened the potential use of 3 d cell-free bacterial supernatants and 3 and 5 d unfiltered bacterial ferments. In addition, we tested two application systems: (i) contact application of the bacterial compounds and (ii) volatile bacterial compounds application. Our findings indicate that the deterrent effectiveness varied with bacterial species, the use of bacterial cell-free supernatants or unfiltered fermentation product, and the culture times. Grapes soaked in the 3 d *X. nematophila* and *P. laumondii* ferments had ~ 55% and ~ 95% fewer eggs laid than the control, respectively. Likewise, the volatile compounds emitted by the 5 d *P. laumondii* fermentations resulted in ~ 100% avoidance of *L. botrana* ovipositional activity for three days. Furthermore, both bacterial fermentation products have larval feeding deterrent effects (~65% of the larva chose the control grapes), and they significantly reduced the severity of damage caused by third instar larva in treated grapes. This study provides insightful information about a novel bacteria-based tool which can be used as an eco-friendly and economical alternative in both organic and integrated control of *L. botrana* in vineyard.

1. Introduction

The vineyard is an important socio-economical crop traditionally linked to a massive amount of pesticide use (Santos et al., 2020). Currently, the sector joined the trend of developing organic management of pests and diseases, keeping the balance between productivity and environmental health (Provost and Pedneault, 2016). Discovering and developing novel bio-tools that cope with the urgent need for pesticide alternatives opens new research lines in pest management science (Raymaekers et al., 2020).

The tortricid *Lobesia botrana* Den. & Schiff. (Lepidoptera: Tortricidae) is considered a global vine pest (Gilligan et al., 2011; Gonzalez, 2010; Varela et al., 2013). This moth achieves three generations on vineyard in temperate areas, while an additional fourth generation is increasingly frequent due to global warming (Amo-Salas et al., 2011; Castex et al., 2018). The insect, *L. botrana,* has preferences for certain host plants, the decision to lay eggs or not and the number of eggs laid on a given substrate, are based on several proximate environmental cues (Torres-Vila et al., 2012). Decisions correlate positively with offspring performance in adverse situations but not with favorable ones (Torres-Vila et al., 2012). Female moths of *L. botrana* have a fascinating olfactory behaviour that allows them to detect the presence of the vine from a great distance (Tasin et al., 2006) or to distinguish between healthy grapes and those infected by fungus (Tasin et al., 2012). Female lays

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single eggs and after hatching, larvae develop on inflorescences, unripe berries and ripening-ripe berries during the respective generation. Individuals from the last generation overwinter as diapausing pupae from autumn to early spring. Adults do not exhibit migratory habits and show reduced active dispersal (Torres-Vila et al., 2006). Grape volatiles, alone or in combination with non-volatile metabolites found on the surface of the grapes and/or visual cues, also function as oviposition stimulants in this insect (Anfora et al., 2009; Ioriatti et al., 2011).

Traditionally, the control of *L. botrana* has been performed by several applications of insect growth regulators or organophosphate insecticides (Ioriatti et al., 2011). Nowadays, farmers are seeking new control alternatives due to the harmful effects of these treatments on non-target organisms and the environment. Pheromone-mediated mating disruption (MD) to control *L. botrana* is a current efficient semiochemical-technique. MD is based on interference of the mate finding process affecting the chance of reproduction of the moth, and with a consequent impact on population dynamics. Techniques such as MD proved that olfactory cues are crucial information for *L. botrana* to choose feeding, mating and oviposition sites, and help them avoid non-host plants (Tasin et al., 2012, 2011, 2006). Identifying novel chemical cues (i.e. bacterial volatile compounds) which drive the ovipositional and trophic interactions through olfactory reception of *L. botrana* and inducing behavioral changes of this pest in vineyard field could be an efficient control strategy.

Bacteria can be an ally in this new approach because they produce a broad-spectrum of ecological activities and are often a source of novel chemical compounds (Flórez et al., 2018; Kajla et al., 2019). In particular, the symbiotic bacteria of entomopathogenic nematodes (EPNs) are becoming one of the chemically best-studied species due to their potential biotechnological interest in the field of pest/disease management (Boemare et al., 1997; Cimen et al., 2022; Koppenhöfer and Gaugler, 2009). *Xenorhabdus* spp. and *Photorhabdus* spp. are γ-proteobacterial species (Enterobacterales: Morganellaceae) characterized by their symbiotic relationship in nature with the infective juveniles (IJs) of certain nematodes in the families Steinernematidae and Heterorhabditidae, respectively, with each partner requiring the other to complete its life cycle (Adeolu et al., 2016; Dillman et al., 2012; Shi and Bode, 2018; Ulug et al., 2014). Although most strains of the bacteria are species-specific and essential for growth and reproduction of their nematode hosts, some of these bacteria can dwell in multiple hosts or even co-exist two symbionts with the same IJs nematode host (Koppenhöfer and Gaugler, 2009; Maher et al., 2021). The nematode/bacterium complex kills a broad range of soil-dwelling insects and decompose their tissues as a food source (Hazir et al., 2022; Shapiro-Ilan et al., 2020). *Xenorhabdus* spp. and *Photorhabdus* spp. assist the nematode (*i*) overcoming prey defenses (Ahmed and Kim, 2018; Bode, 2009; Shi and Bode, 2018; Tobias et al., 2017); and (*ii*) synthesizing defensive compounds (deterrent factors) against animals and microbial competitors to the host cadaver resources (Blanco-Pérez et al., 2017; Grewal et al., 2006; Gulcu et al., 2012; Ulug et al., 2014). Insect cadavers attract different opportunistic organisms and harbour interspecific competition for nutrients by insect scavenger arthropods like ants and by the surrounding microbial community like viruses, con– and hetero- specific bacteria, saprobic fungi, protozoa and/or even nematode competitors (Flórez et al., 2015; Gulcu et al., 2017; Wollenberg et al., 2016). Nematode-killed insects that are<2-days-old may be consumed by opportunistic organisms while the ones that are 4 to 5-days-death or older, are deterred by natural compounds produced by the mutualistic bacteria (Clarke, 2016; Karthik Raja et al., 2021; Zhou et al., 2002). This fact indicates that the bacteria produce most defensive compounds during the following post-exponential phase of growth and some of them act as semiochemical signals, modifying the behaviors of other individuals (deterrence) and having a great impact over transkingdom crosstalk (Calcagnile et al., 2019; Flórez et al., 2015). While the insect-killing compounds fueled research during decades, chemical, evolution and ecological knowledge of defensive symbiont-provided compounds by *Xenorhabdus* spp. and *Photorhabdus* spp. is thus still far lacking (Crawford et al., 2012; Flórez et al., 2015).

The study carried out by Vicente-Díez et al. (2021a) showed that *Xenorhabdus* spp. and *Photorhabdus* spp. natural compounds had insecticidal activity against larval instars of *L. botrana*. Likewise, recent work of Kong et al. (2022) has proved that emissions from EPNs symbiotic bacteria are key players in chemical communication among insects, nematodes, and microbes. These findings laid the groundwork to support the hypothesis that olfactory cues emitted by bacterial nematode symbionts as defensive compounds could have a behavioural deterrent activity against *L. botrana*. In this study, we investigated how the deterrent factors emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* may influence the ovipositional behavior and in feeding site preferences of *L. botrana*. This study aims to provide insightful information about a novel bacteria-based tool which can be used as an eco-friendly and economical alternative in the integrated control of a huge range of crop pests.

2. Material and methods

2.1. Bacterial isolation and fermentation

Bacteria *X. nematophila* (GenBank accession number MW574906) and *P. laumondii* subsp. *laumondii* (GenBank accession number OQ285858) are symbionts of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, respectively. We isolated the bacteria species from their symbiotic EPNs according to Vicente-Díez et al., (2021b). To obtain the bacterial ferment compounds, we inoculated 1 mL of bacterial phosphatase buffered saline (PBS) suspension in 500 mL Erlenmeyer flasks with 250 mL of Triptone Soy Broth (TSB) (VWR Chemicals, Barcelona, Spain). We incubated the flasks on an orbital shaker at 150 rpm, at 25 ± 2 °C, in full darkness for three days. The bacteria metabolism produces some secondary compounds during the exponential bacterial growth phase (approx. during three days after the inoculation). Nevertheless, their secondary metabolism is generally activated during the post-exponential or stationary phase after the bacterial growth (Clarke, 2016). Thus, for the bioassays performed in the preset study, we have employed three different bacterial resources: (*i*) 3-day bacterial cell-free supernatants (3 d-CFSs), (*ii*) 3 d bacterial unfiltered fermentation products (3 d-UFs) and (*iii*) 5 d bacterial unfiltered fermentations (5 d-UFs). During the research process, we selected the best bacterial resource to perform the subsequent test.

To produce the CFSs, we centrifuged the 3 d bacterial culture at 68.905 g (Thermo Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge, Fisher Scientific SL, Madrid, Spain) for 20 min at 4° C (Donmez Ozkan et al., 2019; Hazir et al., 2016). Then, we filtered the liquid supernatant through a 0.22 μm sterile pore filter. We cultured 1 mL of the *X. nematophila* and *P. laumondii* CFSs on petri dish with Nutrient Agar (NA), Bromothymol blue (Alfa Aesar, Kandel, Germany), and 2,3,5-Triphenyl tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates), supplemented with Ampicillin (50 mg/mL) (PanReac AppliChem, ITW Reagents, Barcelona, Spain) in duplicate to verify the absence of bacteria. We also seeded the bacterial pellet obtained after the centrifugation in NBTA plates to check the correct bacterial growth based on dye adsorption, pigmentation and morphology of the colonies (Han and Ehlers, 2001). The TSB was also filtrated to maintain the control treatments under the same conditions. To obtain the 3 d-UFs, we used the product of the bacterial fermentation after three days from the inoculation keeping it at room temperature. Finally, we obtained 5 d-UFs to test the secondary metabolites in the post-exponential bacterial growth phase by keeping the 3 d bacterial fermented flasks at room temperature, close, without agitation, in semidarkness and at 22 °C for 2 additional days. This period allowed the bacteria to produce the secondary metabolites, including the a synthesis of defensive compounds (Kong et al., 2022).

2.2. *Lobesia botrana* rearing and grapes collection

The rearing of *L. botrana* was performed in an environmentally controlled chamber at 22 °C and 60% RH, with 16:8 (L:D) photoperiods, at the Institute of Grapevine and Wine Sciences (ICVV, Logroño, La Rioja, Spain) following the protocol described by Vicente-Díez et al. (2021a). For bioassays, it was necessary to separate larvae of the same age cohort and to separated pupae between males and females. We separate the same larval instars measuring their size (third instar larva: 4.5–5.0 mm). We separated male and female pupas based on the number of abdominal segments (male = 4 segments and female pupae = 3 segments) following the protocol described by Steinitz et al. (2016).

We randomly collected ripening-ripe red grapes (*Vitis vinifera* cv. Tempranillo) from an organic vineyard located in Logroño (La Rioja, Spain, 42° 26′ 39″N and 2° 30́ 54″W), where no fungicide pre-harvest treatment was applied. We selected healthy and homogenous grape berries and randomly assigned them to different bioassays. Before applying any treatment, we disinfected the surface of the grapes by dipping them in 3% (v/v) of sodium hypochlorite (NaClO) solution for 1 min, we washed them with tap water two times and then they were air-dried for ∼ 2 h at the lab conditions.

2.3. Ovipositional-deterrence bioassays

2.3.1. Soaked grapes in bacterial culture compounds

We soaked grape berries with 3 d-CFSs, 3 d-UFs and TSB (as a negative control treatment). We placed 6 grapes of each treatment inside curtain mesh bags, and we used three bags for each treatment (Fig. 1A). We checked that the size of the curtain mesh pores was larger than the moth egg size, to prevent the moths from ovipositing on the surface of the bag. We placed all the experimental units in the rearing chamber under the same conditions. Then, we transferred 10 1- to 3-day-old *L. botrana* adults (five females and five males) to each bag and allowed them to lay eggs for two days. We provided a cotton piece with 10% honey solution to supply food *ad libitum* to the moth. After 24 and 48 h, we registered the number of eggs laid on every single berry. The whole experiment was performed twice (n total = 30 female moths/ treatment).

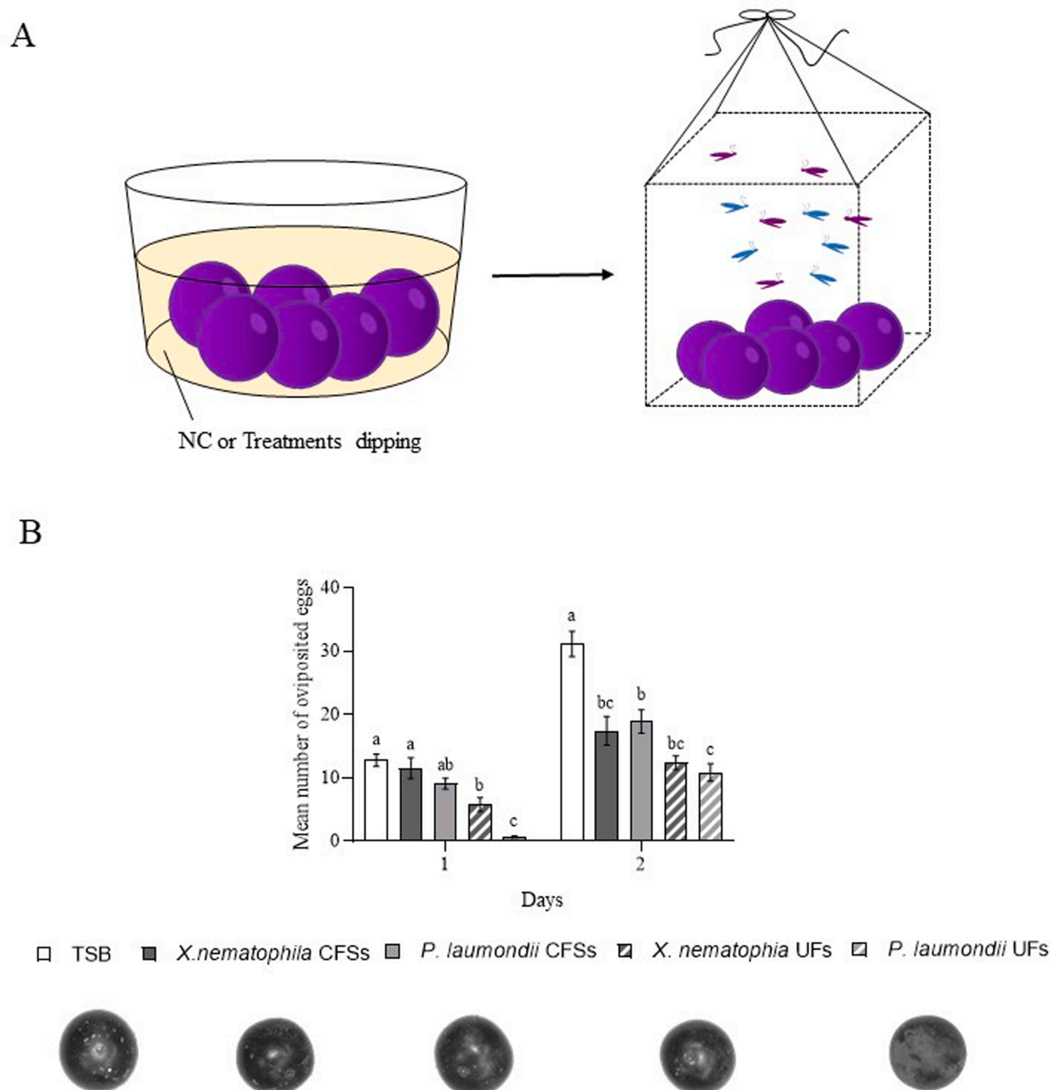

Fig. 1. Ovipositional-deterrence bioassays soaking grapes 3 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* cell-free supernatants (CFSs) and unfiltered fermentations (UFs). (A) The schematic drawing shows the method for testing the deterrent effects used in the respective assays. (B) Mean number of oviposit eggs on each grape. Different lower case letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$).

2.3.2. Grapes exposed to volatile organic compounds (VOCs)

We performed a test to check if the VOCs emitted by bacterial cultures have an effect on the ovipositional behaviour of female grapevine moths. We placed six grape berries inside curtain mesh bags and we used three bags for each treatment per trial. Below each experimental bag, we placed one glass beaker with 25 mL of 3 d *X. nematophila* and *P. laumondii* UFs. Inside each bag, we placed ten 1- to 3-day-old *L. botrana* adults (five females and five males) in each bag. We covered the system with a glass beaker (Fig. 2A). Then, we placed all the experimental systems in a shaker at 60 rpm to ensure the emission of VOCs from the bacterial cultures. After 24 and 48 h, we counted the number of eggs laid on every single berry. A subsequent experiment tested the ovipositional-deterrence activity of 5 d *P. laumondii* UFs, selected as the most promising bacteria strain, using the secondary metabolic volatile compounds produced after the exponential growth phase. This experiment was performed as described before, using three experimental bags per treatment and trial, and the whole experiment was replicated twice (n = 36 grapes/treatment). We registered the number of eggs laid on every single berry each day during 72 h. All the experiments were conducted two times, with new ferment, grapes and insects.

2.4. Feeding source preference bioassays

2.4.1. Soaked grapes in bacterial culture compounds

We conducted dual-choice experiments using manual laboratory olfactometers to assess the effect of *X. nematophila* and *P. laumondii*

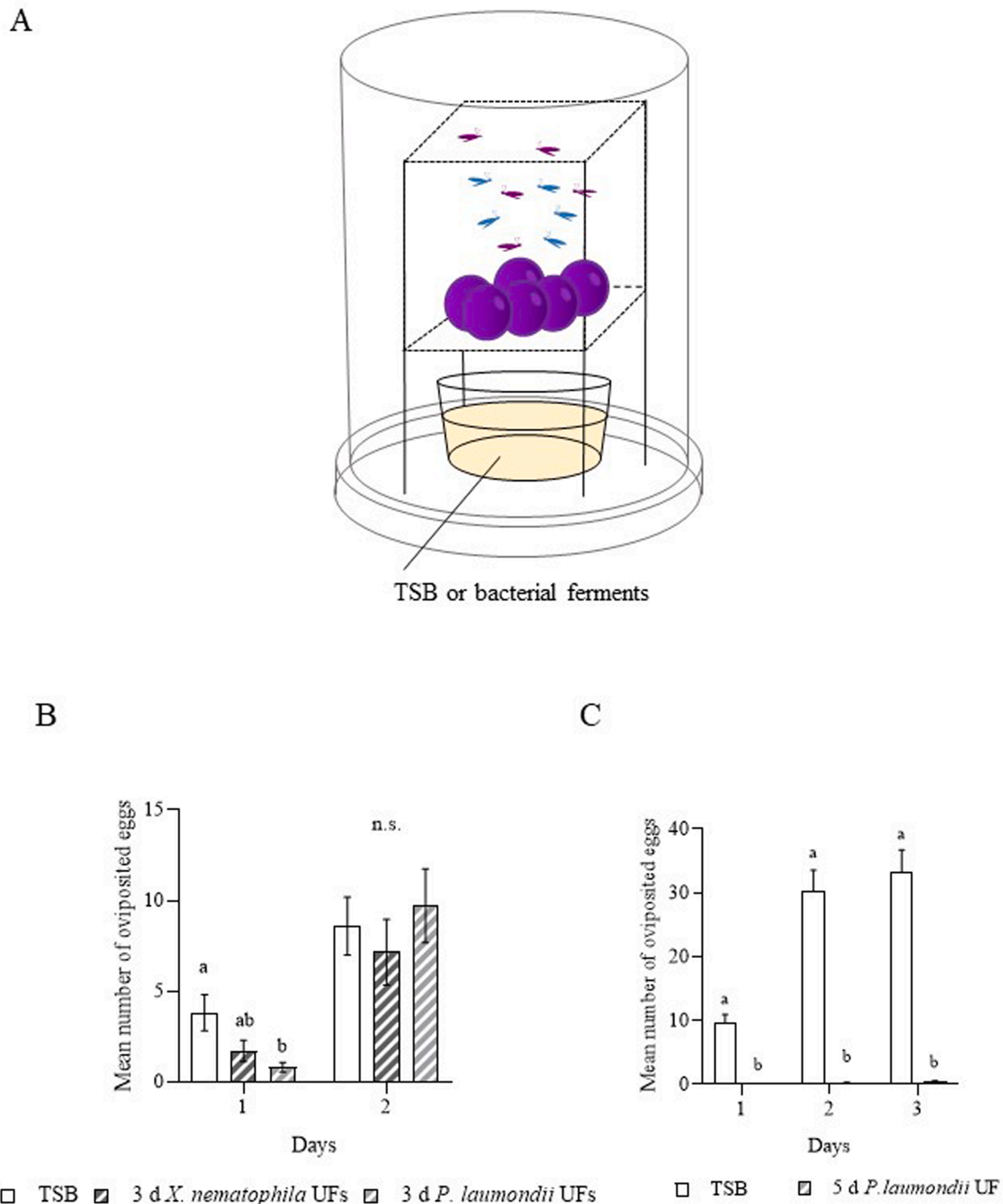

Fig. 2. Ovipositional-deterrence bioassays using bacterial ferment volatiles of 3 d and 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* unfiltered fermentations (UFs). (A) The schematic drawing shows the method for testing the deterrent effects used in the respective assays. (B) The mean number of oviposited eggs on each grape with the semiochemical compounds of 3 d TSB ferment. (C) The mean number of oviposited eggs on each grape with the semiochemical compounds of 3 d TSB ferment. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$).

culture VOCs on *L. botrana* larval behaviour. Previous work by Vicente-Díez et al. (2021a) found that in one-choice feeding, the larva died due to the oral toxicity of *X. nematophila* and *P. laumondii* metabolites. The two-choice system lets us check if the metabolites produced by bacteria modify the feeding behaviour of the larva. We modified 50 mL Falcon tubes by placing one cloth net at 4 cm from the top. We made one hole in one side of the tube (1 cm diameter) at 2 cm from the top, and we connected two modified Falcon tubes with 10 cm polypropylene (Fig. 3A). We soaked grape berries with 5 d *X. nematophila* and *P. laumondii* UFs (as described before) as well as in TSB as a negative control. We weight each of the experimental grapes by using a precision balance. Then, we placed one of those treated grapes over the one-cloth net and one control grape in the other tube. After that, we put five third instar of the same age cohort in the middle of the connecting tube that were starved for 24 h. We placed these experimental units in rearing conditions, and orientations were randomized to account for potential

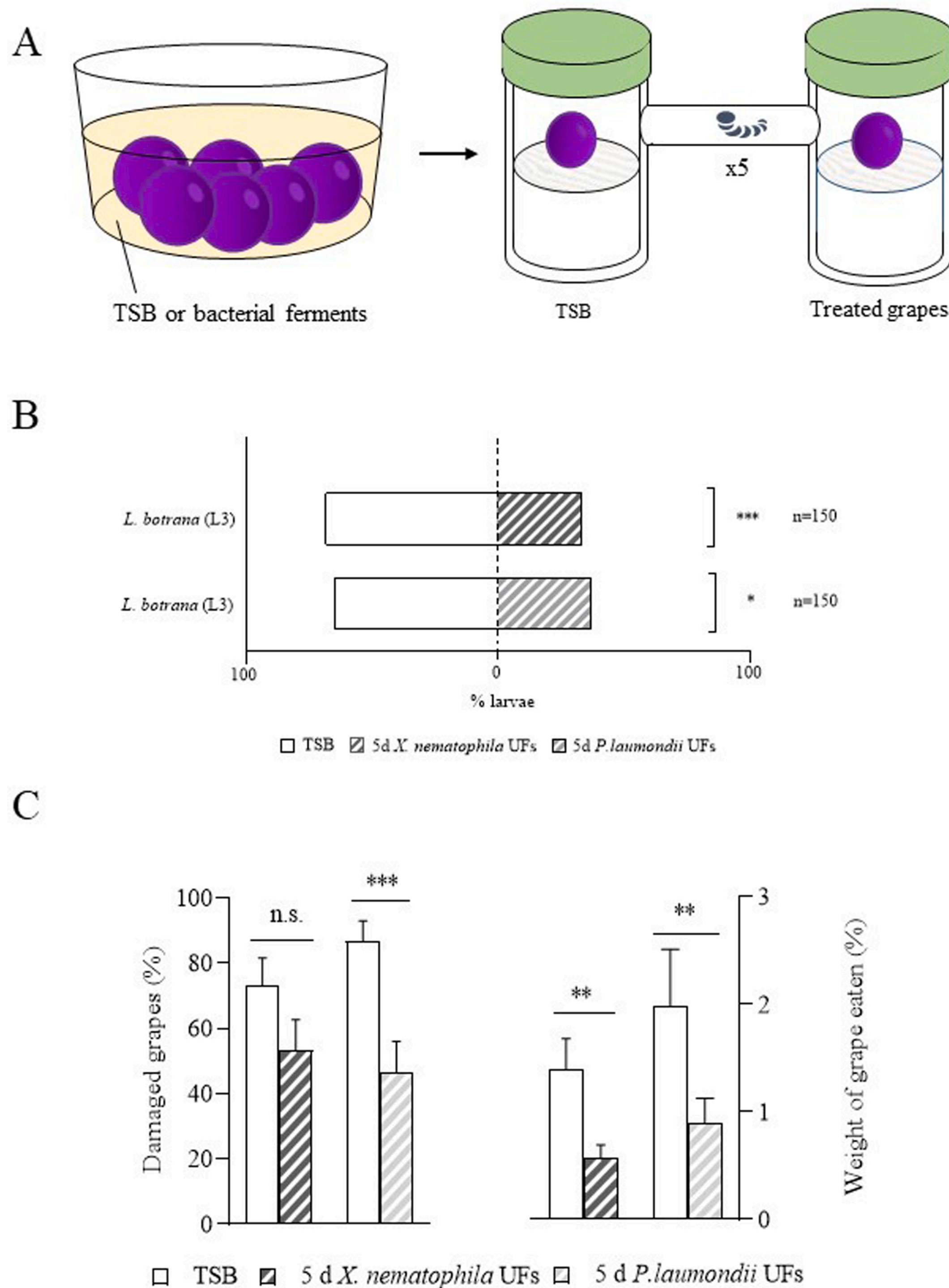

Fig. 3. Feeding source preference bioassay of soaked grapes. Effects of natural products produced by 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* UFs on third larval instar *Lobesia botrana*. (A) The schematic drawing shows the method for testing the deterrent effect used in the respective assays. (B) Larval choice. (C) Grape damage: percentage of grapes with herbivory damage and percentage of grape eaten. Asterisks indicate significant differences at ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s., not significant.

direction bias. After one day, we checked (*i*) the position of the larva (in which tube they were); (*ii*) whether or not there was herbivory damage to the grapes, and (*iii*) the weight loss of grape berries caused by the larval activity. In each trial, we used 10 experimental units (one 2 Falcon tubes-pair), and the experiment was repeated three times (n total = 150 larvae and 60 grape berries).

2.4.2. Grapes exposed to bacterial VOCs

In the same two-choice system described in section 2.4.1, we added 35 mL of 5 d *X. nematophila* or *P. laumondii* TSB ferments on one of the bottom of the Falcon tubes (Fig. 4A). We added 35 mL of TSB as a negative control in the connected-Falcon tube. Over the cloth net, we placed one disinfected grape berry in each tube previously weighted by precision balance. We ensured that none of the bacterial ferments directly interacted with the grape berries. In the middle of the connecting tube, we placed five third-instar *L. botrana* of the same age cohort. We put these experimental units in rearing conditions, and orientations were randomized to account for potential directional bias. After 24 h, we checked (*i*) the position of the larva (in which tube they were), (*ii*) whether or not there was herbivory damage to the grapes, and

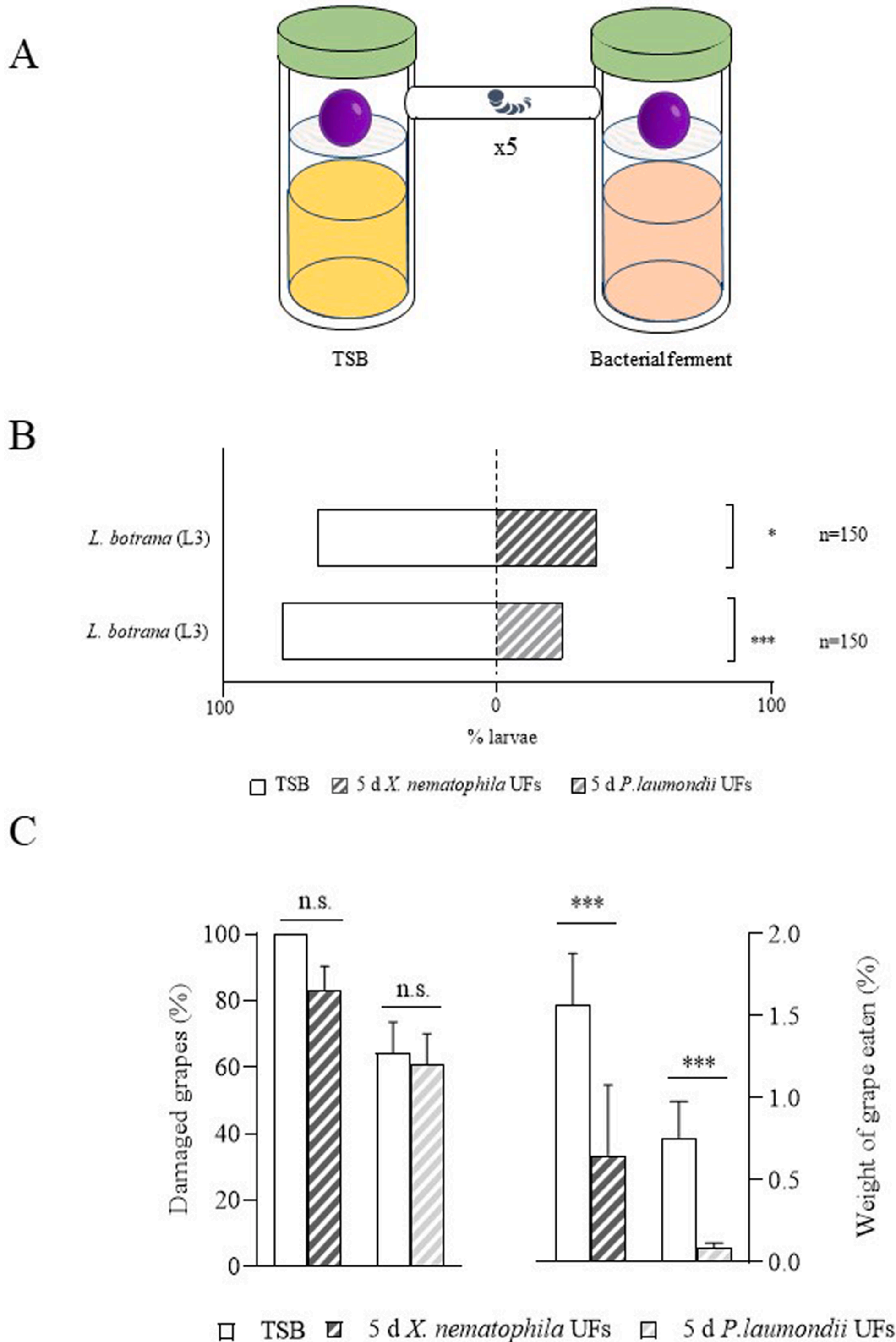

Fig. 4. Feeding source preference bioassay of grapes under bacterial volatiles. Effects of the odours of the 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* cultures against third stage third instar larvae of *Lobesia botrana*. (A) The schematic drawing shows the method for testing the deterrent effect used in the respective assays. (B) Larval choice. (C) Grape damage: percentage of grapes with herbivory damage and percentage of grape eaten. Asterisks indicate significant differences at ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s., not significant.

(*iii*) the weight loss of grape berries caused by the larva. In each trial, we used 10 experimental units (one 2 Falcon tubes-pair), and the experiment was repeated three times (n total = 150 larvae and 60 grape berries).

2.5. Statistical analysis

To analyse the ovipositional deterrence effect of bacterial ferments on *L. botrana*, we ran one-way analysis of variance (ANOVA) followed by means compared by Tukey's test. The proportions of the third stage larvae responding to each treatment was compared by binomial test ($P < 0.05$). We ran general linear models (GLM) with binomial distribution (logit-link function) for the treatment comparisons (treated grapes *versus* untreated ones) to test the presence of damage on the surface of the grape berries. The severity of damages (the percentage of eaten grape) on treated grape berries was also compared to the severity of the damage in the untreated ones (control) using GLM with ordinal logistic. Statistical significance was stablished for $P \leq 0.05$. All data was expressed as the mean ± standard error (SE) for the three replicates in each treatment and the two or three trials combined. We performed these analyses with SPSS 25.0 (SPSS 170 Statistics, SPSS Inc., Chicago, IL, USA). We developed the charts with Prism Graphpad 8.0 (Prism).

3. Results

3.1. Ovipositional deterrence

In the no choice ovipositional experiment, there were significant differences on the number of eggs laid on grapes soaked in the different substances after one ($F_{4, 205} = 20.836$, $P < 0.001$) and two days ($F_{4,205} = 21.811$, $P < 0.001$) (Fig. 1B). In particular, the number of eggs laid over soaked grapes in 3 d bacterial CFS had no significant differences to the grapes soaked in TSB (control) after 24 h. However, the grapes soaked on the 3 d *X. nematophila* and *P. laumondii* UFs had a significant reduction of ~ 55% and ~ 95% number of eggs laid than the control, respectively. After 48 h, all the bacterial treatments caused a significant reduction in the number of eggs laid per grape berries. In particular, the grapes soaked in the 3 d *X. nematophila* or *P. laumondii* UFs reduced 60 and 68% the number of eggs compared with the grapes of the control treatment, respectively.

In the test using bacterial VOCs under the grapes, we observed significant reduction of the numbers of eggs laid over treated grapes after 24 h ($F_{2, 63} = 3.611$, $P < 0.05$) (Fig. 2B). However, this difference was not observed after 48 h ($F_{2, 63} = 0.416$, $P > 0.05$). In detail, female *L. botrana* oviposited significantly fewer eggs on grape clusters in presence of the *P. laumondii* fermentation compared to the grape clusters in presence of TSB (control). The subsequent study evaluating the semiochemical emitted by the 5 d *P. laumondii* UFs showed a limitation of the ovipositional activity of *L. botrana* during the whole length of the study (3 days) (Fig. 2C). In detail, there were no eggs deposited in any grape at 24, 48 and 72 h post-exposure (Day 1: $F_{1, 64} = 36.66$, $P < 0{,}001$; Day 2: $F_{1, 64} = 67.26$, $P < 0{,}001$; Day 3: $F_{1, 64} = 64.316$, $P < 0{,}001$) (Fig. 2C).

3.2. Feeding-deterrence effects

The third instar larva of *L. botrana* significantly preferred grape berries soaked on the control compared with grape berries soaked on *X. nematophila* ($P < 0.001$) or *P. laumondii* ($P < 0.05$) (Fig. 3B.). In particular, the larva chose 67% and 64% of the times the rapes soaked in TSB control than the grapes soaked in 5 d *X. nematophila* and *P. laumondii* UFs, respectively. The number of damaged grapes previously soaked in 5 d *X. nematophila* UFs was not significantly reduced compared to the grape berries soaked in TSB ($X^2 = 2{,}533$, > 0.05), but the percentage of weight grape eaten by the larva was significantly reduced ($X^2 = 5.031$, $P < 0.05$) (Fig. 3C.). In particular, while the control grapes lost weight at ~ 1.4%, in the treated grapes the larva had eaten 0.5% of the total grape weight. The number of grape berries damaged was significantly reduced by *P. laumondii* treatment ($X^2 = 9.52$, $P < 0.01$) and the percentage of grape eaten by the larvae was significantly reduced ($X^2 = 6.441$, $P < 0.01$) (Fig. 3C.). In detail, 46% of grape berries were damaged by the larvae and the severity of the damage was 0.9% of the total weight of the grape.

The third instar of *L. botrana* was significantly deterred by grapes under *X. nematophila* ($P < 0.05$) or *P. laumondii* ($P < 0.001$) VOCs (Fig. 4B.). In particular, in the choice between control and treated grapes, 66% and 73% of larva choose the control grape. The number of grape berries damaged was not significantly reduced by *X. nematophila* ($X^2 = 0.00$, > 0.05) or *P. laumondii* ($X^2 = 0.076$, $P > 0.05$) VOCs (Fig. 4C). However, the percentage by weight of grapes eaten by the larvae was significantly reduced by *X. nematophila* ($X^2 = 23.77$, $P < 0.001$) and *P. laumondii* ($X^2 = 21.170$, $P < 0.001$) culture odors (Fig. 4C). In particular, the percentage of grape eaten by the larvae was 0.65% and 0.08% in the grapes over *X. nematophila* and *P. laumondii*, respectively.

4. Discussion

The present work proves the repellent activity of *X. nematophila* and *P. laumondii* cultures against *L. botrana*. The results show that the bacterial cultures deter the oviposition of the grapevine moth and change its larval feeding preference. Our findings indicate that the ovipositional deterrence effectiveness varied with bacterial species, the use of bacterial cell-free supernatants or unfiltered ferment and the culture age. The deterrent compounds emitted by *P. laumondii* exhibited better ovipositional deterrent activity against *L. botrana* than the compounds emitted by *X. nematophila*. In both cases, the unfiltered bacterial fermentation products showed better anti-ovipositional activity than their respective bacterial cell-free supernatants. Furthermore, the bacterial culture of *P. laumondii* after 5 d showed a better deterrent effect than their fermentations after 3 d. These results are consistent with the recent results reported by Kong et al., (2022), which showed that all bacterial cultures tested of different EPN symbiotic bacteria exhibited the best deterrent effect against *S. frugiperda* larva after 5 d.

Both bacterial deterrent compounds can modify the larval feeding preference, achieving fewer grapes damaged and decreasing significantly the severity of the damage. Both grapes soaked or under 5d bacterial UFs were significantly less attractive to the third instar *L. botrana* in two-choice bioassay. The best feeding deterrent results were obtained with the application of 5 d *P. laumondii* volatiles application, reducing under 10% the weight loss for all the tested grapes. These results are consistent with the anti-ovipositional results found.

At the same time, we tested two application systems of the bacterial deterrent compounds: (i) contact application (soaking grapes on the bacterial metabolites) and (ii) bacterial VOCs application (grapes were placed under bacterial culture volatiles). Our results indicate that the volatiles emitted by *X. nematophila* and *P. laumondii* are able to modulate *L. botrana* behaviour better than a contact application. Future agricultural technologies may benefit from the development of volatile compounds due to advantages related to their easy diffusion and absence of toxic residues.

Symbiotic bacteria of EPN are well-known producers of a wide range of compounds with biologically relevant activities (Bode, 2009; Dreyer et al., 2018; Shi and Bode, 2018). During the last decade, they have been identified as a potential source of insecticidal (Da Silva et al., 2013; Shrestha and Lee, 2012), nematicidal (Abebew et al., 2022; Kusakabe et al., 2022), and acaricidal (Cevizci et al., 2020; Eroglu et al., 2019; Incedayi et al., 2021) metabolites. Nevertheless, their natural deterrent compounds emitted for defence against saprophytes, omnivores, and scavengers have not been widely employed in pest control. The anti-ovipositional effect of the EPN symbiotic bacteria metabolites tested in the present study was previously tested against the calliphorid fly, *Chrysomya albiceps* (Gulcu et al., 2012). The supernatant of *P. luminescens* deterred *C. albiceps* from depositing eggs on meat (Gulcu

et al., 2012). However, its anti-ovipositional effect has not been deeply explored so far and has never been studied about one crop pest.

Furthermore, the previous works of Kajla et al., (2019) and Kong et al., (2022) provided evidence of the potent insect-feeding-deterrent effect of the *Xenorhabdus* and *Photorhabdus* compounds. For a lot of larval pests, the choice of feeding-source conditioned larval development time, larval survival, pupal weight, and female fecundity (Savopoulou-Soultani and Tzanakakis, 1988; Tasin et al., 2012). Our results suggest that potential future application of EPN symbiotic bacterial cultures or their deterrent compounds against *L. botrana* may exploit more than one mode of action and can control its damage in the vineyards. Our results lay the groundwork for research into novel applications of these bacterial deterrent compounds in the development of new repellents against crop pests.

5. Conclusions

The deterrent compounds emitted by EPN symbiotic bacteria have ovipositional deterrence and signal the feeding larval preference. The optimization of the direct agricultural application of these compounds, the possible impact on other biotic and abiotic factors and deeper knowledge of their infective mechanisms have yet to be studied in depth.. In the present study, we have tested the physical application mode and used the volatile fermentation products to explore possible agricultural applications. We consider that the discovery and characterization of these new semiochemicals can significantly contribute to advances in novel bio-tools that can cope with the urgent need for alternatives for farmers and open new research lines in the use of bacterial deterrence factors in crop protection.

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.

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Patent

The results presented herein are part of the patent entitled “Composition of volatile organic compounds obtained from Photorhabdus laumondii subsp. laumondii and uses thereof” (registration reference EP23382199).

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