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Development of biotools for vineyard-associated pest and disease control based on entomopathogenic nematode symbiotic bacteria
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Development of biotools for vineyard-associated pest and disease control based on entomopathogenic nematode symbiotic bacteria

Desarrollo de bioherramientas de control de plagas y enfermedades asociadas con el viñedo basadas en bacterias simbiontes de nematodos entomopatógenos

Tesis Doctoral presentada por
Ignacio Vicente Díez
para optar al título de Doctor por
La Universidad de La Rioja

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DEPARTAMENTO DE AGRICULTURA Y ALIMENTACIÓN
UNIVERSIDAD DE LA RIOJA

Programa de Doctorado Interuniversitario en Enología, Viticultura y Sostenibilidad

Development of biotools for vineyard-associated pest and disease control based on entomopathogenic nematode symbiotic bacteria

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Logroño, 2024

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Por la presente declaran que:

La memoria titulada *Development of biotools for vineyard-associated pest and disease control based on entomopathogenic nematode symbiotic bacteria*, que presenta Ignacio Vicente Díez, Graduado en Biotecnología por la Universidad de Lleida y Máster en Agrobiología Ambiental por la Universidad del País Vasco y la Universidad Pública de Navarra, ha sido realizada en la Universidad de La Rioja bajo su dirección y reúne las condiciones específicas para optar al grado de Doctor como compendio de publicaciones.

Lo hacen constar en Logroño, a 16 de octubre de 2023.

Fdo.: Dra. Raquel Campos Herrera

Fdo.: Dra. Alicia Pou Mir

Esta Tesis ha sido realizada en el Grupo de Investigación In-Vid del Departamento de Viticultura del Instituto de Ciencias de la Vid y del Vino (Gobierno de La Rioja, Consejo Superior de Investigaciones Científicas y Universidad de La Rioja), y apoyada por el Programa para la realización de acciones de integración de tecnólogos e investigadores en empresas y centros de investigación de La Rioja – Programa Becas ADER (2018) de la Agencia de Desarrollo Económico de La Rioja ADER I + D + i (Decisión C/2015/8084); por el contrato predoctoral FPI-UR-2020, Resolución de concesión nº 1150/2020, por el Plan Nacional PID2019-104112RB-I00 "Nuevas estrategias de manejo de plagas de artrópodos persistentes y emergentes en viñedo y su impacto en la calidad de la uva" (SOS-VINE) del Ministerio de Ciencia, Innovación y Universidades y de la Agencia Estatal de Investigación; y por la Ayuda para Estudios Científicos de Temática Riojana 2022 ref. 29/2022 "Desarrollo de un nuevo biofungicida a partir de las bacterias *Xenorhabdus* y *Photorhabdus* para el manejo de *Botrytis cinerea* en el viñedo del Instituto de Estudios Riojanos" (IER-2022). Además, los resultados presentados en los artículos 3 y 5 forman parte de la patente titulada "Volatile organic compounds obtained from *Photorhabdus laumondii* subsp. *laumondii* and uses thereof" [Compuestos orgánicos volátiles obtenidos de *Photorhabdus laumondii* subsp. *laumondii* y sus usos] (Referencia EP23382199).

Durante la realización de la tesis doctoral se completaron estancias en el laboratorio de Integración Metabolómica y Señalización Celular, Bioquímica y Biología Molecular, Unidad Asociada al Consejo Superior de Investigaciones Científicas de la Universitat Jaume I de Castelló del 2 al 5 de marzo de 2021 y del 10 al 17 de marzo de 2022, en el laboratorio de Ecología Evolutiva de las Interacciones Planta-Herbívoro de la Misión Biológica de Galicia (CSIC) (Pontevedra, España) del 1 al 30 de noviembre de 2021 y en el departamento de Ecología y Biología Evolutiva en la Universidad de California (Irvine) del 6 de noviembre de 2022 al 6 de marzo de 2023.

ARTÍCULOS INCLUIDOS EN LA TESIS

De acuerdo con la normativa vigente en la Universidad de La Rioja (Normativa para la defensa de tesis doctoral en la Universidad de La Rioja, aprobada por Consejo de Gobierno de 18 de marzo de 2022 y, específicamente, con su Capítulo V), la tesis se presenta como compendio de publicaciones científicas. Las referencias completas de las mismas se listan a continuación en orden cronológico de publicación. Además, se presenta una copia de los artículos en el apartado 4 "Publication catalogue".

La presente tesis se ha configurado a partir de cinco artículos científicos, todos ellos publicados en revistas internacionales incluidas en los listados *Journal of Citation Reports-Science Edition* (JCR):

- **Vicente-Díez, I.**, Blanco-Pérez, R., González-Trujillo, M. del M., Pou, A., & Campos-Herrera, R. (2021). Insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from their symbiotic bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae) Nymphs. *Insects, 12*(5), 448. https://doi.org/10.3390/insects12050448

  Área: Entomology; IF: **3,141** (2021), **Q1** (17/100)

- **Vicente-Díez, I.**, Blanco-Pérez, R., Chelkha, M., Puelles, M., Pou, A., & Campos-Herrera, R. (2021). Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae). *Insects, 12*(11). https://doi.org/10.3390/insects12111033

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- **Vicente-Díez, I.**, Pou, A., & Campos-Herrera, R. (2023). The deterrent ability of *Xenorhabdus nematophila* and *Photorhabdus laumondii* compounds as a potential novel tool for *Lobesia botrana* (Lepidoptera: Tortricidae) management. *Journal of Invertebrate Pathology, 198*, 107911. https://doi.org/10.1016/j.jip.2023.107911

  Área: Zoology; IF: **3,4** (2021), **Q1** (8/176)

- **Vicente-Díez, I.**, Carpentero, E., Pou, A., & Campos-Herrera, R. (2023). Exploring bacterial cell-free supernatants, unfiltered ferments and crude bacteria uses of *Xenorhabdus* and *Photorhabdus* (Morganellaceae) for controlling *Botrytis cinerea* (Helotiales: Sclerotiniaceae). *Biological Control*, *183*, 105259. https://doi.org/10.1016/j.biocontrol.2023.105259

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- **Vicente-Díez, I.,** Moreira, X., Pastor, V., Vilanova, M., Pou A., & Campos-Herrera, R. (2023). Control of post-harvest gray mold (*Botrytis cinerea*) on grape (*Vitis vinifera*) and tomato (*Solanum lycopersicum*) using volatile organic compounds produced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii*. *BioControl*, *183*, 10212. https://doi.org/10.1007/s10526-023-10212-7

  Área: Entomology; IF: **2,5** (2022), **Q1** (19/100)

Para cada uno de estos estudios, el autor de esta tesis, bajo la supervisión de sus directoras y en colaboración con el resto de coautores, ha desarrollado las siguientes funciones:

- ✓ Análisis del estado del arte.
- ✓ Planteamiento de objetivos y elección de los materiales y métodos.
- ✓ Ejecución de los bioensayos y varias técnicas analíticas descritas en la metodología.
- ✓ Procesamiento de datos y análisis estadístico.
- ✓ Análisis de los resultados y discusión sobre los mismos.
- ✓ Redacción completa de los artículos.

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Abstract

Grapevine is one of the most important socio-economic crops worldwide. However, the vine sector faces numerous challenges today, ranging from climate change and various forms of environmental degradation to severe pest and disease outbreaks. The development of new sustainable tools to address these challenges and protect vineyards is necessary. In this context, symbiotic bacteria of entomopathogenic nematodes, specifically *Xenorhabdus* spp. and *Photorhabdus* spp., emerge as potential biocontrol agents due to their ability to produce bioactive compounds with insecticidal and antifungal properties. We hypothesized that these symbiotic bacteria and their by-products might effectively manage selected pests and diseases that pose significant threats to grapevines. Therefore, the specific objectives to accomplish during this Thesis were as follows: (*i*) to evaluate the insecticidal and antifungal effect of the soluble toxins produced by *Xenorhabdus* and *Photorhabdus* for controlling vineyard pests and diseases; (*ii*) to assess the deterrent and the antifungal activity of the volatile organic compounds (VOCs) emitted by *Xenorhabdus* and *Photorhabdus* fermentations; and (*iii*) to evaluate the effect that bacterial secondary metabolites may have on the defence of the harvested fruit.

We conducted *in vitro* experiments to assess the insecticidal and antifungal properties of various bioproducts derived from *Xenorhabdus* spp. and *Photorhabdus* spp. To obtain these bacterial bioproducts, we inoculated individual colonies of each pure culture in Triptone Soya Broth (TSB) media, maintaining the liquid cultures for 72 hours under continuous orbital shaking (150 rpm) at a temperature of 22 ºC in a light-protected environment. The resulting bacterial by-products included the soluble secondary metabolites (bacterial cell-free supernatants and the unfiltered ferments), the volatile organic compounds (VOCs) emitted from the ferments and the crude bacteria isolates. For our bioassays, we employed two insect species: *Philaenus spumarius*, the primary vector responsible for transmitting *Xylella fastidiosa* in Europe, the causative agent of Pierce's disease in grapevines; and *Lobesia botrana*, one of the most economically significant pests affecting European vineyards. Our disease studies focused on *Botrytis cinerea*, the pathogen responsible for grape rot. Each set of

experiments was conducted under controlled conditions. We employed a randomized complete block design with multiple replications to conduct our experiments. All the experiments were performed 2-3 times to confirm the observations.

The results demonstrated that the soluble secondary metabolites produced by symbiotic bacteria associated with entomopathogenic nematodes possess insecticidal properties against *L. botrana* larvae and *P. spumarius* nymphs and antifungal activity against *B. cinerea* grape rot. Unfiltered bacterial ferments exhibited the highest insecticidal and antifungal capacity compared to bacterial cell-free supernatants studied against *L. botrana* larvae and *B. cinerea*, respectively. Moreover, the crude isolate of the specie *Photorhabdus laumondii* subsp. *laumondii* showed an equivalent antifungal degree to the commercial *Bacillus amyloliquefaciens* (Serenade® ASO fungicide). On the other hand, the so far little studied VOCs produced by the bacteria had a potential control effect on grapevine pests. The VOCs emitted by *Xenorhabdus nematophila* and *P. laumondii* subsp. *laumondii* ferments demonstrated feeding and anti-oviposition deterrent effects against *L. botrana* larvae and adult instars, respectively. Furthermore, the VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferments inhibited > 60 % of *B. cinerea* mycelial growth in *in vitro* tests and limited the lesion area of *B. cinerea* to 0.5 % and 2.2 % on the grapes, respectively. Finally, we tested whether the VOCs may have a preventive effect on the grapes. The results showed that the volatile natural products emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* showed potential for inducing preventive effects on both damaged and intact post-harvest grapes, protecting against potential *Botrytis* rot infection. The mechanisms are still undescribed.

In conclusion, the findings suggest that *Xenorhabdus* and *Photorhabdus* hold significant potential as effective biocontrol agents for managing pests and diseases associated with vineyards. These symbiotic bacteria offer a promising source of new biotools for sustainable viticulture.

Resumen

La vid es uno de los cultivos socioeconómicos más importantes a nivel mundial. Sin embargo, el sector vitivinícola se enfrenta a numerosos desafíos en la actualidad, desde el cambio climático y diversas formas de degradación ambiental hasta graves brotes de plagas y enfermedades. Para afrontar estos retos y proteger los viñedos es necesario desarrollar nuevas herramientas sostenibles. En este contexto, las bacterias simbióticas de nematodos entomopatógenos, específicamente *Xenorhabdus* spp. y *Photorhabdus* spp., emergen como posibles agentes de biocontrol debido a su capacidad para producir compuestos bioactivos con propiedades insecticidas y antifúngicas. Nuestra hipótesis fue que estas bacterias simbióticas y sus subproductos podrían gestionar de manera efectiva ciertas plagas y enfermedades que representan amenazas significativas para las vides. Por tanto, los objetivos específicos de esta Tesis fueron los siguientes: (*i*) evaluar el efecto insecticida y antifúngico de las toxinas solubles producidas por *Xenorhabdus* y *Photorhabdus* para controlar plagas y enfermedades en viñedos; (*ii*) investigar la actividad disuasoria y antifúngica de los compuestos orgánicos volátiles (COVs) emitidos durante las fermentaciones de *Xenorhabdus* y *Photorhabdus*; y (*iii*) evaluar si los metabolitos secundarios bacterianos pueden alterar los mecanismos de defensa de las uvas.

Se llevaron a cabo experimentos *in vitro* para evaluar las propiedades insecticidas y antifúngicas de varios bioproductos derivados de *Xenorhabdus* spp. y *Photorhabdus* spp. Para obtener estos bioproductos bacterianos, se inocularon colonias individuales de cada cultivo puro en medio de cultivo *Triptone Soya Broth* (TSB), manteniendo los cultivos líquidos durante 72 horas bajo agitación orbital continua (150 rpm) a una temperatura de 22 ºC en oscuridad. Los subproductos bacterianos resultantes incluyeron los metabolitos secundarios solubles (sobrenadantes libres de células bacterianas y fermentos sin filtrar), los compuestos orgánicos volátiles (COVs) emitidos por los fermentos y las bacterias crudas aisladas. Para nuestros bioensayos, utilizamos dos especies de insectos: *Philaenus spumarius*, el principal vector responsable de transmitir *Xylella fastidiosa* en Europa, el agente causante de la enfermedad de Pierce en vid;

y *Lobesia botrana*, una de las plagas más significativas económicamente que afecta a los viñedos europeos. En cuanto a las enfermedades del viñedo, se seleccionó *Botrytis cinerea*, el patógeno responsable de la podredumbre de la uva. Los experimentos se llevaron a cabo en condiciones controladas. Se emplearon diseños de bloques completamente al azar con múltiples repeticiones (de 2 a 3 veces) para confirmar las observaciones.

Los resultados demostraron que los metabolitos secundarios solubles producidos por las bacterias simbióticas asociadas a nematodos entomopatógenos poseen propiedades insecticidas contra larvas de *L. botrana* y ninfas de *P. spumarius*, así como actividad antifúngica contra la podredumbre de la uva causada por *B. cinerea*. Los fermentos bacterianos sin filtrar exhibieron la mayor capacidad insecticida y antifúngica en comparación con los sobrenadantes libres de células bacterianas estudiados contra larvas de *L. botrana* y *B. cinerea*, respectivamente. Además, el aislado crudo de la especie *Photorhabdus laumondii* subsp. *laumondii* mostró un grado antifúngico equivalente al del fungicida comercial *Bacillus amyloliquefaciens* (Serenade® ASO). Por otro lado, los COVs emitidos por las bacterias, que hasta ahora han sido poco estudiados, mostraron potencial para el control de plagas en las vides. Los COVs emitidos por los fermentos de *X. nematophila* y *P. laumondii* subsp. *laumondii* demostraron efectos disuasorios para la alimentación y la oviposición contra larvas de *L. botrana* y estados adultos, respectivamente. Además, los COVs emitidos por los fermentos de TSB de *X. nematophila* y *P. laumondii* subsp. *laumondii* inhibieron más del 60 % del crecimiento micelial de *B. cinerea* en pruebas *in vitro* y limitaron el área de lesión de *B. cinerea* al 0.5 % y 2.2 % en ensayos en uvas, respectivamente. Finalmente, se investigó el efecto preventivo de los COVs en las uvas, demostrando que los compuestos volátiles naturales emitidos por *Xenorhabdus* y *Photorhabdus* tenían el potencial de inducir efectos preventivos tanto en uvas dañadas como en uvas sanas después de la cosecha, protegiendo contra posibles infecciones por podredumbre causada por *B. cinerea*. Los mecanismos específicos son aún desconocidos.

En conclusión, nuestros hallazgos sugieren que *Xenorhabdus* y *Photorhabdus* tienen un gran potencial como agentes de biocontrol para el manejo de plagas y enfermedades asociadas a los viñedos. Estas bacterias simbióticas ofrecen una fuente prometedora de nuevas herramientas biológicas para una viticultura sostenible.

1. Introduction

"Mirar desde el prisma de la sostenibilidad de la vida nos lleva a asumir la urgencia en ponernos de acuerdo"

Yayo Herrero. Interviewed by Santiago Canales, Journal *El Salto*, 2018

1.1. Status of viticulture in a global-trade context

1.1.1. Towards sustainability

The current context of viticulture

Viticulture encompasses the cultivation, protection, and harvest of grape-bearing vines in vineyards (Daane et al., 2018). From the early fifties until the mid-eighties, the grape industry experienced massive growth in acreage and value (Brostrom & Brostrom, 2008; Venkitasamy et al., 2019). This development entailed certain challenges, which were similar to those of other annual crops: increasing inputs of agrochemicals, increasing international trade, improving global incomes, and changing policies and remarkable technological innovations in production, storage, and transport (Daane et al., 2018). Currently, the vineyards cover an approximate extension of 7.3 million ha worldwide (~ 0.15 % of the worldwide cropping acreage) and produce around 1 ton of grapes per ha per annum (OIV, 2019) (Figure 1). The winemaking sector uses around 50 % of the cultivated grapes, with the remaining portion being consumed as fresh table grapes and dried grapes, or transformed into non-alcoholic grape juice or grape musts, whether concentrated or not (FAO-OIV, 2016) (Figure 1). The grape industry is a sector of great economic relevance (~32.000 million $ per year) (OIV, 2019), which is particularly important in some regions that often lack other viable economic alternatives and where viticulture plays a crucial cultural role (Masson et al., 2021). In addition to the benefits of grape and wine production, viticulture shapes picturesque landscapes with significant value for tourism and leisure, helping the socio-economic development of wine regions (Getz & Brown, 2006).

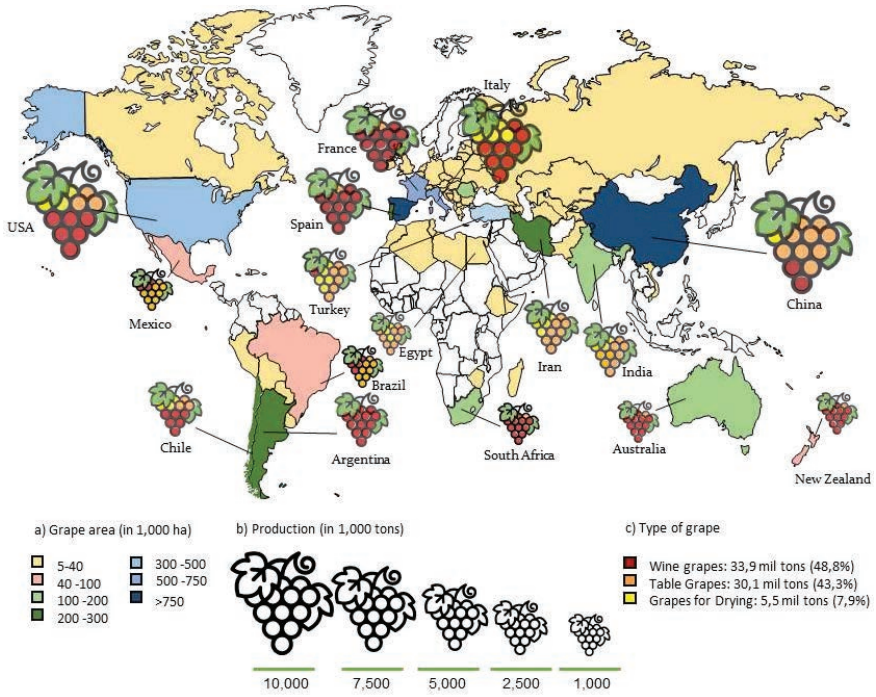

Figure 1. Major grape production worldwide: (a) the total land area dedicated to vineyards, (b) grapefruit production in each of the 16 most productive countries, and (c) the percentage of grape commodities (wine, table fruit or dried fruit) in each of the 16 most productive countries. Map adapted from Daane et al. (2018), with icons based on data from OIV Report (2022).

Grape production has a long history of cultivation and breeding for various soils and climates. Vineyards are present on all continents and climates, from tropical to temperate to desert regions (Figure 1). Lately, there have been changes in grape production patterns in response to factors such as climate change, human land use, and alterations due to pests and diseases, which induce variations in the distribution of vineyards (Hannah et al., 2013). The traditional territorial concentration of production in a limited number of countries, namely Italy, France, the United States, Spain, and Turkey, has been replaced by increased territorial diversity since the late 1980s, both in countries of the northern hemisphere (China, Egypt, India, Mexico, or Turkey) and the southern hemisphere (Chile, South Africa, Brazil, or Peru) (FAO-OIV, 2016). However, predictions in the sector reflect the imminent challenges it will encounter in the short term. Given the

results of Hannah et al. (2013), the suitable area for viticulture may decrease by 19 % to 73 % in different wine-producing regions by 2050 due to climate change. In addition, global warming may cause the establishment of vineyards at higher altitudes, which would increase impacts on upland ecosystems and may lead to natural landscape conversion, such as the relocation of production to higher latitudes in areas like western North America (Hannah et al., 2013; Santos et al., 2020). Furthermore, under future climate conditions, grape-producing regions may face an increased risk of pests and diseases, in principle requiring stronger plant-protection measures, when environmental impacts from phytosanitary treatments need to be reduced (Butt & Copping, 2000; Santos et al., 2020). These predictions highlight the necessity for sustainable adaptation and conservation efforts to anticipate the mentioned direct effects and challenges.

Sustainability in viticulture

Nowadays, agricultural and food systems face many challenges, from climate change and various forms of environmental degradation to the health and welfare of livestock, agricultural workers, and farmers (Santos et al., 2020; Tang et al., 2021). In 2015, the United Nations established the Sustainable Development Goals (SDGs) as a universal call to action to end poverty and protect the planet by 2030 (UN, 2015). However, developing a sustainability framework for the agricultural system requires a clear understanding of the concept of sustainability itself (Baiano, 2021). In 1994, John Elkington introduced the concept of The Triple Bottom Line in hopes of transforming the current financial accounting-focused business system to adopt a more comprehensive approach to measuring impact and success (Elkington, 1998). Following this concept, *sustainable agriculture* emerges as a global strategy encompassing agricultural production and processing systems that can transcend conventional business practices, finding The Triple Bottom Line balance of sustainability that compromises economy, environment, and social well-being (Figure 2).

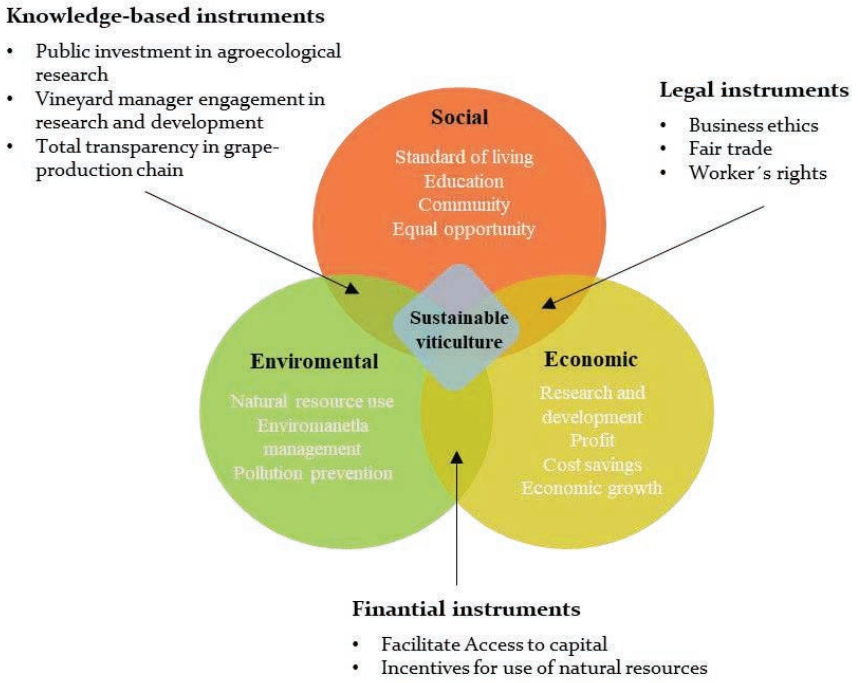

Figure 2. The Triple Bottom Line balance of sustainability that comprises economy, environment, and societal well-being. Adapted from Daane et al. (2018).

This concept was embraced by the International Organisation of Vine and Wine (OIV) which, in 2008, in response to industry and consumer demands, developed a set of guidelines for sustainable vitiviniculture (OIV, 2008). In this Resolution, "sustainable vitiviniculture" is defined as:

> a global strategy on the scale of the grape production and processing systems, incorporating at the same time the economic sustainability of structures and territories, producing quality products, considering requirements of precision in sustainable viticulture, risks to the environment, products safety and consumer health and valuing of heritage, historical, cultural, ecological and landscape aspects. (p. 2)

Since then, the viticulture and winemaking sectors have been at the forefront of these challenges, due to their business nature and historic ecological stewardship (Jones, 2011; Mariani & Vastola, 2015). Over the past decades, according to Moscovici & Reed (2018), the number of sustainability

certifications in the grape industry has quickly grown, including different production models, such as organic or biodynamic, and certifications for wineries, vineyards, or both. These data provide valuable insights into the development of these sustainability measures, their membership levels over time, the procedure of becoming certified as sustainable, and their plans for future certification (Moscovici & Reed, 2018).

In both public discourse and scientific literature, debates about the future sustainability of viticulture are increasingly framed in differentiating between *conventional* and *organic* system models (e.g., Borsato et al., 2020; Ostandie et al., 2022; Puig-Montserrat et al., 2017; Therond et al., 2017). However, understanding what these terms mean can be complex, mainly because the category of conventional agriculture has little analytical acceptance and no legal definition (Sumberg & Giller, 2022). In the present Thesis, *conventional* viticulture refers to intensive grape production with high degree of tillage and agrochemical inputs. In contrast, *organic* viticulture entails the production of high-quality grapes that minimizes the use of inputs in the vineyard and has specific legal requirements, which vary across countries (Provost & Pedneault, 2016; Seufert et al., 2017). Understanding and analysing both models is essential to decide the steps towards the viticulture of the future.

Conventional versus organic viticulture

Conventional viticulture is often considered to have a significant environmental impact that reduces biodiversity and functionality through the use of destructive agricultural practices, such as intensive tillage, high chemical fertilization, and frequent and intensive use of herbicides and other pesticides. On the other hand, organic viticulture is recognised as an agroecological approach to vineyard management, considering the vineyard as an ecosystem where all resources are optimised to maintain a balance and where low inputs are used (Thiollet-Scholtus et al., 2021; Wezel et al., 2020). Despite the widespread knowledge that organic agriculture benefits biodiversity and ecosystem services, while conventional viticulture damages agroecosystems, it is still difficult to draw a single picture of what environmental sustainability in vineyard management means and how to measure it (Ostandie et al., 2022; Thiollet-Scholtus et al., 2021).

Environmental sustainability comparison

Traditionally, environmental sustainability parameters in vineyards have been studied separately (e.g., Blanco-Pérez et al., 2022b; Brunori et al., 2016; Probst et al., 2008), and very few long-term sustainability assessments of viticulture systems have been reported. Most of them were performed at only one experimental site, and none performed a full multivariate analysis to assess the diversity of the systems (Thiollet-Scholtus et al., 2021). Recently, a novel approach has emerged to compare the environmental performance of conventional and organic agricultural systems by studying multiple ecological functions and ecosystem services; that is, its ecosystem multifunctionality (Garland et al., 2021). Recent works explore the ecosystem multifunctionality in the vineyard using four main parameters: vineyard management indicators, water footprint, carbon footprint, and economic performance (Borsato et al., 2020). In line with Lamastra et al. (2016), vineyard management indicators include (*i*) pest and disease management, (*ii*) soil management (erosion and compaction), (*iii*) fertility management (soil organic matter management and fertilizer application), and (*iv*) biodiversity management. The water footprint indicator is an explicit spatial-temporal indicator of freshwater use that depends on climatic conditions, management options, and vineyard features (Lamastra et al., 2014); and the carbon footprint is the amount of direct and indirect CO_2 emissions caused by grape production, which is related to local characteristics, climate, land, agricultural practices, and grape type (Marras et al., 2015). Economic performance will be further developed when discussing the socio-economic comparison.

Focusing first on vineyard management indicators, pest, and disease management in conventional vineyards has lately become an integrated system in most grape countries (Integrated Pest Management, IPM), but it is still a highly pesticide-demanding crop. For example, nowadays, grape production in Spain occupies 5.5 % of the total area devoted to agriculture and consumes nearly 60 % of the total pesticides employed in the country (97 % of them are fungicides) (Ministerio de Agricultultura, Pesca y Alimentación, 2019). These data are similar across the European Union (EU) and there is still limited implementation of the measures mandated by Directive 2009/128/EC, which aims to promote the principles of IPM for a more sustainable use of pesticides and which has been in force for over a

decade (European Court of Auditors, 2020; Lázaro et al., 2021). IPM is a holistic enterprise that emphasizes a systems approach. It is based on agronomic, mechanical, physical, and biological principles, resorting to the selective use of pesticides when addressing situations that cannot be successfully managed with other tools (Barzman et al., 2015). However, the use of tillage, the absence of soil cover, and mineral fertilization, standard tools in conventional viticulture, are significantly detrimental to all soil biodiversity (Karimi et al., 2020). For Borsato et al. (2020), the indicators of soil organic matter, soil compaction, and erosion showed more harmful effects in conventional vineyards than in organic vineyards due to different factors such as the lack of covered surface throughout the year, soil tillage practices, and the absence of grass.

On the other hand, the grape industry also implements an organic production system that minimizes the incidence of disease and pests and, consequently, reduces the use of pesticides, such as copper-based fungicides, without compromising crop productivity (Provost & Pedneault, 2016) (Figure 3). Pest and disease management in organic viticulture is based on increasing biodiversity and promoting the maintenance or improvement of ecosystems. Therefore, organic agriculture has tremendous environmental benefits beyond the management of biotic threats that are easily transferable to small-scale farms, such as providing biological control agents with food and habitat (Provost & Pedneault, 2016). Moreover, organic viticulture can provide further ecosystem services, for example, increased carbon stock in soil and related fauna (Karimi et al., 2020), which enhances benefits for plant growth, and reduces soil erosion and compaction (Brunori et al., 2016; Puig-Montserrat et al., 2017). Finally, different studies have shown how the use of agroecological activities within organic management increases water security and soil water retention, thus saving water, reducing vulnerability to water scarcity, and decreasing the water footprint (Michos et al., 2018). In addition, organic management can reduce the carbon footprint by lowering energy input with higher energy efficiency, which results in lower greenhouse gas emissions (Borsato et al., 2020; Michos et al., 2018). In conclusion, organic management appears to be fully environmentally sustainable, while conventional viticulture, on the other hand, requires many inputs (Figure 3).

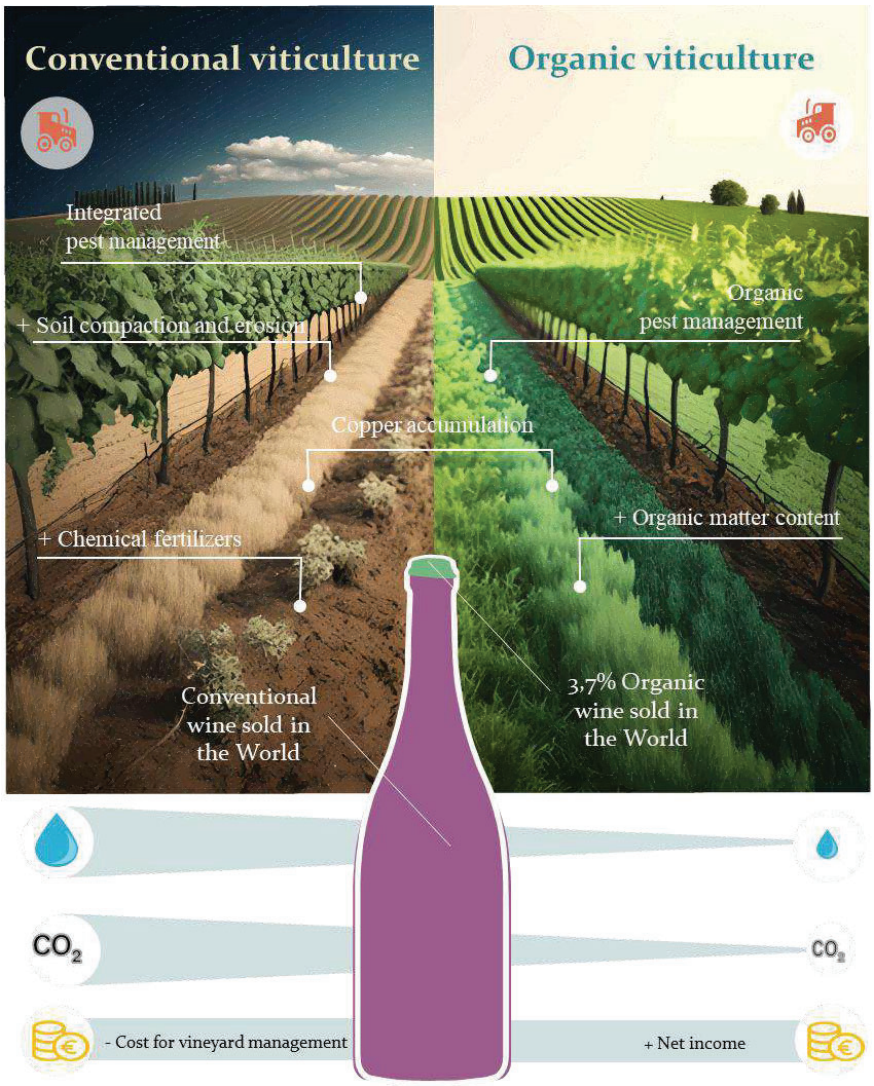

Figure 3. Comparison between conventional and organic viticulture. The vineyard management indicators showed that conventional viticulture inside IPM allows the use of synthetic pesticides (EU Directive 2009/128/EC), while organic pest management is based on organic pest management techniques. Both systems shared the use of inorganic pesticides (copper and sulphur-based pesticides), showing the current accumulation of high amounts of copper in the soil (Karimi et al., 2021). Organic viticulture demonstrates higher organic content (Borsato et al., 2020), soil biodiversity (Karimi et al., 2020; Probst et al., 2008), and benefits aboveground biodiversity (Ostandie et al., 2021a, 2021b, 2022). On the other hand, conventional

viticulture shows higher soil compaction, erosion, and chemical fertilizers (Borsato et al., 2020). The water and carbon footprints are significantly higher in conventional vineyards (Borsato et al., 2020). Finally, conventional management gains a higher net income and marginal benefit due to the lower cost of maintenance (Falcone et al., 2016). Background image created by OpenAI.

Socio-economic comparison

As with any intensive cultivation, conventional viticulture focuses mainly on economic performance, giving little importance to environmental and social issues, as described above. Generally, producers claim that organic viticulture has a lower yield, which might become an obstacle to achieving food security worldwide. However, this fact is not universal, since different studies have shown that organic vineyards, despite producing fewer grapes, have lower production costs due to lower provisioning services compared to conventional systems (Ostandie et al., 2022). The rate of conversion of vineyards to organic production has increased considerably since the beginning of the last twenty years. Approximately, the area of certified organic vineyards increased an average of 13 % per year, while the area of conventional vineyard decreased an average of 0.4 % in the same period (OIV, 2021). One of the factors explaining this intense growth rate is that certified organic viticulture is still a recent phenomenon (OIV, 2021), besides political promotion towards this agricultural model (European Commission, 2020).

Wine industry is the leading economic sector in the grape industry. Currently, wine macro trends have remained stable, and consumption seems to reach a plateau (OIV, 2019). By 2022, the volume sold has increased to 281 million cases, worth 32,900 million dollars. Within these wine statistics, interesting regional and segmental dynamics are affected by broader economic trends of increasing population growth and disposable income, particularly in Asia. And sub-categories within the wine industry—such as rosé and organic wines—have shown remarkable growth, while other segments have lagged behind. Another statistical fact is that although society has increasingly mobilized against conventional viticulture practices, the proportions of cultivated areas have changed very little (Masson et al., 2021). In 2020, around 7.3 % of the world's wine grape vineyards were certified as organic vineyards (a total of 506 million hectares) (Willer et al., 2022). These vineyards have significantly lower

yields on average compared to conventional vineyards, so wine produced from organic grapes likely sits at 3 % of the total. Organic vineyards are found predominantly in Europe, with 431 million hectares giving the continent 80 % of the world's total. Within this, 90 % of Europe's organic vineyards are found in Spain, France, and Italy (Willer et al., 2022). Besides the wine sector, global table grape production and consumption increased from 15.7 million tonnes to almost 27 million tonnes from 2000 to 2014, with the remarkable cases of the People's Republic of China and India being the main drivers of growth (FAO-OIV, 2016). This fact is also reflected in California, where annual per capita table grape consumption grew from 1.8 to 3.5 kg between 1980 and 2001 (Daane et al., 2018).

From the producers' perspective, the priority is to fill the remaining substantial knowledge gaps in terms of perceived environmental and economic benefits and costs. Therefore, further research effort focused on the costs and benefits of different viticultural practices and technical assistance for implementation could provide them with that knowledge. Moreover, specific marketing strategies are needed to enhance consumers' involvement and attitude toward sustainable wine. This would improve understanding and use of sustainability labels and claims, and would raise awareness about some environmental credentials of wine packaging (Mariani & Vastola, 2015). Organic food is generally considered safer than conventional food, which is an important incentive for consumers and their commercial choices. Thus, the decision between organic and conventional wine depends on the consumer's perception of wine quality and their ability to pay a higher price for the product, which depends on consumer segmentation, i.e., societal class of origin (Costa et al., 2016). Finally, the willingness to pay is generally higher for organic wine or food due to the acknowledgement of a product with a supposed better quality and greater attention to the environment (Sandhu et al., 2010).

1.1.2. Pests and pathogens challenges in vineyards

Current context, causes and consequences of the pest and diseases challenges

In natural ecosystems, plants interact with a diverse community of organisms, from arthropods to soil microorganisms (Fernández de Bobadilla

et al., 2021). Some of these interactions benefit plant fitness, such as interactions with pollinators or plant growth-promoting microorganisms. Other interactions negatively impact plant fitness because they involve organisms that consume plant tissues, such as herbivorous insects, or phytopathogens, that can cause diseases. In agroecosystems, herbivorous insects or phytophagous mites become 'pests', and pathogenic fungi, bacteria, and viruses become 'pathogens' via two main modes of evolution, either through previous association with the wild crop ancestor or by host shifts from native plants to introduced crops (Chen & Schoville, 2018). Pathogens and pests are organisms that are harmful to the plant and reduce the yield and quality of agricultural products in crops (Savary et al., 2017). They cause substantial agricultural losses from overall production to physical availability, distribution, economic access, production stability, quality and nutritional value (Savary et al., 2019). Every year, up to 40 % of global food crops are lost to plant pests and diseases, leading to annual agricultural trade losses of more than $220.000 million (FAO, 2021). However, pathogens and pests are an integral part of human-made agroecosystems. This coevolution, in a broad sense, enhances plant defence and protection mechanisms to prevent or mitigate injuries (Moreira et al., 2018) and shift pathogen and pest infectivity and aggressiveness (growth and reproduction rate) (Mooney et al., 2012). Research efforts and understanding of coevolution between cultivated plants and their pests and pathogens need to expand because it is the way for agricultural entomologists and other experts to comprehend the rapid evolution of pests and pathogens in response to control measures and to develop novel control tactics based on this knowledge (Chen & Schoville, 2018).

In the vineyard agroecosystem, especially during the last 70 years, the vine–pathogen or vine-pest coevolution process has been driven and disrupted by two main anthropogenic factors: the intensification of the viticultural system, and the global market (Figure 4). These have presented different challenges in the control of pests and diseases.

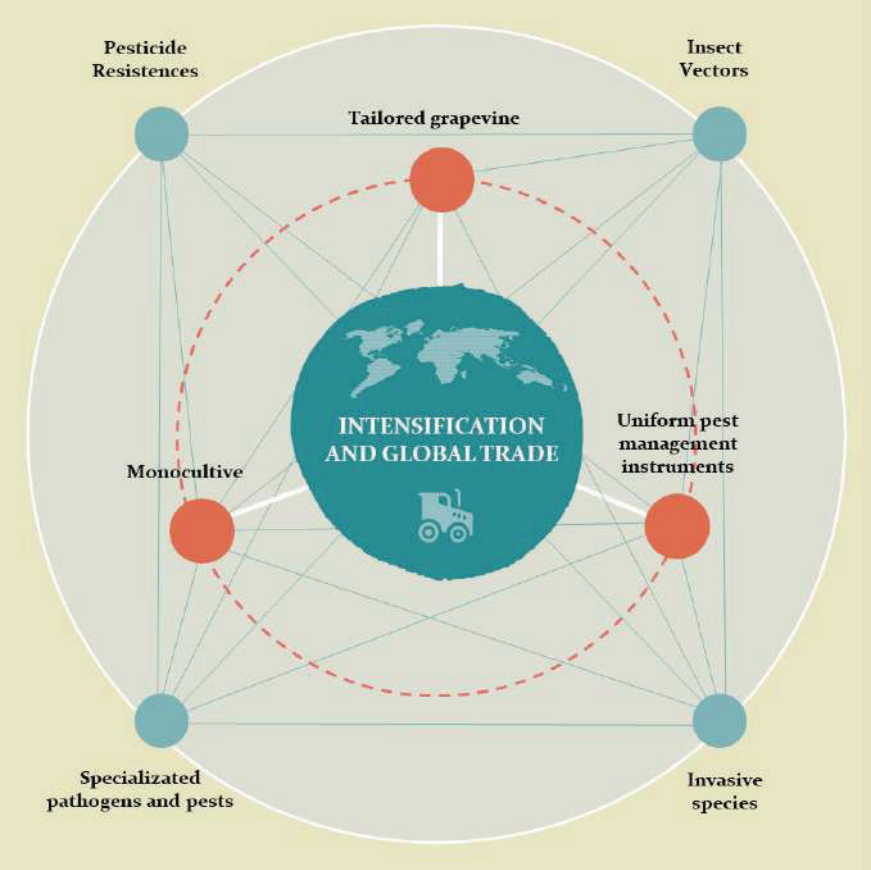

Figure 4. Context, causes and consequences of the current challenges of pest and disease in vineyards (icons from Flaticon).

The intensification of the viticulture system shifted from small-scale and diverse vineyards (generally associated with traditional and organic viticulture) to large-scale, genetically uniform, intensive monoculture vineyards (associated with conventional viticulture). This change has been considered a disruption of co-evolutionary processes. These facts favour outbreaks and intense and large-scale epidemics in vineyards that have become more vulnerable to the attack of phytophagous species. Moreover, conventional intensive vineyards consist mainly of cohorts of genetically adapted vine individuals at the same physiological and phenological stage (Vivier & Pretorius, 2002), where adapted pathogens and pests genotypes can rapidly reproduce. Finally, another critical factor in this

intensification is the uniform and indiscriminate implementation of pathogens and pest management instruments, such as host plant resistance genes or chemical pesticides, which is also a source of instability and outbreaks. For its part, international trade in plant material, machinery, clothing and footwear, grape postharvest products, and winery wastes has induced the expansion of invasive species, which are a great risk to the health of vineyards (Mooney & Cleland, 2001; Simberloff et al., 2013). For these reasons, among the valuable crops recurrently affected by emerging diseases and pests, the grapevine occupies a remarkable place in the history of phytopathology. The intensification of viticulture reduces the total biodiversity and functionality and lead to the extinction of soil biota and aboveground organisms, inducing important changes in the performance of pests and pathogens and making their control challenging (Daane et al., 2018).

Pest and disease challenges

Invasive species

Lasting relationship

The growing international trade in vines, grapes, and equipment increases opportunities for the accidental movement of invasive pests and pathogens. Once landing in the new territory, pathogens and pests can find abundant undefended host plants and disrupt existing pest control programs (Daane et al., 2018). The classic example of this problem in vineyards is *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae). Commonly known as grapevine phylloxera, this pest is an obligate biotroph of *Vitis* spp., capable of feeding on both grapevine roots and leaves and currently present in all wine-growing regions worldwide (Forneck & Huber, 2009). Grape phylloxera was unintentionally introduced into Europe from North America at the end of the nineteenth century (Tello et al., 2019). In susceptible *V. vinifera* (Vitales: Vitaceae) cultivars, grape phylloxera forms abundant root nodosities and tuberosities that occlude the vine vascular system, resulting in loss of leaf surface area and reduced yield. In addition, feeding wounds allow secondary infections by soil-borne pathogens and cause plants to die from secondary infections. The appearance of phylloxera in grapes almost devastated European viticulture and caused the most radical change in viticultural practices in the last two centuries, when grape cultivation

shifted from using own-rooted *V. vinifera* plants to grafting onto partially-resistant non-*vinifera* American *Vitis* spp. or hybrids used as rootstocks.

Another two classic invasive species are *Erysiphe necator* (Erysiphales: Erysiphaceae) and the oomycete *Plasmopara viticola* (Peronosporales: Peronosporaceae). The former causes grapevine powdery mildew and was spread from North America to England in 1845 (Gadoury et al., 2011), while the latter causes the downy mildew and was also introduced from North America to Europe in 1878 through the importation of wild American rootstocks resistant to *Phylloxera* (Gobbin et al., 2006). Both have remained at the centre of disease control efforts ever since and are some of the most harmful grapevine pathogens. On the one hand, *E. necator* is an obligate parasitic of genera within the family Vitaceae, including *Vitis*, *Cissus*, *Parthenocissus* and *Ampelopsis* (Gadoury et al., 2011). The most economically important host is grapevine (*Vitis*), mainly the European grape, *V. vinifera*, which is highly susceptible to powdery mildew. The symptoms of powdery mildew can include red, blotchy areas on dormant canes, chlorotic (loss of greenness) spots on upper surfaces of the leaves, white, powdery or dusty areas, and white, powdery masses settled over the entire berry surface. On the other hand, *P. viticola* is a heterothallic oomycete with a predominantly, if not exclusively, bipolar mating system. The sexual stage includes the fusion of gametes that results in the formation of oospore (Wong et al., 2001). Symptoms on the leaves appear as round yellowish spots (also called "oil spots"). Wind-dispersed sporangia are produced on primary lesions, which, in the presence of foliar moisture, release zoospores that cause secondary infections. The occurrence and number of secondary cycles depend on rainfall events, the presence of dew and lighting conditions; thus, the number of secondary cycles may vary between years or regions (Gessler et al., 2011).

Short-lived relationship

The intensive mechanism and global trade have increased in recent decades, and invasive species still represent new threats even higher than in the previous century, due to changing climatic conditions and international market (Paini et al., 2016). Recent estimates suggest that crop yield losses caused by invasive pests will increase up to 25 % in the European Union (EU) by 2080 (Balog et al., 2017). There are numerous examples in recent

years of the challenges of controlling invasive pests and diseases in vineyards. One of the most important ones is the spotted-wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae) (Asplen et al., 2015). This insect is an invasive Asian pest that was initially detected in Europe and North America in 2008 (Walsh et al., 2011). This fly has a specialized ovipositor that enables it to infest grape varieties that have softer berries, resulting in direct damage and transfer of pathogens that can affect wine quality. Furthermore, *D. suzukii* facilitates *Drosophila melanogaster* (European native species) infestation and sour rot outbreaks in vineyards (Rombaut et al., 2017). Similarly, another example is the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), native from eastern Asia, which emerged as a harmful invasive insect pest in North America and Europe in the 1990s and 2000s, respectively, and was recently reported in Bordeaux vineyards (Delbac et al., 2022). This type of bug is constantly increasing in the south of France (Streito et al., 2020) and is becoming a major concern for French winegrowers. Its adults aggregate in grape clusters during harvest, presenting significant risks to the quality of harvested grapes due to the release of pungent alkaloids (Streito et al., 2020).

As can be observed, in general terms, newer grape-growing regions have fewer arthropod and pathogen problems than older regions. These new regions (e.g., New Zealand, Australia, Canada) are more likely to protect their industry from inadvertent introductions of exotic pests from older regions (e.g., Mediterranean countries). However, exceptions occasionally occur, such as the case of *Lobesia botrana* (Lepidoptera: Tortricidae), the European grapevine moth, which is a relevant pest in European and Middle Eastern vineyards. Recent studies reported *L. botrana* as a new grape pest in the Americas: in Chile (2008) and Argentina (2009) (Gonzalez, 2010; Varela et al., 2013), and in California (2010) (Gilligan et al., 2011). Larvae feed on fruit, causing direct damage and promoting a secondary infection by *Botrytis cinerea* (Helotiales: Sclerotiniaceae). Management of *L. botrana* in California is an excellent example of how co-development of control programs has improved pest management systems and has been essential in identifying, suppressing, and eradicating invasive pests. Coordinated multinational responses to pest invasion have been highly effective. For example, they eradicated the *L. botrana* moth from California vineyards in 2016 by applying *Bacillus thuringiensis* (Bacillales: Bacillaceae) and fruit

extraction techniques. However, *L. botrana* is still present in Argentina and Chile, because this multinational coordination and scientific advice was not followed in the same way as in California.

Finally, another example of invasive species is a Gram-negative bacterium, *Xylella fastidiosa* (Xanthomonadales: Xanthomonadaceae). This pathogen shows a wide range of vectors and host plants, many of which can carry the pathogen for a long time without showing any symptoms. Until recent years, most diseases caused by *X. fastidiosa* had been reported in North and South America. However, in 2013, a widespread infection of olive quick decline syndrome caused by this fastidious pathogen appeared in Apulia (South-Eastern Italy), and several cases of *X. fastidiosa* infection have been reported in other European Countries, aggravating the harmful effects caused by this bacterium (Saponari et al., 2014). In grapevines, *X. fastidiosa* causes Pierce's disease, a lethal grapevine disease that causes significant economic costs (Tumber et al., 2014). In Spain, it was officially detected in a grapevine in Mallorca in May 2017 (Moralejo et al., 2019). Currently, epidemic-risk vineyards with moderate to high growth rates are marginal outside the United States (Giménez-Romero et al., 2022). Nevertheless, there are projections indicating that by 2050, there will be a worldwide extension of regions prone to epidemics, along with slight increases in the rate of disease growth (Giménez-Romero et al., 2022).

Pesticide resistance and declining availability of active substances

Nowadays, vineyards are more susceptible to numerous pathogens and insect pests that significantly reduce the quality and yield of grapes (Daane et al., 2018; Otoguro & Suzuki, 2018). Herbivorous insects have developed sophisticated biochemical defence systems to protect themselves against naturally occurring xenobiotics in their environment (Hawkins et al., 2019; Yang et al., 2021). These systems, which include several families of detoxifying enzymes, are also frequently recruited in the evolution of resistance to synthetic toxins such as chemical insecticides (Yang et al., 2021). Similarly, the emergence of multi-resistant fungal pathogens and the better-publicised threat of antibiotic-resistant bacteria pose a high-level threat to crop protection (Fisher et al., 2018). In addition, long-term use of organic and inorganic (Cu-based) pesticides dramatically accelerates

evolutionary changes in species, especially in commercially important pest and disease organisms (Komárek et al., 2010).

One of the most important examples of fungicide resistance in vineyards is *E. necator*, which is associated with high resistance to sterol demethylation inhibitor fungicides (Savocchia et al., 2004). The results presented by Délye et al. (1997) showed a single mutation leading to the substitution of phenylalanine for tyrosine at codon 136 in 14 alpha-demethylase genes that can make *E. necator* resistant to pesticides. Similarly, *P. viticola* has been reported to acquire resistance to Qo-inhibiting chemical fungicides through single amino acid substitution. The fungus *B. cinerea*, which causes grey mould in grapes, has various mechanisms of resistance to fungicides in the field (Leroch et al., 2011; Leroux et al., 2002). For example, benzimidazole resistance was associated with point mutations at codon 198 (Ben R1) or 200 (Ben R2) of the b-tubulin gene (Leroux et al., 2002). Also, vineyard pests such as *Pseudococcus affinis* (Hemiptera: Pseudococcidae), *P. longispinus* (Hemiptera: Pseudococcidae), and the grape mealybug, *P. maritimus* (Hemiptera: Pseudococcidae), have been reported to have resistance to pesticides (Charles et al., 1993; Flaherty et al., 1982). Economically, there is a large gap between the demand of scientists' alternatives and the actual market need for chemical fungicides alternatives. The market size for these options is very limited compared to the market for chemical fungicides (Seiber et al., 2014). Therefore, grape producers and scientists are always searching for other alternatives to chemical fungicide and pesticide applications for control of fungus in vineyards.

Transmission of grapevine pathogens through insect vectors

In most wine-making regions, the main concern is the transmission of plant pathogens, rather than the damage caused by insect-feeding (Almeida et al., 2013; Giménez-Romero et al., 2022; Hommay et al., 2021). Among the key pathogens are the vector-borne virus species in the family Closteroviridae, *Grapevine leafroll-associated viruses* (GLRaVs), which are present in all grape-growing regions in the world, and primarily affect wine grape varieties (Maree et al., 2013). Several mealybugs such as *P. maritimus*, *Pseudococcus Viburni* (Hemiptera: Pseudococcidae), *Planococcus ficus* (Hemiptera: Pseudococcidae), *Pl. citri* (Hemiptera: Pseudococcidae), and *Parthenolecanium corni* (Hemiptera: Coccidaeare), the main GLRaV

vectors, including common species that have spread to many viticultural regions in the world (Almeida et al., 2013; Hommay et al., 2021). Control is further hampered as mealybugs and viruses can survive in vine roots for years after the vine has been uprooted (Hommay et al., 2021). Another recent example is the vector-borne bacterium *X. fastidiosa*, responsible for Pierce's disease, a lethal grapevine disease that originated in the Americas. To date, the meadow spittlebug xylem fluid-feeding, *Philaenus spumarius* (Hemiptera: Aphrophoridae), has been confirmed to be the main vector of Pierce's disease in European vineyards (Cornara et al., 2018); therefore, its geographic distribution should be taken into account when assessing the risk of Pierce's disease in the EU.

The challenges associated with controlling these pathogen vectors often disrupt sustainability programs, since farmers adopt a zero tolerance for vector species and a greater reliance on insecticides to reduce vectors and slow down the pathogen spread. In these examples, even low vector or pathogen incidence often leads to insecticide applications—even before the life cycle of the vectors, their ecology, or the vector–pathogen–plant interactions are fully understood—, highlighting the increased awareness of the damage caused by invasive species that are also insect vectors.

1.1.3. Sustainable pest and disease management strategies

The current agricultural system, increasingly influenced by the principles of agroecology, seeks to improve farming crops by harnessing ecosystem services, creating beneficial biological interactions between agroecosystem components, and minimizing synthetic and toxic external inputs (Wezel et al., 2020). The current developed pest and disease management programs (e.g., IPM or organic management) are based on indirect and preventative measures followed by more direct and curative measures only when necessary (Zehnder et al., 2007). This evidence has been used for hierarchized pest and disease management strategies based on their impact on agroecosystem health and sustainability (Zehnder et al., 2007) (Figure 5).

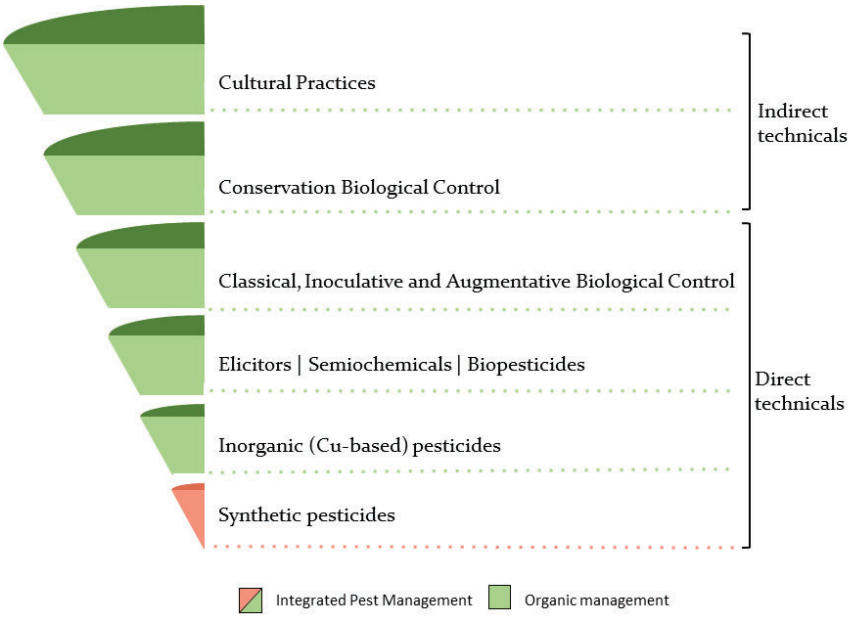

Figure 5. Sustainable pest and disease management strategies. Diagram based on Eilenberg et al. (2001) and Zehnder et al. (2007).

Priority is given to indirect strategies based on preventive actions followed by direct techniques when preventive strategies are insufficient due to the harmful impact of the pest or disease that affects the vineyard. Among the indirect techniques, cultural practices are the basis for sustainable pest and disease management. Their primary objective is to make the crop unavailable to pests in space and time by interfering with oviposition preferences, by host plant discrimination, or by reducing pest survival on the crop by enhancing natural enemies. This strategy is followed by other indirect biological control strategies that can be performed by conservation biological control (Eilenberg et al., 2001). Once indirect control strategies are overcame by pest and disease pressure, direct measures are required. Among these, the most eco-friendly ones are the classical, inoculation, or augmentative biological control, and the use of elicitors, semiochemical cues, and novel pesticides. When all these strategies have failed, farmers have used synthetic pesticides only in conventional viticulture and always under the legal doses (Pertot et al., 2017) (Figure 5).

Cultural practices

Cultural practices are among the oldest techniques used for pest suppression, and many of the preventative practices used in conventional and organic agriculture today have their roots in them (Zehnder et al., 2007). Cultural practices are indirect and preventative measures based on techniques compatible with natural processes such as soil management, resistance of non-transgenic host plant, or good selection of the farming place. These techniques may have opposite effects on different pests, so the selection of specific practices must be based on an overall pest risk assessment (Zehnder et al., 2007). In some cases, cultural practices that can result in soil erosion and environmental degradation (e.g., excessive tillage, summer fallowing, burning of crop residue) are discouraged, but may be allowed under certain circumstances to control specific pests or pathogens (Provost & Pedneault, 2016).

In viticulture, one of the main objectives of cultural practices is to make the vine less attractive to pests. Several approaches have been and continue to be adopted: use of resistant varieties and/or rootstocks (Pertot et al., 2017; Töpfer et al., 2011), vineyard sanitation (such as removal of residuals, canopy management, leaf removal, cutting of wild grapevine and elimination of clusters and pruning of excess wood, or reduction of the cluster compactness), modification of harvest dates, implementation and/or conservation of trap plants, or modulation of irrigation and fertilization schedules (Provost & Pedneault, 2016; Zehnder et al., 2007).

After the introduction of Phylloxera, *E. necator* and *P. viticola* from the Americas to Europe in the late 19th century, the cultivation of traditional *V. vinifera* varieties has no longer been possible without considerable pesticide applications. In viticulture, the first major cultural practice used to reduce the occurrence of an aggressive pest was the grafting of *V. vinifera* varieties onto *Vitis* spp. from North American rootstocks, which show high resistance to Phylloxera (Powell et al., 2013). This practice has been the main control against this pest for almost 150 years, and new rootstocks continue to be bred to constrain the resistance (Powell et al., 2013). The optimal selection of grape varieties is advantageous to successfully control fungal diseases (Pedneault & Provost, 2016) and insect pests by enhancing the

presence of predators in a variety-dependent manner (Ostandie et al., 2021b).

Furthermore, operations such as soil tillage and the use of mulches may contribute to enhancing the insect pest control by favouring the activity of beneficial microorganisms, modulating the dispersion and abundance of pests, and increasing the occurrence and diversity of natural enemies (Blanco-Pérez et al., 2022a, 2022b; Sáenz-Romo et al., 2019). The implementation of an optimal irrigation program can affect vine vigour and, consequently, equally modulate the pest and predatory populations (Irvin et al., 2016). Finally, vineyard sanitation, such as canopy management, leaf removal, cutting and bunch removal and pruning of remaining woods, and reduction of the bunch compactness, are other ways to reduce pest and disease incidence in organic vineyards (Caffi et al., 2013).

Biological control strategies

Biological control is based on reducing diseases and pests through the activity of biological control agents. Thus, Eilenberg et al. (2001) restrict the term *biological control* to the use of living organisms, and outline four biological control strategies: (*i*) *conservation* biological control, (*ii*) *classical* biological control, (*iii*) *inoculation* biological control, and (*iv*) *augmentative* biological control. *Conservation* biological control is an indirect and preventative measure based on modifying the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests. In the same cases, it can be difficult to clearly distinguish between cultural control and conservation biological control. Eilenberg et al. (2001) consider that conservation biological control is practiced when specific natural enemies are protected and enhanced to obtain control of specified pests, while cultural control tends to directly target the pest population and not the biological control agent. *Classical, inoculation*, and *augmentative* biological controls are direct and curative techniques that are necessary when indirect measures are not enough to ensure the vineyard's health.

Conservation biological control

Conservation biological control in agroecosystems requires a landscape management perspective due to the abundance and diversity of increasing

natural enemies in response to varied conservation measures, including plant and habitat diversification, reduction in cropping intensity, and increased landscape complexity (Begg et al., 2017; Tscharntke et al., 2007). Conservation biological control management seeks to maximize ecosystem services, such as pest regulation, by enhancing the impact of the natural enemy through the manipulation of plant-based resources in the landscape. Conservational biological control measures in viticulture are broad. For example, annual cover crops are widely employed in vineyards that bloom quickly in a given year (Sáenz-Romo et al., 2019) and can then be cut or incorporated into the soil to provide soil nutrients. Cover crops reduce the grapevine's vegetative vigour and its susceptibility to grey mould and downy mildew (Valdés-Gómez et al., 2008, 2011). Furthermore, they provide several vital resources for parasitoids and predatory arthropods, such as permanent vegetation cover suitable for over-wintering, refuge from disturbance, and resources such as alternative prey, pollen and nectar (Nicholls et al., 2000; Sáenz-Romo et al., 2019). This tactic is most common in regions with regular rainfall that favours the growth of grasses and broadleaf cover crops, such as clover, which provide resources for beneficial insects and are easily managed with tilling, mowing, or herbicides.

Classical, inoculation, and augmentative biological control

Classical biological control is based on the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control. Inoculative biological control is based on the deliberate release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently. In contrast, augmentative or inundative biological control consists in using living organisms to control pests when control is achieved exclusively by the released organisms, often in high concentrations and with ephemeral activity.

Different examples of classical, inoculative and augmentative biological control strategies have been performed in vineyards. An example is the use of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae), which has been used around the world to suppress *Pl. citri* and *Pl. ficus* (Daane et al., 2004; Fallahzadeh et al., 2011). Another example is the use of the egg parasitoid *Trichogramma* sp. against different species of grape berry moths

such as *Endopiza viteana* (Lepidoptera: Tortricidae) or *L. botrana* (Nagarkatti et al., 2002). Furthermore, application of *Trichoderma* species to grapevine pruning wound surfaces reduces wound infection by trunk pathogens that cause grapevine decline (e.g., *B. cinerea*) (Mutawila et al., 2016). Likewise, Phytoseiid mites (Acari: Tetranychidae, Phytoseiidae) are well-known biological control agents of phytophagous Tetranychid spider mites such as *Tetranychus urticae* or *Eutetranychus carpini* (Prischmann et al., 2002; Sáenz-Romo et al., 2019). Another possible control agent proposed in recent years is the foliar application of entomopathogenic nematodes for the management of aerial arthropod pests (Campos-Herrera et al., 2021).

Elicitors

Elicitors are stress stimuli capable of inducing defence responses in plants which are similar to those induced by pathogen infection, but without any detrimental effects on the plant (Cabrera-De la Fuente et al., 2018; Delaunois et al., 2014; Thakur & Sohal, 2013). There are two type of elicitors: (*i*) physical elicitors, such as light, salinity, or temperature, which produce injury (Thakur & Sohal, 2013); and (*ii*) chemical elicitors, either of biotic origin, such as oligosaccharides, yeast derivatives, or protein fragments (Lemaitre-Guillier et al., 2021); or of abiotic origin, such as metal ions (Thakur & Sohal, 2013) (Figure 6). Elicitor-induced plant resistance against diseases is an attractive strategy to reduce the use of synthetic agrochemicals because they trigger plant defence reactions, resulting in antimicrobial production and/or cell-wall reinforcement that stops or limits subsequent pathogen infection (Garcia-Brugger et al., 2006).

The use of elicitors as alternatives to agrochemicals in the prevention of grape diseases and infections has also a great impact on the quality components of the grape (Gutiérrez-Gamboa et al., 2019). Numerous studies intending to improve wine aroma quality have researched the effects of different elicitors on the volatile compositions of grapes, such as chitosan (Gutiérrez-Gamboa et al., 2019). However, its impact varies depending on several factors such as grape cultivar, type of elicitor, and dose (Salifu et al., 2022). Physical and abiotic elicitors trigger the plant's production of benzothiadiazole or methyl Jasmonate to respond to the stress stimuli (Ding et al., 2002). Among these types of elicitors, silica (Si) has recently been

found to directly enhance induced resistance in plants attacked by pests, by acting as a signal to induce systemic chemical defences in plants. On the other hand, the efficacy and mode of action of some biological origin elicitors have already been reported in grapevine, especially for *Trichoderma fungi* (Lazazzara et al., 2021; Palmieri et al., 2012) or *Bacillus* bacterial species (Ongena et al., 2007; Pérez-García et al., 2011) having a positive effect on increasing the vine's defences.

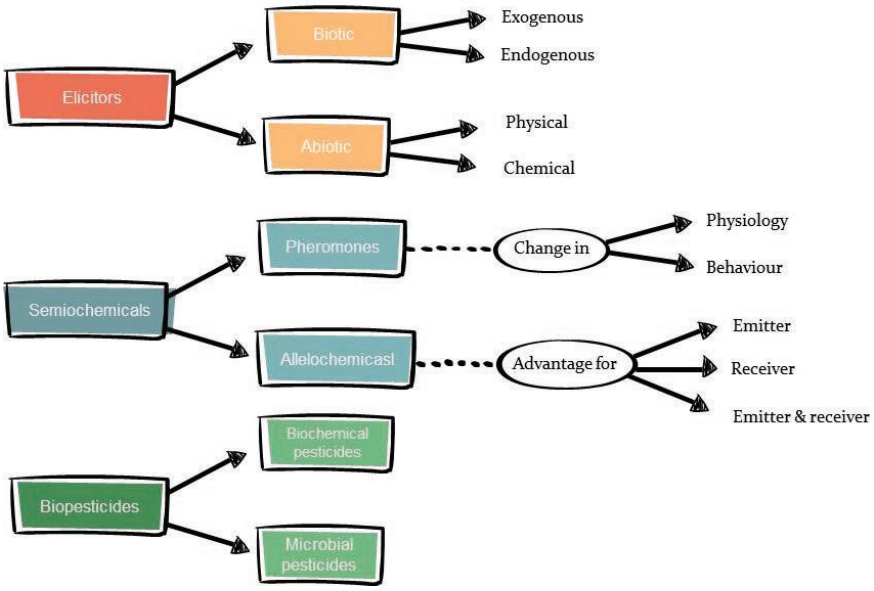

Figure 6. Classification of elicitors, semiochemicals and biopesticides based on the normative of the European Union, based on Kost (2008) and Vicente-Díez et al. (2023).

Semiochemicals

The use of chemical signals for the management of pests and diseases is one of the most promising alternatives in organic crops. *Semiochemicals* are compounds involved in chemical communication between organisms (Kost, 2008) (Figure 6). They are divided into pheromones and allelochemicals. Pheromones are chemical signals that carry information from one individual to another member of the same species (interspecific). These include sex attractants, trail-marking compounds, alarm substances, and other intraspecific messages. On the other hand, allelochemicals are signals

that travel from one organism to a member of a different species. These include defensive signals such as repellents, compounds used to locate suitable host plants, and a vast array of other substances that regulate interspecific behaviours (Kost, 2008).

In their natural habitat, semiochemicals are involved in many interactions between the different trophic levels, involving insects, plants and hosts for parasitoids or prey for predators (Leroy et al., 2011). In vineyards, pheromone mating disruption fits well into an area-wide pest management approach and was successfully used to improve control of two crucial pests of Tortricid moth (*L. botrana* and *Eupoecilia ambiguella*) (Ioriatti & Lucchi, 2016). The most important example of using sex pheromones for mating disruption was with the grapevine moth *L. botrana*. Field development of this tool occurred in Europe, where *L. botrana* mating disruption is now applied on ~140,000 hectares, or about 3–4 % of the grape-growing area, providing a highly selective and environmentally acceptable control tool (Benelli et al., 2023; Shapira et al., 2018). On the other hand, the use of allelochemicals has been much less researched and implemented in agriculture (Calcagnile et al., 2019). Nevertheless, additional evidence supports the hypothesis that allelochemicals play a role in trans-kingdom interactions and can play a significant part in future pest and disease management.

Biopesticides

Biopesticides are one of the most promoted tools in pest management as a possible alternative to synthetic pesticides (Figure 6). The European Union defined biopesticides as products derived from a biological origin and distinguishes only two categories: biological control products and microbial biological control agents; however, it does not recognise genetically modified plants as biopesticides (Vicente-Díez et al., 2023). The European regulatory framework legislation on genetically modified food and feed is the strictest worldwide (EU, 2003). Biopesticides currently comprise a small part (~5 %) of the global crop protection market, valued at 3.000 million dollars in 2015 (Damalas & Koutroubas, 2018). However, biopesticide use steadily increases by 10 % yearly (Kumar & Singh, 2015). More than 200 products have been sold in the US market, compared to only 60 similar products in the EU, due to its lengthy, expensive, and cumbersome approval

procedures of biopesticides compared to the rest of the world (Balog et al., 2017; Damalas & Koutroubas, 2018; Scheepmaker et al., 2019).

Biopesticides can be used in organic and conventional agriculture and enhance agroecological systems. Compounds derived from natural sources (plant, animal, bacteria, fungi, and other origins) have the potential to be used for food safety and crop protection due to their antimicrobial properties against a broad range of pathogens and pests (Gyawali & Ibrahim, 2014). Nevertheless, over 90 % of microbial biopesticides are derived from the single bacterium *B. thuringiensis* (Bacillales: Bacillaceae) (*Bt*) and several sub-species of *B. thuringiensis* (Lacey et al., 2015). These bacterial species produce insecticidal proteins during the sporulation phase as parasporal crystals (also called δ-endotoxins), predominantly comprising one or more proteins (Cry and Cyt toxins), thus disrupting the gut of the ingested insect. These completely biodegradable toxins are highly specific for their target insects but innocuous to humans, vertebrates, and plants (Bravo et al., 2007). In vineyards, *Bt* has been employed mainly for the control of *L. botrana* (Ifoulis & Savopoulou-Soultani, 2009) and its *Bacillus*–based products are used for the control of *B. cinerea* (e.g., *Bacillus amyloliquefaciens*) (Jacometti et al., 2010). The results obtained by Bae et al. (2004) showed that *B. thuringiensis* did not inhibit *Saccharomyces cerevisiae* in agar culture or during alcoholic fermentation of grape juice. Although it inhibited malolactic bacterium, *Oenococcus oeni* (Lactobacillales: Leuconostocaceae) in agar culture, it did so during mixed cultures in liquid medium.

Inorganic (copper- and sulphur-based fungicides) and synthetic pesticides

The prolonged sulphur and copper applications are related to the adverse effects on non-target arthropods (Reiff et al., 2021) and the accumulation of copper in the topsoil of many vineyards (Karimi et al., 2021; Rusjan et al., 2007). Copper has been used in viticulture for more than 150 years, at a rate of up to 80 kg ha^{-1} per year, to combat downy mildew of grapevine (Rusjan et al., 2007). The recent meta-analysis by Karimi et al. (2021) showed that microbial activity decreased by 30 % when more than 400 kg of Cu ha^{-1} per year was applied. Currently, the use of copper fungicides in organic and conventional agriculture is limited to 4 kg ha^{-1} per year in most European

countries (EFSA, 2018). Despite its unfavourable ecotoxicological profile (Flemming and Trevors, 1989), copper is still tolerated in organic agriculture and viticulture in acknowledgement of its unique properties as a wide-spectrum fungicide and bactericide. Likewise, fungicides based on other mineral elements such as sulphur and lime are approved in organic production and are frequently used with or without copper (Provost & Pedneault, 2016). Sulphur is commonly used to control powdery mildew caused by *E. necator* and eriophyid mites on grapes (Griffith et al., 2015). In 2001, approximately 176 million kg of sulphur was used on grapes in the United States, almost five times the amount applied on 24 other fruit and vegetable crops surveyed by the National Agricultural Statistics Service in 2001 (USDA, 2001). At the moment, it is demonstrated that viticulture's future depends on the availability of copper and sulphur, unless alternatives are identified (Karimi et al., 2021).

On the other hand, few new synthetic insecticides or fungicides have been registered for use in vineyards during the past decade (Daane et al., 2018). Furthermore, recent works, such as the results of Janssen & van Rijn (2021), showed that pest resurgence due to pesticide applications will increase average pest densities throughout a growing season when effective natural enemies are present. In the past few years, more selective and less disruptive insecticides, including some neonicotinoids, insect growth regulators, and tetramic acids, have replaced the broad-spectrum insecticides in many grape-producing regions. For example, for lepidopteran pests, ecdysone agonists such as methoxyfenozide provide selective disruption of moth development, with activity on multiple life stages of key vineyard pests and with low impact on natural enemies (Carlson et al., 2001; Sáenz De Cabezón-Irigaray et al., 2005). In the same way, the tetramic acid spirotetramat has shown excellent activity against challenging pests, including mealybugs, phylloxera, and scales (Brück et al., 2009), resulting in reduced spread of grapevine leafroll-associated virus, with limited non-target effects on some natural enemies (Daane et al., 2018). On the other hand, the willingness to reduce the use of chemical pesticides is especially limited when it comes to fungicides. Despite the regulatory framework of the European New Green Deal, the amount of fungicides sold annually in the EU increased by up to 11 % in the period 2011–2018 (Lázaro et al., 2021). Alternatives to synthetic fungicides have been developed for *B.*

cinerea management in vineyards (Jacometti et al., 2010). However, they are still far from being a natural alternative if current production and quality levels are to be maintained.

1.2. Entomopathogenic nematode symbiotic bacteria

1.2.1. *Xenorhabdus* and *Photorhabdus* genera

The bacteria *Xenorhabdus* (Thomas & Poinar, 1979) and *Photorhabdus* (Boemare et al., 1993) have been recently classified within the family Morganellaceae, belonging to the order 'Enterobacteriales' (Adeolu et al., 2016). This order is a large and diverse group of Gram-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria within the class Gammaproteobacteria (Figure 7).

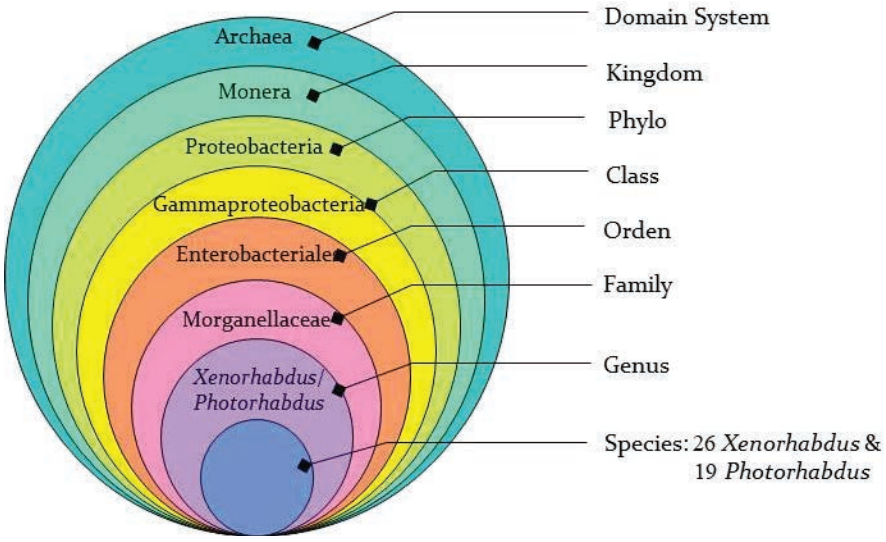

Figure 7. *Phylogeny of Xenorhabdus* and *Photorhabdus*. In line with Adeolu et al. (2016), N. E. Boemare et al. (1993), and Thomas & Poinar (1979).

Members of this group inhabit a large number of different ecological niches, both soil and water, and create associations with living organisms, including plants, insects, other animals, and even humans (Janda & Abbott, 2015). The whole-genome-based phylogeny and taxonomy classification system has

recently recognised 26 species of the genus *Xenorhabdus* and 19 species of the genus *Photorhabdus* (Machado et al., 2018; Sajnaga & Kazimierczak, 2020).

The life cycle of *Xenorhabdus* and *Photorhabdus*

Xenorhabdus and *Photorhabdus* are among the best studied microorganisms due to their complex dualistic life-cycle (Cao & Goodrich-Blair, 2017; Eckstein & Heermann, 2019; Murfin et al., 2015) and the interest in their application as a source of novel chemical compounds (ffrench-Constant et al., 2007; Shi et al., 2022). *Xenorhabdus* and *Photorhabdus* have two types of relationships during their life cycle: they are mutualists on nematodes of the family Steinernematidae and Heterorhabditidae, respectively, and pathogens of soil-dwelling arthropods (ffrench-Constant et al., 2003; Forst & Nealson, 1996; Milstead, 1979). The life cycle of *Xenorhabdus* and *Photorhabdus* begins from the non-feeding stage of the nematode, known as the free-living infective juvenile (IJ). The IJs stay in the soil and carry their associated microbiota (Stock, 2015) (Figure 8A). Nematodes of the genus *Steinernema* carry, among other microorganisms (Ogier et al., 2020), the mutualistic bacteria *Xenorhabdus* in a specialized vesicle called "the receptacle", placed in the anterior part of their intestine (Bird & Akhurst, 1983; Forst & Clarke, 2002). However, *Heterorhabditis* nematodes, which do not have such a specialized structure, use their intestinal lumen to harbour *Photorhabdus* until they find a host to infect (Boemare, 2002). IJs enter the target arthropod (often immature stages such as larva or pupa) via natural openings or rarely through the insect cuticle. Inside the host, the IJs release the bacteria into the insect host's blood cavity (hemocoel) (Figure 8A).

Once inside the host, the bacteria must successfully accomplish three distinct roles: (*i*) rapid killing of the insects in the face of the host's cellular and humoral immunity, (*ii*) production of nutrients from the cadaver to facilitate growth and development of the nematode, and (*iii*) colonization and growth within the IJ stage of the nematode (Goodrich-Blair & Clarke, 2007) (Figure 8B). First, the bacteria replicate and kill the insect by suppressing the insect's immune responses and inducing its septicaemia (Seo et al., 2012). The bacteria then protect the prey cadaver against food competitors from soil living organisms, including bacteria, fungi, protozoa, or other nematodes (Boemare, 2002; Gulcu et al., 2012) (Figure 8C). The

nematodes sense a "food signal" of enough bacterial biomass being available, and subsequently recover into adults. Finally, when the food source is depleted, a new generation of IJs receive the bacterial symbiont (transmission) before the nematodes leave the dead arthropod in search of new prey (Ciche et al., 2008; Somvanshi et al., 2012) (Figure 8D). The insecticidal activity of the entomopathogenic nematodes mostly depends on the pathogenicity of their bacteria symbiont. Mutualistic symbiosis is highly specific, with nematodes only developing in the presence of their cognate bacterial species (Han & Ehlers, 2001; Hurst et al., 2015), whereas a variety of different hosts can be killed by just 50–200 bacterial cells within one or two days. While the bacterium can also kill the insects in the absence of nematodes when injected directly into the hemocoel, in nature the bacteria benefits from the nematode as a vector for transportation to a new prey and has not been isolated at a free-living putative stage (Stock, 2015) (Figure 8).

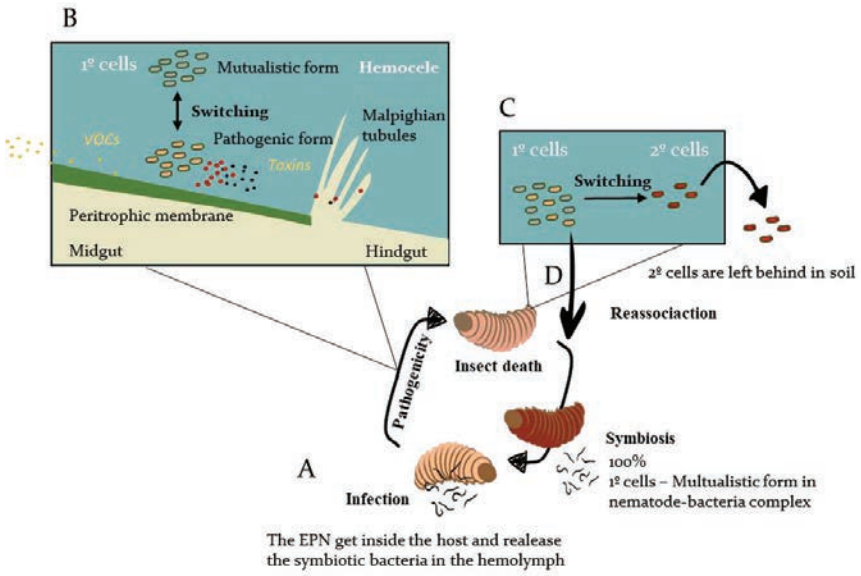

Figure 8. Life-cycle of *Photorhabdus* symbiotic bacteria, based on Eckstein & Heermann (2019).

Phenotypic switching and heterogeneity in *Xenorhabdus* and *Photorhabdus*

The bacteria *Xenorhabdus* and *Photorhabdus* experience predictable and unpredictable environmental variability as part of their natural life cycle as an insect pathogen, transmitted by their mutualistic nematode host (Cao & Goodrich-Blair, 2020). These bacteria control their life cycle stages and host interactions by molecular mechanisms regulated by promoter inversion switches (Cao & Goodrich-Blair, 2020; Eckstein & Heermann, 2019; Somvanshi et al., 2012). There is a trade-off between pathogenicity and mutualism in both bacteria, suggesting that the transition between these interactions is under regulatory control. The phenomenon of phenotypic variation, in which different subpopulations of cells express distinctive and potentially adaptive characteristics, contributes to microbial adaptation to a lifestyle that includes rapidly changing environments (Cao & Goodrich-Blair, 2017). Despite their similar lifestyle and close phylogenetic origin (Tailliez et al., 2010), the bacterial species *Xenorhabdus* and *Photorhabdus* differ significantly in the nematode host range, symbiotic strategies for parasite success, arrays of released antibiotics and toxins, and the molecular components of the regulatory networks controlling pathogenicity and mutualism behaviour (Bode, 2009).

Among the genus *Xenorhabdus*, the species *X. nematophila* is a well-studied model of symbiosis and of how these may contribute to the evolution of microbial population heterogeneity and anticipatory behaviour (Cao & Goodrich-Blair, 2020). The species *X. nematophila* is mutualist of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) nematodes and pathogenic of arthropods. Different studies have proved that *X. nematophila* exhibits phenotypic variation between the mutualistic nematode and the insect virulence phenotypes. Its life cycle starts within the IJ stage nematode that carries *X. nematophila* into the insect hosts. In the work of Cao & Goodrich-Blair (2020), it was proved that the virulence (V) and mutualistic (M) bacterial phenotypes occur reciprocally depending on the levels of the Lrp transcription factor: high-Lrp expressors are M+V-, while low- Lrp expressors are V+M-. On the other hand, the basic principle of the phenotypic bacteria switching and heterogenecity is exemplified by *Photorhabdus luminescens* symbiont of *Heterohabditis bacteriophora* (Rhabditida: Heterorhabditidae). The review by Eckstein & Heermann

(2019) showed that phenotypic heterogenicity is highly distinct in *P. luminescens*. The bacteria exist in two phenotypic forms that differ in various morphological and phenotypic traits and are therefore distinguished as primary (1°) and secondary (2°) cells. The 1° cells are bioluminescent and pigmented, produce several secondary metabolites and exo-enzymes, and support the nematode growth and development. These cells possess the ability to live inside both the nematode and the host. The 2° cells lack all these 1°-specific phenotypes. The entomopathogenic nematodes carry 1° cells in their upper gut and release them into the insect's hemolynpha after slipping inside. During insect infection, up to half the number of 1° cells undergo phenotypic switching and become 2° cells. Since 2° cells can no longer live in nematode symbiosis, they cannot re-associate with their symbiosis partners after the infection and therefore remain in the soil (Regaiolo et al., 2020). The phenotypic switching in *P. luminescens* must be tightly regulated since a high switching frequency would lead to a complete break-down of the nematode-bacteria life cycle. It has been reported that HexA is involved in the regulation of this pathogen–symbiont transition (Joyce et al., 2011). Furthermore, Eckstein et al. (2021) showed that XRE-like transcriptional regulators are also involved in this process. Recently, Dominelli, Jäger, et al. (2022) demonstrated that phenotypic differences in *P. luminescens* 1° and 2° cell cultures are not caused by mutations or genetic rearrangements in the genome but actually emerge from phenotypic heterogeneity. Future work is required to fully understand this molecular regulation of this phenotypical switch.

1.2.2. *Xenorhabdus* and *Photorhabdus*: source of novel compounds

Diversity of compounds

Throughout the complex life cycle of these bacteria, the control of their interactions is regulated by the production of conserved and unique molecules (Shi et al., 2022). The most studied period for their production is inside the infected insect, where the bacteria generate soluble and/or volatile secondary metabolites (Bode, 2009) to perform diverse biological and ecological functions. Secondary metabolism is the cluster of metabolic pathways and the products of metabolism that are not absolutely required for the survival of the organism (Clarke, 2016). Secondary metabolism is

generally associated with the post-exponential or stationary phase of bacterial growth when nutrients are limited and the metabolic flux is redirected away from the production of new bacterial cells (i.e., primary metabolism) to the production of secreted metabolites (i.e., secondary metabolism) (Clarke, 2016). However, there is no reason why secondary metabolites cannot also be produced during bacterial growth, and it is now clear that secondary metabolism in bacteria is regulated by a complex network of pathway-specific and global regulators that link primary and secondary metabolism (Cimermancic et al., 2014). The function of the resulting secondary metabolites during the life cycle of *Xenorhabdus* and *Photorhabdus* is not fully understood. However, these molecules may act as signals to modulate the interaction between the bacteria and the symbiotic nematode-the insect host- and other organisms in the ecosystem (Clarke, 2016; Stock, 2015).

Secondary metabolites of *Xenorhabdus* and *Photorhabdus* include non-ribosomal peptides, polyketides, and/or hybrid natural products that are synthesized using non-ribosomal peptide synthetase (NRPS), polyketide synthetase (PRS), or similar enzymes, such as cell wall degrading enzymes (e.g., *chitinase*) (Dominelli, Platz, et al., 2022) (Figure 9). The biosynthetic gene clusters (BGCs) for the synthesis of these compounds can range up to 6.5 % of the bacterial genome (Tobias et al., 2017). They are recognised as novel sources of new pesticide/drug compounds that can serve as lead molecules for the design and synthesis of new alternatives that could help with the challenge of pesticide resistance in crops and antibiotic resistance in veterinary and human health (Cimen et al., 2022; Lanois-Nouri et al., 2022; Shi & Bode, 2018). The huge diversity of the secondary metabolites produced by these bacteria has the evolutionary role of guaranteeing the success of the bacteria-nematode interaction, performance, and survival. The most classic example of these metabolites is isopropylstilbene. Although the biosynthesis of stilbenes is widespread in plants, the only non-plant organism known to produce this type of compound is *Photorhabdus luminescens*, which produces small amounts of 2-ethyl-5-[(E)-2-phenylvinyl]benzene-1,3-diol during the post-exponential phase of bacterial growth to help the nematode development (Joyce et al., 2008).

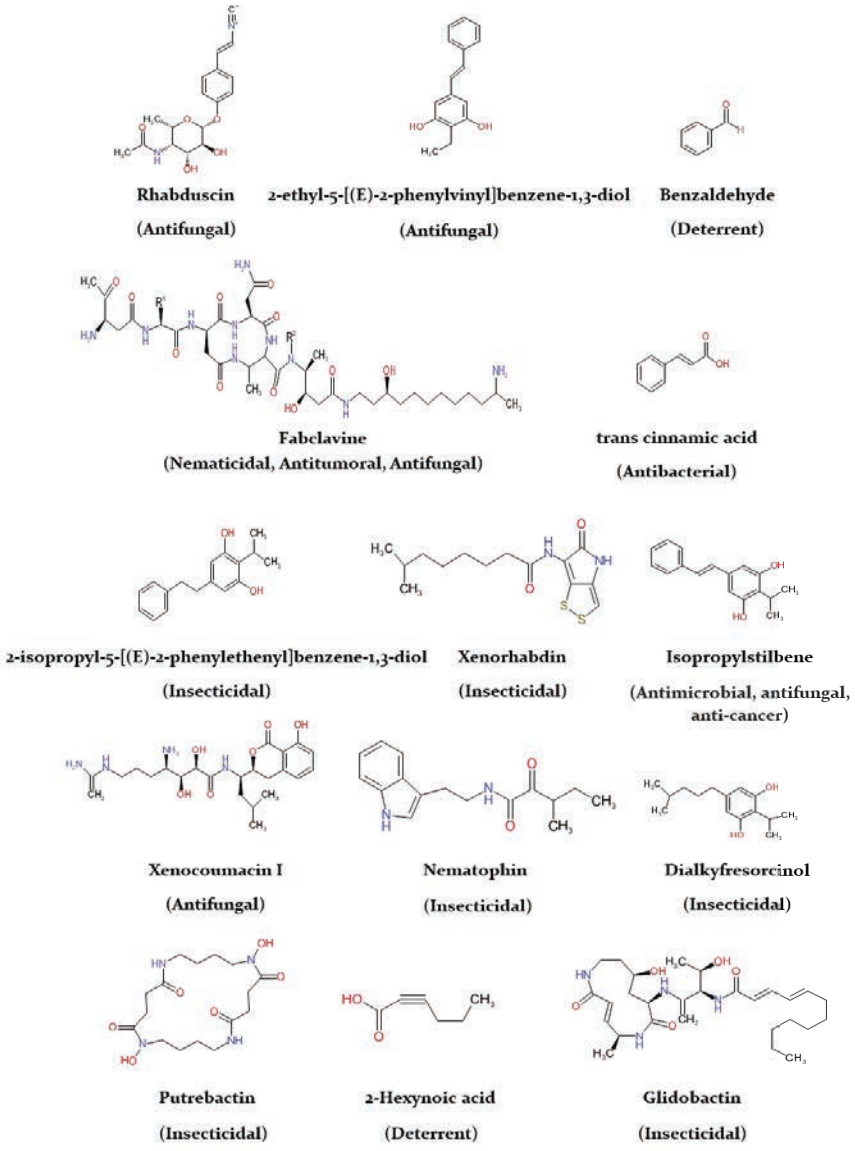

Figure 9. Chemical structure of natural products produced by *Xenorhabdus* and *Photorhabdus* bacterial symbionts of entomopathogenic nematodes and their described activity. Drawn with the Add-In Chem4Word.

For the life cycle of the bacterium-nematode complex, these bacteria have acquired an extend number of BGCs that produce compounds for specific biological functions: (*i*) to inhibit several of the insect immune pathways, (*ii*) antimicrobial compounds to easily keep out any competitors that would otherwise survive in the insect carcass and outcompete the symbiotic pair, (*iii*) scavenger deterrent factors to protect the insect cadaver against any opportunistic organism, and (*iv*) signal molecules to recover the mutualistic relationship (Fenton et al., 2011; Gulcu et al., 2012; Hillman & Goodrich-Blair, 2016; Tobias et al., 2018). Among others, from *Xenorhabdus* spp., the discovered peptides have been nematophin, xenorhabdin, xenortide, xenocoumacin, xenotetrapeptide, benzylidenacetone, rhabduscin, rhabdopeptide, fabclavine, ambactin, cabanillasin, indole, szentiamide, and peptide array X-linking (PAX) (Dreyer et al., 2018; Tobias et al., 2018) (Figure 9). On the other hand, from *Photorhabdus* spp., the reported compounds are anthraquinone pigments, rhabduscin, β-lactam carbapenem, darobactin, transcinnamic acid, trans-stilbenes, phototemtide, mevalagma- peptides, and isopropylstilbenes (Bozhüyük et al., 2017). They all perform different functions. Recently, studies are beginning to focus on the novel volatile deterrent compounds emitted by these symbiotic bacteria that can fortify EPNs' field performances and be of interest in feeding deterrence and ovipositional deterrence of pests in crops.

Diversity of mechanisms against invertebrate immune system response

Invertebrate pests have two responses against a possible attack of the EPNs-bacteria complex: physicochemical barriers and immune response (Beckage, 2008). Much of the EPNs' success in parasitism, overcoming the physicochemical barrier, and the first immune response of the invertebrate host is due to their ability to disguise themselves from host's immune defences. However, little is understood about the mechanisms that underpin these host–parasite interactions. Current research has described that this tactic involves the synthesis and release of molecules as excreted-secreted products (e.g., ShK-Domain-Containing Protein family proteins or fatty-acid-and-retinol-binding proteins) into the host tissue during the invasion process (Lima et al., 2022; Parks et al., 2021). These molecules are able to evade, suppress, or modulate host immune responses in order to persist and spread in the host (Cooper & Eleftherianos, 2016).

Once the nematode enters the host, *Xenorhabdus* or *Photorhabdus* reach the invertebrate host hemolyph and quickly activate the innate response pathway, a non-specific response that tries to eliminate the antigen, regardless of its nature (Tobias et al., 2018) (Figure 10). The innate immune system possesses pathogen pattern recognition receptors (PRRs), which are used to recognise pathogenic patterns. These receptors are activated upon recognition of pathogen-associated molecular patterns (PAMPs). The location of these PRRs is diverse, although Toll-like receptors (TLRs) are highly conserved. The Toll pathway is involved in recognition of fungal and Gram-positive bacterial pathogens.

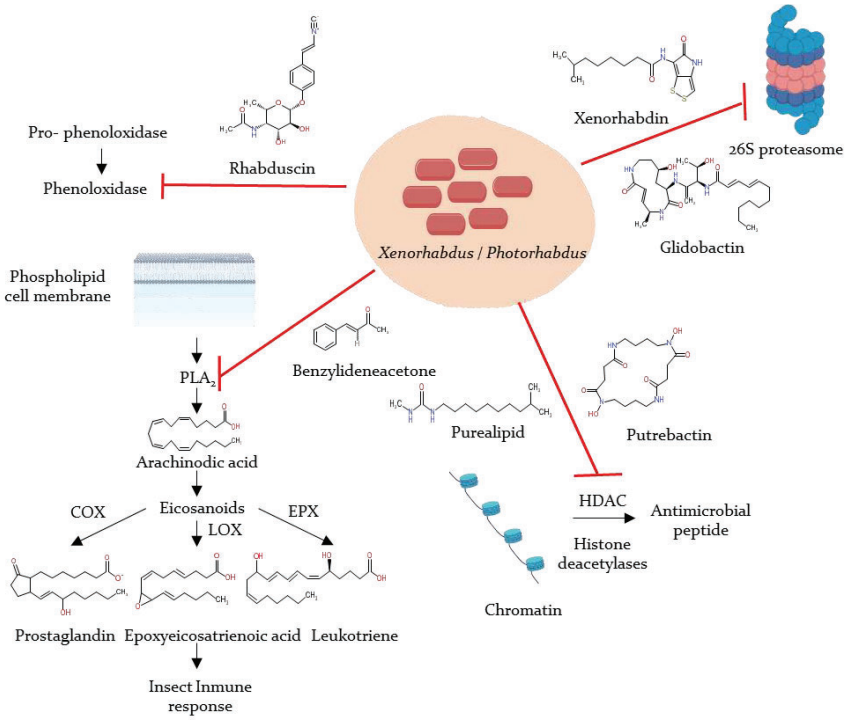

Figure 10. Summary of natural products synthesised by *Xenorhabdus* and *Photorhabdus* that act to repress different facets of the insect immune response, based on Tobias et al. (2018). Drawn with the Add-In Chem4Word.

On the other hand, the Imd pathway is essential for recognition and response to Gram-negative bacterial infection (Gottar et al., 2002; Lemaitre et al., 1995). PRRs activation triggers the activation of cellular and humoral

responses, such as hemocytes activation (Strand, 2008), activation of synthesis of eicosanoid, phenoloxidase activation (Cerenius & Söderhäll, 2004), proteasome activation, and Production of Antimicrobial Peptides activation (AMPs) (Beckage, 2008). *Xenorhabdus* and *Photorhabdus* have developed elegant strategies that allow them to inhibit several of these insect immune pathways by producing a wide variety of secondary metabolites.

Many of the natural products produced by *Xenorhabdus* and *Photorhabdus* have direct functions, specifically targeting different facets of nematode development or the insect immune system. These adaptations have allowed the bacteria to thrive in a unique environment and become versatile and highly efficient insect pathogens. Some of the main functions altered by these metabolites are: Eicosanoid inhibition, Phenoloxidase Inhibition, Proteosome Inhibition, or the suppression of antimicrobial peptides (Darsouei et al., 2017; Mollah & Kim, 2020; Seo et al., 2012; Tobias et al., 2018), as further displayed in Figure 10. Understanding the different mechanisms of action of the toxins produced by *Xenorhabdus* and *Photorhabdus* is crucial for their development as biopesticides and for attempting to avoid the possible emergence of resistance.

1.2.3. Potential application of symbiotic bacteria in agriculture

Since 1980, a large number of potential applications of biological activities derived from *Xenorhabdus* and *Photorhabdus*, along with their secondary metabolites, have been described (Cimen et al., 2022). Recent research on the Web of Science with the keywords "*Xenorhabdus*" AND "*Photorhabdus*" AND "control" AND "alternative" (http://apps.webofknowledge.com/; accessed on 24 Jan 2023) shows that an increasing number of articles (61) positioned these bacteria as an alternative tool for the control of pest and disease in different farming crops. Their antibacterial (Akhurst, 1980; Furgani et al., 2008; Muangpat et al., 2020), antifungal (Chacón-Orozco et al., 2020; Cimen et al., 2021; Fang et al., 2014; Fang et al., 2011; Hazir et al., 2016; Wang et al., 2022), antiprotozoal (Grundmann et al., 2014; Gulsen et al., 2022; Zhao et al., 2018, 2020), insecticidal (Da Silva et al., 2020; Dreyer et al., 2018), nematicidal (Abebew et al., 2022; Kusakabe et al., 2021;

Sayedain et al., 2019) and acaricidal (Eroglu et al., 2019; Incedayi et al., 2021) effects on organisms have been tested.

One of the best examples is the broad exploration of its use as an insecticide. In a very intuitive way, researchers began to focus on the potential of these bacteria and their secondary metabolites in the control of various crop pests, given the direct relationship with their natural behaviour within the EPN-bacteria symbiotic complex. In the review by Sandhi & Reddy (2019), it is reported that *Xenorhabdus* species can infect species of both Lepidoptera (Ji & Kim, 2004; Kalia et al., 2014; Khandelwal et al., 2004; Vicente-Díez et al., 2021a) and Hymenoptera (Dudney, 1997) when injected artificially into their bodies in the laboratory or by oral toxicity of their secondary metabolites. Similarly, species of *Photorhabdus* are virulent against a wide range of insects, including species of Lepidoptera (Jallouli et al., 2013; Vicente-Díez et al., 2021b), Coleoptera (Ansari et al., 2003; Batalla-Carrera et al., 2016), Thysanoptera (Gerritsen et al., 2005), Orthoptera (Mahar et al., 2004), Hymenoptera (Bowen & Ensign, 1998; Vicente-Díez et al., 2021a; Zhou et al., 2002), and Diptera (Da Silva et al., 2013) in the laboratory when hosts are infected orally or by injection. Similarly, acaricidal activity has progressively gained attention as well. Eroglu et al. (2019) reported the toxic effects of the secondary metabolites produced by *Xenorhabdus* spp. and *Photorhabdus* spp. against various developmental stages of *T. urticae*. However, few field studies have so far reported the virulence of the foliar application of *P. luminescens* and *X. nematophila* against pests in different crops (Abdel-Razek, 2003; Jallouli et al., 2013; Mohan et al., 2003, 2004).

Another limitation to be highlighted is that few researchers have examined the effect of the symbiotic bacteria or their secondary metabolites on non-target arthropods regardless of their EPNs. Lalitha et al. (2012) studied the effect of *P. luminescens* (the symbiotic bacteria of *H. bacteriophora* strain PDBC Hbb1) on pupae and adults of the egg parasitoid *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae), and eggs and larvae of the predator *Chrysoperla zastrowi sillemi* (Neuroptera: Chrysopidae). There were no physical changes in the eggs, larvae, and adults of *T. chilonis* and *C. zastrowi sillemi*, and no significant reduction was observed in egg hatch, adult emergence, or parasitism from the bacteria. Mohan & Sabir (2005) tested the virulence of *P. luminescens* (isolated from

H. indica) against *T. chilonis* and *Trichogramma japonicum* (Hymenoptera: Trichogrammatidae) inside eggs of their host *Corcyra cephalonica* (Lepidoptera: Pyralidae) in the laboratory at the rate of 1×10^5 cells per ml and reported a 84 % reduction in emergence of *Trichogramma* adults from treated host eggs.

Another potential attribute of these bacteria for their implementation in agriculture is their capacity to produce environmental signals (semiochemicals) as deterrent factors (Raja et al., 2021). Zhou et al. (2002) demonstrated that *X. nematophila* and *P. luminescens* produce compound(s) that deter scavengers such as ants and could thus protect nematodes from being eaten during reproduction within insect cadavers. Despite the diversity of bacterial metabolites and the biological relations mediated by them, which form a huge reservoir of resources with potential applicative interest, the application of deterrent factors has so far only been marginally explored (Gulcu et al., 2012, 2018; Jaffuel et al., 2021; Kajla, 2020; Kajla et al., 2019; Kong et al., 2022), and their practical use is found at an early stage, especially for improving crop tolerance and quality.

Another area of research that has received limited attention so far is the capacity to communicate with plants, specifically through root colonization as an endophyte or via the influence of secondary metabolites, which can serve as elicitors or biostimulants for plants or post-harvest fruits. In a greenhouse setting, Hazir et al. (2016) showed that the application of cell-free supernatants of *Xenorhabdus* or *Photorhabdus* had no phytotoxic effect on various plants, including eggplant (*Solanum melongena,* Solanales: Solanaceae), pepper (*Capsicum annuum,* Solanales: Solanaceae), tobacco (*Nicotiniana tabacum,* Solanales: Solanaceae), tomato (*Solanum lycopersicum,* Solanales: Solanaceae), peach (*Prunus persica,* Rosales: Rosaceae), and pecan (*Carya illinoinensis,* Juglandales: Juglandaceae). Furthermore, Regaiolo et al. (2020) investigated the response of the secondary variant of *P. luminescens* to plant root exudates and its interaction with microorganisms in the rhizosphere, revealing a distinct interaction between *P. luminescens* and plant roots and demonstrating that a specific interaction of *P. luminescens* with plant roots might exist. Furthermore, Lai et al. (2020) showed that treatment with *P. luminescens* enhanced the activities of defence enzymes (peroxidase, superoxide dismutase and catalase) and induced a significant increase in the trehalose

content in the fruit pulp while a significant decrease in malondialdehyde (MDA), hydrogen peroxide (H_2O_2) accumulations in pericarp. These findings indicated that, the application of *P. luminescens* enhanced the defence related mechanism and non-enzymatic antioxidant system (trehalose, MDA, ROS and H_2O_2) of litchi against fruit decay.

Finally, the last potential applications of the symbiotic bacteria in agriculture are the production of genetically altered bio-insecticides, which include recombinant entomopathogens (Karabörklü et al., 2018) and the controversial production of transgenic resistance plants based on the genomic information of *Xenorhabdus* and *Photorhabdus* (ffrench-Constant et al., 2007). Advances in biotechnology (recombinant technology) may contribute to the production of insect-resistant plants and other biotechnologically enhanced bio-control agents (Karabörklü et al., 2018). So far, not much progress has been made in these areas for *Xenorhabdus* and *Photorhabdus*, but it is an idea that is gaining momentum as the challenges facing agriculture worsen.

1.3. *Xenorhabdus* and *Photorhabdus* – potential based tool for vineyard management

1.3.1. A multipurpose of beneficial performance

Vineyard managers on all continents are increasingly facing new challenges to guarantee yield and quality in the grape industry (Daane et al., 2018). Among the complex challenges previously reported, the global warming and biodiversity crisis are the principal triggers to induce relevant changes in the sector (Venios et al., 2020). Even though IPM is currently performed in most vineyards all over the world, viticulture is still a highly pesticide-demanding crop, essentially because of the high fungus infections and the invasive species. For these reasons, the viticulture sector is searching for new technologies and working in coordination programs to face these upcoming challenges. The sector's eco-sustainability (environmental – social - economic) requires moving vineyards towards more diverse agroecosystems, in which indirect pest and disease control measures and novel biotechnological techniques will be necessary.

In this scenario, this Thesis has studied the potential use of *Xenorhabdus* spp. and *Photorhabdus* spp. in the viticulture sector due to its huge range of biological functions in the agroecosystem (Figure 11).

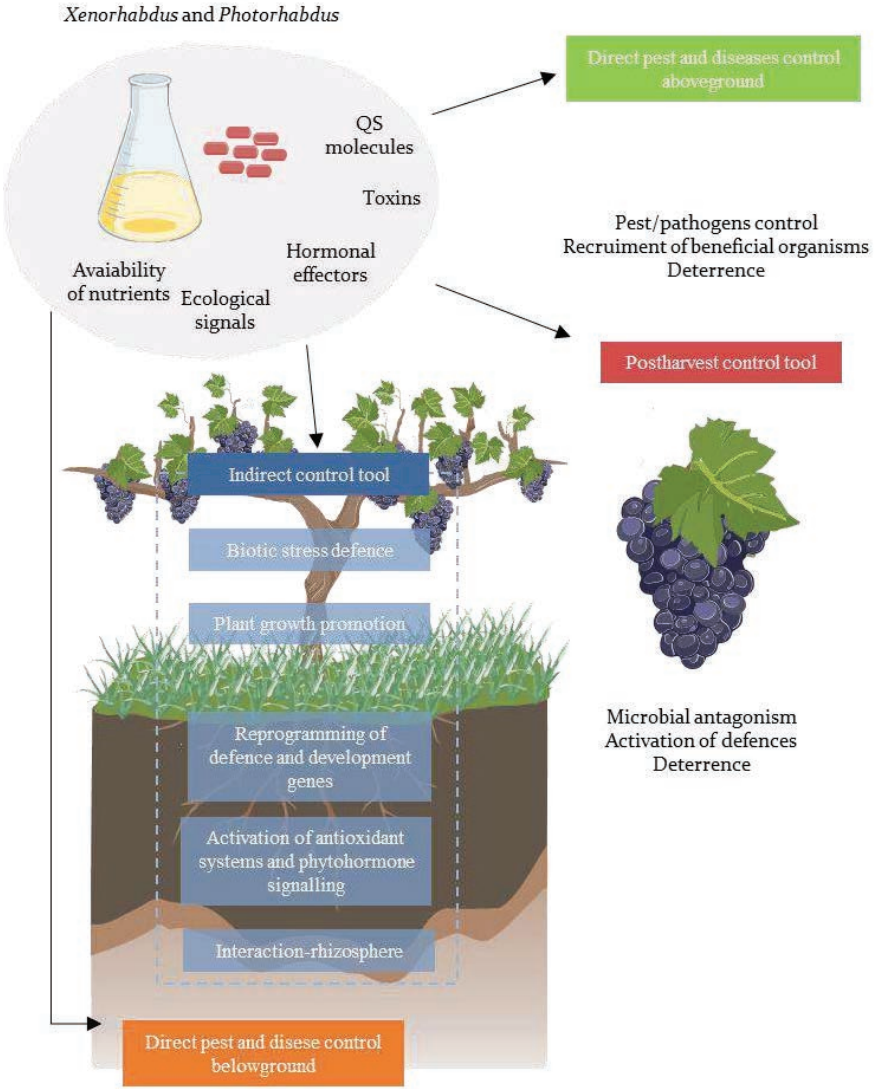

Figure 11. *Xenorhabdus* and *Photorhabdus*: multipurpose, plant-beneficial microorganisms for eco-sustainable agriculture. Drawn using icons from Flaticon.

Recent studies by Blanco-Pérez et al. (2022a, 2022b, 2020) showed how different cultural practices (the use of cover crops and mulching) and pest management techniques (integrate or organic) modulate the presence of EPNs in the vineyards. Furthermore, Campos-Herrera et al. (2021) conceptualized how the application of EPNs and natural products derived from their symbionts can be an alternative tool against some of the main fungal and arthropods pests present aboveground in next generation viticulture, placing a framework of exploration for those approaches.

Technologies to produce ideal EPN symbiotic bacteria products for viticulture should exploit its multipurpose assets by selecting species or strains that have potential biological control of pest and disease in the field and post-harvest, root interaction of the vine and the rhizosphere, and that can induce resistance to disease and/or promote plant growth (Figure 11).

1.3.2. Sources of natural products

Among these multipurpose beneficial performances associated with *Xenorhabdus* or *Photorhabdus* described above, the most widely studied has been the use of soluble and volatile (VOCs) bioactive compounds they produce. Recent research has mainly focused on obtaining, optimising and using these natural products (Booysen & Dicks, 2020; Da Silva et al., 2020; Kong et al., 2022).

A single strain of *Xenorhabdus* or *Photorhabdus* may produce a broad range of antibiotic and insecticidal extracellular bioactive compounds (Dreyer et al., 2018). When producing antibiotics, biochemistry engineering conditions are known to be critical for the production of secondary metabolites by microorganisms (Jiang & Zengyi, 2011). Even small changes in the culture medium can alter the quantities of certain compounds and modify the general metabolic profile of microorganisms, microbial cellular networks, and fine-tuning of physiological capabilities. Thereby, the industry can develop viable strains for producing natural and non-natural value-added compounds. Different studies have enhanced the antibiotic activity of *Xenorhabdus* spp. and *Photorhabdus* spp. and optimized different bacterial strains. Factors such as ambient temperature, pH, rotation speed, inoculation concentration, medium volume flask, fermentation time, aeration, and batch or continuous stirred tank reactor or other media properties may influence gene expressions and increase the production of

antimicrobial interest compounds. For example, Wang et al. (2011) tested the production of antibiotics of *Xenorhabdus bovienii* YL002 by optimizing the medium using the response surface methodology. Their results pointed out that an optimized medium by adding glycerol and soy peptone increased antibiotic activity by 38 %. However, previous one-factor-at-a-time assays with *X. nematophila* YL001 identified glucose and peptone as the best carbon and nitrogen sources that significantly affected antibiotic production (Wang et al., 2008). Recently, Booysen et al. (2021) suggested that the secondary metabolism of *X. khoisanae* J194 may be regulated by oxygen, water activity, or both. The dissolved oxygen level, tested only for *X. nematophila* YL001, was optimal when shifted during fermentation from 70 % after the first 18 h to 50 % for the remaining 54 h (Dreyer et al., 2018). Booysen & Dicks (2020) summarized common trends for all studies at pH from 6.0 to 8.24, temperature range of 25–32 ºC, rotary speed of 150–220 rpm, inoculation volume of 4–15 %, a medium volume of 54–100 ml/250 ml flask, and a fermentation time of 54–72 h. Keskes et al. (2021) investigated the optimization of the culture conditions for enhancing *Photorhabdus temperata* biopesticide production using wastewater (WS4) as raw material and proved its viability. In this line, and in the context of the SDGs, research linking the production of biopesticides within a circular economy results very promising.

1.3.3. Target vineyard pest and diseases: description and relevance

We focused on three primary biological threats among the many pests and diseases that impact vineyards: Pierce's disease, grape moths, and grapevine rot. In this context, we provide a brief overview of their biology, ecology, and current management methods. This discussion sets the stage for considering the utilization of *Xenorhabdus* spp. and *Photorhabdus* spp. as direct biological tools.

Pierce's diseases: *Philaenus spumarius – Xylella fastidiosa*

The xylem-inhabiting bacterium *X. fastidiosa* is responsible for Pierce's disease, a lethal grapevine disease, and also for diseases in many economically important crops, such as citrus, almond, coffee and olive trees (Schneider et al., 2020). The meadow spittlebug *P. spumarius*, never

considered a pest in Europe, raised the attention of scientists and stakeholders after the discovery of its main role in the transmission of *X. fastidiosa* strain ST53 to olive trees, discovered as the first reported European outbreak of the bacterium, occurred in Apulia (South Italy) in 2013 (Cornara et al., 2018; Saponari et al., 2014). This xylem sapfeeding insect has a univoltine life cycle and the eggs can diapause over winter for more than one hundred days, although adults survive if the climate is appropriate. Both the nymphal and adult instars of *P. spumarius* can inoculate the pathogen *X. fastidiosa* to healthy plants immediately after acquiring it by feeding on the xylem of infected plants.

As highlighted by Cornara et al. (2018), strategies to manage diseases associated with *X. fastidiosa* should include tactics aimed at (*i*) reducing vector populations, and (*ii*) diminishing sources of inoculum available to the vector. Given the absence of universally-applicable solutions, it is imperative to consider the ecological context and population dynamics of *P. spumarius* across various sites and crop systems. Regarding controlling *P. spumarius* populations, strategies should concentrate on two stages of the insect's life cycle: nymphs and recently emerged non-infective adults capable of moving toward *X. fastidiosa* source plants. Additionally, utilising biological control agents, such as entomopathogenic nematodes, or natural products for their management will serve as pivotal tools for effectively and sustainably handling the *P. spumarius*–*X. fastidiosa* complex. Ultimately, control measures should be executed across the most expansive geographical range.

Grapevine moth: *Lobesia botrana*

Tortricid *L. botrana* is considered a global vine pest (Benelli et al., 2023; Gilligan et al., 2011; Varela et al., 2013). This moth achieves three generations in vineyards in temperate areas, while an additional fourth generation is becoming more frequent due to global warming (Amo-Salas et al., 2011; Castex et al., 2018) (Figure 12A, B, C, D, E). The *L. botrana* 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). Good decisions are positively correlated with offspring performance in adverse situations but not in favourable ones (Torres-Vila et al., 2012). The female *L. botrana* moths have an extraordinary

olfactory behaviour that allows them to detect the presence of the vine from a great distance (Tasin et al., 2006) or to distinguish between intact grapes and those infected by fungus (Tasin et al., 2012). The female lays single eggs and, after hatching, the larvae develop in inflorescences, unripe and ripe berries during the respective first, second or third generation, causing the main damage in grapevine (Figure 12F). Individuals of 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).

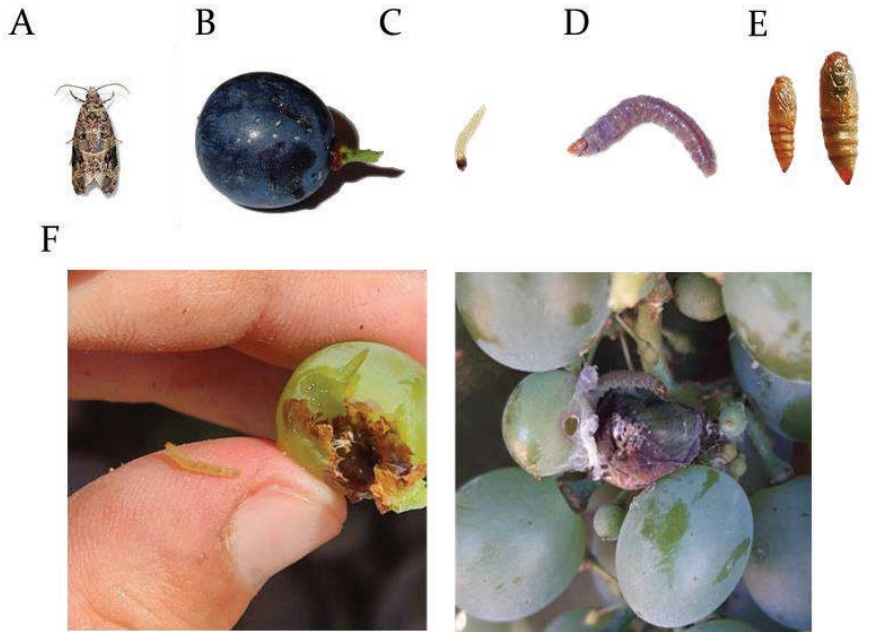

Figure 12. *Lobesia botrana* developmental stages and distinctive damage in field: A) adult, B) newly laid egg, C) L1 instar larva, D) L5 instar larva, E) male and female pupae, F) characteristic grape damage caused by *Lobesia botrana* larvae.

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 claiming new control alternatives due to the harmful effects of these treatments on non-

target organisms and on the environment. Pheromone-mediated mating disruption (MD) to control *L. botrana* is a current efficient semiochemical-technique. MD is based on the interference in the mate finding process affecting the chance of reproduction of the moth, with the consequent impact on its population dynamics. Techniques such as MD proved that olfactory cues are crucial information for *L. botrana* to choose feeding, mating and oviposition sites, and helps them avoid non-host plants (Tasin et al., 2006, 2011, 2012) (Table 1).

Table 1. Overview of biological control agents and biotechnical control tools against grapevine moths in viticulture and the facultative use of entomopathogenic nematodes and theirs symbiotic bacteria based products.

				Principal target	**References**
Biological control agents	Entomopathogenic fungi		*Beauviera basiana*	Pupae	López Plantey et al. (2019)
			Metarhizium spp. (*M. robertsii*; *M. anisopliae*)	Larval instars	López Plantey et al. (2019), Sammaritano et al. (2018)
	Bacterias		*Bacillus. thuringiensis*	Larval instars Adults	Ioriatti et al. (2011)
	Artropods	Predadora	*Chrysoperla* sp.	Eggs Pupae	Castex et al. (2018)
		Parasitoids	*Trichogramma* sp.	Eggs	Pérez Moreno et al. (2000)
			Dibrachis sp.	Pupae	
	Entomopathogenic nematodes		*Steinernema* sp. *Heterohabditis* sp.	Larval instars Pupae	**This thesis**
Biotechnical control tools	Pheromone-mediated Malting Disruption (MD)			Adults	Ioriatti et al. (2011), Shapira et al. (2018)
	Insecticidal-plants extracts		*Bifora radians*	Larval instars	Gökçe et al. (2011)
	Insecticidal-fungiae based products		*Trichoderma* sp.	Larval instars	Woo et al. (2022)
	Insecticidal- bacterium based product		*Bacillus thuringiensis*	Larval instars	Ifoulis & Savopoulou-Soultani (2009)
			Xenorhabdus spp. *Photorhabdus* spp.	Larval instars	**This thesis**

Furthermore, different effective measures to manage *L. botrana* exist based on classical biological control methods and on the use of eco-friendly biotechnical tools (Table 1). For example, *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) is a natural enemy of *L. botrana* due to its parasitic eggs (Castex et al., 2018), and *B. thuringiensis* (Bt) is a well-known effective bio-insecticidal bacteria (Glare et al., 2017). In addition, Bt produces several active compounds that are associated with pests and disease control, such as zwittermicin A and acyl homoserien lactonase (Zhou et al., 2008). Identifying novel chemical cues (e.g., bacterial volatile compounds) that drive ovipositional and trophic interactions through olfactory reception of *L. botrana*, inducing behavioural changes of this pest in the vineyard field, could be an efficient control strategy (Table 1).

Grapevine rot: *Botrytis cinerea*

The fungus *B. cinerea* is a necrotrophic wound fungus that causes gray mold on a wide range of fruits, vegetables, and ornamental plants worldwide (Jacometti et al., 2010). Despite the wide range of hosts, it is most destructive on mature or senescent tissues of dicotyledonous plants (Yigal et al., 2004). It usually entry to such tissues at an earlier stage in crop development. Then, it remains quiescent for a considerable period (as mycelia and/or conidia) before rapidly rotting tissues when the humidity and temperature conditions are favourable and the host physiology changes (Elad et al., 2007; Williamson et al., 2007).

The fungus *B. cinerea* causes massive losses in some field- and greenhouse-grown horticultural crops (e.g., tomato crops) before the harvest, or even at the seedling stage in some hosts. Moreover, *B. cinerea* is the main cause of postharvest fruit and vegetable decay during the supply chain (Elad et al., 2007). Fungal spores are generally present on the surface of pre-harvest fruit and vegetables, and during post-harvest handling, and a suitable environment can drive to spore germination.

The *B. cinerea* costs are diffuse because its damage occurs in different stages of the production and retail chain and are difficult to estimate. Nevertheless, global expenses of *Botrytis control* (cultural measures, botryticides, broad-spectrum fungicides, biocontrol) easily surmount 1000 million € per year, which highlight the exceptional importance of this pathogen (Dean et al., 2012). *Botrytis* rot is one of the

main causes of losses in viticulture before and after harvest worldwide (Fillinger & Elad, 2016). Elmer & Michailides (2007) estimated losses due to *B. cinerea* in the grape system to be around 2,000 million $ per year in the US, even though the world market for *Botrytis* control products is estimated at 15-25 million $ in this country (Elmer & Michailides, 2007). Grapes affected by *Botrytis* rot are of low value for winemaking, not only because of weight loss but also because of interference with fermentation and change in the flavour and colour of the wine (e.g., increased sensitivity to oxidation or decreased foamability in sparkling wines) (Marchal et al., 2020; Pearson & Goheen, 1988) (Figure 13).

Figure 13. Damage of *Botrytis cinerea* over red grapevine cluster.

Traditionally, winegrowers have managed this disease by combining cultural and chemical practices, including synthetic fungicides and copper-containing products (Fillinger & Elad, 2016). Alternative methods for controlling *B. cinerea* have included the adoption of appropriate rootstocks (Coutos-Thévenot et al., 2001), pruning and defoliation to improve canopy aeration, and sanitation practices aimed at eliminating sources of *B. cinerea* inoculum (Jacometti et al., 2007). Additionally, biological control agents, such as filamentous fungi from the genera *Trichoderma*, *Ulocladium* and *Gliocladium*, bacteria from the genera *Bacillus* and *Pseudomonas*, as well as yeasts from the genera *Pichia* and *Candida*, have been commonly used in the fight against *B. cinerea* (Elmer & Michailides, 2007). Grape growers are increasingly seeking for more sustainable and environmentally aware disease control, with consequent reductions in fungicide applications (Howell, 2001).

2. Objectives

"'Would you tell me, please, which way I ought to go from here?'
'That depends a good deal on where you want to get to',
said the Cat"

Lewis Carroll, in *Alice's Adventures in Wonderland*, 1865

The main challenge of agriculture nowadays is to reconcile the production of goods that meet human and global trade demands with the conservation of ecosystem services and biodiversity in agricultural lands, through the reduction of inputs and the use ecological tools (Wezel et al., 2020). As ambitious as it is necessary, this purpose is tightly linked to the development of new biotechnological tools that reduce their impact on the agroecosystem while maintaining biodiversity and environmental health (Villaverde et al., 2014).

Conventional vineyards remain highly demanding for pesticides, although several key principles have been established to reduce their use (Barzman et al., 2015) and the legislation for the registration of new active substances is becoming increasingly restrictive (Directive 2009/128/EC). Vineyard managers are requesting new biotools that guarantee the yield and quality of the grape industry while protecting human and agroecosystem health. This thesis was grounded on the hypothesis that by investigating diverse approaches to utilize the symbiotic bacteria of entomopathogenic nematodes, it could be possible to settle the basis for the development of effective tools for managing the target pests and diseases in vineyards, which present substantial threats to grapevines. To this end, the specific objectives to accomplish were:

1. To evaluate the insecticidal and antifungal effect of the soluble secondary metabolites produced by *Xenorhabdus* and *Photorhabdus* to control pests and diseases in the vineyards.

2. To evaluate the deterrent and the antifungal activity of the volatile organic compounds (VOCs) emitted by *Xenorhabdus* and *Photorhabdus* ferments as a novel strategy for controlling pests and diseases based on semiochemical signals.

3. To evaluate the effect that bacterial secondary metabolites may have on the defence of the harvested fruit.

In this Thesis, we focused the study on one emergent insect-vector *P. spumarius* and the persistent worldwide pest *L. botrana*. In addition, we selected the pathogen *B. cinerea* as a model to evaluate the potential use of these bacteria and their bioproducts in crop and post-harvest approaches.

3. Material and Methods

"What the hand does, the mind remembers"

María Montessori

3.1. Production of bioactive compounds from bacteria

We selected four EPN-bacteria complexes species for addressing the possible management of selected biotic threats: *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora*. EPNs were cultured in last-instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) reared at the Instituto de Ciencias de la Vid y del Vino (Logroño, Spain). The IJs were recovered in tap water upon emergence, stored at 12–14 ◦C, and used within two weeks after harvest. We employed freshly recovered IJs for isolating the symbiotic bacteria.

Throughout all the experiments conducted in this Thesis, bacterial isolation and fermentation were consistently executed using the same protocol. The symbiotic bacteria associated with the tested EPN species comprised three *Xenorhabdus* species (*X. bovienii*, *X. nematophila*, and *X. kozodoii)* and one *Photorhabdus* species (*P. laumondii* subsp. *laumondii*) (Table 2) (Figure 14). For the bacteria isolation, we exposed~500 IJs of each EPN species (inoculated in 100 µL of distilled water) to 5 % NaClO for 2–5 min. Then, after thoroughly washing with distilled water, we mechanically disaggregated them in a 50:50 (v/v) suspension of distilled water and nutritive broth (NB, VWR, Chemicals, Barcelona, Spain), employing sterile blue pestles assembled in a pellet mixer (VWR International, Lutterworth, UK). Then, we seeded 50 µl of each nematode–bacterium complex suspension on three Petri dishes with Nutrient Agar (NA), Bromothymol blue (Alfa Aesar, Kandel, Germany), and 2,3,5-Triphenyl tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates) (Figure 14). We stored the Petri dishes for 48 h under controlled conditions (25 ± 2 ºC, 20 % RH in the dark) before selecting those colonies with morphology associated with most *Xenorhabdus* and *Photorhabdus* species (rounded, smooth margins, and colorant absorption capacity). To obtain pure colonies, we seeded them in NTBA plates.

Table 2. Entomopathogenic nematode (EPN) and symbiotic bacterial species tested in the following bioassays.

EPN species	Population	ITS-Gen Bank accession	Bacterial species	16S-Gen Bank accession
Steinernema feltiae	RM-107	MW480131	*Xenorhabdus bovienii*	MW467374
Steinernema carpocapsae	All	MW574913	*Xenorhabdus nematophila*	MW574906
Steinernema riojaense	RM-30	MK503133	*Xenorhabdus kozodoii*	MW467375
Heterorhabditis bacteriophora	RM-102	MW480132	*Photorabhdus laumondii* subsp. *laumondii*	OQ285858

Subsequently, we inoculated single colonies of each pure culture in Triptone Soya Broth (TSB) (VWR Chemicals, Barcelona, Spain), maintaining the liquid cultures for 16 h under rotatory agitation (150 rpm) at 22 ºC in in a light-protected environment. We used an aliquot of each suspension to verify the absence of the catalase enzyme of the *Xenorhabdus* strains. Additionally, we checked the morphology of bacilli and its mobility under a microscope using the flagellum. A second aliquot of each bacterial suspension was concentrated and saved for DNA extraction, performed with the Speedtools tissue DNA extraction kit (Biotools, Madrid, Spain), and the rest stored at -80 ºC in aliquots of 300–400 µL in 30–35 % glycerol. For the molecular identification, we used universal primers to amplify the 16S rDNA region following the protocol described by Enright et al. (2003). All runs contained a negative control by adding mQ water (Milli-Q Water System, Millipore S.A., Molsheim, France) instead of DNA template. Hereafter, the PCR was verified through electrophoresis. Later, individual bands were cut and cleaned (SpeedTools Tissue DNA Extraction kit, Biotools, Madrid, Spain), sequenced (Macrogen), aligned with the software Geneious (R.6.1.5., Biomatters, Inc., Auckland, New Zealand), compared to reported sequences using Blast (http://blast.ncbi.nlm.nih.gov), and submitted to Genbank (Table 2).

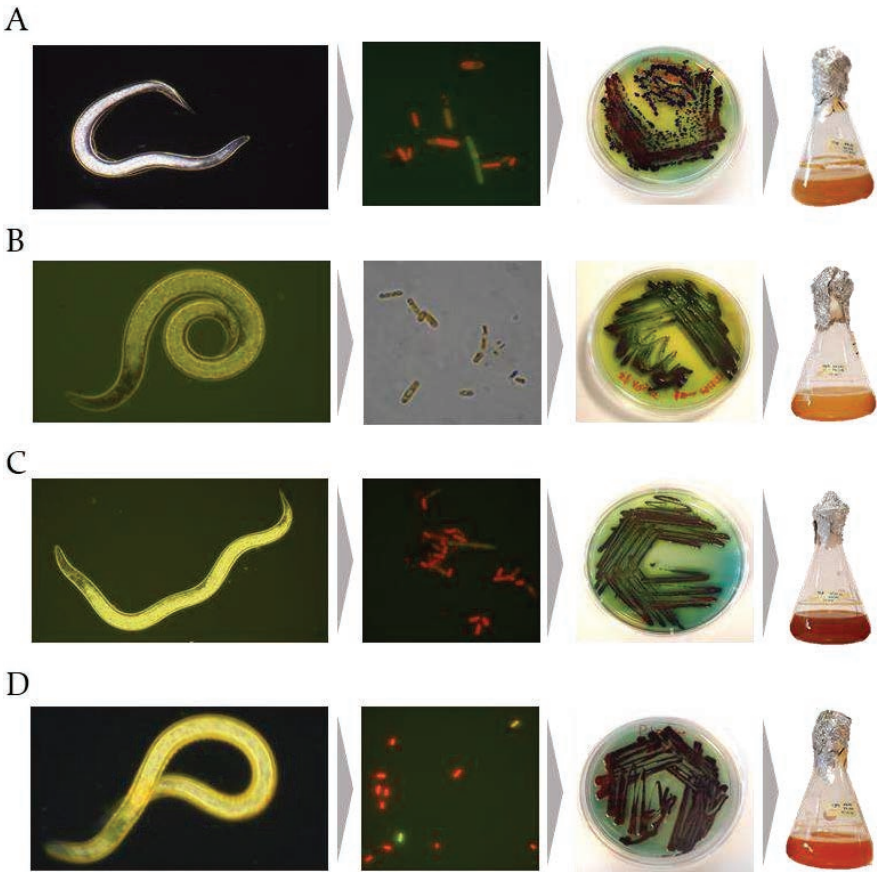

Figure 14. For all rows, from left to right: infective juveniles of entomopathogenic nematode (EPN), symbiotic bacteria, NBTA agar plate cultures of each bacterium, and TSB bacterial fermentation. A) *Steinernema carpocapsae – Xenorhabdus nematophila*; B) *Steinernema feltiae – Xenorhabdus bovienii*; C) *Steinernema riojaense – Xenorhabdus kozodoii*; D) *Heterorhabditis bacteriophora – Photorhabdus laumondii* subsp. *laumondii*. Microscopy images of EPNs and symbiotic bacteria were captured using acridine orange staining and fluorescence microscopy, supervised by PhD Antonio Guillén in October 2023 at the Institute of Grape and Wine Sciences (La Rioja).

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 TSB (Figure 15). We incubated the flasks on an orbital shaker at 150 rpm, at 25± 2 ºC, in full darkness for three days. The bacteria metabolism produces some secondary compounds during the

exponential bacterial growth phase (approximately during three days after the inoculation). Nevertheless, their secondary metabolism is generally activated during the post-exponential or stationary phase following bacterial growth (Clarke, 2016).

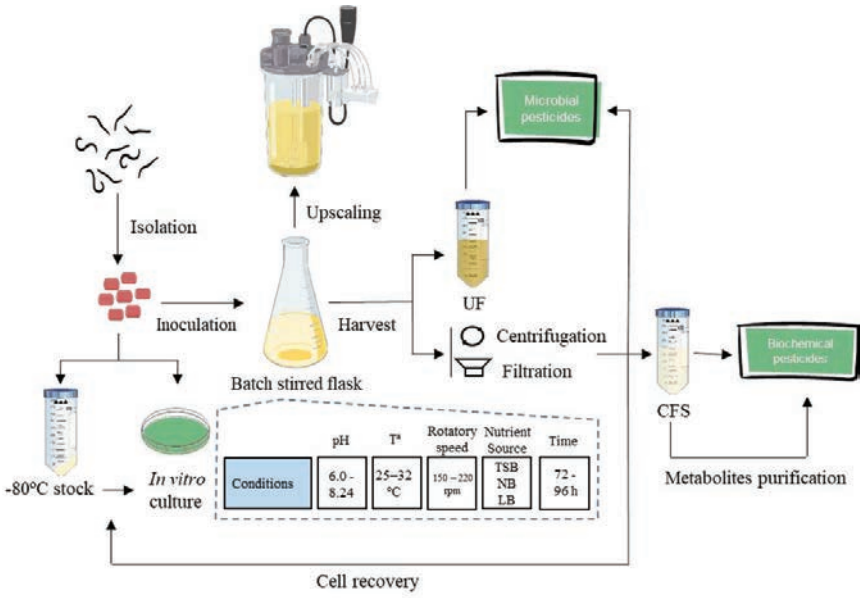

Figure 15. Flowchart of the biotechnological process developed to obtain bioproducts from the entomopathogenic nematode symbiotic bacteria.

To produce the cell-free supernatants (CFSs), we centrifuged the 3-day-old (3-d onwards) bacterial ferments at 68.905 g (Thermo Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge, Fisher Scientific SL, Madrid, Spain) for 20 minutes at 4° C. 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 NTBA dishes 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 & Ehlers, 2001). The TSB were also filtrated to maintain the control treatments under the same conditions. To obtain the 3-d unfiltered ferments, we used the product of the bacterial ferment after three days from the inoculation keeping it at room temperature. Finally, for some experimental bioassays of bacterial VOCs, we obtained 5-d unfiltered ferments to test the secondary

metabolites in the post-exponential bacterial growth phase by keeping the bacterial fermented flasks at room temperature, close, without agitation, in semidarkness and at 22 ºC for 2 additional days. This period allows the bacteria to carry out secondary metabolites and the synthesis of defensive compounds (Kong et al., 2022).

3.2. Collection, rearing and cultivation of selected pest and diseases

3.2.1. Collecting *Philaenus spumarius*: insect vector of Pierce's diseases

Since an patent (CSIC) protects the rearing of *P. spumarius* (Morente et al., 2018), we carried out all the experiments using *P. spumarius* nymphs collected in the field. In periodic samplings during April-May 2019–2020, we collected plants with signs of foam, mostly *Carduus acanthoides* (Asterales: Asteraceae), in weeds adjacent to vineyards located in La Grajera (Logroño, La Rioja, Spain, 42º29'14"N 2º30'7"W) and belonging to the Government of La Rioja (Figure 16). In the laboratory, we kept the plant material at room temperature and under natural light conditions until the collection of the nymphs of *P. spumarius* for the experiments on the same day of capture.

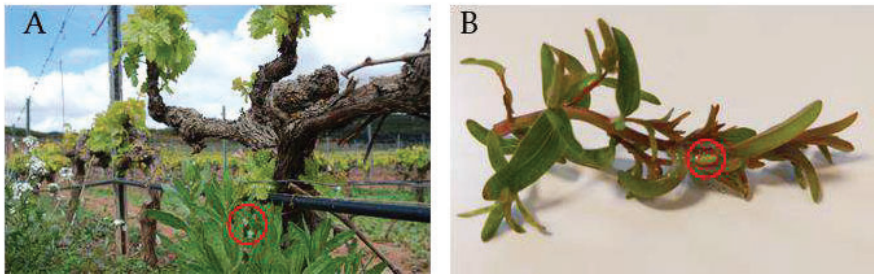

Figure 16. Signs of *Philaenus spumarius* presence in the field: A) Presence of foam next to a grapevine; and B) *Philaenus spumarius* on an adventitious weed collected adjacent to a vineyard.

3.2.2. Rearing grapevine moth: *Lobesia botrana*

The rearing of *L. botrana* took place within a controlled environment chamber at the Institute of Grapevine and Wine Sciences (ICVV, Logroño, La Rioja, Spain), maintaining conditions at 22 ºC with a relative humidity of 60% and a photoperiod of 16 hours of light and 8 hours of darkness. The initial population of *L. botrana* used to assess the pathogenicity of entomopathogenic nematodes (EPNs) was sourced from the Public University of Navarra (Spain). However, due to COVID-19 lockdown restrictions, new specimens were utilized for the bioassays examining the insecticidal properties of bacterial natural products. These specimens were provided by PhD Ally Harari from the Department of Entomology at the Volcani Center in Israel. Additionally, the rearing optimization process was carried out per the instructions and protocol provided by PhD César Gemeno from the University of Lleida (Spain).

The *L. botrana* larvae were reared on a semi-artificial diet modified from that of Ivaldi-Sender (1974) (Figure 17). Pupae were sexed following the protocol described by Steinitz et al. (2016) and kept in separate environmental chambers with unrestricted access to a 10 % sucrose in water dilution. For the bioassays, it was necessary to separate larvae of the same age cohort and pupae between males and females. We separated the same larval instars by measuring their size (third instar larva: 4.5-5.0 mm). We separated the male and female pupae based on the number of abdominal segments (male = 4 segments and female pupae = 3 segments) following the protocol described by Steinitz et al. (2016).

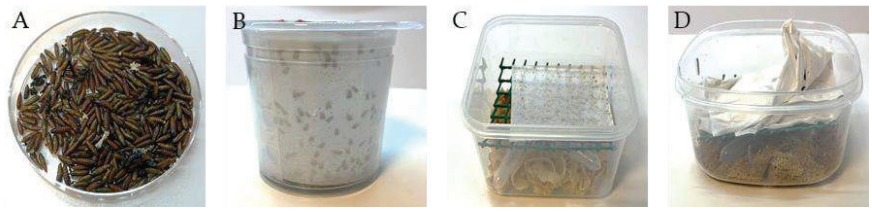

Figure 17. *In vitro* rearing protocol of the grapevine moth, *Lobesia botrana*: A) *Lobesia* pupae; B) Adult moths ovipositing on wax paper; C) *Lobesia* eggs arranged on artificial diet; and D) L5 larvae ready to pupate.

3.2.3. Cultivating and maintaining grapevine rot: *Botrytis cinerea*

The fungus *B. cinerea* was isolated from a contaminated grape cluster in the La Rioja wine region and transferred to Potato Dextrose Agar (PDA) (VWR, Leuven, Belgium) medium. For the bioassay, the pathogenic fungi were grown in Petri dishes with PDA medium by seeding a plug of agar with mycelium in active growth, and the *B. cinerea* was grown at 25 ºC for three days. The conidia were removed from these plates, and a suspension was prepared with sterile phosphate-buffering saline (PBS, pH = 7.4) at a concentration of 1×10^7 conidia/mL via cell counting method in a Neubauer counting chamber. We stored the pathogen population at -80 ºC in glycerol (20 %). Furthermore, we confirmed the identification as *B. cinerea* using molecular tools following the approach described by Bueno-Pallero et al. (2020). We compared the sequences using Blast (http://blast.ncbi.nlm.nih.gov) and those submitted to Genbank (Accession number MZ544643).

3.3. Plant material

We randomly collected ripe red grapes (*Vitis vinifera* cv. Tempranillo) and tomato fruits and leaves (*Solanum lycopersicum* cv. Sweet Million) from an organic field located in Logroño, La Rioja, Spain (42º29'14"N 2º30'7"W). Both fruits and leaves were cultivated under organic management, with no pre-harvest fungicide treatments applied. We carefully selected intact, healthy, and uniform fruit and leaves for the various bioassays and allocated them randomly to different treatments. To sterilize the surfaces of the plant material, we immersed them in a 3% (v/v) sodium hypochlorite (NaOCl) solution for 1 minute, followed by a thorough rinse with distilled water. Subsequently, we allowed them to air-dry for approximately 2 hours.

3.4. Experimental designs and statistical analysis

We conducted *in vitro* bioassays to assess the insecticidal and antifungal properties of the different bioproducts derived from *Xenorhabdus* spp. and

Photorhabdus spp. Overall, we employed a randomized complete block design with multiple replications to conduct our experiments. Appropriate controls were designated in all the studies, using same number of replicates than in the corresponding treatments. All of the experiments were performed under laboratory conditions, with 22 ºC, 60 % RH and specific conditions of photoperiod, if required. The experiments were performed 2-3 times (new materials, organisms) to confirm results. Variables were transformed, if required, before statistical analysis to evaluate the effect of the factors ($P < 0.05$).

Specific design of each experiment and data transformation is described in the following catalogue of publications. Overall, statistical analyses were conducted using SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA) for the relevant statistical tests based on the specific analyses required for each experiment. Graphs present the means with standard errors of the mean (SEM). Graphs and visual representations were generated using GraphPad Prism version 8.0.0 for Windows, Graphpad Software, San Diego, California, USA.

4. Publication catalogue

Insects

Insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from their symbiotic bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae) Nymphs

Vicente-Díez, I., Blanco-Pérez, R., González-Trujillo, M. del M., Pou, A., & Campos-Herrera, R.

Insects

Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae)

Vicente-Díez, I., Blanco-Pérez, R., Chelkha, M., Puelles, M., Pou, A., & Campos-Herrera, R.

Journal of Invertebrate Pathology

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

Vicente-Díez, I., Pou, A., & Campos-Herrera, R.

Biological Control

Exploring bacterial cell-free supernatants, unfiltered ferments and crude bacteria uses of *Xenorhabdus* and *Photorhabdus* (Morganellaceae) for controlling *Botrytis cinerea* (Helotiales: Sclerotiniaceae)

Vicente-Díez, I., Carpentero, E., Pou, A., & Campos-Herrera, R.

BioControl

Control of post-harvest gray mold (*Botrytis cinerea*) on grape (*Vitis vinifera*) and tomato (*Solanum lycopersicum*) using volatile organic compounds produced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii*

Vicente-Díez, I., Moreira, X., Pastor, V., Vilanova, M., Pou, A., & Campos-Herrera, R.

4.1. Publication 1

Insecticidal Effect of Entomopathogenic Nematodes and the Cell-Free Supernatant from Their Symbiotic Bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae) Nymphs

Ignacio Vicente-Díez, Rubén Blanco-Pérez, María del Mar González-Trujillo, Alicia Pou, Raquel Campos-Herrera

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Insecticidal Effect of Entomopathogenic Nematodes and the Cell-Free Supernatant from Their Symbiotic Bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae) Nymphs

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Simple Summary: The disease caused by *Xylella fastidiosa* affects economically relevant crops such as olives, almonds, and grapevine. Since curative means are not available, its current management principally consists of broad-spectrum pesticide applications to control vectors like the meadow spittlebug *Philaenus spumarius*, the most important one in Europe. Exploring environmentally sound alternatives is a primary challenge for sustainable agriculture. Entomopathogenic nematodes (EPNs) are well-known biocontrol agents of soil-dwelling arthropods. Recent technological advances for field applications, including improvements in obtaining cell-free supernatants from EPN symbiotic bacteria, allow their successful implementation against aerial pests. Here, we investigated the impact of four EPN species and their cell-free supernatants on nymphs of the meadow spittlebug. First, we observed that the exposure to the foam produced by this insect does not affect the nematode virulence. Indeed, direct applications of certain EPN species reached up to 90–78% nymphal mortality rates after five days of exposure, while specific cell-free supernatants produced 64% mortality rates. Overall, we demonstrated the great potential of EPN and cell-free supernatant of their symbiont bacteria applications against this vector, opening new venues to develop novel biopesticides for integrated management practices and organic productions.

Abstract: The meadow spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae) is the primary vector of *Xylella fastidiosa* (Proteobacteria: Xanthomonadaceae) in Europe, a pest–disease complex of economically relevant crops such as olives, almonds, and grapevine, managed mainly through the use of broad-spectrum pesticides. Providing environmentally sound alternatives to reduce the reliance on chemical control is a primary challenge in the control of *P. spumarius* and, hence, in the protection of crops against the expansion of its associated bacterial pathogen. Entomopathogenic nematodes (EPNs) are well-known biocontrol agents of soil-dwelling arthropods. Recent technological advances in field applications, including improvements in obtaining cell-free supernatant from their symbiotic bacteria, allow their successful implementation against aerial pests. Thus, this study aimed to evaluate, for the first time, the efficacy of EPN applications against nymphal instars of *P. spumarius*. We tested four EPN species and the cell-free supernatant of their corresponding symbiotic bacteria: *Steinernema feltiae–Xenorhabdus bovienii*, *S. carpocapsae–X. nematophila*, *S. riojaense–X. kozodoii*, and *Heterorhabditis bacteriophora–Photorhabdus laumondii* subsp. *laumondii*. First, we showed that 24 and 72 h exposure to the foam produced by *P. spumarius* nymphs did not affect *S. feltiae* virulence. The direct application of steinernematid EPNs provided promising results, reaching 90, 78, and 53% nymphal mortality rates after five days of exposure for *S. carpocapsae*, *S. feltiae*, and *S. riojaense*, respectively. Conversely, the application of the cell-free supernatant from *P. laumondii* resulted in nymphal mortalities of 64%, significantly higher than observed for *Xenorhabdus* species after five days of exposure. Overall, we demonstrated the great potential of the application of specific EPNs and cell-free supernatant of their symbiont bacteria against *P. spumarius nymphs*, introducing new opportunities to develop them as biopesticides for integrated management practices or organic vineyard production.

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Keywords: biocontrol; *Heterorhabditis*; *Photorhabdus*; *Steinernema*; sustainable agriculture; *Xenorhabdus*; *Xylella fastidiosa*

1. Introduction

The xylem-inhabiting Gram-negative bacterium *Xylella fastidiosa* (Proteobacteria: Xanthomonadaceae) can damage several relevant crops that affect the global farming economy. The main problem associated with these diseases is the obstruction of the xylem, with symptoms ranging from leaf marginal necrosis and leaf abscission to dieback, delayed growth, and death of plants through insufficient water flow [1,2]. The current forecast for the expansion and severity of these diseases, named as the grapevine Pierce's disease (PD) or the Olive Quick Decline Syndrome (OQDS), may increase shortly [1–3], but they are characterized by symptoms often similar to water stress [4]. The bacterium *X. fastidiosa* is known to colonize crops of different climatic zones worldwide. Its presence has already been reported in several countries in the EU, including Italy (west coast of Salento Peninsula, Apulia, and the Argentario, Tuscany), France (the island of Corsica and the Provence-Alpes-Côte d'Azur region), Portugal (district of Porto), and Spain (Madrid, Alicante, and the Balearic Islands) [5].

The meadow spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae) is considered the principal vector of *X. fastidiosa* in Europe and an emergent threat for several perennial crops, including vineyards and olive and almond groves [6,7]. This xylem sap-feeding insect has a univoltine life cycle and the eggs can diapause over winter for more than one hundred days, although adults survive if the climate is appropriate [8]. The eggs usually hatch in early spring, and the five nymphal instars feed on plant shoots covered by a mucilaginous foam [9] that serves as a barrier that allows the diffusion of O_2 from the surrounding atmosphere [10]. A recent study completed in the Iberian Peninsula has shown that this spittlebug mainly occurs in the spring season on herbaceous ground vegetation in olive groves across Southern, Eastern, and Central Spain and Northeastern Portugal [11]. However, it is likely that their populations increase and expand due to climate change [12,13]. The adults emerge after 5–8 weeks to start mating in late spring to early summer and, depending on weather conditions, oviposition begins in early November or later depending on the region [14]. The mucilaginous foam is known to protect the nymphs from desiccation and high temperatures [15] and could also fulfill other biological functions. For example, bioassays with cercopid nymphal foam revealed that it could protect them from some predators because they can be repellent or produce irritation [16]. Both the nymphal and adult instars of *P. spumarius* can inoculate the pathogen *X. fastidiosa* to healthy plants immediately after acquiring it by feeding on the xylem of infected plants [7].

Since there are currently no curative means for the control of the diseases caused by *X. fastidiosa* [4], such as PD in grapevines or OQDS in olive groves, the management of these diseases focuses on its vectors [2], mainly based on chemicals, particularly on neonicotinoids' and pyrethroids' products [17]. For the biological control of *P. spumarius*, there are only a few reports involving entomopathogenic fungi [18], some parasitoids, and natural predators such as wasps and spiders [19]. Under the current paradigm of severe restrictions in the use of pesticides for pest control [20,21], there is an urgent need for more biologically sound and low impact practices [22]. In this context, it is crucial to search for efficient biotools and new alternative management strategies based on biological control agents and natural compounds compatible with integrated management practices (IPM) and organic production.

Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are well-known biological control agents that become entomopathogens in symbiosis with γ-Proteobacteria species in the genera *Xenorhabdus* and *Photorhabdus*, respectively [23–26]. Their non-feeding free-living infective juvenile (IJ) stage survives in the

soil, searching for a suitable host. Once located, IJs penetrate within the hemocoel to release the symbiont bacteria. The nematode–bacterium complex overcomes the host's immune response, allowing the bacteria to proliferate exponentially and killing the arthropod by septicemia within 48–72 h after infection [23]. The IJs develop into adults and reproduce, feeding on their partner bacteria and degraded host tissues until the resources deplete. The second-stage juveniles then molt to IJs, incorporate some of the symbiont bacteria, and emerge from the host into the soil to begin a new cycle [27,28]. During this process, *Xenorhabdus* and *Photorhabdus* bacteria produce a diversity of natural products (NPs), such as phage-derived bacteriocins, colicin E3-type killer proteins, and insect toxin complexes, that kill the host and defeat other microbes competing for food sources [29–31]. These NPs, present in the cell-free supernatant, exhibit toxicity against many pests [32].

Traditionally, the application of EPNs was limited to the biological control of arthropod pests that inhabit agricultural soils [33,34]. Advances in application and formulation technologies allow their use against aerial pests [35]. The use of EPNs could be an alternative to manage *P. spumarius*'s nymphs. A previous study reported that the use of native EPN species produced high nymphal mortality rates (62–73%) against the species *Philaenus simulans* and *P. teapana* in sugar cane fields [36]. However, it is unknown if *P. spumarius* control can also be effective by EPN applications. In addition, whether the foam produced by the nymphs may be a suitable environment for EPN survival is still unknown. Furthermore, even if the aerial application of cell-free supernatant is becoming a novel system to control different pests [37], it has not yet been tested against any species of spittlebug nymphs. Thereby, we hypothesized that the foam produced by *P. spumarius* nymphs might not affect EPNs, and EPN activity against *P. spumarius* nymphs will be species-specific. Similarly, we expect that the NPs of the cell-free supernatant obtained from EPN symbiont bacteria will affect them during their feeding activity, causing death. The objectives of this study were (i) to investigate the effect of the foam produced by *P. spumarius* on EPN activity and to evaluate (ii) the EPN virulence and (iii) the symbiont bacterial cell-free supernatant's toxicity against *P. spumarius* nymphs.

2. Materials and Methods

2.1. Collecting and Rearing of Organisms

Since an ongoing patent (CSIC) protects the rearing of *P. spumarius* [38], we performed all the experiments using nymphs of *P. spumarius* collected in the field. In periodic samplings during April-May 2019–2020, we collected plants with signs of foam, mostly *Carduus acanthoides* (Asterales: Asteraceae), in weeds adjacent to vineyards located in 'La Grajera' (Logroño, La Rioja, Spain, 42°26′ N and 2°30′ W) and belonging to the Government of La Rioja. In the laboratory, we kept the plant material at room temperature and under natural light conditions until the collection of the nymphs of *P. spumarius* for the experiments on the same day as the capture.

We evaluated four EPN species against nymphs of *P. spumarius*: *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora* (Table 1). EPNs were cultured in last-instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) reared at the Instituto de Ciencias de la Vid y del Vino (Logroño, Spain). The IJs were recovered in tap water upon emergence, stored at 12–14 °C, and used within two weeks after harvest [39]. We completed the molecular identification of all EPN populations following the procedures described by Blanco-Pérez et al. [40]. Briefly, we mechanically disaggregated ~500 IJs employing sterile blue pestles assembled in a pellet mixer (VWR International, Lutterworth, UK). Then, we extracted the DNA with the Speedtools tissue DNA extraction kit (Biotools, Madrid, Spain), analyzed it for quality and quantity using a Nanodrop system (Thermo Scientific 2000C spectrophotometer, provided by Actylab, Logroño, Spain), and stored it at −20 °C until use. For each EPN species, the ITS rDNA region was amplified using universal primers and following the protocols described by Campos-Herrera et al. [41]. All runs contained a negative control by adding mQ water (Milli-Q Water System, Millipore S.A., Molsheim, France) instead of DNA template. Hereafter, the PCR was verified through electrophoresis

in 2% agarose gel in TBE (pH 8.0 ± 0.1) to ensure the expected PCR size. Later, individual bands were cut and cleaned (SpeedTools Tissue DNA Extraction kit, Biotools, Madrid, Spain), sequenced, aligned with the software Geneious (R.6.1.5., Biomatters, Inc., Auckland, New Zealand), compared to reported sequences using Blast (http://blast.ncbi.nlm.nih.gov), and submitted to Genbank (Table 1).

Table 1. Entomopathogenic nematode (EPN) and symbiotic bacterial species tested against nymphs of *Philaenus spumarius*.

EPN Species	Population	ITS-GenBank Accession	Bacterial Species	ITS-GenBank Accession
Steinernema feltiae	RM-107	MW480131	*Xenorhabdus bovienii*	MW467374
Steinernema carpocapsae	All	MW574913	*Xenorhabdus nematophilus*	MW574906
Steinernema riojaense	RM-30	MK503133	*Xenorhabdus kozodoii*	MW467375
Heterorhabditis bacteriophora	RM-102	MW480132	*Photorabhdus laumondii* subsp. *laumondii*	MW574908

The symbiotic bacteria associated with the tested EPN species comprised three *Xenorhabdus* species (*X. bovienii*, *X. nematophila*, and *X. kozodoii*) and one *Photorhabdus* species (*P. laumondii* subsp. *laumondii*) (Table 1). To isolate them, we exposed~500 IJs of each EPN species (inoculated in 100 µL of distilled water) to 5% NaClO for 2–5 min. Later, after thoroughly washing with distilled water, we mechanically disaggregated them in a 50:50 (*v*/*v*) suspension of distilled water and nutritive broth (VWR, Chemicals, Barcelona, Spain), employing sterile blue pestles assembled in a pellet mixer. Then, we seeded 50 µL of each nematode–bacterium complex suspension on three Petri dishes with Nutrient Agar (NA), Bromothymol blue (Alfa Aesar, Kandel, Germany), and 2,3,5-Triphenyl tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates) [42], supplemented with Ampicillin (50 mg/mL) (PanReac AppliChem, ITW Reagents, Barcelona, Spain). We stored the Petri dishes for 48 h under controlled conditions (25 ± 2 °C, 20% RH in the dark) before selecting those colonies of morphology associated with most *Xenorhabdus* [43] and *Photorhabdus* [31] species (rounded, smooth margins, and colorant absorption capacity). To obtain pure colonies, we seeded them in NTBA plates. Subsequently, we inoculated single colonies of each pure culture in Triptone Soya Broth (TSB) (VWR Chemicals, Barcelona, Spain), also supplemented with Ampicillin (50 mg/mL), maintaining the liquid cultures for 16 h under stirring (150 rpm) at 22 °C in the dark. We used an aliquot of each suspension to verify the absence of the catalase enzyme of the *Xenorhabdus* strains [27]. Additionally, we checked under a microscope the morphology of bacilli and its mobility using the flagellum. A second aliquot of each bacterial suspension was concentrated and saved for DNA extraction, performed with the Speedtools tissue DNA extraction kit (as described above), and the rest stored at −80 °C in aliquots of 300–400 µl in 30–35% glycerol. We used universal primers to amplify the 16S rDNA region [44].

2.2. Production of Cell-Free Supernatant from the Symbiotic Bacteria of Entomopathogenic Nematodes

The bacterial stock was initiated from a single colony of each of the four bacteria, inoculated in liquid media, and grown for 16 h at 25 °C ± 2 °C in darkness under agitation at 150 rpm. Aliquots of 500 µL were stored at −80 °C for each bacterium. Then, we inoculated 100 µL of the aliquots to produce cell-free supernatant in 250 mL of TSB (two 500 mL Erlenmeyer per bacteria). A volume of 50 µL was also seeded in NBTA plates to verify the growth of pure bacteria. We incubated the Erlenmeyer on a shaker for three days under aerobic and dark conditions, at 150 rpm and 25 ± 2 °C, in darkness. Subsequently, we centrifuged the bacterial media at 25830 g and 4 °C for 40 min, and the supernatant

was filtered through a 0.22 µm sterile pore filter. An aliquot of this filtrate was cultured on NBTA plates to verify the absence of bacteria. The filtrate was defined as cell-free supernatant and subsequently used in toxicity tests. The TSB media used as controls were also filtrated to follow the same protocols as treatments. The material was used immediately upon filtration.

2.3. Evaluation of Entomopathogenic Nematode Virulence after Exposure to Foam Produced by *Philaenus spumarius*

We evaluated the IJ virulence when exposed to the foam produced by the nymphs of *P. spumarius* for 24 and 72 h for *S. feltiae* and only for 24 h for *H. bacteriophora*. We employed two 24-multi-well trays (Corning, NY, USA) per treatment, using 12 interleaved wells per tray. In each well, we added 0.5 g of sterilized sand (pure sand, Vale do Lobo, Loulé, Portugal), 1 cm^2 of a leaf of *C. acanthoides* (Finca de La Grajera, La Rioja, Spain), and the volume of foam corresponding to (approximately) the production of a single nymph of *P. spumarius*. Immediately after, 20 µL of water with 3 IJs was inoculated inside the foam. The control treatments followed the same procedure but without the presence of the foam. In addition, we included two treatments without nematode application, one with only water and another with foam only, as controls in the subsequent study of infectivity against *G. mellonella*. After incubation under controlled conditions (80% RH, 20 °C/16 h light, and 14 °C/8 h dark, on-ramp/for 24 or 72 h), we added *G. mellonella* larvae to each well. We checked the larval mortality daily for six days. The experiment was conducted twice with freshly produced foam, plant material, larvae of *G. mellonella*, and nematode cultures.

2.4. Evaluation of Entomopathogenic Nematode Virulence and Bacterial Cell-Free Supernatant Toxicity against *Philaenus spumarius*

We placed five nymphs of *P. spumarius* (using hairbrush 000 sizes) in 55 mm Petri dishes (n = 10) with two filter papers (Whatman no.1) arranged on the inner faces. The final volume applied per filter paper was 500 µL. First, to favor nymph settlement, we moistened them with distilled water, 400 µL for the EPN virulence test and 450 or 425 µL (depending on the selected supernatant dilution, see below) for the cell-free supernatant toxicity test. Then, in the EPN test, we inoculated a total of 75 IJs per Petri dish, applied half on the top and half on the bottom filter paper in 100 µL suspension. In the case of the cell-free supernatant toxicity test, we applied 50 or 75 µL of the supernatant (to obtain a concentration of 1:10/1: 6.67 metabolite concentration) to each of the filter papers. In all the cases, we included control treatments containing only water or equal proportions of sterile and filtered culture media. We also included for the cell-free supernatant experiment the mixed treatments *X. bovienii* + *X. nematophila* (1:1) and *X. nematophila* + *P. laumondii* subsp. *laumondii* (1:1) to study the interaction of their metabolites. All the plates, closed with parafilm, were incubated under controlled conditions with an increase in temperature to simulate regional spring temperatures (±60% RH, 20 °C/10 h light and 14 °C/14 h darkness) (https://www.larioja.org/agricultura/es/informacion-agroclimatica/red-estaciones-agroclimaticas-siar) (accessed on 29 March 2021). We applied 50 µL of a sucrose solution (1 g in 10 mL distilled water) per Petri dish every two days to allow their feeding. We checked the nymphal mortality daily for six days. The experiment was conducted twice with freshly produced bacterial cell-free supernatant, nematodes, and insects.

2.5. Statistical Analyses

We ran general linear models (GLM), with a binomial distribution (logit-link function), for the pair-treatment comparisons (control versus treatment) testing the impact of the foam produced by P. *spumarius* on EPN virulence against G. mellonella last-instar larvae as well as the IJ virulence and bacterial cell-free supernatant toxicity on nymphs of *P. spumarius*. To evaluate the nature of the combination of NPs (antagonistic, no-interaction/additive, or synergistic), we followed the formulae proposed by Shapiro-Ilan et al. [45] and Ansari et al. [46]. We compared the expected and observed nymph mortalities for each single

NP and mixed NP. The expected mortalities (M_E) were calculated as $M_E = M_{T1} + [M_{T2} \times (1 - M_{T1})]$ when different NPs were combined. We ran an χ^2 test for the expected and observed mortalities [i.e., $\chi^2 = (M_{T1T2} - M_E)^2/M_E$, where M_{T1T2} is the observed mortality for each single NP]. These values were matched with the χ^2 table for one degree of freedom ($p = 0.05$) so that $\chi^2 < 3.8415$ indicated additive interaction and $\chi^2 > 3.8415$ non-additive (antagonist or synergist) interaction. Thus, the interaction was considered synergistic if $M_{T1T2} - M_E > 0$, and antagonistic if $M_{T1T2} - M_E < 0$ [45,46]. We performed all analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), using $p < 0.05$ for assessing statistical differences. We used least-square means ± SE as descriptive statistics.

3. Results

The foam produced for nymphs of *P. spumarius* affected neither EPN pathogenicity nor *G. mellonella* larvae, independently of the EPN species evaluated or the time of exposure (Figure 1).

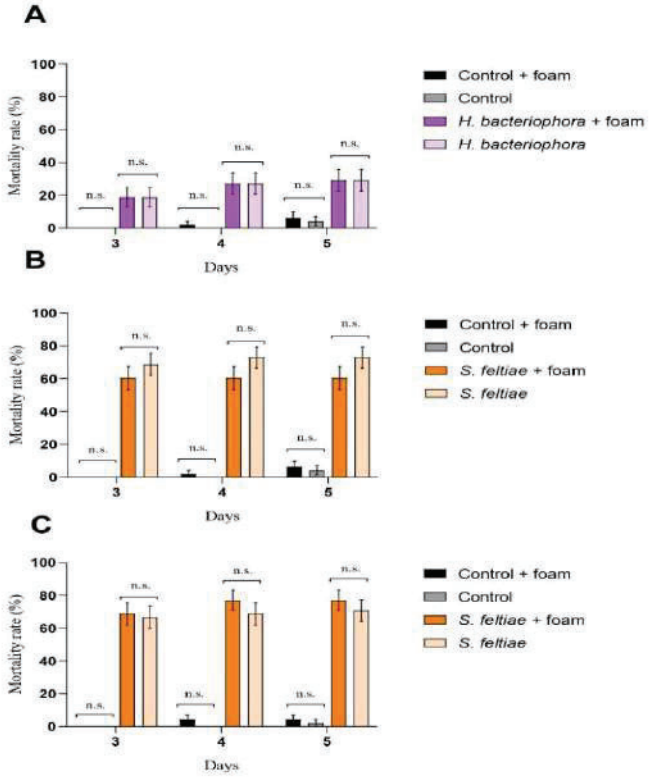

Figure 1. Entomopathogenic nematode pathogenicity against *Galleria mellonella* larvae after exposure to the foam produced for *Philaenus spumarius*. Cumulative larval mortality (three to five days) at (**A**) 24 h exposure for *Heterorhabditis bacteriophora*, (**B**) 24 h exposure for *Steinernema feltiae*, and (**C**) 72 h exposure for *S. feltiae*. No significant differences (n.s.) ($p < 0.05$) in general linear model testing within pair-treatment comparisons of exposure and no exposure to foam were found. Values are least-square means ± SE.

We reported high nymphal mortality rates for the three steinernematid species for all revised days, particularly for *S. feltiae* and *S. carpocapsae* IJs, while we did not observe differences in the mortalities due to *H. bacteriophora* and control (absence of IJs) treatments (Figure 2; Supplementary Material, Table S1). On the other hand, the nymph mortality was strongly dependent on the initial concentration of cell-free supernatant applied: the application of 1:10 dilutions, except for a few cases, significantly increased nymph mortality rates compared to control treatments for all counting days (Figure 3), while 1:6.67 dilutions did not (Figure 4; Supplementary Material, Table S1). Contrary to our observations for IJ inoculations, we reported the highest mortality rates for the application of 1:10 dilution cell-free supernatant from *P. laumondii* subsp. *laumondii*, the symbiont bacteria isolated from *H. bacteriophora*. (Figure 3). For the natural products derived from *Xenorhabdus* spp., we observed differences only for the cell-free supernatant from *X. nematophilus* (isolated from *S. carpocapsae*) for all counting days and from *X. kozodoii* (isolated from *S. riojaense*) at day three and four after application (Figure 3; Supplementary Material, Table S1). Both cell-free supernatant combinations resulted in additive effects (Table 2).

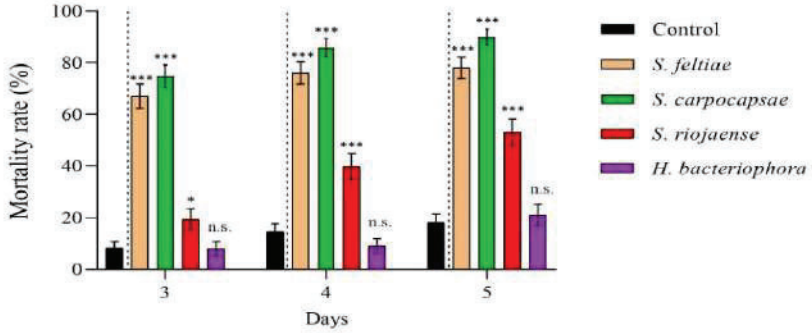

Figure 2. Entomopathogenic nematode (EPN) pathogenicity against *Philaenus spumarius* nymphs. Cumulative larval mortality (three to five days) for the EPN species *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, *Heterorhabditis bacteriophora*, and the absence of nematodes (control). Asterisks indicate significant differences at *** $p < 0.001$, * $p < 0.05$, and n.s., not significant, for generalized linear models testing within pair-treatment comparisons of inoculations and no inoculations (control) of EPNs. Values are least-square means ± SE.

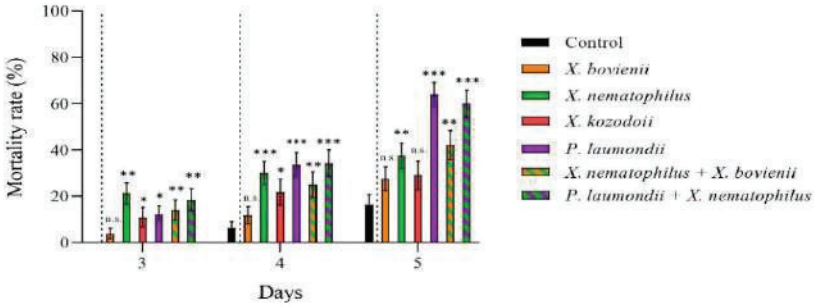

Figure 3. Cell-free supernatant 1:10 diluted against *Philaenus spumarius* nymphs. Cumulative larval mortality (three to five days) for the symbiont bacterial species *Xenorhabdus bovienii*, *X. nematophilus X. kozodoii*, *Photorhabdus laumondii*, and the combinations of *X. nematophilus* + *X. bovienii* and *P. laumondii* + *X. nematophilus*. Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and n.s., not significant, for generalized linear models testing within pair-treatment comparisons of inoculation and no inoculation (control) of cell-free supernatants. Values are least-square means ± SE.

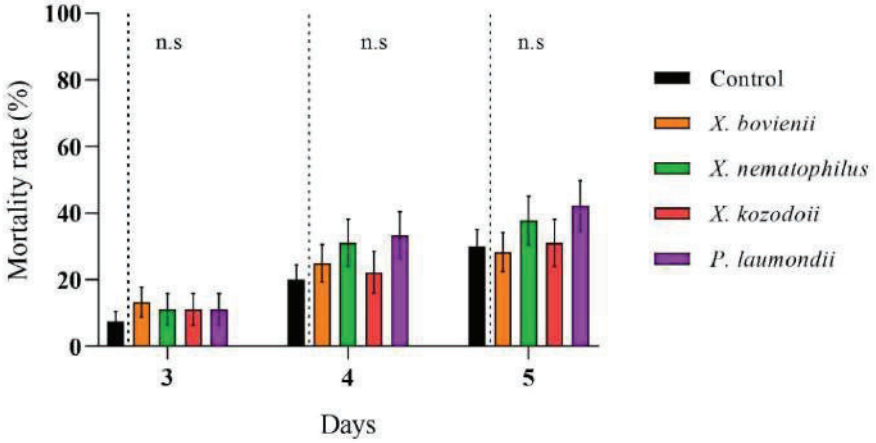

Figure 4. Cell-free supernatant 1:6.67 diluted against *Philaenus spumarius* nymphs. Cumulative larval mortality (three to five days) for the symbiont bacterial species *Xenorhabdus bovienii*, *X. nematophilus X. kozodoii*, *Photorhabdus laumondii*, and the combinations of *X. nematophilus* + *X. bovienii* and *P. laumondii* + *X. nematophilus*. No significant differences (n.s.) ($p < 0.05$) for general linear model testing within pair-treatment comparisons of inoculation and no inoculation (control) of cell-free supernatants were found. Values are least-square means ± SE.

Table 2. Interactions of the mixed cell-free supernatant applications *Xenorhabdus bovienii* + *X. nematophilus* and *X. nematophilus* + *Photorhabdus laumondii*. Expected mortality (ME) calculated as ME = MT1 + [MT2 x (1 − MT1)], where MT1 and MT2 are the observed mortality rates (%) recorded for single cell-free supernatant applications. Interactions were based on χ^2 ratio between expected and observed mortalities ($\chi 2$ = (MT1T2 − ME)2/ME, where MT1T2 is the observed mortality for each single application).

Combinations	Observed Mortality (%)	Expected Mortality (%)	χ2	Interaction
X. nematophilus + *X. bovienii*	42	43	3.20	Additive
P. laumondii + *X. nematophilus*	60	68	2.33	Additive

4. Discussion

This study shows the potential of EPNs and the application of their symbiont bacterial cell-free supernatant to control nymphs of *P. spumarius*. First, we observed that the foam produced by *P. spumarius* nymphs did not affect EPN virulence after 24 and 72 h of exposure, despite previous records on the nature and function of this foam. Other studies proposed that cercopid foam creates a microhabitat that protects against desiccation, extreme temperatures, and predatory and parasitic enemies [15]. Indeed, the only parasite of cercopid nymphs reported is a nematode in the family Mermithidae [47]. In this line, laboratory bioassays showed that some natural spittlebug foams, and a synthetic mixture composed of representative compounds identified in it, are repellent to ants and produce topical irritation in cockroaches [16]. However, our results showed that the foam was not deleterious to IJs. Since the foam could also protect the applied IJs and facilitate their movement to locate the nymphs, our results suggest that the direct application of EPN suspensions in the spit–nymph complex might be compatible and hence a promising method to control the pest in crops.

It is noteworthy that the opposite results were obtained for the insecticidal effect against *P. spumarius* nymphs for IJs and cell-free supernatants of the same EPN species. Thus, after five days of exposure, we reported over 80% nymphal mortality rates for *S. carpocapsae* and *S. feltiae* IJ applications, while they did not reach 50% for the NP applications

of their symbiont bacteria. Conversely, the pathogenicity observed for *H. bacteriophora* IJs was not significantly higher than that obtained for the controls, while their NP applications surpassed 60% mortality at five days of exposure. These results illustrate the differences between EPN species in their efficiency when locating and penetrating susceptible hosts, and how the environment might modulate their virulence. For example, temperature is a factor that affects EPN infectivity and reproduction [48]. The virulence studies against *P. spumarious* nymphs were performed in a temperature range of 14–20 °C to simulate typical spring changes in La Rioja (Spain). These changes in the temperature could favor the activity of certain species. For example, *S. feltiae* infection can be achieved from 8 to 30 °C and reproduction from 10 to 25 °C, while the *H. bacteriophora* range included higher values, from 10 to 32 °C and 15 to 30 °C, respectively [48]. The limited infectivity observed for *H. bacteriophora* IJs could be due to the stress when the temperature decreased to 14 °C. Surprisingly, *S. carpocapsae* IJs exhibited high mortality rates against *P. spumarius* nymphs even if the temperature range for successful infectivity and reproduction was similar to that reported for *H. bacteriophora*. It is plausible that the differences in virulence observed in this study are due to the broader/limited range of temperature for infectivity and reproduction that could characterize the selected population. Moreover, several EPN species could likely show better compatibility with this host. Regardless of whether the best infectivity is related to the EPN population employed, the fit with the host, or a combination of both, our results showed the compatibility of certain EPN species to fight against the nymphs of *P. spumarius*.

On the other hand, we verified, for the first time, the insecticidal activity of *Xenorhabdus* and *Photorhabdus* cell-free supernatants against *P. spumarius* nymphs when ingested orally. Nymphal mortality was observed the day after the application of the sucrose suspension that allowed them to feed. The wide variety of products released by EPN symbiont bacteria perform different functions for the nematode–bacteria complex. The toxicity of cell-free supernatants extracted from EPN symbiont bacteria against an ample range of insects is well known [49]. Indeed, *Xenorhabdus* [50] and *Photorhabdus* [51,52] display different gene clusters related to their bioactivity that, when combined, establish a suitable niche to survive and reproduce within the host cadaver. This diversity of natural compounds makes them a powerful tool for exploring new bioproduct development to be used as biopesticides. However, additional studies are required to establish the specific compounds responsible for the insecticidal effect on selective targets and, in particular, *P. spumarius*.

To improve the insecticidal effect of individual bacterial cell-free applications, we combined and tested the cell-free supernatant proceeding from different symbiont bacterial species. We observed that none of the two mixed treatments, *X. bovienii* + *X. nematophila* and *X. bovienii* + *P. laumondii* cell-free supernatants (1:1), enhanced the insecticidal impact over the prevailing metabolite, showing a final additive effect. Further investigation to enhance this activity might warrant attention. For example, the natural product generation might differ if two or more bacteria species are combined at the beginning of the fermentation. Moreover, different proportions to the 50:50 investigated herein can increase nymph mortality. In this line, our results revealed the importance of fine-tuning for bacterial cell-free supernatant applications. Thus, we observed that 1:10 dilution applications were bioactive against *P. spumarius* nymphs, while, at a slightly higher concentration (1:6.67), the possible insecticidal effect was masked by the TSB oral toxicity observed in the controls. Hence, additional studies are required to select the best bacterial NPs, concentration rates, and application procedures to optimize the use of this promising biotool.

To the best of our knowledge, no previous study relates the direct application of EPNs and the use of NPs from their symbiotic bacteria to control the same pest. Since *P. spumarius* is the most relevant vector of *X. fastidiosa* in the EU, there is an urgent need to provide tools to reduce its propagation, particularly in organic production, for which the use of pesticides is strictly limited. Furthermore, EPN implementations to fight this vector–disease complex are highly viable as there are numerous commercial products based on them [53]. However, additional studies are required to evaluate the impact of EPN or cell-free supernatant

application on *P. spumarius* nymphs infected with *X. fastidiosa*. Advances in this knowledge will contribute to extending the strategies currently proposed by the EU, focused on host removal, vector control, and restrictions on the production and transport of plant materials, for the eradication or containment of this disease.

5. Conclusions

Our results showed that the foam produced by nymphs of the spittlebug *P. spumarius* did not affect EPN virulence. Indeed, *steinernematid* IJs caused significant nymphal mortality rates while *H. bacteriophora* not. Moreover, the cell-free supernatant obtained from their symbiont bacteria showed toxicity against *P. spumarius* nymphs, particularly for *Photorhabdus* species. The knowledge gained herein has opened a new avenue for advances in innovative approaches to complement traditional strategies. These natural products are promising biopesticides that require a deep understanding due to their broad potential for controlling arthropod pests in sustainable agriculture. Therefore, further research is needed to isolate, identify, and characterize the metabolites produced by the EPN symbiotic bacteria, but also to prove that their application will be safe for non-target organisms, plants, and the environment before being used as biopesticides.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects12050448/s1, Table S1. Results from generalized linear mixed models testing within pair-treatment comparisons (treatment vs. controls) for the impact of entomopahtogenic nematodes (EPNs) and cell-free supernatants (SM) of their symbiont bacteria (applied at two concentrations) on *P. spumarius* nymphs. Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and n.s., not significant.

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4.1.1. Supplementary materials

Table S1. Results from generalized linear mixed models testing within pair-treatment comparisons (treatment vs. controls) for the impact of entomopahtogenic nematodes (EPNs) and cell-free supernatants (SM) of their symbiont bacteria (applied at two concentrations) on *P. spumarius* nymphs. Asterisks indicate significant differences at $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and n.s., not significant.

		Day 3		Day 4		Day 5	
	Pair-treatment comparison (treatment vs. control)	χ^2	P	χ^2	P	χ^2	P
EPNs	*S. feltiae*	70,491	***	76,652	***	72,865	***
	S. carpocapsae	83,603	***	91,33	***	86,051	***
	S. riojaense	5,963	*	18,398	***	30,077	***
	H. bacteriophora	0,012	n.s.	1,726	n.s.	0,299	n.s.
SM 1 : 10	*X. bovienii*	1,063	n.s.	1,39	n.s.	2,78	n.s.
	X. nematophilus	11,988	**	12,499	***	8,53	**
	X. kozodoii	5,092	*	6,284	n.s.	2,99	n.s.
	P. laumondii	6,581	*	15,442	***	34,42	***
	X. nematophilus + *X. bovienii*	7,233	**	8,581	**	10,99	***
	X. nematophilus + *P. laumondii*	10,128	**	15,088	***	27,19	***
SM 1 : 6.67	*X. bovienii*	1,265	n.s.	0,496	n.s.	0,46	n.s.
	X. nematophilus	0,462	n.s.	1,923	n.s.	0,787	n.s.
	X. kozodoii	0,459	n.s.	0,086	n.s.	0,017	n.s.
	P. laumondii	0,459	n.s.	2,685	n.s.	1,886	n.s.

4.2. Publication 2

Exploring the Use of Entomopathogenic Nematodes and the Natural Products Derived from Their Symbiotic Bacteria to Control the Grapevine Moth, *Lobesia botrana* (Lepidoptera: Tortricidae)

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Exploring the Use of Entomopathogenic Nematodes and the Natural Products Derived from Their Symbiotic Bacteria to Control the Grapevine Moth, *Lobesia botrana* (Lepidoptera: Tortricidae)

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Simple Summary: The European grapevine moth (EGVM) *Lobesia botrana* (Lepidoptera: Tortricidae) attacks vineyards in Europe, the Middle East, and North and South America. Global movement toward sustainable agriculture urges the development of environmentally friendly tools that can replace traditional pesticides. Entomopathogenic nematodes (EPNs) are well-known biological control agents against various arthropod pests. The EPNs act together with symbiotic bacteria that produce natural products with insecticidal potential. Novel formulations and application technology allow their application against aerial pests, including those associated with vineyards. This study investigated the viability of four EPN species and their corresponding bacteria derivates (unfiltered ferment, UF, or cell-free supernatant, CFS) against EGVM (larval and pupa instars). The results revealed that all EPN species killed various EGVM larval stages. Killing pupae required a higher number of IJs than controlling larvae. *Steinernema carpocapsae* registered the most promising results, killing ~50% L1 and >75% L3/L5 in 2 days. The use of the bacterial bioactive compounds achieved similar results, with UF registering higher activity than CFS. Overall, we demonstrated that both EPN and bacterial bioproducts have a great potential to control EGVM in sustainable viticulture. Further research in co-formulation with adjuvants is required to ensure their survival in the aboveground grapevine areas.

Abstract: The European grapevine moth (EGVM) *Lobesia botrana* (Lepidoptera: Tortricidae) is a relevant pest in the Palearctic region vineyards and is present in the Americas. Their management using biological control agents and environmentally friendly biotechnical tools would reduce intensive pesticide use. The entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are well-known virulent agents against arthropod pests thanks to symbiotic bacteria in the genera *Xenorhabdus* and *Photorhabdus* (respectively) that produce natural products with insecticidal potential. Novel technological advances allow field applications of EPNs and those bioactive compounds as powerful bio-tools against aerial insect pests. This study aimed to determine the viability of four EPN species (*Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora*) as biological control agents against EGVM larval instars (L1, L3, and L5) and pupae. Additionally, the bioactive compounds from their four symbiotic bacteria (*Xenorhabdus bovienii*, *X. nematophila*, *X. kozodoii*, and *Photorhabdus laumondii* subsp. *laumondii*, respectively) were tested as unfiltered ferment (UF) and cell-free supernatant (CFS) against the EGVM larval instars L1 and L3. All of the EPN species showed the capability of killing EGVM during the larval and pupal stages, particularly *S. carpocapsae* (mortalities of ~50% for L1 and >75% for L3 and L5 in only two days),

followed by efficacy by *S. feltiae*. Similarly, the bacterial bioactive compounds produced higher larval mortality at three days against L1 (>90%) than L3 (~50%), making the application of UF more virulent than the application of CFS. Our findings indicate that both steinernematid species and their symbiotic bacterial bioactive compounds could be considered for a novel agro-technological approach to control *L. botrana* in vineyards. Further research into co-formulation with adjuvants is required to expand their viability when implemented for aboveground grapevine application.

Keywords: cell-free supernatant; grape; *Heterorhabditis*; *Photorhabdus*; *Steinernema*; secondary metabolites; unfiltered ferment; *Xenorhabdus*

1. Introduction

The vineyard agroecosystem is one of the main study perennial crops, covering 7.5 M ha worldwide [1]. Controlling the principal pests and diseases is crucial for maintaining qualitative and quantitative production standards [2]. Conventional viticulture continues to be the most pesticide-consuming agricultural system even though worldwide interest in organic farming has increased significantly since the last decade [3]. Organic wine production aims at producing high-quality grapes and wines while minimizing the use of inputs and improving environmental care. The control of pests [4,5] and diseases [6] needs new biotechnological approaches that facilitate this possibility.

Even the accepted mean use of synthetic insecticides in conventional viticulture, grapevine moths are severe damage agents for grapes worldwide, causing yield losses and quality reduction. *Lobesia botrana* Denis & Schiffernüller (Lepidoptera: Tortricidae), known as the European grapevine moth (EGVM), is a relevant pest in European and the Middle Eastern vineyards. Current studies have reported EGVM as a new grape pest in the Americas: in Chile (2008) and Argentina (2009) [7,8] and in California (2010) [9]. Furthermore, global warming could have two critical collateral effects on the management of this pest. First, all of the suitable areas for *Vitis vinifera* are at risk of EGVM pest presence [10,11]. On the other hand, warming-derived phenological shifts imply a higher impact of first-generation EGVM and increased voltinism [12,13], increasing the land range EGVM's damaging effects. The first larval generation of the season usually attacks inflorescence, while later generations cause damage to the fruits. In addition to direct damage on berries, pest occurrences result in disease due to the interconnected relationships in the entire agroecosystem. For example, the presence of larvae encourages bunch rot development (causal agents being *Aspergillus*, *Alternaria*, *Rhizopus*, *Cladosporium*, *Penicillium*, and *Botrytis*), which results in severe qualitative and quantitative damages [2,9,14].

Due to the mentioned progressive EGVM expansion and all of the damage that can they can cause to vineyards by grey mold induction [10], the interest in controlling EGVM is more pressing than ever [15]. Different effective measures to manage EGVM exist based on classical biological control methods and on the use of eco-friendly biotechnical tools (Table 1). For example, *Trichogramma* spp. is a natural enemy of EGVM due to its parasitic eggs [16], and *Bacillus thuringiensis* (*Bt*) is a well-known effective bio-insecticidal bacteria [6,17]. In addition, *Bt* produces several active compounds that are associated with pests and disease control, such as zwittermicin A and acyl homoserien lactonase [18]. Moreover, in organic viticulture, the pheromone-mediated mating disruption (MD) can be used against EGVM [19,20]. This environmentally friendly technique, which uses non-target effects, employs high doses of the pest´s synthetic sex pheromone in vineyard plots to interfere with its reproduction. Even though MD has some handicaps such as socio-economic challenges that hinder the introduction of this approach among the other grower tools, it requires pretreatment with conventional insecticides that have been endorsed by IPM regulations and needs at least a 5 ha extension to be effective [20].

Table 1. Overview of biological control agents and biotechnical control tools against grapevine moths in viticulture and the facultative use of entomopathogenic nematodes and their symbiotic bacteria-based products.

			Principal Target	References
Biological control agents	Entomopathogenic fungi	*Beauviera bassiana*	Pupae	López Plantey et al., 2019 [15]
		Metarhizium spp. (*M. robertsii*; *M. anisopliae*)	Larvae	López Plantey et al., 2019; Sammaritano et al., 2018 [15,21]
	Bacterias	*B. thuringiensis*	Larvae Adults	Ioriatti et al., 2011 [20]
	Artropods — Predator	*Chrysoperla sp.*	Eggs Pupae	Castex et al., 2018 [16]
	Artropods — Parasitoids	*Trichogramma* sp. *Dibrachis* sp.	Eggs Pupae	Pérez Moreno et al., 2000 [22]
	Entomopathogenic nematodes	*Steinernema* sp. *Heterohabditis* sp.	Larvae Pupae	Current research
	Pheromone-mediated Malting Disruption (MD)		Adults	Ioriatti et al., 2011; Shapira et al., 2018 [19,20]
Biotechnical control tools	Insecticidal-plants extracts	*Bifora radians*	Larvae	Gökçe et al., 2011 [23]
	Insecticidal-bacterium based product	*Bacillus thuringiensis*	Larvae	Ifoulis and Savopoulou-Soultani, 2004 [24]
		Xhenorhabdus sp. *Photorhabdus* sp.	Larvae	Current reserch

The implementation of good integrated pest management (IPM) that enhances the presence of biological control agents could facilitate an overlap between pests and their natural enemies. In addition, expanding current biotechnological control tools is needed, especially in organic crop management (Table 1). In this context, entomopathogenic nematodes (EPNs) are good candidates with probed virulence toward numerous arthropod pests [25,26]. Their non-feeding, free-living infective juvenile (IJ) stage can penetrate the hemocoel of the host and can release a mutualistic enteric γ-Proteobacteria (genera *Xenorhabdus* for steinernematids and *Photorhabdus* for heterorhabditids). The Phase-I symbiotic bacteria overcome the host's immune response through the secretion of a wide variety of biologically active compounds. These natural products (NPs) have broad-spectrum activity that can result in two effects: (1) toxicity effects (insecticidal, fungicidal, antibiotic activity) [27] and (2) deterrent effects that allow the EPN to protect the cadaver by deterring opportunistic and scavenger organisms [28]. The bacterial growth increases exponentially, resulting in the death of the arthropod by cause of septicaemia within 48–72 h of infection. Inside the cadaver, the EPNs feed on their partner bacteria and the degraded host tissues. Due to the resources being depleted, second-stage juveniles develop to the IJ stage, incorporate some of the symbiotic bacteria, and exit the insect cadaver by the thousands into the soil to start a new cycle [26,29–31].

In the context of modern viticulture, we considered that the use of EPNs as well as the use of the bioactive compounds that are obtained by their symbiotic bacteria could be an additional alternative to chemical treatments (Table 1) [32]. Although one of the limitations for the use of the EPN against the EGVM is their main distribution in the aboveground part of the vineyard, the current biotechnological improvements in the aerial application of EPNs has broadened the range of target pests, including this tortricid species [33]. Previous studies have shown the compatibility of EPN aerial applications against various tortricid species such as *Cydia pomonella* (L.) and *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) [34–38]. Furthermore, a recent study has shown the high virulence of two EPN species (*S. yirgalemense* and *S. jeffreyense*) against tortricid larvae in the species *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae), a sporadic pest in vineyards in South Africa [39]. However, to date, there is no information about the compatibility of EPNs against the widespread EGVM. In addition, it is still unknown whether the natural products produced by their symbiotic bacteria can control EGVM.

This study aimed to explore their use against various larval instars of *L. botrana* (L1, L3, and L5) and their pupal stage. This study settles the basis for the long-term goal of developing new bio-tools that provide an efficient alternative for the integrated management of EGVM.

2. Materials and Methods

2.1. Insects and Nematode Rearing

The EGVM population used to test EPNs was obtained from the Public University of Navarra (Spain), but for the test with natural products generated by the bacterial symbionts, we had to employ new specimens (because of the COVID-19 lockdown), which were supplied by Dra. Ally Harari (Department of Entomology, Volcani Center, Israel). All individuals were reared in an environmentally controlled chamber at 22 ± 1 °C and 60 ± 10% RH, with 16:8 (L:D) photoperiods, at the Institute of Grapevine and Wine Sciences (ICVV, Logroño, La Rioja, Spain). Under these conditions, we placed 20–30 adults into one transparent truncated conical cup with one piece of honey-soaked cotton (1:10 water-diluted) as a source of nutrients. Every 2–3 days, the eggs that had been laid all over the plastic surface were collected and combined from all of the adult cups in the rearing boxes with filter paper on the bottom and pieces of a semisynthetic diet (Supplementary Material, Table S1). We checked larval growth 2–3 days per week, adding food as needed while they completed their five larval instars. Lastly, we removed the pupae in order to start the ovipositional protocol with new adults. The same larval cohort age was employed for each experimental trial.

The EPN populations that were evaluated, *Steinernema feltiae* RM-107, *S. carpocapsae* ALL, *S. riojaense* RM-30, and *Heterorhabditis bacteriophora* RM-102 (Table 2), were cultured in *Galleria mellonella* (Lepidoptera: Pyralidae) larvae, which had also been reared at ICVV in an environmentally controlled chamber at 28 ± 1 °C and 20 ± 10% RH without a photoperiod and using an artificial diet (Supplementary Material, Table S2). The IJs were recovered in tap water upon emergence, stored at 12–14 °C, and used within two weeks of harvest.

Table 2. *Steinernema* and *Heterorhabditis* species and their symbiotic bacteria species (*Xenorhaddus* and *Photorhabdus*) tested for their effects as biocontrol agent against *Lobesia botrana*.

Entomopathogenic Nematodes Species	Population	ITS-Gen Bank Accession Number	Bacterial Species	ITS-Gen Bank Accession Number
Steinernema feltiae	RM-107	MW480131	*Xenorhabdus bovienii*	MW467374
Steinernema carpocapsae	All	MW574913	*Xenorhabdus nematophila*	MW574906
Steinernema riojaense	RM-30	MK503133	*Xenorhabdus kozodoii*	MW467375
Heterorhabditis bacteriophora	RM-102	MW480132	*Photorabhdus laumondii* subsp. *laumondii*	MW574908

2.2. Symbiotic Bacterial Isolation and Natural Products Generation

Three *Xenorhabdus* species (*X. bovienii*, *X. nematophila*, and *X. kozodoii*) and *Photorhabdus laumondii* subsp. *laumondii* were isolated from their respective mutualistic EPN species (Table 2), following the protocols of Vicente-Díez et al. (2021) [40]. Briefly, we first cleaned ~500 IJs of each EPN population by immersion in 5% NaClO for 2–5 min and then washing them with distilled water (three times) before bacterial extraction. Then, we mechanically disaggregated the IJs in a 50:50 (*v*/*v*) suspension of distilled water and nutritive broth (VWR, Chemicals, Barcelona, Spain) using sterile blue pestles (15 s) that had been assembled to a Kontes™ Pellet Pestle™ motor (DWK Life Sciences GmbH, Mainz, Germany). For each EPN species, we seeded 50 µL of this nematode–bacterium complex suspension on three Petri dishes with Nutrient Agar (NA, VWR, Dorset, UK), Bromothymol blue (Alfa Aesar, Kandel, Germany), 2,3,5-Triphenyl tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates), and Ampicillin (50 mg/mL) (PanReac AppliChem, ITW Reagents, Barcelona, Spain). After 48 h, we selected a colony in Phase I from the NBTA medium to generate pure bacterial cultures by further subculturing them in new NBTA plates. All of

the bacterial strains were refreshed weekly into another NBTA plate, checking for purity based on morphology and color.

We obtained the bioactive compounds produced by *Xhenorhabdus* and *Photorhabdus* by inoculating single colonies of each bacterium into two Erlenmeyers with 250 mL of Tryptone Soya Broth (TSB) (VWR Chemicals, Barcelona, Spain). We incubated this culture at 150 rpm and 25 ± 2 °C in darkness for three days to obtain the unfiltered ferments (UF). Finally, we used one of the containers to generate cell-free supernatants (CFS). First, we centrifuged the bacterial suspension at 68.905× *g* (Thermo Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge, Fisher Scientific SL, Madrid, Spain) for 20 min at 4 °C. Then, the supernatant was filtered through a 0.22 µm sterile pore filter [40]. An aliquot of this filtrate was cultured on NBTA plates in duplicate to verify the absence of bacteria. The pellet obtained after the centrifugation was also cultured in NBTA plates to check that the bacteria were still in Phase-I. The TSB used as controls were also filtrated to maintain all treatments under the same conditions.

2.3. Larvicidal and Pupicidal Assays

The larvicidal activity of IJs and bacterial products (UF and CFS) was tested against different EGVM larvae instars following the same methodology. We performed independent assays for each combination of EGVM larval stage and EPN/bacteria product. The experimental unit was a Petri dish (55 mm diam.) covered with one Whatman no.1 filter paper, with each containing five larvae of the corresponding instar and diet (to ensure food *ad libitum*, see details below). The dish was closed tightly with Parafilm and incubated in a growth chamber under controlled conditions (22 °C, 60% RH, and 16L:8O). Each treatment (EPNs, UF, CFS, and their corresponding controls) comprised six Petri dishes (30 insects per treatment in group of five per dish), and each experiment was performed twice (at different times) with freshly produced IJs, UF/CFS stocks, and insects. Larval mortality was checked daily for five days.

For the EPN assays against L1, L3, and L5 instars, we added ~1 cm^3 of semisynthetic diet as a source of nutrients. Each EPN population was applied in a volume of 400 µL in a final concentration of 10 IJs/cm^2 using distilled water in the control treatments. In addition, based on the preliminary results, we performed a lethal concentration (LC) response test against L5 instars for the most virulent EPN populations (*S. carpocapsae* and *S. feltiae*). In this case, the concentrations were 10, 5, 2, and 1 IJs/cm^2 in a final volume of 400 µL (only distilled water for negative controls). All of the tests were conducted in the same controlled conditions as those reported before. On the other hand, we tested the toxicity of the natural products against the L1 and L3 instars. In this study, L5 was excluded because the larvae did not eat enough for any visible effect on the mortality to be observed. In the same experimental unit as the one described before, we replaced the semisynthetic diet with UF and CFS products that had been thickened with the addition of 0.1% agar bacteriologic (ITW reagents, Panreac, Barcelona, Spain) and supplied with 0.05% Methyl 4-hydroxybenzoate (Nipagina) (Sygma Aldrich, Barcelona, Spain) to avoid contamination in the diets. Specifically, we placed five hundred milligrams of each solidified medium diet in each Petri dish (filtered TSB was used for control treatments).

To test the pupacidal activity of the IJs, we employed two 24-multi-well trays (Corning, New York, NY, USA) per treatment using 12 interleaved wells per tray. In each selected well, we added 1 g of sterilized sand (pure sand, Vale do Lobo, Loulé, Portugal) and one EGVM pupa (no sexual dimorphism accounted). Immediately after, we inoculated 50 or 100 IJs in a final volume of 200 µL (only distilled water for negative controls). We checked how many moths had hatched daily for ten days. Each experiment was performed twice with freshly produced IJs, pupae, and subtracts.

2.4. Statistical Analysis

We ran general linear models (GLM) with a binomial distribution (logit-link function) for the pair treatment comparisons (control *versus* treatment) to test the impact of the IJ

and bioactive compound (UF and CFS) virulence on the EGVM larval and pupal instars. We performed a Probit analysis to calculate the lethal concentration (LC) that could kill 50 and 90% of the population (LC_{50} and LC_{90}) and the regression line slope. We performed all of the analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), using $p < 0.05$ to assess the statistical differences. We used least-square means ± SE as descriptive statistics.

3. Results

3.1. Larvicidal Effect by Entomopathogenic Nematodes

The bioassays for the larvicidal effects of the IJs against the L1, L3, and L5 EGVM instars showed that the EPN species *S. feltiae* and *S. carpocapsae* produced significantly higher mortality rates than the controls for all of the larval instars, while *S. riojaense* and *H. bacteriohora* only showed significant mortality rates against L5 (Figure 1; Supplementary Material, Table S3). The highest and fastest larval mortality rates were observed for *S. carpocapsae*, particularly against L3, reaching >75% mortality in 24 h (Figure 1b; Supplementary Material, Table S3). The concentration–mortality test for L5 showed that less than 1 IJ of *S. carpocapsae* was required for LC_{50}, while 5 IJs was estimated to be necessary for *S. feltiae* (Table 3).

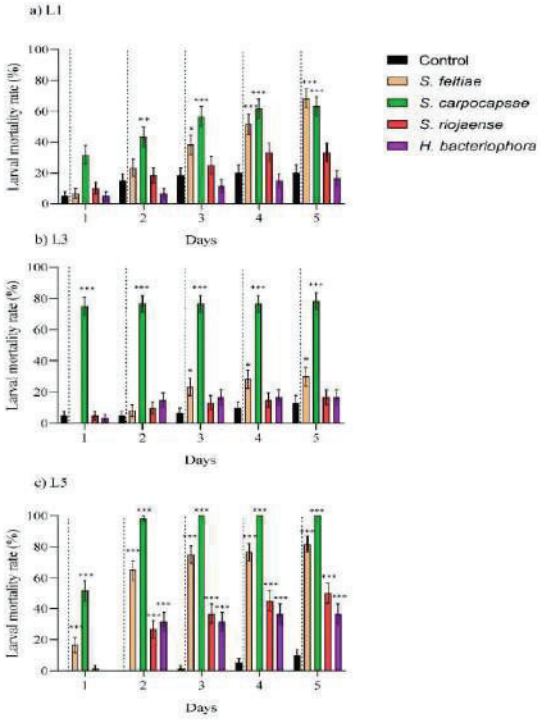

Figure 1. Virulence of the entomopathogenic nematodes species *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora* against various larval instars the European grapevine moth, *Lobesia botrana*. **(a)** First instar (L1), **(b)** third instar (L3), and **(c)** fifth instar (L5). Data are presented in days (from 1 up to 5 days, x-axis) and measured as larval mortality rate (%) (y-axis). Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Values are least-square means ± SE.

Table 3. Lethal concentration (LC) responses against L5 larval stages of the European grapevine moth (EGVM), *Lobesia botrana* estimated for the entomopathogenic nematodes species *Steinernema feltiae* (Sfe) and *Steinernema carpocapsae* (Sca).

Day	Treatment	Tested Stage of EGVM	Number of Insect Tested	Slope ± SE	LC_{50} (95% Confidence Intervals)	LC_{90} (95% Confidence Intervals)	X^2
3	Sfe	L5	300	0.918 ± 0.222	5.229 (3.469–10.297)	130.362 (38.089–3766.676)	1.383
	Sca	L5	300	2.112 ± 0.623	0.352 (0.047–0.629)	1.424 (0.954–2.074)	1.398
4	Sfe	L5	300	1.001 ± 0.223	3. 692 (2.499–5.821)	70.318 (26.189–801.379)	4.395
	Sca	L5	300	3.248 ± 1.073	0.530 (0.110–0.769)	1.315 (1.024–1.887)	0.065

3.2. Larvicidal Effect by Bioactive Compounds Generated by the Symbiotic Bacteria

The CFS derived from the four symbiotic bacteria were toxic when ingested by both of the EGVM larval instars (Figure 2; Supplementary Material, Table S4). Against L1, the mortality rates exceeded 50% and 90% after two and three days, respectively (Figure 2a), while for L3, up to 4–5 days were needed to reach comparable numbers (Figure 2b). Similarly, the ingestion of UF products from both symbiotic bacteria were toxic against the L1 (over 80% in two days) and L3 (over 60% in three days) larval instars (Figure 3; Supplementary Material, Table S5).

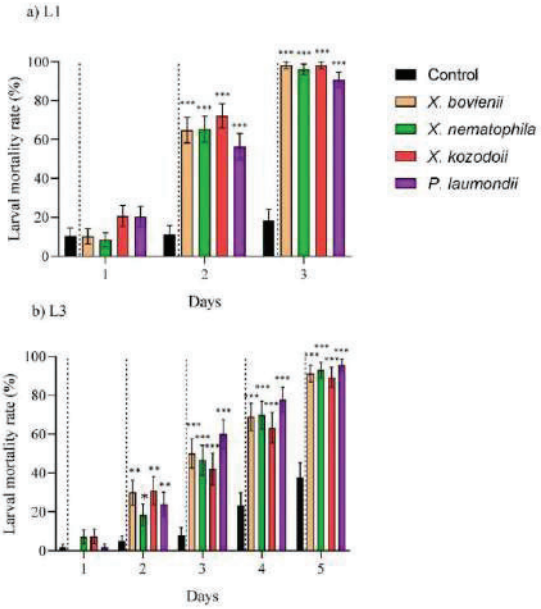

Figure 2. Toxic effect of the natural products produced by the symbiotic bacteria *Xenorhabdus bovienii*, *X. nematophila*, *X. kozodoii*, and *Photorhabdus laumondii* included in the cell-free supernatants tested against various larval instars of the European grapevine moth, *Lobesia botrana*. **(a)** First instar (L1) and **(b)** third instar (L3). Data are presented in days (from 1 up to 3 or 5 days, x-axis) and measured as larval mortality rate (%) (y-axis). Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Values are least-square means ± SE.

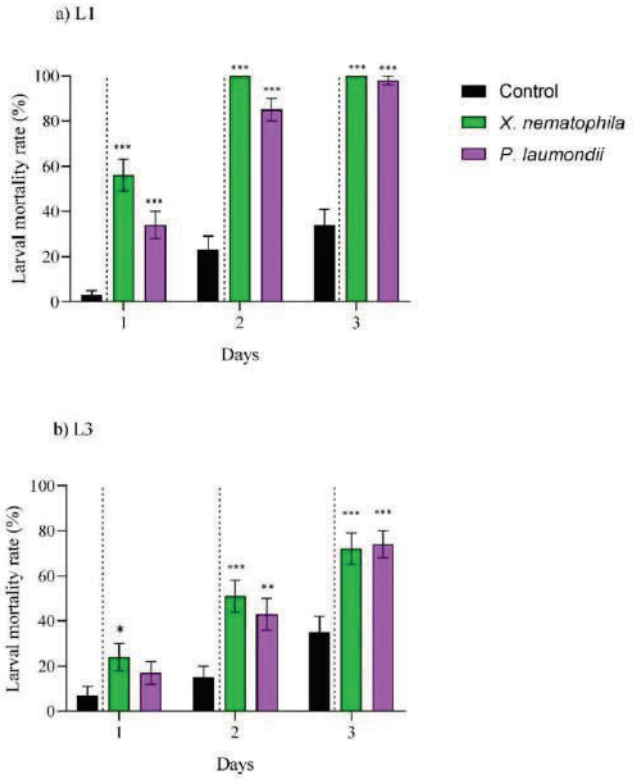

Figure 3. Toxic effect of the natural products produced by the symbiotic bacteria *Xenorhabdus nematophila* and *Photorhabdus laumondii* present in the unfiltered ferment (UF) against various larval instars the European grapevine moth, *Lobesia botrana*. **(a)** First instar (L1) and **(b)** third instar (L3). Data are presented in days (from 1 up to 3 days, x-axis) and measured as larval mortality rate (%) (y-axis). Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Values are least-square means ± SE.

3.3. Pupicidal Effect by the Entomopathogenic Nematodes

For the 50 IJ applications, there were not significant differences compared to the controls for *S. feltiae*. However, the EGVM adult emergences were below 50% for *S. carpocapsae* (Figure 4a) only. Duplicating the concentration to 100 IJs per host, the EPN species *S. feltiae*, *S. riojaense*, and *H. bacteriophora* reduced the adult emergences to 46, 33 and 56%, respectively, while they did not improve the efficiency of *S. carpocapsae*, resulting in a higher adult emergence than the one observed at 50 IJs/cm^2 (Figure 4b; Supplementary Material, Table S6).

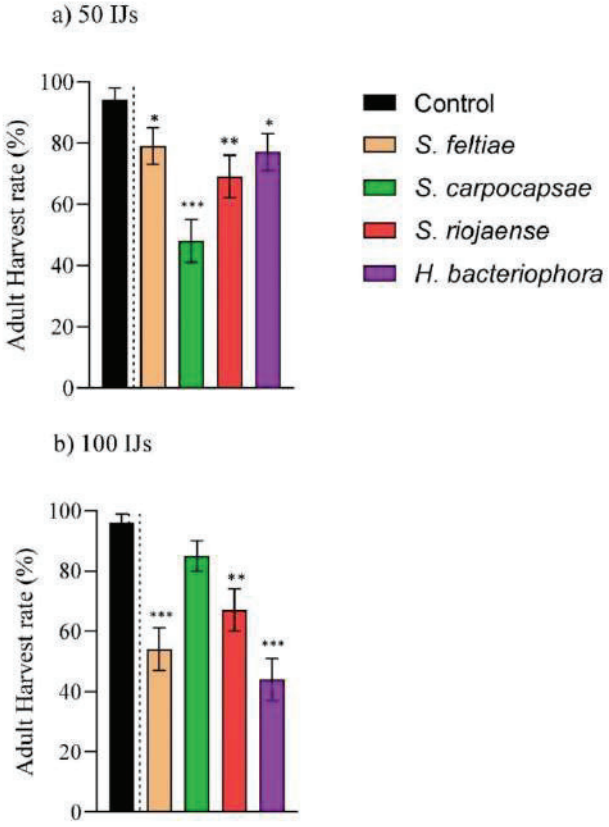

Figure 4. Virulence of infective juveniles (Ijs) of the *Steinernema feltiae*, *S. carpocapsae*, *S. riojaense*, and *Heterorhabditis bacteriophora* species against pupae of the European grapevine moth, *Lobesia botrana*. Concentration of (**a**) 50 IJs and (**b**) 100 IJs per pupa. Asterisks indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Values are least-square means ± SE.

4. Discussion

4.1. Entomopathogenic Nematodes as Biological Control Agents against Larvae and Pupae of the European Grapevine Moth

This study showed that the EPNs could be effective biological control agents against EGVM larvae and pupae in vineyards. In agreement with previous studies against other tortricid species, including *L. vanillana*, EPN virulence differed among nematode species [35,38,39,41,42]. As observed for *C. pomonella* [38], our *S. carpocapsae* population resulted in being the most virulent against the various larval and pupal stages. However, the virulence varied depending on the larval instar, with L1 being the least susceptible, which was probably due to size reasons and may have been too small for EPN. Bastidas et al. (2014) [43] showed that EPNs have limited efficacy against microarthropod hosts that are ~0.5 mm size. On average, *L. botrana* L1 is 0.9–1.5 mm long, while L3 and L5 are 4.5–5.0 and 10.0–11.0 mm, respectively. Consequently, the L1 can present smaller natural

openings that limit colonization by IJs [43]. EPN species with a small-sized IJ such as *S. carpocapsae* can overcome this physical barrier [44]. In addition, L1 and L3 are instars that actively search for food and move intensely in the experimental arena. Hence, EPNs with an ambusher (*S. carpocapsae*) or intermediate (*S. feltiae*) searching behavior might be favored, while nematodes that are expected to display a cruiser behavior (*H. bacteriophora* and *S. riojaense*) can obtain limited results [45,46]. The reduced size of the host linked to the EPN cruiser behavior can explain the low larval mortality observed for *H. bacteriophora* and *S. riojaense*, only reaching ~40% mortality against L5 after five days of exposure. On the contrary, *S. carpocapsae* and *S. feltiae* registered 100% and 80% L5 mortality, respectively, at the same time exposure. Overall, the efficacy of our EPN populations at 48 IJs per host against *L. botrana* obtained similar results to those observed for two South African EPN species against *L. vanillana* but employed 100 IJs per host [39]. Indeed, the efficacy of these two EPN species in the 50% lethal concentration estimations against L5 EGVM is notorious. *S. carpocapsae* only required 0.3 Ijs/cm^2, and *S. feltiae* only required 5.2 IJ/cm^2 in only three days.

Although the pupal stage is less conductive for EPNs [35,39,41,42], our results showed that when employing high IJ concentrations, EGVM adult emergences can be significantly restricted. As for the larval stages, *S. carpocapsae* resulted in the most virulent species, reducing the adult emergence to below 50% when applied at the concentration of 50 IJs per host (~50% pupal mortality if corrected with the control emergence). On the other hand, the species *S. feltiae* and *H. bacteriophora* required double the concentration (100 IJs/host) to achieve similar values, while *S. riojanese*, which has a bigger IJ size [46], only registered ~65% emergence rates. The efficacy of various EPN species against pupa of *C. pomonella* using 50 IJs per host ranged from 20–75% in terms of pupal mortality [41], which is a similar pattern to the one observed for our populations at the same concentration (20–50% pupal mortality if converted from adult emergence). However, compared to the closely related species *L. vanillana*, with ~15% pupal mortality or less, depending on the EPN production system [39], the results obtained for *L. botrana* are promising. In addition, we observed that the presence of EPNs drove miniature EGVM adult emergences from the pupae (I. Vicente-Díez, personal observation). A recent study has shown that the presence of EPNs can alter developmental times and changes in the risk of death of the non-susceptible pupal stage of *Delia antiqua* (Diptera: Anthomyiidae) [47]. As such, this possible alteration in size as well as potential alterations in other metabolic parameters might be of interest in the context of the preventive and biological control of EGVMs. Further research is required to confirm and characterize this non-lethal effect.

4.2. Natural Products Derived from Xenorhabdus and Photorhabdus Have Toxic Effect on Larvae

The CFS and UF products obtained from the symbiotic EPN bacteria exhibited high toxicity against the L1 and L3 EGVM instars, arising as novel biotech tools against this particular pest. In the evaluation of the effect against various pests and pathogens, CFS was the most prevalent system [27,40,48,49]. However, Bussaman et al. (2009) [50] also showed the potential of UF against the mushroom mite *Luciaphorus perniciosus* (Acari: Pygmephoridae), also reporting the non-lethal effect of reducing pest fecundity. Similarly, Steyn et al. (2021) [42] showed that the UF application caused significantly higher egg mortality on *T. leucotreta* than in the control treatment, although the mechanism behind this effect is unknown. Still, to the best of our knowledge, no previous studies analyzed the efficacy of CFS and UF products against the same target. We have demonstrated for the first time that the use of UF products derived from the bacterial species *X. nematophila* and *P. laumondii* can lead to a faster and stronger effect against L1 and L3 instars than their CFS. Today, there are different commercial biopesticides that are classified according to the active substance: (*i*) micro-organisms, (*ii*) biochemicals, and (*iii*) semiochemicals [51]. Despite the massive range of possibilities that exist in the development of this new-biotechnological control approach, *B. thuringiensis* (Table 1) products developed for control of agricultural insect pests (e.g., EGVM) are the most widely spread, representing approximately 95%

of micro-organisms that are used for pest control [52]. Research on *Xenorhabdus* and *Photorhabdus* based products increase their range of action as biopesticides, biofungicides, or bioacaricials [27,49,53]. Their demonstrated oral toxicity against larval instars of EGVM makes them a potential novel biopesticide, with the UF products as a promising area of exploration for new biocompounds and activities.

5. Conclusions

The principal challenges facing all agriculture and especially the grape industry are intensive pesticide use, invasion by new pests/diseases, and climate change [11]. Enhancing good practices for pest, disease, and disease vector management would help address these challenges [40,54]. By maintaining the biodiversity of the vineyard agroecosystem, natural enemies of arthropods can contribute to crop protection [55–57]. In this study, we compared different EPN populations as facultative biological control agents against larval and pupal instars of the key grape pest, EGVM. It is likely that EPNs might control other stages such as adults and eggs, as shown for other tortricids [41,42]. In addition, future viticulture reclaims innovative biotechnical tools that maintain annual crop production while the progressive reduction of chemical supplies becomes legislated. The use of microorganisms such as *Xenorhabdus* spp. and *Photorhabdus* spp. offer promising and environmentally friendly strategies for conventional and organic viticulture worldwide [3,40,58]. Advances in the aerial application of EPNs, the characterization of specific active compounds, and the evaluation of their efficacy and potential risk for other biocontrol agents and the environment will allow the adoption of this technology by growers in a near future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects12111033/s1, Table S1: Ingredients and quantity of the artificial diet for *Lobesia botrana* larvae.; Table S2: Ingredients and quantity of the artificial diet for *Galleria mellonella* larvae.; Table S3: Results from generalized linear mixed models testing within pair-treatment comparisons of control and each entomopathogenic nematode tested.; Table S4: Results from generalized linear mixed models testing within pair-treatment comparisons of control and each bacterial-cell free supernatant tested.; Table S5: Results from generalized linear mixed models testing within pair-treatment comparisons of control and each bacterial unfiltered-ferment tested.; Table S6: Results from generalized linear mixed models testing within pair-treatment comparisons of control and each entomopathogenic nematode tested at 10 days.

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4.2.1. Supplementary materials

Table S1. Ingredients and quantity of the artificial diet for *Lobesia botrana* larvae.

Ingredients	Quantity
Main ingredients	
Corn flour	65 g
Wheat germ	78 g
Beer yeast	65 g
Corn oil	2 ml
Vitamin source	
Ascorbic acid	6,4 g
Preservative	
Benzoic acid	2 g
p-Hydroxybenzoic acid esters (Nipagina)	2 g
Chlortetracycline hydrochloride (Potency 900µg/mg)	0,75 g
Fumagilina 2% (Nosapiol-B)	0,3 g
Agar	20 g
Water	1.1000 ml

Table S2. Ingredients and quantity of the artificial diet for *Galleria mellonella* larvae.

Ingredients	Quantity
Dry dog food	932 g
Water	532 g
Honey	267 g
Glycerine	267 g
Wheat bran	500 g

Table S3. Results from generalized linear mixed models testing within pair-treatment comparisons of control and each entomopathogenic nematode tested.

Asterisks indicate significant at ***P<0.001, **P<0.01, *P<0.05, n.s., not significant.

	L1										L3										L5									
	D1		D2		D3		D4		D5		D1		D2		D3		D4		D5		D1		D2		D3		D4		D5	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
S. feltiae	0.151	n.s.	1.342	n.s.	5.88	*	12.96	***	27.834	***	0	n.s.	0.533	n.s.	6.37	*	6.418	*	4.876	*	12	***	111.429	***	158.256	***	136.123	***	128.552	***
S. carpocapsae	13.257	n.s.	11.51	**	18.527	***	21.224	***	22.791	***	51.051	***	52.989	***	52.943	***	50.146	***	48.232	***	64.138	***	3540	***	3540	***	1140	***	540	***
S. riojaense	1.072	n.s.	0.24	n.s.	0.785	n.s.	2.721	n.s.	2.721	n.s.	0	n.s.	1.072	n.s.	1.471	n.s.	0.682	n.s.	0.261	n.s.	1.017	n.s.	21.818	***	29.564	***	32.542	***	28.235	***
H. bacteriphora	0	n.s.	2.135	n.s.	1.043	n.s.	0.519	n.s.	0.223	n.s.	0.208	n.s.	3.261	n.s.	2.874	n.s.	1.15	n.s.	0.261	n.s.	0	n.s.	0	n.s.	14.544	***	16.718	***	11.646	**

Table S4. Results from generalized linear mixed models testing within pair-treatment comparisons of control and each bacterial-cell free supernatant tested. Asterisks indicate significant at ***P<0.001, **P<0.01, *P<0.05, n.s., not significant.

	L1						L3							
	D1		D2		D3		D1		D2		D3		D4		D5	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
X. bovienii	0.001	n.s.	30.8	***	43.02	***	0	n.s.	11.287	**	19.595	***	18.11	***	25.179	***
X. nematophila	0.12	n.s.	30.903	***	48.486	***	1.927	n.s.	4.573	*	16.815	***	18.381	***	25.322	***
X. kozodoi	2.354	n.s.	38.325	***	43.102	***	2.032	n.s.	11.212	**	13.397	***	12.993	***	20.796	***
P. laumondi	2.234	n.s.	23.385	***	49.618	***	0	n.s.	7.686	**	26.876	***	25.469	***	27.141	***

Table S5. Results from generalized linear mixed models testing within pair-treatment comparisons of control and each bacterial unfiltered-ferment tested. Asterisks indicate significant at ***P<0.001, **P<0.01, *P<0.05, n.s., not significant.

	L1						L3					
	D1		D2		D3		D1		D2		D3	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
X. nematophila	31.268	***	41.257	***	32.625	***	5.516	*	13.586	***	12.572	***
P. laumondi	15.593	***	41.441	***	28.88	***	2.564	n.s.	8.82	**	14.075	***

Table S6. Results from generalized linear mixed models testing within pair-treatment comparisons of control and each entomopathogenic nematode tested at 10 days. Concentration of 50 IJs and 100 IJs per pupa Asterisks indicate significant at ***P<0.001, **P<0.01, *P<0.05, n.s., not significant.

	50 IJs		100 IJs	
	X^2	P	X^2	P
S. feltiae	4.255	*	19.225	***
S. carpocapsae	22.179	***	2.97	n.s.
S. riojaense	9.286	**	12.101	**
H. bacteriophora	5.2	*	25.882	***

4.3. Publication 3

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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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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ABSTRACT

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° 30 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).

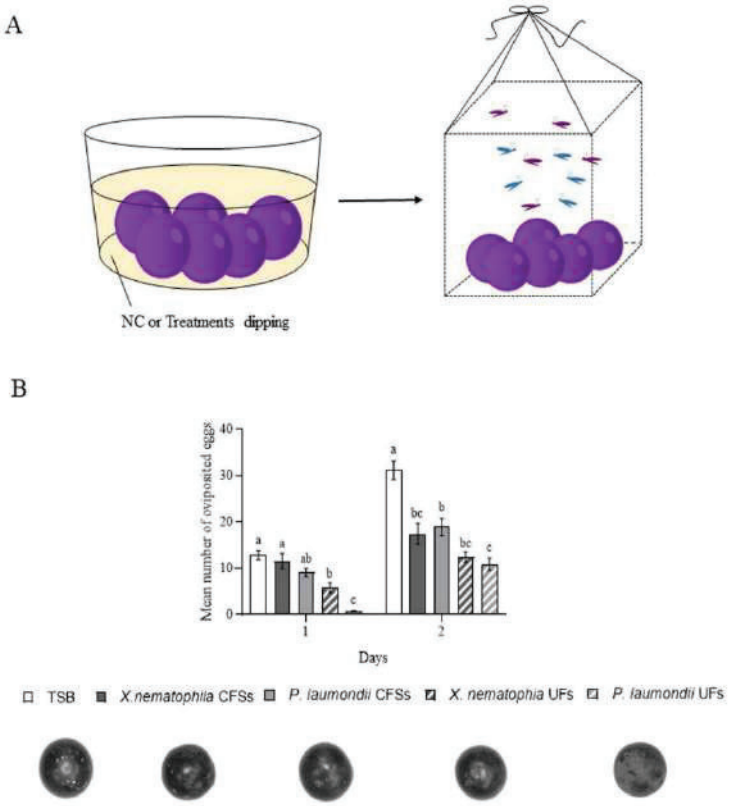

Fig. 1. Ovipositional-deterrence bioassays soaking grapes 3 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* cell-free supernatants (CFSs) and unfiltered fermentations (UFs). (A) The schematic drawing shows the method for testing the deterrent effects used in the respective assays. (B) Mean number 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 oviposition-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*

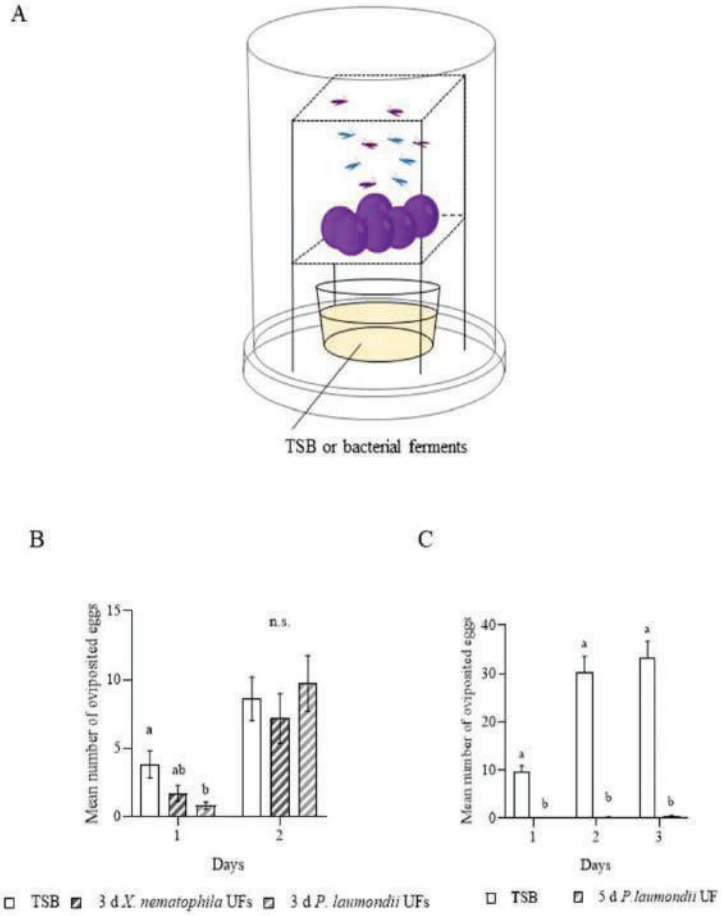

Fig. 2. Oviposition-deterrence bioassays using bacterial ferment volatiles of 3 d and 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* unfiltered fermentations (UFs). (A) The schematic drawing shows the method for testing the deterrent effects used in the respective assays. (B) The mean number 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

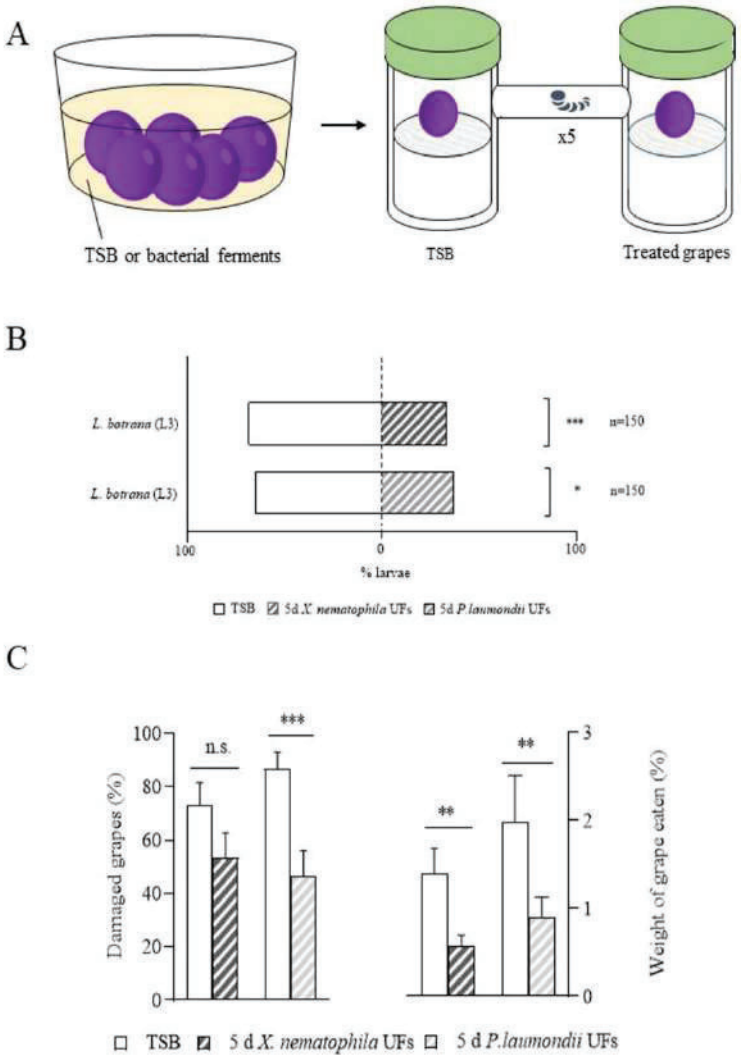

Fig. 3. Feeding source preference bioassay of soaked grapes. Effects of natural products produced by 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* UFs on third larval instar *Lobesia botrana*. (A) The schematic drawing shows the method for testing the deterrent effect used in the respective 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

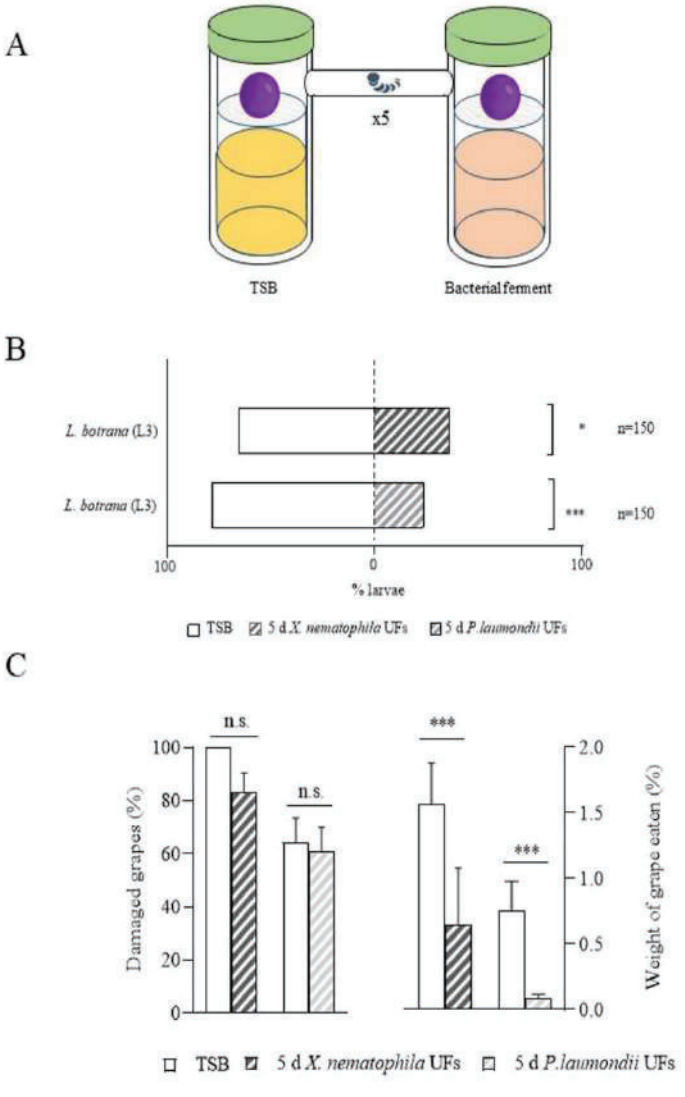

Fig. 4. Feeding source preference bioassay of grapes under bacterial volatiles. Effects of the odours of the 5 d *Xenorhabdus nematophila* and *Photorhabdus laumondii* cultures against third stage third instar larvae of *Lobesia botrana*. (A) The schematic drawing shows the method for testing the deterrent 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 untrented 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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4.4. Publication 4

Exploring bacterial cell-free supernatants, unfiltered ferments and crude bacteria uses of *Xenorhabdus* and *Photorhabdus* (Morganellaceae) for controlling *Botrytis cinerea* (Helotiales: Sclerotiniaceae)

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Exploring bacterial cell-free supernatants, unfiltered ferments and crude bacteria uses of *Xenorhabdus* and *Photorhabdus* (Morganellaceae) for controlling *Botrytis cinerea* (Helotiales: Sclerotiniaceae)

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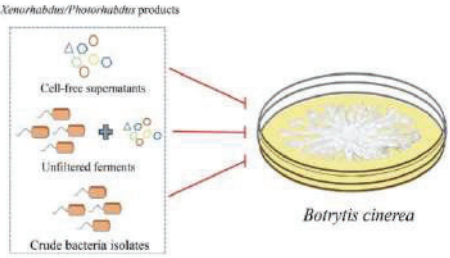
HIGHLIGHTS

- *Xenorhabdus/ Photorhabdus* bacteria/ products explored against *Botrytis cinerea*.
- *X. nematophila* cell-free supernatant inhibited 82% *B. cinerea* growth.
- *X. nematophila* unfiltered ferments inhibited 100% *B. cinerea* growth.
- *P. laumondii*-bacteria inhibited the growth of *Botrytis* in *in vitro* conditions.
- *P. laumondii* bacteria and *Bacillus amyloliquefaciens* had similar control capability.

GRAPHICAL ABSTRACT



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ABSTRACT

The pathogen *Botrytis cinerea* (Helotiales: Sclerotiniaceae) is a wound necrotrophic fungus that causes significant losses in fruits and vegetables worldwide. The entomopathogenic nematode (EPN) symbiotic bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp., are well-known associated biological control agents that produce a diverse range of natural antifungal compounds. This study aimed to evaluate the efficacy of different control strategies against *B. cinerea* using: (*i*) EPN symbiotic bacterial cell-free supernatants, (*ii*) unfiltered ferments and (*iii*) crude bacteria isolates. The antifungal efficacy of *X. bovienii*, *X. nematophila*, *X. kozodoii* and *P. laumondii* subsp. *laumondii* cell-free supernatants obtained after the bacterial fermentations were tested *in vitro* at two different concentrations (10% and 20%). Furthermore, the antifungal effect of *X. nematophila* and *P. laumondii* unfiltered ferments were tested *in vitro*, and their dissuasive effect was also tested over tomato leaves. Finally, the antifungal capacity of the crude *X. nematophila* and *P. laumondii* isolate was tested comparing their effect with the fungicide effect of the commercial *Bacillus amyloliquefaciens* (Serenade® ASO fungicide). The results showed that employing the bacterial cell-free supernatants or the unfiltered ferments could have different antifungal efficacy against this pathogen. Applying *X. nematophila* cell-free supernatant and unfiltered ferments at 20% concentration resulted in the highest inhibition effect compared with the control (distilled water), 82% and 100%, respectively. Furthermore, *P. laumondii*-isolate can control the growth of *Botrytis* in *in vitro* conditions, showing

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no significant differences with the efficacy of *Bacillus amyloliquefaciens* in a four-day experiment. Overall, this study builds on a better understanding of the effects of these novel biocontrol agents against *B. cinerea* and helps to develop an innovative formulation of these bacterial products as an efficient biocontrol tool.

1. Introduction

The pathogen *Botrytis cinerea* (Helotiales: Sclerotiniaceae) is a wound necrotrophic fungus that causes grey mold on more than two hundred species of plants, including agricultural crops, leading to significant losses in fruits and vegetables worldwide. Despite the wide range of hosts, it is most destructive on mature or senescent tissues of dicotyledonous plants (Yigal et al., 2004). It usually entry to such tissues at an earlier stage in crop development. Then, it remains quiescent for a considerable period (as mycelia and/or conidia) before rapidly rotting tissues when the humidity and temperature conditions are favourable and the host physiology changes (Elad et al., 2007; Williamson et al., 2007). The fungus *B. cinerea* causes massive losses in some field- and greenhouse-grown horticultural crops (i.e. tomato crops) before the harvest, or even at the seedling stage in some hosts. Moreover, *B. cinerea* is the main cause of postharvest fruit and vegetable decay during the supply chain (Elad et al., 2007). Fungal spores are generally present on the surface of pre-harvest fruit and vegetables, and during post-harvest handling, and a suitable environment can drive to spore germination. The *Botrytis* costs are diffuse because its damage occurs in different stages of the production and retail chain and are difficult to estimate. Nevertheless, global expenses of *Botrytis* control (cultural measures, botryticides, broad-spectrum fungicides, biocontrol) easily surmount 1000 million € per year, which highlight the exceptional importance of this pathogen (Dean et al., 2012).

The control of *B. cinerea* is challenging because it has a variety of modes of attack, diverse hosts as inoculum sources, and it can survive as mycelia and/or conidia or for extended periods as sclerotia in crop debris (Yigal et al., 2004). The extended use of synthetic fungicides has been the most common control method during the past decades (Oliver and Hewitt, 2014). However, the European Green Deal aims to change the crop protection paradigm, reducing the use of chemical pesticides by half in 2030, enhancing the use of different control treatments and diverse active principles (European Commission, 2020). In recent decades, biocontrol agents are one of these tools promoted in pest management as possible alternatives to synthetic pesticides. Furthermore, it has been demostrated that under field conditions, micro-organisms have more potential for use than macro-organisms as biocontrol agents because they are easier to store and transport, and can be bulked up under laboratory conditions (Veres et al., 2020).

So far, most commonly biological control agents studied against *B. cinerea* are filamentous fungi from the genera *Trichoderma*, *Ulocladium* and *Gliocladium*, bacteria from the genera *Bacillus* and *Pseudomonas* and yeasts from the genera *Pichia* and *Candida* (Jacometti et al., 2010). However, the search for new control agents remains a priority and new organisms are beginning to be investigated (Raymaekers et al., 2020). Previous studies proved that the bacteria symbiont of entomopathogenic nematodes (EPNs), *Xenorhabdus bovienii* YL002 and *X. nematophila* ALL produces antimicrobial compounds with potential for controlling grey mould rot on tomato plants and leaf scorch of pepper (Fang et al., 2011, 2014). Likewise, Vicente-Díez et al (submitted) showed that the volatile organic compounds (VOCs) emitted by *X. nematophila* ALL and *P. laumondii* subsp. *laumondii* 102 inhibit ~100 % of *botrytis* mycelial growth on the postharvest *Vitis vinifera* var Tempranillo for winemaking-grapes. These bacterial VOCs have direct (fungicide activity) and preventive antagonistic effect on the pathogen. Thus, the EPN symbiotic bacteria has become a potential alternative for controlling *B. cinerea*. However, the efficacy of the different EPN symbiotic bacteria species or strains and their different ways of use for fungal control has not been thoroughly studied.

Based on those previous works, we hypothesize that different *Botrytis* control strategies can be developed from the EPN symbiotic bacteria whose efficacy will depend on the bacterial species and the bacterial products used. Therefore, the aim of this study was to evaluate the efficacy of different EPN symbiotic bacterial products in controlling *Botrytis* mycelial growth. The specific objectives were: (*i*) to evaluate the antifungal efficacy of bacterial cell-free supernatants (CFSs) of four EPN symbiotic bacteria species (*X. bovienii*, *X. nematophila*, *X. kozodoii* and *P. laumondii* subsp. *laumondii*) *in vitro* conditions at two concentrations (10% and 20%); (*ii*) to investigate the antifungal effect of *X. nematophila* and *P. laumondii* unfiltered ferments (UFs) at *in vitro* conditions and their dissuasive effect over tomato leaves; and (*iii*) to study the antifungal capacity of the crude isolated *X. nematophila* and *P. laumondii* bacteria, comparing their effect with the fungicide effect of the commercial *Bacillus amyloliquefaciens* (Serenade® ASO fungicide).

2. Material and methods

2.1. Biological resources

We isolated the strain of *B. cinerea* from a contaminated tomato from an organic cultivar in La Rioja (Spain) and we transferred it to Potato Dextrose Agar (PDA) (VWR, Leuven, Belgium) medium. We molecularly confirmed identification as *B. cinerea* following the approach described by Bueno-Pallero et al. (2020). We compared the sequences using Blast (https://blast.ncbi.nlm.nih.gov) and those submitted to Genbank (Accession number MZ544643). For the bioassay, the pathogenic fungi were grown in Petri dishes with PDA medium by seeding a plug of agar with mycelium in active growth and we let the *B. cinerea* growth at 25 °C for three days. From these plates, the conidia were removed, and a suspension was prepared with sterile phosphate-buffering saline (PBS, pH 0 7,4) at a concentration of 1×10^7 conidia/mL via cell counting method in Neubauer counting chamber.

We isolated three *Xenorhabdus* and one *Photorhabdus* species from their symbiotic entomopathogenic nematode (EPN) to test their antifungal activity against *B. cinerea* (Table 1). We performed the isolation process following the protocol described by Vicente-Díez et al. (2021). For all the bioassays described in the present work, we obtained the secondary metabolites by Tryptone Soya Broth (TSB) fermentation during 3 d at 150 rpm, in darkness and at 25 °C. After this time, we kept the bacterial cultures at 4 °C for their use as unfiltered ferment (UF). We obtained the cell-free supernatant (CFS) by centrifuging this bacterial culture at 68.905 × g (g-force or relative centrifugal force, rcf). (Thermo Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge, Fisher Scientific SL, Madrid, Spain) for 20 min at 4 °C. Then, we filtered the supernatant through a 0.22 μm sterile pore filter (Nonsterile, PES Syringe Filters, Branchia, Labbox LabwarE, S.L. Barcelona, Spain). After this process, we

Table 1
Xenorhabdus and *Photorhabdus* species and their symbiotic nematodes associate.

Bacterial species	EPN species	Population
Xenorhabdus bovienii	*Steinernema feltiae*	RM-107
	Steinernema carpocapsae	All
Xenorhabdus nematophila	*Steinernema riojaense*	RM-30
Xenorhabdus kozodoii	*Heterorhabditis bacteriophora*	RM-102
Photorhabdus laumondii subsp. *laumondii*		

cultured 50 μL of this filtrate on Nutrient Agar (NA, VWR, Dorset, UK), Bromothymol blue (Alfa Aesar, Kandel, Germany), 2,3,5-Triphenyl Tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates) in duplicate to verify the absence of bacteria. Finally, we recovered bacterial pellet obtained after the centrifugation with a 1 μL seed loop to test the antifungal capacity of crude bacteria isolate as it is explained in the section 2.4. We filtered the TSB to be used as controls to maintain all treatments under the same conditions.

The *Bacillus amyloliquefaciens* (former *subtilis*) QST 713 strain used in the present study was isolated from the commercial product Serenade®ASO. This product is characterized to have a broad-spectrum biofungicide action and has been approved for use in the European Union (Reg. (EC) No. 839/2008). First, we took 1 mL from the commercial product in one Eppendorf tube and centrifuged the bacterial suspension. Then, we removed the supernatant and suspended the pellet with 1 mL of PBS (the whole process was repeated three times). Then, we inoculated 1 mL of the bacterial suspension in 250 mL of Nutrient Broth (NB) in one 500 mL Erlenmeyer, and the NB bacterial fermentation was performed during 3 d at 150 rpm in darkness at 25 °C. Finally, we recovered the crude isolate bacteria following the protocol described before for EPN symbiotic bacteria.

The tomato plants *Solanum lycopersicum* L. Money Maker variety were grown from seeds under greenhouse conditions (22 ± 1 ◦C and 60 ± 10% RH, with 16:8, L:D photoperiods), at the Institute of Grapevine and Wine Sciences (ICVV, Logroño, La Rioja, Spain) during three weeks. Then, the leaves were cut and arranged for the experiment as described in section 2.3.

2.2. Antifungal activity of entomopathogenic nematodes bacterial symbionts cell-free supernatants

We evaluated the effects of CFSs of four EPN bacteria symbionts (*X. bovienii*, *X. nematophila*, *X. kozodoii* and *P. laumondii* subsp. *laumondii*) on the mycelial growth of *B. cinerea*. We mixed 10 or 20 mL of each bacterial cell-free supernatants with 90 mL (10%, proportion 1:10 final) or 80 mL (20%, proportion 1:5 final) of autoclaved PDA that cooled down to 60 °C, respectively. Then, we pooled 10 mL of each mix into Petri dishes (9 cm diam.). Once the media solidified, we pipetted 20 μL suspension of 10^7 spores/mL of *B. cinerea* prepared in Gamborg B-5 (Sigma-Aldrich, St. Louis, MO, USA) in the middle of the plate. The controls were mixed with distilled autoclaved water and with TSB at the same proportion that the CFSs. All the experimental units were incubated at 60% RH, 22 ± 1 °C, 16:8 L: D photoperiod. A total of n = 15 plates were studied for each treatment in two different trials (n total = 30). We assessed the mycelial growth area (mm^2) by measuring the fungus growth using image analysis with the Image J® program (v. 1.50i, MD, USA) four days after starting the experiment (the day in the mycelial growth of the controls occupy the plate completely).

2.3. Antifungal activity of Xenorhabdus nematophila and Photorhabdus laumondii unfiltered ferments and their dissuasive effect over tomato leaves

We evaluated the antifungal activity of *X. nematophila* and *P. laumondii* UFs on the mycelial growth of *B. cinerea*. We mixed the bacteria fermentation suspension into Petri dishes (9 cm diam.) with PDA (20%). Then, we pipetted 20 μL suspension of 10^7 spores/mL of *B. cinerea* prepared in Gamborg B-5 in the middle of the plate. The controls comprised the mixture of distilled autoclaved water and TSB at the same proportion that the UFs. The mycelial growth area (mm^2) of each treatment was calculated as described before.

For the dissuasive assay over tomato leaves, the second and third leaf from the tip were detached from three weeks old plants following the protocol described by Miazzi et al. (2010). We disinfected the leaves surface by dipping them in 3 % (v/v) of sodium hypochlorite (NaOCl) solution for 1 min, washed them with distilled water and then air-dried them for ~2 h. Then, we placed singly Petri dishes (9 cm diam.) containing 10 mL of the Agar medium, immersing the petiole in the substrate. Using a steel needle, we spot-inoculated each leaf in 10 points, avoiding veins, and we placed 1 cm^2 PDA with *Botrytis* from four days old colonies of a single fungal isolate. We incubated in the same conditions described above. Four days later, we detached new leaves and performed the disinfected protocol. After that, we dipped them in the *X. nematophila* or *P. laumondii* unfiltered ferments or in TSB as control. Then, we placed them in the same Petri dishes with the infected tomato leaves. Fifteen leaves per treatment were used, and the experiment was performed twice. We assessed the mycelial growth area (mm^2) by measuring the fungus growth compared with the size of the leave using image analysis with the Image J® program four days after starting the experiment. The infection rate was calculated by dividing the infected area by the total leaf area.

2.4. Antifungal activity of isolated bacteria compared with commercial Bacillus amyloliquefaciens QST 713 (Serenade)

One centimeter from the edge of the PDA plate (5.5 cm diam.), 0.1 mL of crude bacterial isolated were inoculated with a seeding loops and 20 μL of the pathogen were applied in the opposite direction. The plates were incubated at 25 °C for three days. As control, we left a plate without any application. We assessed the mycelial growth area (mm^2) by measuring the fungus growth using image analysis with the Image J® program four days after pathogen inoculation. The experimental unit was each Petri dish, and the experimental design was completely randomized with ten replicates. The whole experiment was performed twice.

2.5. Statistical analysis

We analyzed the inhibitory effect of bacterial CFSs, UFs and bacteria-isolate on mycelial growth of *B. cinerea* by one-way analysis of variance (ANOVA). Means are shown with standard errors of the mean (SEM), and a comparative analysis was performed using a Tukey test for significance analysis (HSD) at $P < 0.05$. We performed these analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA). All figures were built using Graph Pad 8.

3. Results

3.1. Antifungal effect of bacterial cell-free supernatants on Botrytis cinerea mycelial growth

The bacterial CFSs showed statistically significant inhibitory effect on the mycelial growth of *B. cinerea* at 10% ($F_{5, 174} = 90.835$, $P < 0.001$) and 20% ($F_{5, 239} = 102.71$, $P < 0.001$) concentrations four days after pathogen inoculation (Fig. 1). In particular, we found that *X. nematophila* reduced −60 and −80 % of *Botrytis* mycelial growth compared with water at 10% and 20% concentration in the media, respectively. Likewise, *P. laumondii* showed an inhibitory effect (~40 %) for both concentrations compared to water treatment. TSB control inhibited the fungal mycelial growth, even though there was not more inhibition at higher concentration applied. Therefore, we selected *X. nematophila* and *P. laumondii* as the most efficient bacterial strains for subsequent bioassays.

3.2. Antifungal effect of unfiltered bacterial ferments on Botrytis cinerea mycelial growth and their dissuasive effect in tomato leaves

The UFs showed statistically significant inhibitory effect on the mycelial growth of *B. cinerea* at 20% ($F_{3, 76} = 273.53$; $P < 0.001$) four days after pathogen inoculation (Fig. 2A.). All the treatments and the TSB control showed an inhibitory effect compared with the distilled water. In particular, we found that *X. nematophila* reduced ~100 % of

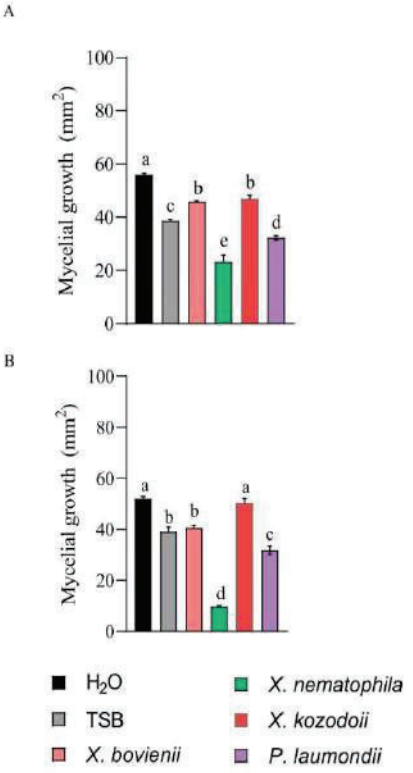

Fig. 1. Mycelial growth (mm^2) of *Botrytis cinerea* induced by *Xenorhabdus bovienii*, *X. nematophila*, *X. kozodoii* and *Photorhabdus laumondii* cell-free supernatants (CFS) in Potato Dextrose Agar (PDA). **(A)** Antifungal effect of bacterial cell-free supernatants 1:10 (CFS:PDA). **(B)** Antifungal effect of bacterial cell-free supernatants 1:5 (CFS:PDA). Data are expressed as mean ± SEM. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$).

botrytis mycelial growth at 20% of concentration in the media. Likewise, *P. laumondii* showed an inhibitory effect (~60 %) compared with the distilled water control at this concentration.

The UFs showed a significant dissuasive effect on the mycelial growth of *B. cinerea* over tomato leaves ($F_{2, 86} = 20.47$; $P < 0.001$) (Fig. 2B.). In particular, *X. nematophila* and *P. laumondii* unfiltered ferments reduced the *B. cinerea* mycelial growth over treated tomato leaves (Fig. 2C.) by 88% and ~100%, respectively.

3.3. Antifungal effect of crude bacteria isolates on Botrytis cinerea mycelial growth

The resuspended bacteria showed a significant antifungal effect on the mycelial growth of *B. cinerea* ($F_{3, 86} = 220.683$; $P < 0.001$) four days after pathogen inoculation (Fig. 3A). In particular, we found that *P. laumondii* and *B. amyloliquefaciens* seeded over the plate reduced ~75 and 88% of *botrytis* mycelial growth, respectively (Fig. 3B.). Nevertheless, the bacterium *X. nematophila* did not show inhibitory capacity on the fungus. Indeed, the presence of the bacteria on the plate promoted the mycelial growth in four days (Fig. 3A,B).

4. Discussion

In agreement with our hypothesis, employing the bacterial CFSs or the UFs could result on different antifungal efficacy against *B. cinerea*. In addition, different species showed variability in the control potential against this fungus, with *X. nematophila* and *P. laumondii* being the bacteria with the highest potential for growth inhibition among the four evaluated. Furthermore, *P. laumondii*-isolate controlled the growth of *Botrytis* in *in vitro* conditions, showing in four days no significant differences with the efficacy of *B. amyloliquefaciens* commercial product. These results are in agreement with the enormous potential of the *Xenorhabdus* and *Photorhabdus* bacteria, and their expected production of a variety of toxins with antifungal activity against the phytopathogen *B. cinerea*. As shown by Fang et al. (2011, 2014), these natural compounds have a great potential to control *B. cinerea* in different fruit and vegetable crops, but still, developing an adequate formulation of these bacterial products, including CFSs, UFs or crude cell isolate, is crucial to get an efficient biological control tool.

The secondary metabolites released by bacteria of the genera *Xenorhabdus* and *Photorhabdus* into the media and used as CFS are well-known active compounds with antifungal capacity (Chacón-Orozco et al., 2020; Hazir et al., 2016). Indeed, similar results were obtained by Hazir et al. (2016) using four *Xenorhabdus* species and two *P. luminescens* strains against *Fusicladium carpophilum* (peach scab), *F. effusum* (pecan scab), *Monilinia fructicola* (brown rot), *Glomerella cingulate* (anthracnose) and *Armillaria tabescens* (root rot), concluding that, overall, *Xenorhabdus* spp. exhibited a stronger antifungal effect compared with supernatant of *Photorhabdus* spp. The reason for differential suppressive abilities between the different EPN symbiotic bacteria species and strains may lie in production of different active compounds among the genus *Xenorhabdus* and *Photorhabdus* and the different species and strains (Bode, 2009).

The use of the unfiltered ferments, including the bacteria and the secondary metabolites, released to the media resulted as the most efficient formulation against the *B. cinerea*. Similar pattern was observed against the insect *Lobesia botrana* (Lepidoptra: Tortricidae), where the UF resulted more efficient against the larvae than the CFS (Vicente-Díez et al., 2021). However, these results contrast with the study by Bussaman et al. (2012), who performed a screening in the acaricidal activities of *X. stokiae* UFs and CFSs against *Luciaphorus* sp. Then, Bussaman et al. (2012) showed that the UFs were slightly less effective than its CFS, suggesting that *X. stokiae* was more likely to release its metabolites with acaricidal activities to the surrounding culture media. Whether these differences were due to the bacteria species, the application method or the differences in the host (acari vs insect) are unknown. Also, in agreement with Fang et al. (2014), *X. nematophila* and *P. laumondii* UFs showed a dissuasive effect against *B. cinerea* protecting the surface of the treated tomato leaves. Therefore, the use of the whole product generated in the UFs has the great potential for their use in integrated control of this pathogen in tomato crops.

Finally, we evaluated the potential use of the crude bacteria isolated in an approach similar to the current commercial product based on *Bacillus* spp. We observed that *P. laumondii*-isolate inhibited *Botrytis* mycelial growth while *X. nematophila*-isolate did not control the growth of the fungi in comparison with the control. Few researchers have examined the effect of the free-living forms of *Xenorhabdus* and *Photorhabdus* as control agents (Sandhu and Reddy, 2019). Until recent findings, scientific observations suggested that there was little likelihood that the symbiotic bacteria could effectively control pests when used alone (Burnell and Stock, 2000). Nevertheless, recent studies have proved the phenotypic heterogeneity of bacteria of the genus *Photorhabdus* that have a second putative lifestyle out of their nematode symbiont and remain in the soil (Eckstein and Heermann, 2019). Furthermore, current transcriptomic analysis has proved that *P. luminescens* responds to the external signals (e.g. root exudates) driving the expression of various genes, for example, those involved in chitin degradation, biofilm regulation, flagella formation, and type VI

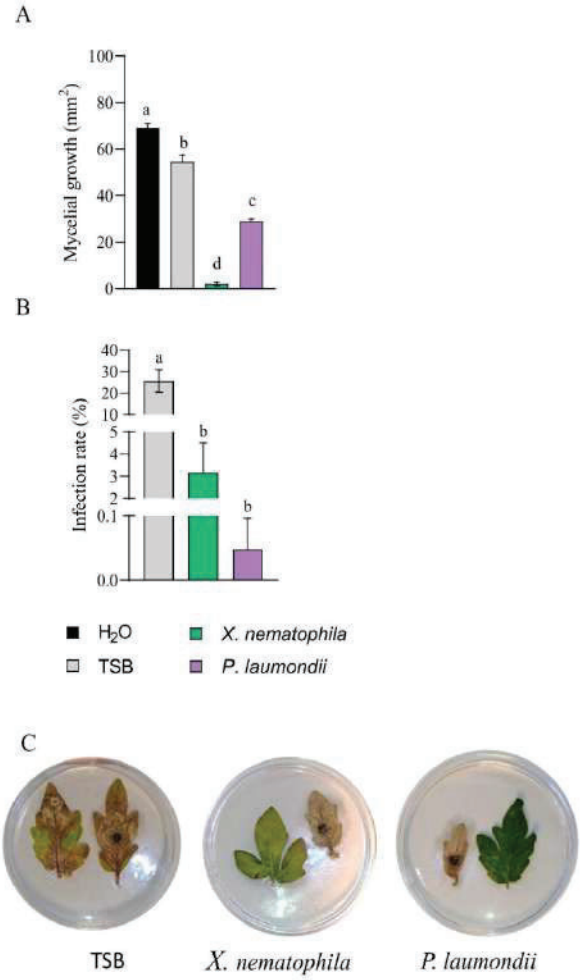

Fig. 2. Mycelial growth (mm2) and Infection rate (%) of *Botrytis cinerea* induced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* unfiltered ferments (UFs) after 4 days post-inoculation in Potato Dextrose Agar (PDA). **(A)** *B. cinerea* mycelial growth induced by *X. nematophila* and *P. laumondii* TSB unfiltered ferments 1: 5 (UFs:PDA). **(B)** Dissuasive effect of *X. nematophila* and *P. laumondii* against *B. cinerea* over tomato leaves. **(C)** Display of the fungal growth in each treatments four days after infection. Data are expressed as mean ± SEM. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$).

secretion system (Regaiolo et al., 2020). These recent evidence help to understand the positive results found by the use of *P. laumondii*-isolate against *B. cinerea*. On the other hand, in agreement with Bussaman et al. (2012), up to date, there is no evidence that any *Xenorhabdus*-isolate can control pests or pathogens when applied to free-living cells.

5. Conclusions

Different forms of use of the EPN symbiotic bacteria fermentation have variable effectivity controlling the mycelial growth of *B. cinerea*. On the one hand, among the screened bacteria studied herein, *X. nematophila* UFs showed the highest antifungal effectiveness. On the other hand, *P. laumondii*-isolate could control the fungal growth during four days as the commercial produced based on *B. amylolyquefancies*. This study illustrates the possible uses of the bacteria with various formulations as a plant-protecting organism in agriculture. Indeed, the development of these novel bacteria-based tools can be used as an eco-friendly and economical alternative in the integrated control of diseases by reducing the amount and number of chemical fungicide applications in agricultural crops, in line with the current paradigm of reduction of pesticide such as the EU Green Deal (European Commission, 2020). More greenhouse and field application studies are required to determine the possible modulation effects in the plant and under natural conditions.

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, to ensure accessibility to any researcher.

CRediT authorship contribution statement

Ignacio Vicente-Díez: Conceptualization, Methodology,

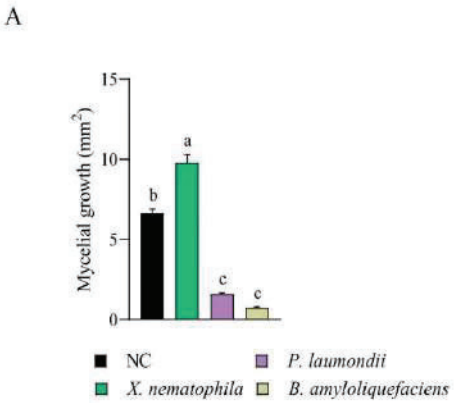

Fig. 3. Impact of *Xenorhabdus nematophila*, *Photorhabdus laumondii*, *Bacillus amyloliquefaciens* isolated bacteria in the *Botrytis cinerea* growth. **(A)** Mycelial growth (mm^2) of *B. cinerea* in the presence of *X. nematophila*, *P. laumondii*, and *B. amyloliquefaciens* isolated bacteria. **(B)** Display of the fungal growth in each treatments four days after infection. Data are expressed as mean ± SEM. Different lowercase letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$).

Investigation, Writing – original draft, Visualization, Data curation, Resources, Writing – review & editing. **Elizabeth Carpentero:** Methodology, Writing – review & editing. **Alicia Pou:** Resources, Writing – review & editing, Funding acquisition. **Raquel Campos-Herrera:** Conceptualization, Resources, Writing – review & editing, Funding acquisition.

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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Authors' contributions

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

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4.5. Publication 5

Control of post-harvest gray mold (*Botrytis cinerea*) on grape (*Vitis vinifera*) and tomato (*Solanum lycopersicum*) using volatile organic compounds produced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii*

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Control of post-harvest gray mold (*Botrytis cinerea*) on grape (*Vitis vinifera*) and tomato (*Solanum lycopersicum*) using volatile organic compounds produced by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii*

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Abstract Post-harvest fruit and vegetable rot produced by *Botrytis cinerea* (Helotiales: Sclerotiniaceae) causes significant reductions in food availability and drastically increases economic losses. The use of microbial-based tools for pathogen management holds promise. In particular, volatile organic compounds (VOCs) emitted by microbes (e.g., bacterial compounds) are becoming increasingly more frequent as an alternative to chemical and physical treatments. In this study, we performed three laboratory experiments to investigate the effects of VOCs emitted by two gram-negative entomopathogenic bacteria, *Xenorhabdus nematophila*, and *Photorhabdus laumondii* subsp. *laumondii*, on the infection and growth of the pathogenic mold *B. cinerea* on post-harvest red grapes and tomatoes. In addition, we evaluated the preventive effects of these bacterial VOCs against pathogens in post-harvest wounded and intact grapes. Overall, VOCs emitted by *X. nematophila* and *P. laumondii* limited the lesion area of *B. cinerea* to 0.5% and 2.2%, respectively, on the grapes. Similarly, VOCs emitted by *X. nematophila* and *P. laumondii* limited the lesion area of *B. cinerea* to 0.5% and 0.02%, respectively, in tomatoes. In addition, the emission of VOCs by both bacteria showed strong preventive fungal effects. In particular, VOCs emitted by *P. laumondii* reduced to 13% *B. cinerea* incidence in damaged grapes exposed to VOCs. Moreover, intact grapes exposed to VOCs emitted by *X. nematophila* and *P. laumondii* decreased *B. cinerea* incidence by 33%. This study provides insightful information about a potential novel bacteria-based tool that can be used as an alternative in the integrated control of post-harvest diseases.

Keywords Antifungal compounds · Entomopathogenic bacteria · Post-harvest management · *Solanum lycopersicum* · *Vitis vinifera*

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Introduction

The United Nations Food and Agriculture Organization (FAO) estimates that post-harvest of fruit and vegetables is the highest among all types of food losses, reaching up to 40% (FAO 2019). Recent estimations indicate that loss at the retail and consumer levels in the USA includes 6.7 M kg of fruit and 10.6 M kg of vegetables per year, adding up to a loss of ~ US$ 40,000 million (Buzby et al. 2011; Buzby and Hyman 2012). Storage, transport, and household waste are the most critical loss points in the fruit and vegetable supply chains, owing largely to inadequate use of bulk packaging and management (Watada et al. 1996). These conditions cause abiotic stresses such as extreme temperatures, desiccation, mechanical injury, low O_2, and high CO_2 percentage that often result in food loss (Toivonen and Hodges 2011). In addition, fruit and vegetables are highly perishable because, once harvested, they can also suffer biotic stresses such as infections of wound-invading necrotrophic pathogens (Sharma et al. 2009) that compromise both quantity and quality (Delgado et al. 2017).

Several chemical and physical tools have been used to reduce post-harvest losses of fruit and vegetables due to fungal pathogen infections, but their efficiency, economic, and environmental costs are intensely debated (Romanazzi et al. 2012; De Simone et al. 2020). For instance, synthetic fungicides have proven to provide long-lasting control of many target plant pathogens and still contribute heavily to disease control in conventional farming (Oliver and Hewitt 2014). However, their widespread use has triggered severe environmental problems due to their persistence in the air, soil, water, and food, as well as the development of pathogen resistance (Narayanasamy 2006; Gyawali and Ibrahim 2014). As a result, European Union (EU), through the European Green Deal, aims at reducing the use of chemical fungicides by half by 2030, recommending their limited application, adopting prevention measures, and pushing non-chemical control methods (European Commission 2020). Alternatively, physical technologies such as variations in temperature, UV-C irradiation, pressure, or changing atmospheric composition can increase fruit and vegetable resistance against abiotic and biotic stresses after harvesting. Although these methods are often considered non-harmful and residue-free emerging technologies, they involve high energy inputs and costs (Usall et al. 2016). Overall, there has been a pressing need for developing environmentally friendly and economical methods for the management of pathogen infections in fruit and vegetables after harvesting.

The use of microbial-based tools for pathogen management can provide new alternatives. In this sense, various defense-related phytohormones, biological elicitors, and non-organic elicitors have been used as biopesticides against plant pathogens and thus might be also useful on detached fruit (Sharma et al. 2009; Poveda 2021). In particular, volatile organic compounds (VOCs) emitted by microbes (e.g., bacteria) are emerging as an alternative to conventional chemical and physical treatments, mostly in circumstances where direct contact between the pathogen and its antagonist is not practical (Tilocca et al. 2020; Poveda 2021). Bacterial VOCs might increase toxicity against fungal pathogens in post-harvest fruit (Mari et al. 2016) and/or induce fruit defense response (Romanazzi et al. 2016). Unfortunately, the mechanisms underlying these antagonistic effects are still poorly understood. In-depth investigations are thus needed to investigate the antifungal activity, efficacy, and preventive effects of bacterial VOCs in controlling pathogen infections in harvested fruit (Cellini et al. 2021).

Grape and wine processing industries yearly generate around 5–9 M kg of solid waste worldwide, which constitutes 20–30% of processed materials (Schieber et al. 2001). Likewise, tomato is the second most consumed vegetable in the world (Savatović et al. 2012) and its industrial processing generates a considerable amount of waste (10–30% of their raw weight; Rahmatnejad et al. 2009). A critical problem in these industries is the losses and waste generated by post-harvest fungal pathogens due to the lack of proper handling methods and infrastructure (Calicioglu et al. 2019).

The gram-negative entomopathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. produce a huge range of bioactive compounds (peptides, polyketides, and toxins) with antibacterial (Böszörményi et al. 2009; Muangpat et al. 2020), antifungal (Fang et al. 2011, 2014; Chacón-Orozco et al. 2020; Alforja et al. 2021; Cimen et al. 2021; Li et al. 2021), insecticidal (ffrench-Constant et al. 2007; Vitta et al. 2018; Vicente-Díez et al. 2021a, b) and nematicidal (Kusakabe et al. 2021; Abebew et al. 2022) activity.

However, the application of these bacterial VOCs to reduce the impact of fungal pathogens has been poorly explored, and their practical use is at an early stage (Crawford et al. 2012; Flórez et al. 2015; Kajla et al. 2019). In this study, we investigated the effects of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* on the infection and growth of the pathogenic mold *Botrytis cinerea* on post-harvest red grapes and tomatoes. In addition, we evaluated the preventive effects of these bacterial VOCs against this pathogen in post-harvest wounded and intact grapes. Overall, this study contributes to a better understanding of the effects of these bacterial VOCs on one pathogen infection in two post-harvest fruit systems, illustrating the potential of this new tool to reduce post-harvest losses in the context of current global agriculture and economy.

Materials and methods

Bacteria isolation and volatiles organic compounds generation

We isolated *X. nematophila* (region 16S rDNA, GenBank accession number MW574906) and *P. laumondii* subsp. *laumondii* (region 16S rDNA, GenBank accession number OQ285858) from their symbiotic entomopathogenic nematode (EPNs) (Supplementary Material 1, Table S1) as described by Vicente-Díez et al. (2021a). We inoculated bacterial strains on Petri dishes with Nutrient Agar (NA), Bromothymol blue (Alfa Aesar, Kandel, Germany), and 2,3,5-Triphenyl tetrazolium chloride (TTC, VWR, Chemicals, Barcelona, Spain) (NBTA plates). We ensured to use the bacteria in the primary and active form based on dye adsorption, pigmentation, and morphology, as described Han and Ehlers (2001). We refreshed the bacteria weekly into another NBTA plate. To ensure the purity and activity for all the experimental trials, we observed the bacterial movement in the microscope and plated in new NBTA dishes, confirming morphology and uniformity.

We obtained natural VOCs derived from *X. nematophila* and *P. laumondii* subsp. *laumondii* by inoculating one single bacteria colony from the NBTA plates in 500 ml Erlenmeyer flasks with 250 ml of Triptone Soya Broth (TSB) (VWR Chemicals, Barcelona, Spain). We incubated the flasks on an orbital shaker at 150 rpm and 25 ± 2 °C in darkness for three days until reaching the saturation of the medium. We verify the purity and activity of the ferments by checking the normal movement of the bacteria under the microscope and ensuring normal growth in NBTA plates. Although some secondary metabolites are produced during the exponential phase, the secondary metabolism is generally activated during the post-exponential or stationary phase of the bacterial growth (Clarke 2016). For that reason, we kept the bacterial ferments inside the Erlenmeyer flask at room temperature, without agitation, close and in darkness condition. We used the VOCs produced at the third day.

Botrytis cinerea isolation and identification

We isolated the strain of *B. cinerea* from a contaminated grape cluster in the wine region of La Rioja, Spain and transferred to Potato Dextrose Agar (PDA) (VWR, Leuven, Belgium) medium. For the bioassays, we prepared conidia following standardized protocols (Supplementary Material 2). We stored the pathogen strain at − 80 °C in glycerol (25%). Furthermore, we confirmed identification as *B. cinerea* by molecular tools following the approach described by Bueno-Pallero et al. (2020). We compared the ITS1 genetic region sequences using Blast (http://blast.ncbi.nlm.nih.gov) and those submitted to GenBank (Accession number MZ544643).

Antifungal activity of VOCs emitted by bacteria

We evaluated the effects of VOCs emitted by both bacteria (*X. nematophila* and *P. laumondii* subsp. *laumondii*) on the mycelial growth of *B. cinerea*. We used the double plate method (Fig. 1a) as a proof of concept bioassay (Raymaekers et al. 2020). Briefly, in the top-plate (55 mm diam. Petri dish) we applied 6 µl of *B. cinerea* 1×10^7 conidia ml^{-1} of Gamborg B-5 (Sigma-Aldrich, St. Louis, MO, USA) solution in the middle of 6 ml of PDA medium. In the base-plate, we added 5 ml of *Xenorhabdus* and *Photorhabdus* three days-old TSB ferments. Consequently, we exposed the pathogen fungus to the VOCs generated by the bacteria without physical contact. Control treatments were distilled water and TSB in the base-plate instead of each of the bacterial ferments. Each treatment

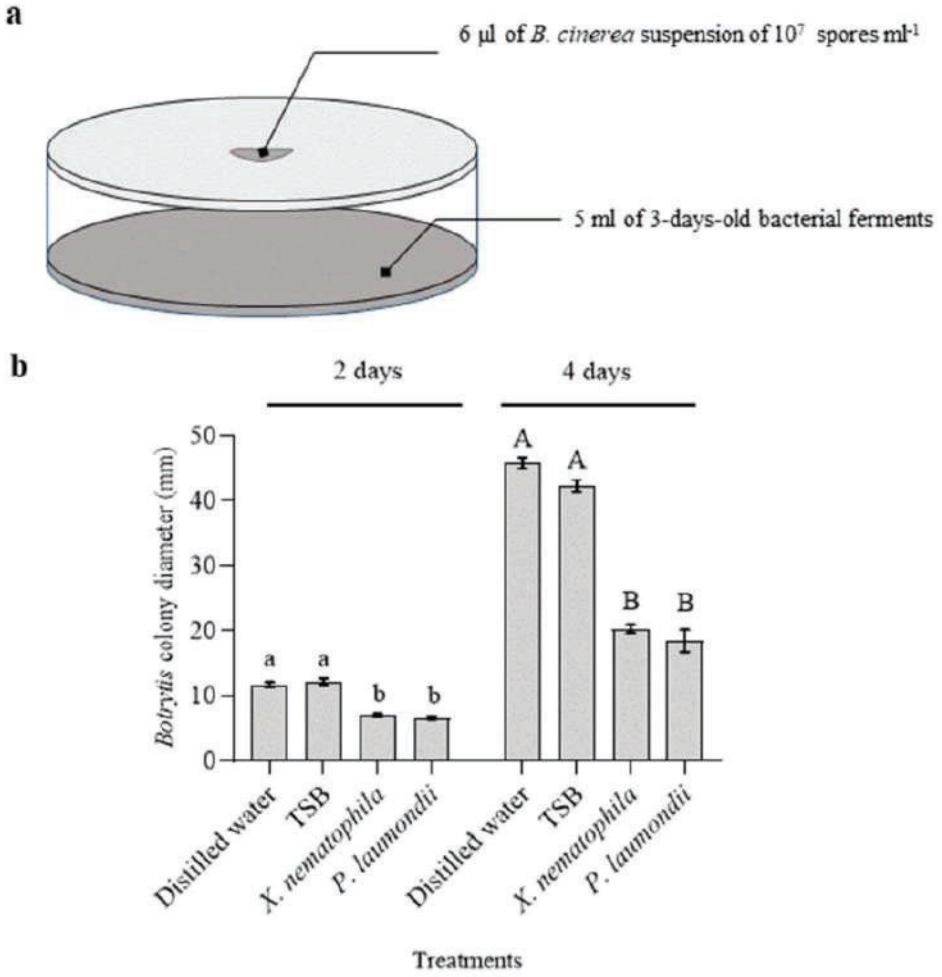

Fig. 1 In vitro antifungal effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* mycelial growth. **a** Schematic diagram displaying the methodological approach for testing in vitro the antifungal activity of VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii*. **b** Inhibition of *B. cinerea* mycelial growth induced by VOCs of three-day-old *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferments. Each treatment comprises 15 replicates and there was two independent trials per study (total n per treatment = 30). The error bars represent SE, and different case letters (lower, after two days, and capital, after four days) represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P < 0.05$)

comprised fifteen replicates and we conducted the same experiment twice, with new material, ferment and fungus preparation. We incubated all the experimental units at 60% RH, 22 ± 1 °C, and a 16:8 L:D photoperiod for four days. We daily recorded colony diameters (cross directions) until

the control treatment covered 100% of the medium surface inside the dish (four days after pathogen infection).

To analyze the antifungal activity of bacterial VOCs on *B. cinerea*, first, we tested the goodness of fit against normal distribution, and then, we performed a one-way ANOVA with four levels (distilled water control, TSB control, *X. nematophila* ferment, and *P. laumondii* subsp. *laumondii* ferment) on the diameter of *B. cinerea* colonies two and four days after pathogen inoculation. We subsequently conducted a post-hoc multiple comparison test using Tukey's method at a significance level of 5%. We performed these analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA).

Efficacy of VOCs emitted by bacteria in controlling pathogen infection in grapes and tomatoes

We randomly collected ripe red grapes (*Vitis vinifera* cv. Tempranillo) and tomatoes (*Solanum lycopersicum* cv. Sweet Million) from an organic field located in Logroño (La Rioja, Spain, 42° 29′ 14″ N, 2° 30′ 7″ W). We cultured both fruits under organic management and without applying any pre-harvest fungicide treatments. We selected intact, healthy, and homogenous fruit and randomly assigned them to different treatments. Before inoculation and treatment application, we disinfected the fruit surface by dipping them in 3% (v/v) of sodium hypochlorite (NaOCl) solution for 1 min, washed them with distilled water and then air-dried them for ~ 2 h. We performed artificial wounding using a sterile pipette tips to make 5 mm deep and 3 mm wide wounds (one wound for each grape or tomato) along the berry equatorial areas. We inoculated each wound with 6 µl drop of 1×10^7 conidia ml^{-1} of *B. cinerea*. We placed grape berries and tomatoes on plastic packaging trays over 7 mm net (Figs. 2a, 3a). Below each net with fruit. For the grapevine experiment, we included a 90 mm diameter Petri dish with 10 ml of three days ferment *X. nematophila* or *P. laumondii* subsp. *laumondii* TSB ferments (Fig. 2a). In the case of the tomatoes, we included a container (40 mm high × 90 mm diam.) filled with 25 ml of the same ferments (Fig. 3a). In both experiments, we used TSB as control. To create a humid environment, we placed 5 ml of distilled water on cavity trays. We incubated the trays on orbital shaking 60 rpm at 22 °C and 95% RH in darkness during four days after pathogen application to provide favorable conditions for the post-harvest onset of the disease. We evaluated diseases incidence (fungal infection or not) for all the treatments in fifteen grape fruit (three groups with five grapes) and eight tomatoes (two groups of four cherries), and we conducted the same experiment twice (for a total of 30 and 16 fruit per treatment, respectively). We simultaneously assessed the relative lesion area (mm^2) by measuring the fungus growth area and the total fruit area using image analysis with Image J® program (v. 1.50i, MD, USA) four days after pathogen inoculation (Vicente-Díez et al. 2023).

To investigate the efficacy of bacterial VOCs over the incidence and the severity of *B. cinerea* infection in grapes and tomatoes, first, we tested the goodness of fit against normal distribution, and then, we performed a one-way ANOVA testing for the effect of bacterial VOCs (three levels: TSB control, *X. nematophila* ferment and *P. laumondii* subsp. *laumondii* ferment) on the percentage of fruit infected and the relative measure of the fungal growth four days after pathogen infection. We subsequently conducted a post-hoc multiple comparison test using Tukey's method at a significance level of 5%. In the case of the relative lesion area, we firstly transformed percentage data using the arcsine transformation to meet normality. We performed these analyses with SPSS 25.0.

Preventive effect of VOCs emitted by bacteria in controlling pathogen infection in wounded and intact grapes

We performed the subsequent studies only in grape as a proof of concept approach. We arranged surface-disinfected red grapes in plastic trays over a wire net above 10 ml of three days bacterial ferments placed in 90 mm Petri dish (without physical contact). For each approach (wounded or intact grapes), the experimental design was as described in section "Efficacy of VOCs emitted by bacteria in controlling pathogen infection in grapes and tomatoes", using fifteen grape fruit (three groups with five grapes) per treatment and conducting the same experiment twice (for a total of 30 fruit per treatment, respectively). To evaluate the preventive effect of bacterial VOCs on damaged grapes, we made the wounds as described in the section "Efficacy of VOCs emitted by bacteria in controlling pathogen infection in grapes

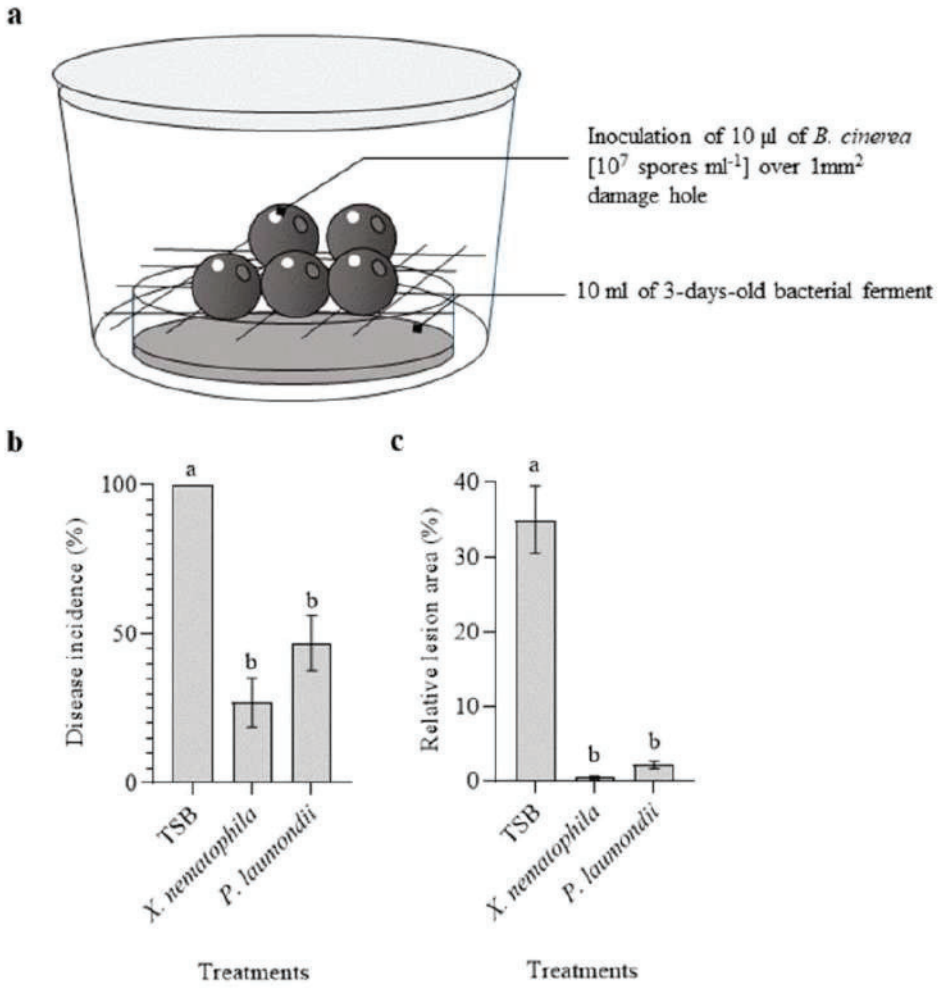

Fig. 2 In vivo antifungal effect of VOCs emitted by three-day-old *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* TSB ferments against *Botrytis cinerea* bunch rot on grapes four days after infection. **a** Schematic diagram showing the methodological approach for testing antifungal activity of the VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii*. **b** Disease incidence. **c** Relative lesion area caused by *B. cinerea* mycelial growth. Different lower-case letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P<0.05$). Each treatment comprises 15 replicates and there was two independent trials per study (total n per treatment = 30). Values are means of each treatment and vertical bars indicate SE

and tomatoes". Then, we arrange the grapes inside the plastic trays with the bacterial ferments (Fig. 4a). We did not wound grapes in the preventive effect bioassays with intact grapes (Fig. 5a). We exposed all grapes to bacterial volatiles at 60 rpm orbital agitation, with a 16:8 L:D photoperiod and 22 °C for 72 h. After VOCs

exposure, we removed the bacterial ferment and placed a piece of 1 cm^3 of four-day-old *B. cinerea* active culture in the base plate (Figs. 4b, 5b). We kept high RH inside of the plastic packing by adding 5 ml of distilled water in the base of the plastic tray. For the bioassay with intact grapes, we wounded the grapes at this time to facilitate disease incidence. We assessed disease incidence by counting the number of infected grapes and the disease severity using an 1-to-4 ordinal scale following Parafati et al. (2015), slightly modified. The diseases severity scale was: 1 (no visible symptoms: 0%); 2 (soft rot: ≤25%); 3 (mycelial growth: 25–75%); and, 4 (sporulation: > 75%) (see Supplementary Material 3). As described by Parafati et al. (2015), we calculated average fruit disease severity for its graphical representation. The final value was expressed as percentage as in Parafati et al. (2015). We collected all data four days after pathogen infection.

To investigate the preventive efficacy of bacterial VOCs over the incidence of *B. cinerea* infection in wounded and intact grapes, first, we tested the goodness of fit against normal distribution. Thereafter, we ran a one-way ANOVA testing for the effect of bacterial VOCs (three levels: TSB control, *X. nematophila* ferment and *P. laumondii* subsp. *laumondii* ferment) on the percentage of infected grapes four days after pathogen infection. We subsequently conducted a post-hoc multiple comparison test using Tukey's method at a significance level of 5% (SPSS Statistics 25.0).

Results

Antifungal activity of VOCs emitted by bacteria

Volatile organic compounds emitted by three-days-old bacterial ferments significantly affected the mycelial growth of *B. cinerea* two ($F_{3,116}=68.69$, $P<0.001$) and four ($F_{3,116}=167.50$, $P<0.001$) days after pathogen infection. In particular, we found that VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) reduced *B. cinerea* colony diameter to 41% and 44%, respectively, in two days, reaching a reduction of 56% and 60%, respectively after four days (Fig. 1b).

Efficacy of VOCs emitted by bacteria in controlling pathogen infection in grapes and tomatoes

Volatile organic compounds emitted by three-days-old bacterial ferments significantly reduced *B. cinerea* incidence compared with TSB control ($F_{2,87}=28.03$, $P<0.001$) and the relative lesion damage in red grapes four days after infection ($F_{2,87}=96.24$, $P<0.001$). In particular, VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) limited the disease incidence on grapes to 27% and 47%, respectively (Fig. 2b). Similarly, VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) reduced 99% and 94%, respectively, the relative lesion area on grapes (Fig. 2c).

In tomatoes, the three-days-old *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferments significantly reduced *B. cinerea* incidence compared by control by 50% and 94%, respectively ($F_{2,45}=32.09$, $P<0.001$) (Fig. 3b). Also, the relative lesion damage in tomatoes was reduced 75% and 99%, respectively, four days after pathogen infection ($F_{2,45}=33.61$, $P<0.001$) (Fig. 3c).

Preventive effect of VOCs emitted by bacteria in controlling pathogen infection in grapes

We tested the preventive effect of the bacterial ferments using wounded and intact red grapes. Volatile organic compounds emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* significantly reduced *B. cinerea* incidence on harvested wounded grapes compared to the TSB control treatment four days after pathogen infection ($F_{2,87}=21.16$, $P<0.001$). We found that VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) reduced 44 and 84% of *B. cinerea* incidence on wounded grapes after four days of pathogen infection, respectively (Fig. 4c). In addition, although disease severity increased over time, the preventive treatment with bacterial VOCs reduced significantly the overall disease severity on wounded grapes compared to the TSB control treatment four days after pathogen infection ($P<0.001$). In particular, VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) kept 65% and 80% of the grapes without *B. cinerea* symptoms until four days after pathogen infection (Fig. 4d).

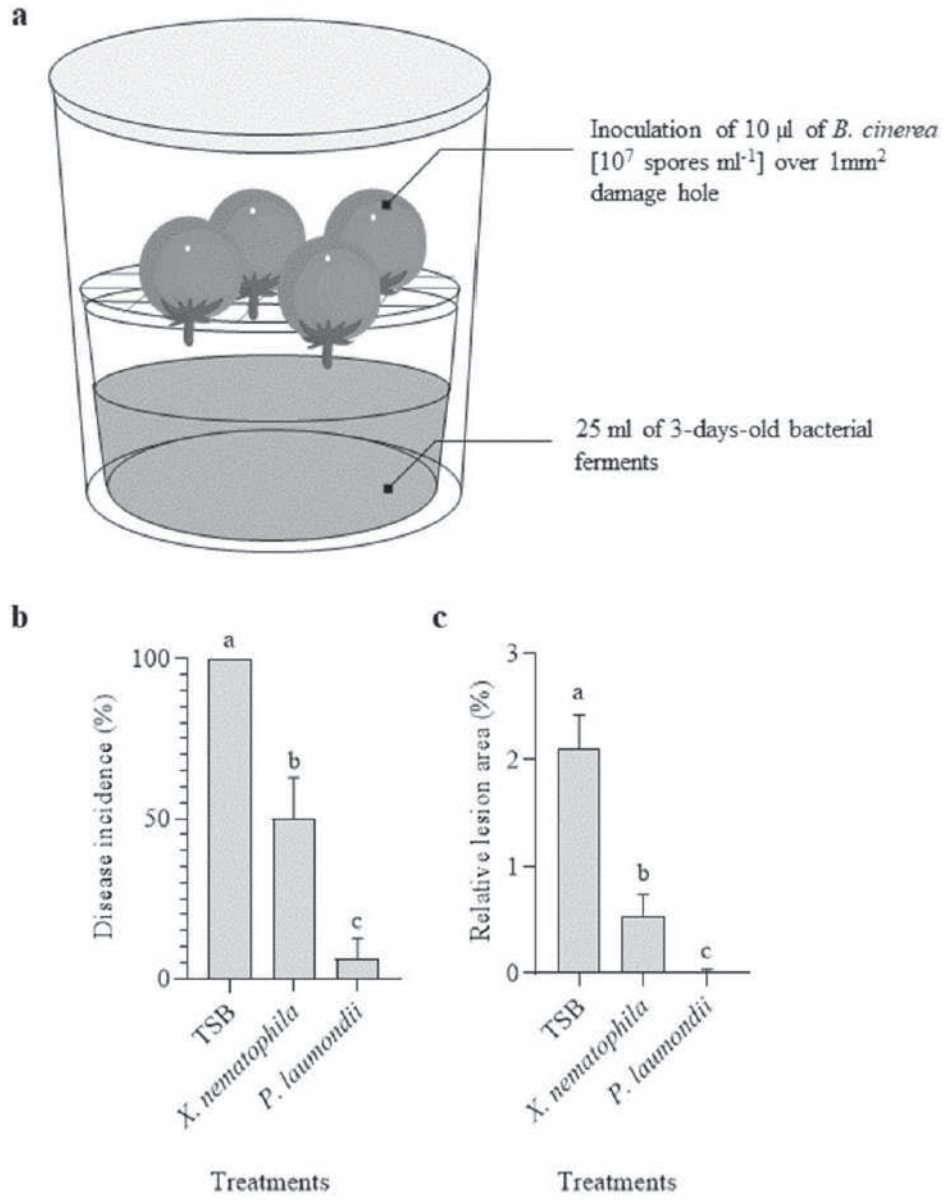
a
Inoculation of 10 µl of B. cinerea [10^7 spores ml^-1] over 1mm^2 damage hole
25 ml of 3-days-old bacterial ferments
b
Disease incidence (%)
100
50
0
a
b
c
TSB
X. nematophila
P. laumondii
Treatments
c
Relative lesion area (%)
3
2
1
0
a
b
c
TSB
X. nematophila
P. laumondii
Treatments

Fig. 3 In vivo antifungal effect of VOCs emitted by three-day-old *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* TSB ferments against *Botrytis cinerea* bunch rot on tomatoes four days after infection. **a** Schematic diagram showing the methodological approach for testing antifungal activity of the VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii*. **b** Disease incidence. **c** Relative lesion area caused *by B. cinerea* mycelial growth. Different lower-case letters represent statistically significant differences between treatments according to Tukey's multiple comparison test ($P<0.05$). Each treatment comprises eight replicates and there was two independent trials per study (total n per treatment = 16). Values are means of each treatment and vertical bars indicate SE

We tested also the possible changes in the fruit modulated by the bacterial ferments using intact grapes. The VOCs emitted by bacteria significantly decreased *B. cinerea* incidence on intact grapes four days after pathogen infection ($F_{2,87}=14.73$, $P<0.001$). In particular, VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) reduced 82 and 62% *B. cinerea* incidence on healthy grapes four days after pathogen infection, respectively (Fig. 5c). In addition, bacterial VOCs significantly reduced *B. cinerea* severity on intact grapes four days after pathogen infection ($P<0.001$). In particular, VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* (vs. control) kept 90 and 65% of the grapes without *B. cinerea* symptoms four days after pathogen infection, respectively (Fig. 5d).

Discussion

Soil-dwelling bacteria *X. nematophila* and *P. laumondii* subsp. *laumondii* emitted VOCs with antifungal activity against the saprophytic pathogen *B. cinerea*. This property has a great potential to control *B. cinerea* in harvested red grapes and tomatoes if fruit quality is not affected. Furthermore, our results showed that *B. cinerea* had less incidence and growth on grapes if treated with these compounds previous to the fungal attack, suggesting that the bacterial VOCs might modulate changes in the fruit that can trigger better resistance to fungal infection. Despite the presence of a high concentration of CO_2 might also contribute to reducing the growth of the fungus (Teles et al. 2014), a recent study by Kong et al. (2022) revealed that a rich and complex blend of VOCs emitted by *Xenorhabdus* and *Photorhabdus*, potentially also similar to that produced by our bacterial strains, contributes to the inhibition of growth and reducing the damage caused by fungal attack. Although promising, still, the subsequent development and scale-up of this novel bacteria-based tool are required to provide an economical alternative in the integrated control of post-harvest diseases that might contribute to reducing the amount and number of chemical fungicide applications during the food supply chain.

The VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferments inhibited > 60% of *B. cinerea* mycelial growth in in vitro tests. As far as we know, this study is the first showing an inhibitory effect of VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferment using a dual plate system to create a medium without contact between the pathogen and the biological control agent (Raymaekers et al. 2020). Previous work by Chacón-Orozco et al. (2020) found that *Xenorhabdus szentirmaii* produces secondary metabolites with inhibitory effects on the mycelial growth of the phytopathogenic fungi *Sclerotinia sclerotiorum*. However, their methodology did not create a medium without contact between the bacterial secreted metabolites and the pathogen and, therefore, it cannot be proven that the effect was due to VOCs as we showed in our experiments.

Traditionally, many studies on the antimicrobial activities of *Xenorhabdus* spp. and *Photorhabdus* spp. secondary metabolites have been performed through in vitro assays with nutrient medium (Fang et al. 2011; Lai et al. 2020). These assays often over- or under-estimate antifungal activity compared with in vivo tests working with harvested fruit. Pathogenic infection is a well-regulated phenomenon that requires cross-talk between the host (fruit or vegetables) and the pathogen through signals located on the external surfaces of cells (Raymaekers et al. 2020). The disturbance of cell membranes by antifungal compounds often leads to interference with such signals, which could eventually fail a fungal infection. For this reason, results might drastically differ depending on whether they are performed on an artificial medium or on natural fruit. Our test in harvested fruit (red grapes and tomatoes) showed that the VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* TSB ferments drastically reduced

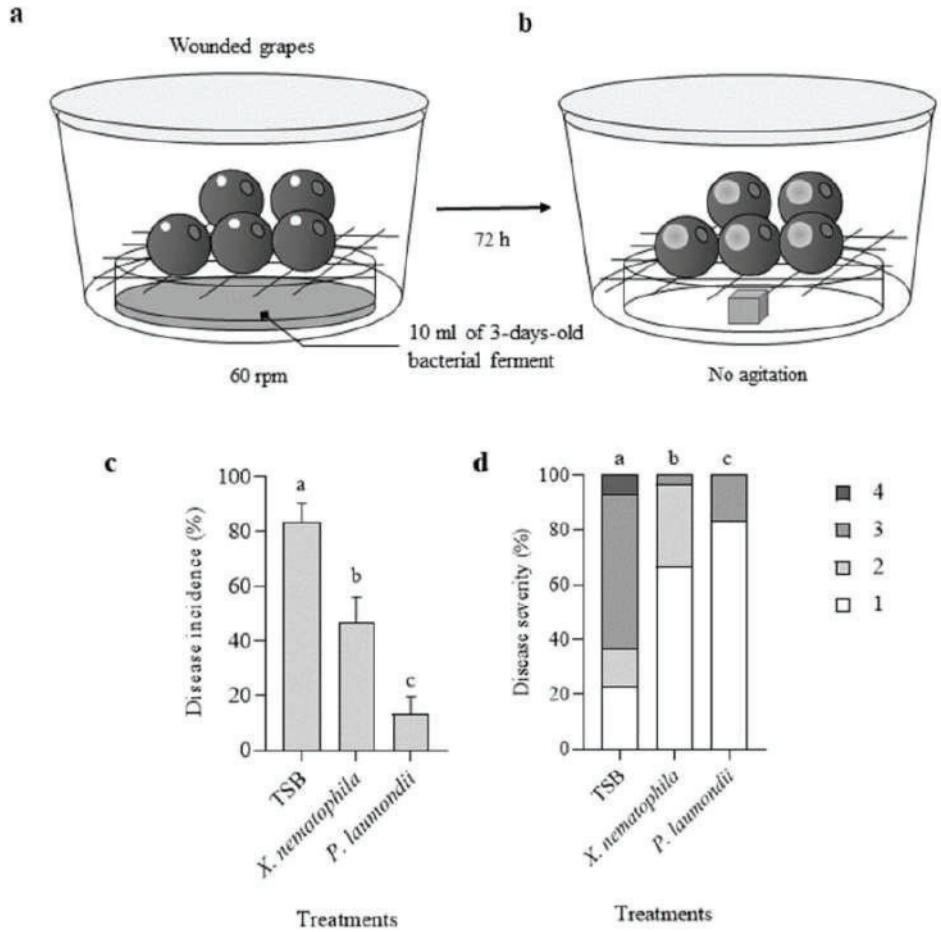

Fig. 4 In vivo antifungal preventive effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* bunch rot on wounded grapes. **a** Schematic diagram showing the methodological approach for testing antifungal preventive activity of the VOCs of *X. nematophila* and *P. laumondii* subsp. *laumondii*. **b** Exposure to the fungus after incubation with the VOCs. **c** Disease incidence. **d** Disease severity caused by *B. cinerea* conditioned by the different treatments. The diseases severity scale was: 1 (no visible symptoms: 0%); 2 (soft rot: ≤25%); 3 (mycelial growth: 25–75%); and 4 (sporulation: > 75%). Each treatment comprises 15 replicates and there was two independent trials per study (total n per treatment = 30). The error bars represent SE, and different lower-case letters represent statistically significant differences between treatments analyzed on disease rating classes ($P < 0.05$)

the incidence and growth of *B. cinerea*. Although the possible mechanisms producing such an effect would require further research, Lai et al. (2020) observed that the application of *Photorhabdus luminescens* enhanced the defensive mechanism and non-enzymatic antioxidant system of detached litchi, delaying the browning and the decay of the fruit (Lai et al. 2020). Therefore, it is plausible that, in our study, *X.*

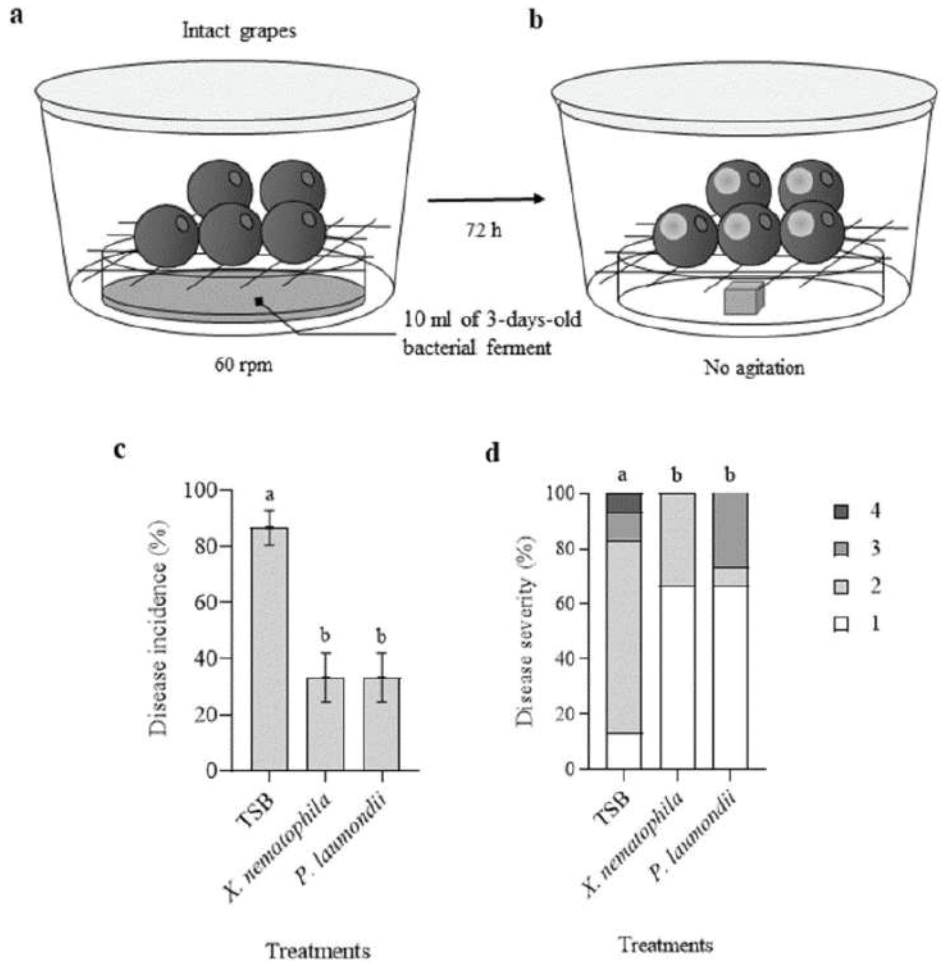

Fig. 5 In vivo antifungal preventive effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* bunch rot on intact grapes. **a** Schematic diagram showing the methodological approach for testing antifungal protective activity of the VOCs of *X. nematophila* and *P. laumondii* subsp. *laumondii*. **b** Exposure to the fungus after incubation with the VOCs. **c** Disease incidence. **d** Disease severity caused by *B. cinerea* conditioned by the different treatments. The diseases severity scale was: 1 (no visible symptoms: 0%); 2 (soft rot: ≤25%); 3 (mycelial growth: 25–75%); and 4 (sporulation: >75%). Each treatment comprises 15 replicates and there was two independent trials per study (total n per treatment = 30). The error bars represent SE, and different lower-case letters represent statistically significant differences between treatments analyzed on disease rating classes ($P < 0.05$)

nematophila and *P. laumondii* subsp. *laumondii* can follow similar routes that will require further transcriptomic, metabolomic, and enzymatic experiments to confirm the exact mechanisms.

As with the wounded grape evaluation, we found that both intact and wounded grapes treated previously with the bacterial ferment drastically reduced *B. cinerea* infection. Still, the mechanisms involved in this phenotype remain unknown. One possibility might be that the VOCs can induce a response in the fruit defenses. Indeed, plant hormones, plant extracts, microorganisms, and abiotic stimulants activate defense responses in grapes against *B. cinerea* infection (Jacometti et al. 2010; Romanazzi et al. 2016). In particular, different microbial biological control agents (e.g., Filamentous fungi from the genera *Trichoderma*, *Ulocladium*, and *Gliocladium*; bacteria from the genera *Bacillus* and *Pseudomonas*; and yeasts from the genera *Pichia* and *Candida*) have been reported to increase fruit resistance against postharvest diseases (Spadaro and Droby 2016; Dukare et al. 2019).

The use of *Xenorhabdus* or *Photorhabdus* is still in its infancy due to their phenotypic and phase variation complexity (Han and Ehlers 2001; Clarke 2016; Dominelli et al. 2022). More widespread is the use of their secondary metabolites, earned by the filtration of the bacterial ferments in different medium cultures to reduce the growth of fruit fungal phytopathogens (Yang et al. 2011; Fang et al. 2014; Hazir et al. 2016). Among the diverse array of bioactive metabolites produced by beneficial microorganisms, bacterial VOCs are getting a potential applied interest due to their broad range of positive effects (easy renewability, biodegradability, great diversity of compounds, non-toxicity) on plant and fruit resistance (Parafati et al. 2015; Mari et al. 2016; Cellini et al. 2021), as well as the restrictions on the widespread use of synthetic fungicides (Mari et al. 2016). So far, this study showed that soil-dwelling nematode symbionts *Xenorhabdus* and *Photorhabdus* can be explored as beneficial microorganisms to control post-harvest fruit decay. Our future research will be aimed at identifying the specific VOCs emitted by *X. nematophila* and *P. laumondii* subsp. *laumondii* responsible for this antifungal activity, as well as unraveling how these VOCs might lead to modulating the post-harvest fruit at the level of secondary metabolism.

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

Declarations

Competing interests 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.

Patent The results presented herein are part of the patent entitled "Volatile organic compounds obtained from *Photorhabdus laumondii* subsp. *laumondii* and uses thereof" (Reference EP23382199).

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Xoaquín Moreira is a tenured scientist from Misión Biológica de Galicia at the Spanish National Research Council (CSIC). He is interested in the ecological and evolutionary processes that occur across different trophic levels (plants, herbivores and natural enemies), and predict how future global change might influence not only each species individually, but the various interactions as a whole.

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Mar Vilanova is a tenured scientist at the Instituto de las Ciencias de la Vid y del Vino (ICVV) from the Spanish National Research Council (CSIC). She has a background in volatile composition and sensory characterization of *Vitis vinifera* grapes and wines. She is a specialist on how the grape responses to biotic and abiotic stresses, with emphasis on how to improve the grape quality by controlling stress in the plant, and stimulating glycosylated forms.

Alicia Pou is a tenured scientist at the Instituto de las Ciencias de la Vid y del Vino (ICVV) from the Spanish National Research Council (CSIC). Her research interests are mainly related to grapevine physiology. Recently, she is investigating the role of different soil management techniques as an integral part of the plant physiological performance, grape quality, and microbial communities living with/within plants.

Raquel Campos-Herrera is a tenured scientist at the Instituto de las Ciencias de la Vid y del Vino (ICVV) from the Spanish National Research Council (CSIC). She uses entomopathogenic nematodes as a model system to addresses the long-term challenge of reducing dependence on traditional agrochemicals in pest control, with special emphasis on the vineyard, focusing on two specific challenges (1) developing pest control bio-tools, and (2) understanding how agronomic management impacts agroecosystems to identify the most sustainable actions.

5. Results and Discussion

"The powerful play goes on,
and you may contribute a verse"

Walt Whitman, in *Leaves of Grass*, 1855

5.1. *Xenorhabdus* and *Photorhabdus* soluble bioproducts: insecticidal and antifungal activity

5.1.1. Insecticidal effects against pest and insect vectors of grapevines

The first of the three objectives of this Thesis was to evaluate the insecticidal and antifungal effect of the soluble secondary metabolites produced by *Xenorhabdus* and *Photorhabdus* in order to control pests and diseases in vineyards. To achieve this, we evaluated the potential control of secondary metabolites of symbiont bacteria on grapevine pests like *L. botrana*, and disease-vector insects such as *P. spumarius*. It is well documented that the natural products extracted from EPN symbiont bacteria exhibit toxicity against a wide range of insects (Da Silva et al., 2020; Eroglu et al., 2019; ffrench-Constant et al., 2007; Jallouli et al., 2013). However, its potential use in vineyard management had not been previously explored until these bioassays, described in Publication 1 and Publication 2.

For the control of *P. spumarius* nymphs, our results showed that natural products derived from *P. laumondii* subsp. *laumondii* caused the highest mortality rates for the application of 1:10 dilution cell-free supernatant (~60 %). On the other hand, in the genera *Xenorhabdus*, we observed insecticidal activity only for the cell-free supernatant obtained from *X. nematophila* (~37 %). Nevertheless, *X. kozodoii* and *X. bovienii* did not increase nymphal mortality after five days of application with regard to the TSB control. For the bioassays performed against *L. botrana* larvae instars (L1, L3 and L5), our results showed that the cell-free supernatants derived from the four symbiotic bacteria were toxic when ingested by larval instars. Our results showed that the mortality rates exceeded 50 % and 90 % at two and three days compared to L1, respectively. For L3, it took up to

four or five days to reach comparable numbers. Furthermore, for *L. botrana,* we tested the use of the *X. nematophila* and *P. laumondii* unfiltered ferments (UFs). The ingestion of UF products from both symbiotic bacteria was toxic against the L1 (over 80 % in two days) and L3 (over 60 % in three days) larval instars.

These results demonstrate the insecticidal toxicity of the soluble metabolites produced by EPN symbiont bacteria against an important pest and an insect vector in vineyards, highlighting their potential as a source for developing novel biopesticides (Figure 18). When designing bioassays and considering the upscaling of toxin production from EPN symbiotic bacteria, it is essential to accurately identify the target organism for control and to gain a comprehensive understanding of its biology and environmental interactions, including factors such as its feeding behaviour. This knowledge serves multiple objectives, including investigating the mode of toxin entry, analysing the mode of action, exploring diversification in the active compounds, and guiding the purification of key compounds, among others.

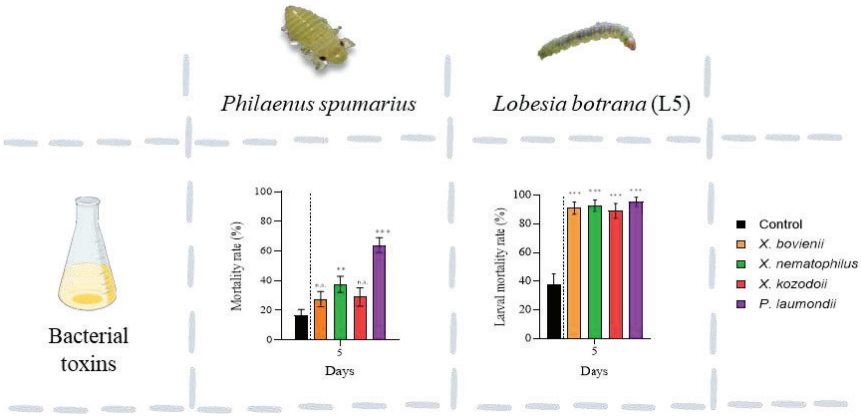

Figure 18. Insecticidal effect of *Xenorhabdus* and *Photorhabdus* toxins against *P. spumarius* and *L. botrana* (L5). Data from Publication 1 and Publication 2.

For instance, Cevizci et al. (2020) reported that bioactive acaricidal compounds produced by *Xenorhabdus szentirmaii* and *X. nematophila* exhibit the highest efficacy when the entire integument of *T. urticae* comes in contact with it, compared to contact with the ventral side only. In our research, we have worked with *P. spumarius*, which is a xylem-feeding insect

(Cornara et al., 2018), and *L. botrana* larvae, known for their biting behaviour characterized by short jaws featuring four short apical sensilla and a short palpus (Benelli et al., 2023). Consequently, when considering these various studies collectively, it becomes evident that these metabolites can effectively control a wide array of arthropod pests, including sap-feeding insects and chewers. This broad spectrum can be advantageous in its application because it is a way of controlling different target pests with only one product.

Moreover, we assessed the insecticidal efficacy of bacterial cell-free supernatants and unfiltered fermentations in these bioassays with *L. botrana*. We demonstrated that the use of unfiltered ferments derived from the bacterial species *X. nematophila* and *P. laumondii* can lead to a faster and stronger effect against L1 and L3 instars than their cell-free supernatants. The outcomes align with the concept advanced by Marrone (2023), suggesting that individual metabolites may exhibit relatively low activity levels but manifest heightened potency when produced in combination. Consequently, regulatory frameworks mandating the isolation and purification of individual metabolites for risk assessment may offer limited utility compared to alternative approaches. This notion warrants careful consideration, particularly given the prevalent focus within research and development endeavours on acquiring isolated molecules designed to fulfil specific functions. As evidenced in our experiments, it is plausible that the purification processes could potentially diminish toxicity, thus underscoring the need to account for such factors when formulating and advancing these bioproducts.

5.1.2. Antifungal effects against *Botrytis cinerea*

The antifungal capacity of *Xenorhabdus* and *Photorhabdus* secondary metabolites has been widely studied during the last years (Cimen et al., 2021; Fang et al., 2014; Fang et al., 2011; Yang et al., 2011). During our bioassays, included in Publication 4, we focused on the efficacy of different control strategies against *B. cinerea*, using *Xenorhabdus* and *Photorhabdus* bacterial cell-free supernatants, unfiltered ferments and crude bacteria isolates (Figure 19A, B, and C). As we show and discuss in section 5.2.2., the bioassays carried out with the bacterial volatile organic compounds (VOCs) have also demonstrated the antifungal capacity against *B. cinerea*.

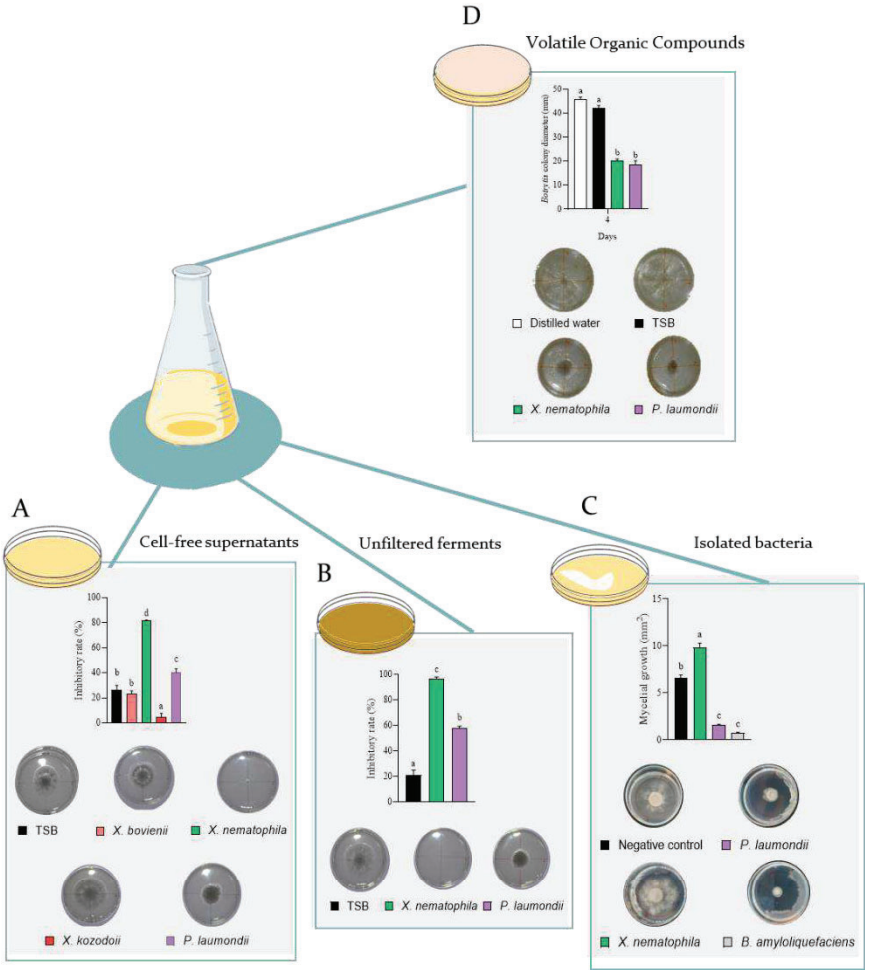

Figure 19. *In vitro* antifungal activity of bacterial cell-free supernatants, unfiltered ferments, volatile organic compounds, and isolated bacteria. A) Inhibitory mycelial growth rate effect by bacterial cell-free supernatants 1:5 (cell-free supernatants: PDA). B) Inhibitory mycelial growth rate effect by bacterial unfiltered ferments 1:5 (unfiltered ferments: PDA). C) Impact of *X. nematophila*, *P. laumondii*, *Bacillus amyloliquefacien*s isolated bacteria in the *B. cinerea* growth. D) Antifungal effect of VOCs emitted by *X. nematophila* and *P. laumondii* against *B. cinerea* mycelial growth.

The antifungal efficacy of *X. bovienii*, *X. nematophila*, *X. kozodoii* and *P. laumondii* subsp. *laumondii* cell-free supernatants obtained after the bacterial fermentations were tested *in vitro* following the protocol

described by Fang et al. (2011). The results obtained showed that *X. nematophila* and *P. laumondii* were the most effective species for the control of *B. cinerea* achieving >80 % and >40 % inhibition rates. Furthermore, the antifungal effect of *X. nematophila* and *P. laumondii* unfiltered ferments were tested *in vitro*, and their dissuasive effect was also tested over tomato leaves. The *X. nematophila* unfiltered ferments resulted in 100 % fungal inhibitory rate. On the other hand, the *P. laumondii*-isolate can control the growth of *B. cinerea* under *in vitro* conditions, showing no significant differences in four days with the efficacy of the commercial product *Bacillus amyloliquefaciens* (Serenade® ASO fungicide). The results showed that the use of bacterial cell-free supernatants, unfiltered ferments or isolated bacteria have different antifungal efficacy against this pathogen.

The results obtained from the experiments reported in this Thesis agree with previous studies (Fang et al., 2014; Fang et al., 2011) regarding the effectiveness of *Xenorhabdus* and *Photorhabdus* bioproducts in controlling *B. cinerea* infections. From a crop protection perspective, diseases often pose a more significant challenge than entomological issues in vine-growing regions, as indicated by the frequency of fungicide applications each year to combat downy mildew (*P. viticola*), powdery mildew (*E. necator*), and bunch rot (*B. cinerea*) (Bostanian et al., 2012). Consequently, the development of new biotechnological tools and research into biological disease control agents has gained increasing importance, particularly in the case of fungi such as *B. cinerea*, through the utilization of antagonistic microorganisms, either before or after infection (Armijo et al., 2016; Parafati et al., 2015). In this line, our research proves that the metabolites produced by *X. nematophila* and *P. laumondii* employed as CFSs or UFs have potential as biofungicides. Additionally, it is noteworthy that, for the first time, our study reveals that bacteria belonging to the genus *Photorhabdus* may possess the capability to compete as control agents against certain diseases in a life stage outside of its nematode symbiont. This discovery holds potential developing novel biopesticides utilizing bacteria belonging to the genus *Photorhabdus*. Presently, only in vitro effects against *B. cinerea* within a Petri dish have been observed, thus necessitating additional research to investigate this prospective application comprehensively.

5.2. *Xenorhabdus* and *Photorhabdus* volatile compounds: deterrent and antifungal activity

Beneficial microorganisms have long been used as a source of natural pest control products (Arthurs & Dara, 2019). Among the chemically best-studied microbial compounds are those produced by *Xenorhabdus* and *Photorhabdus* (Flórez et al., 2015). The main focus has been on toxin production to detect biopesticidal molecules, mostly soluble compounds, but much less attention has been paid to other kinds of molecules, such as VOCs, which are related to inter- and intraspecific communication, deterrent factors, etc. These volatile compounds have recently acquired special attention due to their broad-range effects as deterrent, antifungal, biostimulant, or plant-growth promotion agents (Cellini et al., 2021).

5.2.1. Deterrent activity

Xenorhabdus and *Photorhabdus* natural deterrent compounds emitted for defence against opportunistic scavenger insect have not been widely employed in pest control (Gulcu et al., 2018). In this Thesis, as part of Objective 2, we have explored their use as feeding deterrents and anti-ovipositional signals against *L. botrana* larvae and moth. 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 numerous larval pests, the choice of feeding-source conditioned larval development time, survival, pupal weight, and female fecundity (Savopoulou-Soultani & Tzanakakis, 1988; Tasin et al., 2012). Furthermore, the anti-ovipositional effect of the EPN symbiotic bacteria metabolites was previously tested against the calliphorid fly, *Chrysomya albiceps* (Diptera: Calliphoridae), proving that the supernatant of *P. luminescens* deterred *C. albiceps* from depositing eggs on meat (Gulcu et al., 2012). However, both feeding and ovipositional deterrents seem to be effective tools in crop protection.

Publication 3 shows the effect of the allelochemical signals produced by *X. nematophila* and *P. laumondii* on moth ovipositional behaviour and feeding preference of *L. botrana* larvae. We tested 3-d cell-free supernatants and 3-d and 5-d unfiltered ferments. In addition, we tested two application systems: (*i*) contact application of bacterial compounds, and

(*ii*) application of volatile bacterial compounds. Our findings indicate that deterrent effectiveness varied with bacterial species, but also depend on the use of bacterial cell-free supernatants, unfiltered fermentation product, and their corresponding culture times. Specifically, grapes soaked in the 3-d *X. nematophila* and *P. laumondii* ferments had ~55 and ~95 % fewer eggs laid than the control, respectively. Likewise, 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 deterrent effects on larval feeding (~65 % of the larva chose the control grapes), and significantly reduced the severity of third-instar larval damage to treated grapes.

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. Specifically, *L. botrana* has begun to be effectively managed using pheromone-mediated mating disruption, proving the efficacy of semiochemical signals in field. In addition to this, we suggest that allelochemical signals may also serve as an effective semiochemical tool, thereby diversifying the mechanisms to control pests. Our results lay the groundwork for research into novel applications of these bacterial deterrent compounds in the development of new repellents against crop pests.

5.2.2. Antifungal activity

Another aspect that remains relatively unexplored in the field of bacterial VOCs is their potential role as antifungal tools. As shown in Publication 5, we investigated the effects of *X. nematophila* and *P. laumondii* VOCs on the infection and growth of the pathogenic mould *B. cinerea*. Results showed that VOCs emitted by *X. nematophila* and *P. laumondii* (vs. control) reduced ~40 and ~60 % of *B. cinerea* colony diameter two and four days after pathogen infection, respectively (Figure 19D). Moreover, the results showed that *X. nematophil*a and *P. laumondii* VOCs inhibit ~100 % of *B. cinerea* mycelial growth in grapes. Similarly, these bacterial VOCs inhibit ~70 % and 100 % of *B. cinerea* mycelial growth in cherry tomatoes.

The use of VOCs produced by bacteria has many advantages such as not leaving residues or not showing the appearance of resistances (Delgado et al., 2021). However, whether those VOCs also have an effect on

direct and indirect resistance signals is still poorly understood. Verifying the mechanisms of action behind these phenotypes will be critical to determine the holistic impact of these new biotools (Booysen & Dicks, 2020; Cellini et al., 2021). Recently, Wang et al. (2022) explored the fungal inhibitory mechanism of VOCs produced by *X. bovienii*, analysing the changes that occur at the level of gene expression in the fugal pathogen *Fusarium solani* (Hypocreales: Nectriaceae). Their results showed that the expression of many genes involved in the cell cycle, meiosis, and DNA replication were downregulated. Therefore, further research would be required to understand all the potential effects these VOCs can have over target pathogens. As stated by Cellini et al. (2021), bacterial VOCs will likely be an essential tool for crop protection in the near future.

5.3. *Xenorhabdus* and *Photorhabdus* effect on fruit

Microbial-based tools, particularly bacterial VOCs, are becoming increasingly popular as an alternative to chemical and physical postharvest treatments (Delgado et al., 2021). In Publication 5, we evaluated the effects of *Xenorhabdus* and *Photorhabdus* VOCs on intact and damaged postharvest grape. Our results showed that both intact and damaged grapes treated preventively with the bacterial volatiles had less incidence and damage caused by *B. cinerea* afterwards. In particular, in damaged grapes, VOCs emitted by *P. laumondii* were reduced below 20 % *B. cinerea* incidence. Similarly, VOCs emitted by both bacteria decreased *B. cinerea* incidence by 50 % in intact harvested grapes. In contrast to the results shown in section 5.2.2, the effect of volatiles was not tested on the pathogen, but on the fruit, proving that *Xenorhabdus* and *Photorhabdus* secondary metabolites have an effect on fruit.

As far as we know, only the work reported by Lai et al. (2020) has studied the effect of EPN symbiotic bacteria on postharvest fruit. Specifically, they used a suspension *P. luminescens* on postharvest litchi, and their findings demonstrated that it enhanced the defence-related mechanism and non-enzymatic antioxidant system litchi against fruit decay. In our study, we used bacterial VOCs instead to investigate the effect on fruit resistance against rot and proved their enhancement. Thus, the

potential effect of *Xenorhabdus* and *Photorhabdus* VOCs was studied on fruit for the first time. However, we have not yet been able to comprehend the specific defence mechanisms that are activated or if other biochemical mechanisms are involved. Therefore, future research is required.

5.4. Outlooks

The development of new biotools and strategies for crop protection is essential. The potential of *Xenorhabdus* and *Photorhabdus* bacteria for pest and disease management in agriculture has been amply demonstrated by research over the last decades (Da Silva et al., 2020; Dreyer et al., 2018). The studies included in this Thesis highlight their potential usefulness in the management of vine pests and diseases. However, future research on symbiotic bacteria of entomopathogenic nematodes in the agricultural world, and in the vine sector specifically, is required to fill the current knowledge gaps by exploring the following areas:

i. Finding and identifying all metabolites and their function

Significant progress has been made in identifying natural products from entomopathogenic bacteria during the last ten years (Tobias et al., 2017). Nevertheless, several open questions remain unanswered, as pointed out by Shi & Bode (2018): What is the molecular mode of action of each natural product? How can phenotypic switching be controlled so that the production of that bioproduct can be stable? Does the natural-product profile define the range of hosts, or can it be broadened when new natural products or their derivatives are produced? How is the molecular strategy of nematodes to protect themselves against bacterial compounds that are cytotoxic to insects and other nematodes? How do the bacteria detect diverse environments and how quickly can they react to the change, which is proved to be fundamentally the switch from mutualistic to pathogenic symbiosis?

Novel tools have been developed in the fields of bioinformatics (gene cluster identification and prediction of natural product production), analytics (particularly mass spectrometry methods), and structural and molecular biology (manipulation of biosynthetic gene clusters and

heterologous expression methods), such as the easyPACId (easy Promoter Activated Compound Identification) method (Challinor & Bode, 2015; Gulsen et al., 2022). These developments hold great promise for the discovery of novel bioactive compounds produced by *Xenorhabdus* and *Photorhabdus* and answer the raised questions. When combined with rapid improvements in sequencing technology, these advancements may usher in a new era of research in microbial natural products, automating several laborious steps of the past.

Concerning the results obtained in this Thesis, identifying semiochemicals, mainly the VOCs produced by *Xenorhabdus* and *Photorhabdus* with deterrent and antifungal activity, is crucial in developing this research line. As far as we know, few studies have conducted analyses of VOCs produced by bacteria with insect deterrent functions (Jaffuel et al., 2021; Kajla et al., 2019; Kong et al., 2022), and no previous studies have shown its antifungal capacity. Moreover, these chemical analyses do not coincide with the identified compounds, perhaps due to differences in the materials and methods used for their acquisition and the bacterial strains employed. In the coming years, given the significance that the functions of bacterial volatile compounds are likely to have (Cellini et al., 2021), identifying these compounds will be of great importance. It will be a way to continue and complete the results obtained during the bioassays shown.

ii. Understanding ecology and application

Assuming that natural products are aimed at fulfilling a specific role in a particular ecological context, a good knowledge of the ecosystem functions carried out by *Xenorhabdus* and *Photorhabdus* are crucial for understanding the activities of these compounds. These bacteria play essential roles in various ecological processes as has been described above. However, some of the ecological functions of symbiont bacteria have not been extensively studied so far. As described by Jones et al. (2017), entomopathogenic nematode symbiotic bacteria are able to produce multimodality activities, as the cues are in different sensory modalities. For example, once the *Heterorhabditis-Photorhabdus* complex infects a host, infected individuals turn red, produce a chemical defence and bioluminescence, and smell strongly at various stages of the infection process (Crawford et al., 2012; Jones et al., 2017). All these signals have been referred to as “scavenger

deterrent factors" (Gulcu et al., 2012). However, the mechanisms behind these deterrent factors have not been well studied, and their potential application is still in the early stages (Kajla et al., 2019; Kong et al., 2022).

Another feature of enormous ecological importance, but little explored so far, is the possible free-living cycle assigned to *P. luminiscense* (Eckstein & Heermann, 2019). The potential for the genus *Photorhabdus* to have a free-living existence in the soil and potential interactions with plant roots represents an intriguing area of research that warrants further investigation. While our understanding of this aspect is still limited, it is hypothesized that *Photorhabdus* spp. may establish interactions with plant roots during its soil-dwelling phase (Regaiolo et al., 2020). This interaction could potentially involve the production of specific compounds or mechanisms that contribute to plant growth and defence. However, extensive research is needed to elucidate the intricacies of these interactions, including the identification of specific molecules involved, the mechanisms of communication, and the ecological consequences for both the bacterium and the plant.

Every step we take in understanding the nematode-bacteria complex opens the door to new potential applications. For example, our experiments, as presented in Publication 5, evaluated the effects of *Xenorhabdus* and *Photorhabdus* VOCs on postharvest fruit defences. These experiments demonstrated that both intact and damaged grapes treated preventively with bacterial volatiles experienced reduced incidence and damage caused by *B. cinerea* afterward. This finding suggests that the multiple defences systems that the bacteria have developed for the protection of the cadaver, including producing chemical defence compounds, can be employed for inducing the defences, for example, of the postharvest fruit and vegetables during the supply chain. However, additional information is needed regarding this ecological function, including a refinement of the timing at which the bacteria commence the production of these compounds or conducting field experiments to assess their functionality. Furthermore, studies must elucidate the defence mechanism and determine whether this fruit defence can impact quality conditions. Experiments that establish connections between ecosystem functions and potential applications are essential.

iii. Studying non-target effects

It is imperative to prioritize future research efforts towards the study of non-target effects of these beneficial bacteria (Sandhi & Reddy, 2019). Despite the limited number of studies conducted thus far, the potential impacts of entomopathogenic nematodes symbiotic bacteria toxins on non-target organisms cannot be disregarded, including their possible effect on humans (Mulley et al., 2015). The existing knowledge of these effects ranges from negligible to harmful, with instances of transient and localized mortality in non-target organisms (Sandhi & Reddy, 2019). To comprehensively assess the ecological implications and potential risks associated with the utilization of EPN symbiotic bacteria, rigorous investigations specifically focusing on non-target effects are essential.

For instance, in employing *Xenorhabdus* and *Photorhabdus* to manage pests and diseases in grapevines, the investigation of non-target effects holds particular significance. A substantial portion of the industry's added value stems from the quality of the wine derived from these crops. Consequently, it is imperative to examine whether the bioproducts do not influence the grape's organoleptic attributes or disrupt the yeast population during spontaneous fermentation. These are matters that warrant investigation prior to the development of commercial products.

iv. Scaling up the bioproduction of bioactive compounds

To achieve commercialization of products derived from *Xenorhabdus* and *Photorhabdus*, a series of essential steps must be followed, considering both the legal pathway to obtain necessary permits as biopesticides and the biotechnological scaling-up process. Firstly, it is crucial to comply with regulatory requirements and seek approval from relevant regulatory authorities (Villaverde et al., 2014). To this end, conducting comprehensive and rigorous studies to demonstrate the safety and efficacy of the biopesticides is essential. The data from laboratory experiments and field trials should be compiled and submitted as part of the regulatory dossier. The regulatory authorities will assess the potential environmental and human health impacts and evaluate the effectiveness of the product.

On the other hand, the process of industrial scale-up still requires numerous advances to achieve marketable products. Although the

possibility of discovering novel antimicrobial compounds from *Xenorhabdus* spp. is promising, methods need to be developed to produce these compounds at much higher concentrations (Dreyer et al., 2018). This may be difficult, as most of these antimicrobial compounds are produced non-ribosomally and are thus not a single gene product (Tobias et al., 2017). However, increased production will follow as we gain more insight into the control of the metabolic pathways. Moreover, developing suitable fermentation protocols for large-scale production, optimizing growth conditions (Booysen et al., 2021; Jiang & Zengyi, 2011), and improving the extraction and formulation methods to ensure stability and efficacy of the final product (Kim & Ko, 2021; Lanois-Nouri et al., 2022) are key points to achieve the industrialization of these bioproducts. Additionally, stringent quality control measures must be implemented to ensure consistency and purity.

In agricultural systems, particularly viticulture, there is a growing demand for novel and more sustainable biotools to manage pest and disease issues, as explained previously. Based on the bioassays conducted in this Thesis, it can be concluded that the symbiotic bacteria associated with entomopathogenic nematodes represent a substantial reservoir of bioactive compounds with potential utility in vineyard management. This thesis lays the foundation for the development of these new tools for three grapevine issues, and has great potential for other pests and diseases of grapevine and other crops. Nevertheless, scaling up this production and its commercialization remain a distant prospect, thus underscoring the need for continued research funding and development efforts in the coming years.

6. Conclusions

"Things do not change: we change"

Henry David Thoreau, in *Walden*, 1854

The main conclusions derived from the studies presented in this Thesis were the following:

1. The secondary metabolites produced by the entomopathogenic nematode (EPN) symbiont bacteria can potentially directly control target vineyard arthropod pests and disease-vector insects.

2. The soluble and volatile secondary metabolites produced by the EPN symbiont bacteria have the potential for direct antifungal activity against *Botrytis* grape rot.

3. Unfiltered bacterial ferments have the highest antifungal capacity compared to cell-free supernatants and isolated bacteria.

4. The crude bacteria of *Photorhabdus laumondii* has the same antifungal capacity as commercial *Bacillus amyloliquefaciens* (Serenade® ASO fungicide)

5. The allelochemical signals emitted by the *Xenorhabdus* and *Photorhabdus* ferments have feeding and anti-ovipositional deterrent action against the larval and adults instars of *Lobesia botrana*, respectively.

6. The volatile organic compounds (VOCs) produced by *Xenorhabdus* and *Photorhabus* can induce preventive effects in damaged and intact post-harvest grapes protecting them for possible *Botrytis* rot infection.

7. Overall, *Xenorhabdus* and *Photorhabdus* and their by-products are potential tools for controlling pests and diseases associated with vineyards.

6.1. Conclusiones

Las principales conclusiones resultantes de los estudios presentados en esta Tesis fueron las siguientes:

1. Los metabolitos secundarios producidos por las bacterias simbiontes de los nematodos entomopatógenos (NEPs) pueden potencialmente realizar un control directo de las plagas de artrópodos y los insectos vectores de enfermedades de la vid.

2. Los metabolitos secundarios solubles y volátiles producidos por las bacterias simbiontes de los NEPs tienen una actividad antifúngica directa contra la podredumbre de la uva causada por *Botrytis cinerea*.

3. Los fermentos no filtrados tienen una mayor capacidad antifúngica en comparación con los sobrenadantes libres de células, las bacterias aisladas y los compuestos orgánicos volátiles.

4. *Photorhabdus laumondii* de forma aislada tiene la misma capacidad antifúngica que el producto comercial basado en *Bacillus amyloliquefaciens* (Serenade® ASO).

5. Las señales aleloquímicas emitidas por los fermentos de *Xenorhabdus* y *Photorhabdus* tienen acción disuasoria frente a la fuente de alimentación y a la oviposición contra los estadios larvarios y los adultos de *Lobesia botrana*, respectivamente.

6. Los compuestos orgánicos volátiles (COVs) producidos por *Xenorhabdus* y *Photorhabdus* pueden inducir efectos preventivos en uvas postcosecha dañadas e intactas, protegiéndolas de una posible infección por podredumbre gris o *Botrytis*.

7. En general, *Xenorhabdus* y *Photorhabdus* y sus productos derivados son potenciales herramientas para el control de plagas y enfermedades asociadas a la vid.

7. Supplementary scientific activity

Completed during the PhD progress

7.1. Publications

7.1.1. JCR Scientific publications

- Campos-Herrera, R., González-Trujillo, M. del M., **Vicente-Díez, I.**, Carpentero, E., Puelles, M., Vaquero, E. Čepulytė, R., **2023**. Exploring entomopathogenic nematodes for the management of *Lobesia botrana* (Lepidoptera: Tortricidae) in vineyards: Fine-tuning of application, target area, and timing. *Crop Protection, 174*. https://doi.org/10.1016/j.cropro.2023.106392

  Discipline: Agronomy; IF: **2,8** (2022), **Q2** (24/88)

- Blanco-Pérez, R., **Vicente-Díez, I.**, Pou, A., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., **2022**. Organic mulching modulated native populations of entomopathogenic nematode in vineyard soils differently depending on its potential to control outgrowth of their natural enemies. *J. Invertebr. Pathol, 192*. https://doi.org/10.1016/j.jip.2022.107781

  Discipline: Zoology; IF: **2,795** (2021), **Q1** (25/176)

- Blanco-Pérez, R., **Vicente-Díez, I.**, Ramos-Sáez de Ojer, J.L., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., **2022**. Organic viticulture enhanced the activity of native entomopathogenic nematodes in DOCa Rioja soils (North of Spain). *Agric. Ecosyst. Environ., 332*. https://doi.org/10.1016/j.agee.2022.107931

  Discipline: Agriculture/Multidisciplinary; IF: **6,576** (2021), **Q1** (5/59)

- Campos-Herrera, R., **Vicente-Díez, I.**, Galeano, M., Chelkha, M., González-Trujillo, M. del M., Puelles, M., Labarga, D., Pou, A., Calvo, J., Belda, J.E., **2021**. Intraspecific virulence of entomopathogenic nematodes against the pests *Frankliniella occidentalis* (Thysanoptera:

Thripidae) and *Tuta absoluta* (Lepidoptera: Gelechiidae). *Journal of Nematology, 53,* 1-14. https://doi.org/10.21307/jofnem-2021-102

Discipline: Zoology; IF: **1,3** (2022), **Q2** (81/176)

- Campos-Herrera, R., **Vicente-Díez, I.**, Blanco-Pérez, R., Chelkha, M., González-Trujillo, M. del M., Puelles, M., Čepulitė, R., Pou, A., **2021**. Positioning entomopathogenic nematodes for the future viticulture: exploring their use against biotic threats and as bioindicators of soil health. *Turkish J. Zool., 45*, 335–346. https://doi.org/10.3906/zoo-2106-40

  Discipline: Zoology; IF: **0,932** (2021), **Q4** (148/174)

- Chelkha, M., Blanco-Pérez, R., **Vicente-Díez, I.**, Bueno-Pallero, F.Á., Amghar, S., El Harti, A., Campos-Herrera, R., **2021**. Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi. *J. Invertebr. Pathol., 184*, 107620. https://doi.org/10.1016/j.jip.2021.107620

  Discipline: Zoology; IF: **2,795** (2021), **Q1** (25/176)

- Blanco-Pérez, R., Sáenz-Romo, M.G., **Vicente-Díez, I.**, Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., **2020**. Impact of vineyard ground cover management on the occurrence and activity of entomopathogenic nematodes and associated soil organisms. *Agric. Ecosyst. Environ., 301*, 107028. https://doi.org/10.1016/j.agee.2020.107028

  Discipline: Agriculture/Multidisciplinary; IF: **5,567** (2020), **Q1** (1/56)

- Bueno-Pallero, F.Á., Blanco-Pérez, R., **Vicente-Díez, I.**, Martín, J.A.R., Dionísio, L., Campos-Herrera, R., **2020**. Patterns of occurrence and activity of entomopathogenic fungi in the algarve (Portugal) using different isolation methods. *Insects, 11*, 1–18. https://doi.org/10.3390/insects11060352

  Discipline: Entomology; IF: **2,769** (2020), **Q1** (18/102)

- Půža, V., Campos-Herrera, R., Blanco-Pérez, R., Jakubíková, H., **Vicente-Díez, I.**, Nermuť, J., **2020**. *Steinernema riojaense* n. sp., a new

entomopathogenic nematode (Nematoda: Steinernematidae) from Spain. *Nematology, 22*, 825–841. https://doi.org/10.1163/15685411-00003343

Discipline: Zoology; IF: **1,442** (2020), **Q3** (88/175)

- Blanco-Pérez, R., Bueno-Pallero, F.Á., **Vicente-Díez, I**., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., **2019**. Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode Steinernema feltiae. *J. Invertebr. Pathol. 164*, 5–15. https://doi.org/10.1016/j.jip.2019.04.002

  Discipline: Zoology; IF: **2,074** (2019), **Q1** (18/166)

7.1.2. Outreach publications

- **Vicente Díez, I.**, Pou, A., Campos-Herrera R., **2023**. ¿Qué son las sustancias semioquímicas? ¿cómo pueden contribuir en el control de plagas de la vid como la polilla del racimo (*Lobesia botrana*)? *Ae, 52*, 28-29.

- Blanco-Pérez, R., **Vicente Díez, I.**, Ramos Sáez de Ojer, J.L., Marco Mancebón, V.S., Pérez Moreno, I., Campos-Herrera R., **2023**. Manejo del viñedo en la D.O.Ca. Rioja y salud del suelo: los nematodos entomopatógenos como organismos modelo. *Enoviticultura, 81*, 4-13.

- **Vicente-Díez, I.**, Vaquero, E., Pou, A., Campos-Herrera, R., **2022.** Las bacterias simbiontes de los nematodos entomopatógenos como fuente de nuevos antibióticos. *SEM@foro, 74*, 6-7.

- **Vicente-Díez I.**, Pou, A., Raquel Campos-Herrera, R., **2022.** Alternativas al uso de pesticidas contra vectores transmisores de la enfermedad de Pierce y la polilla del racimo. *Tierras, 306*, 52-58.

- Blanco-Pérez, R., Sáenz Romo, M.G., **Vicente-Díez, I.**, Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., **2021.** Cubiertas vegetales y conservación de poblaciones nativas de nematodos entomopatógenos en viña. *Cuaderno de Campo, 65*, 34-39.

- Chelkha, M., Blanco-Pérez, R., Bueno-Pallero, F.A., **Vicente-Díez, I.**, Amghar, S., El Harti, A., Campos-Herrera, R., **2020.** Coexistencia de dos organismos beneficiosos del suelo: ¿Pueden las lombrices de tierra alterar la actividad beneficiosa de los nematodos entomopatógenos como agentes de control biológico? *Ae, 39*, 26-27.

- Campos-Herrera, R., Blanco-Pérez, R., **Vicente-Díez, I.**, **2020**. Nematodos entomopatógenos en el control biológico de ácaros e insectos. *Cuaderno de Campo, 63*, 34-39.

- **Vicente-Díez, I.**, Sáenz Romo, M.G., Blanco-Pérez, R., Ibáñez-Pascual, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., **2019**. Impacto de la implantación de cubiertas vegetales en la presencia de agentes de control biológico en viñedos. *Viticultura, 3555*, 1844-1850.

- Blanco-Pérez, R., Sáenz Romo, M.G., **Vicente-Díez, I.**, Ibáñez-Pascual, S., Martínez-Villar, E., Pérez-Moreno, I., Marco-Mancebón, V.S., Campos-Herrera, R., **2018**. Cubiertas vegetales en viña y su impacto en la riqueza y actividad de los nematodos entomopatógenos. *Ae, 34*, 28-29.

7.1.3. Book chapters

- **Vicente-Díez, I.**, Pou, A., Campos-Herrera, R., **2023**. *Xenorhabdus*- and *Photorhabdus*-based products: status and future perspective in agriculture. In O. Koul (Ed.), Development and Commercialization of Biopesticides Costs and Benefits (1st ed., pp. 81–93). Elsevier.

7.2. Seminars and conference presentations

7.2.1. Seminars

- **Vicente-Díez, I.** *Xenorhabdus* y *Photorhabdus*: bio-herramientas para el control de plagas y enfermedades de la vid. Seminarios internos del ICVV. Instituto de Ciencias de la Vid y el Vino (ICVV). May 3rd 2023. Logroño, Spain.

- **Vicente-Díez, I.,** Pou, A., Campos-Herrera, R. Avances en el empleo de nematodos entomopatógenos y derivados de sus bacterias para el manejo de plagas de la vid. 5° Simposio Chileno de control biológico Instituto de Investigaciones Agropecuarias. Chile. August 31st 2022. Santiago de Chile, Chile.

7.2.2. Presentations at International Conferences

- **Vicente-Díez, I.,** Pou, A., Campos-Herrera, R. The symbiotic bacteria of entomopathogenic nematodes as tools for food protection**. SIP 2023 - 55th Annual Meeting of the Society of Invertebrate Pathology, University of Maryland, Maryland (USA). (From July 30 - August 3, 2023,)**

  Oral Presentation

- **Vicente-Díez, I.,** Blanco-Pérez, R., Chelkha, M., Pou, A., Campos-Herrera, R. Plasticity in the use *Xenorhabdus nematophila* and *Photorhabdus laumondii* against *Botrytis cinerea*. **ICN 2022 - 7th International Congress of Nematology, Antibes Juan-Les-Pins (France). (From 1 to 6 May 2022)**

  Poster Presentation

- Blanco-Pérez, R., **Vicente-Díez, I.,** Ramos, J.L., Marco-Mancebón V.S., Pérez-Moreno I., Campos-Herrera, R. Could agricultural conservation practices enhance the activity of entomopathogenic nematodes in vineyards? **ICN 2022 - 7th International Congress of Nematology, Antibes Juan-Les-Pins (France). (From 1 to 6 May 2022)**

  Poster Presentation

- **Vicente-Díez, I.,** Blanco-Pérez, R., Chelkha, M., Puelles, M., Pou A., Campos-Herrera, R. *Steinernema carpocapsae* and *Xenorhabdus nematophila* based products for the control of the grapevine moth and the grey mold in vineyards**. 53th Virtual Annual Meeting of Society of Invertebrate Pathology. (From 28 June to 2 July 2021)**

  Poster Presentation

- Blanco-Pérez, R., **Vicente-Díez, I.,** Ramos, J.L., Marco-Mancebón V.S., Pérez-Moreno I., Campos-Herrera, R. Impact of differentiated vineyard management on the activity of entomopathogenic nematodes in La Rioja (Spain). **53th Virtual Annual Meeting of Society of Invertebrate Pathology. (From 28 June to 2 July 2021)**

  Poster Presentation

- González-Trujillo. M.M., Čepulitè, R., **Vicente-Díez I.**, Blanco-Pérez, R., Chelkha, M., Puelles M., Gámez A., Ramos J.L., Campos-Herrera R., Screening of adjuvants to enhance the entomopathogenic nematode survival and adherence after aerial application on greapvine leaves. **53th Virtual Annual Meeting of Society of Invertebrate Pathology. (From 28 June to 2 July 2021)**

  Poster Presentation

- Blanco-Pérez, R., **Vicente-Díez, I.**, Marco-Mancebón, V.S., Pérez-Moreno, I., Pou, A., Campos-Herrera, R. Impact of mulching on the activity of entomopathogenic nematode community in DOCa Rioja vineyards (Spain). **Entomological Society of America (ESA) International Branch Virtual Symposium. (From 26 to 28 April 2021)**

  Poster Presentation

- **Vicente-Díez, I.,** González-Trujillo M.M., Galeano M., Chelkha M., Belda, J.E., Calvo, J., and Campos-Herrera, R. Virulence of entomopathogenic nematodes against two aerial pests: *Frankliniella occidentalis* (Thysanoptera: Thripidae) and *Tuta absoluta* (Lepidoptera: Gelechiidae): intra- and interspecific variability. **Virtual Conference of the Society of Nematologists. (From 15 to 16 December 2020)**

  Poster Presentation

- **Vicente-Díez, I.,** González-Trujillo M.M., Galeano M., Chelkha M., Belda J.E., Calvo J., and Campos-Herrera R. Enhancing organic viticulture: insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from *Xenorhabdus* and *Photorhabdus* bacteria

against *Philaenus spumarius* (Hemiptera: Aphrophoridae), vector of *Xylella Fastidiosa* (Proteobacteria: Xanthomonadaceae)**. Virtual Conference of the Society of Nematologists. (From 15 to 16 December 2020)**

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- Chelkha, M., Blanco-Pérez, R., **Vicente-Díez, I.**, González-Trujillo,M. M., Amghar, S., El Harti, A., and Campos-Herrera, R. Unraveling earthworms impact over entomopathogenic nematode infectivity: general trend or species-specific dependent? **Virtual Conference of the Society of Nematologists. (From 15 to 16 December 2020)**

  Poster Presentation

- Blanco-Pérez, R., Bueno-Pallero, F.A., **Vicente-Díez, I.**, Marco-Mancebón V.M., Pérez-Moreno I., and Campos-Herrera, R. *Steinernema feltiae* scavenging behavior: offspring fitness is modulated by various insect cadaver scenarios. **52nd Annual Meeting of Society of Invertebrate Pathology, Valencia (Spain). (From 28 July to 1 August 2019)**

  Poster Presentation

7.2.3. Presentations at National Conferences

- **Vicente-Díez,** I., Pou, A., Campos-Herrera R. Reducing pesticides: new strategies for vine protection. **X Doctoral Conference and V Scientific Outreach Conference of Group 9 Universities. (From 31 May to 2 June 2023)**

  Oral and Poster presentation

- **Vicente-Díez,** I., Pou, A., Campos-Herrera R. Biotech solutions for the agriculture of the future. **IX Doctoral Conference and IV Scientific Outreach Conference of Group 9 Universities. (From 18 to 20 May 2022)**

  Oral and Poster presentation

- **Vicente-Díez, I.**, Blanco-Pérez, R., Moreno, A., Fereres, A., Campos-Herrera, R. Infective capacity of the entomopathogenic nematode *Steinernema feltiae* (Rhabditida: Steinernematidae) against nymphs of *Philaenus spumarius* (Linnaeus) (Hemiptera: Aphrophoridae), vector of *Xylella fastidiosa* (γ-Proteobacteria: Xanthomonadaceae) in Europe. **11th National Congress of Applied Entomology. (Del 4 to 8 November 2019)**

  Poster presentation

- Blanco-Pérez R., Sáenz-Romo M.G., **Vicente-Díez I.**, Ibáñez-Pascual S., Martínez-Villar E., Pérez-Moreno I., S. Marco-Mancebón V., Campos-Herrera R. Impact of cover crops in Rioja vineyards on the natural distribution and activity of entomopathogenic nematodes. **XIII Organic Agriculture Congress (SEAE). (From 4 to 17 November 2018)**

  Poster presentation

- **Vicente-Díez, I.**, Sáenz-Romo, M.G., Veas-Bernal, A.; Carvajal-Montoya, L.D.; Martínez-García, H., Ibáñez-Pascual, S.; Marco Mancebón, V.S.; Martínez-Villar, E. y Pérez-Moreno, I. Impact of plant cover implementation: effect on insect predators of grapevine pests. **XIII Organic Agriculture Congress (SEAE). (From 4 to 17 November 2018)**

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