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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*

Ignacio Vicente-Díez · Xoaquín Moreira · Victoria Pastor · Mar Vilanova · Alicia Pou · Raquel Campos-Herrera

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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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I. Vicente-Díez · M. Vilanova · A. Pou · R. Campos-Herrera (✉)
Instituto de Ciencias de la Vid y del Vino (ICVV), Gobierno de La Rioja, CSIC, Universidad de La Rioja, Finca La Grajera, Crta. Burgos Km. 6 Salida 13 Lo-20, 26007 Logroño, Spain
e-mail: raquel.campos@icvv.es

X. Moreira
Misión Biológica de Galicia (MBG-CSIC), Apartado de Correos 28, 36080 Pontevedra, Galicia, Spain

V. Pastor
Metabolic Integration and Cell Signaling Laboratory, Biochemistry and Molecular Biology Section, Unidad Asociada al Consejo Superior de Investigaciones Científicas, Department of Biology, Biochemistry and Natural Sciences, Universitat Jaume I, Avda. Vicent Sos Baynat, s/n, 12071 Castelló de la Plana, Spain

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

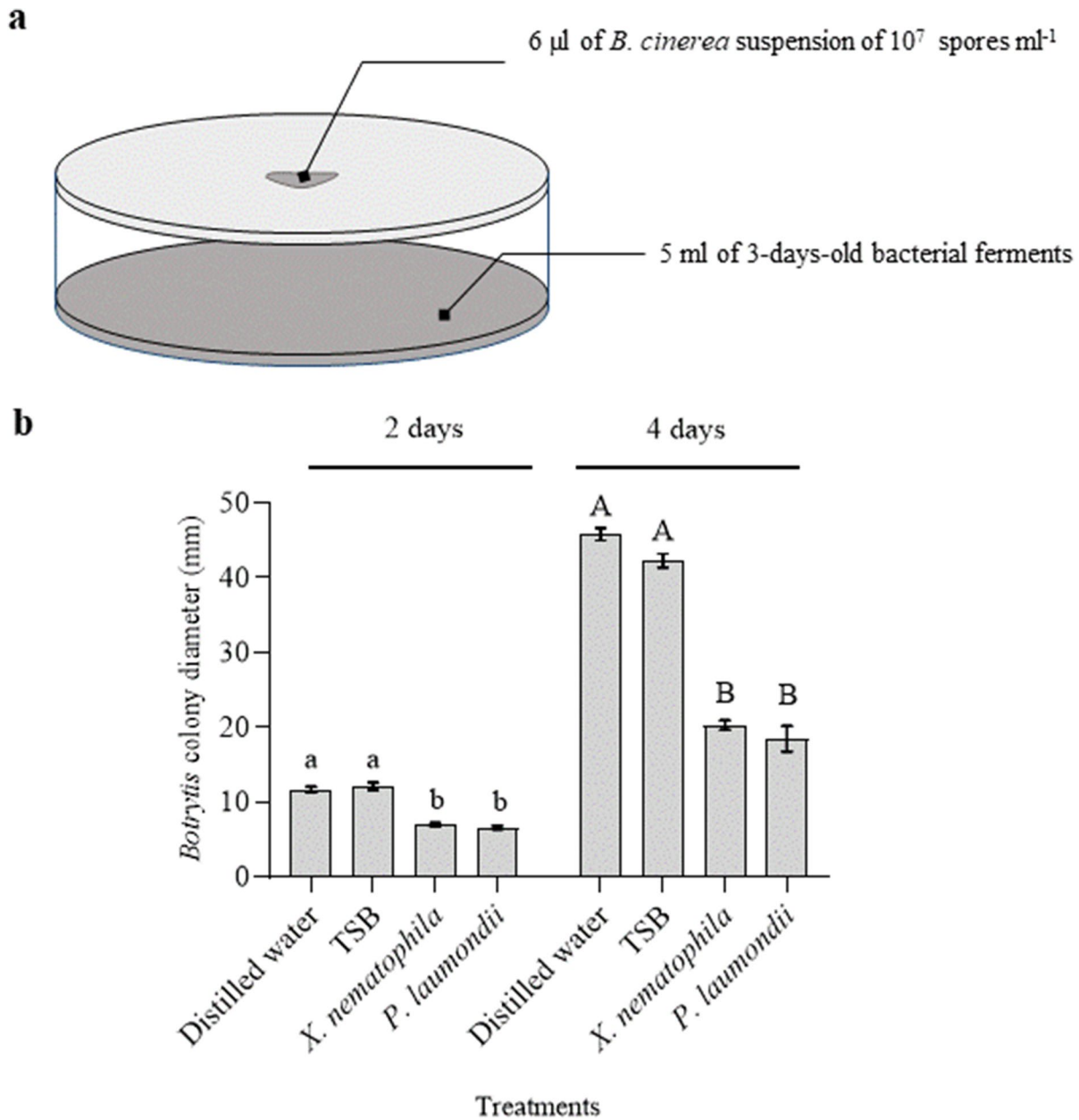


Fig. 1 In vitro antifungal effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* mycelial growth. **a** Schematic diagram displaying the methodological approach for testing in vitro the antifungal activity of VOCs emitted by *X. 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

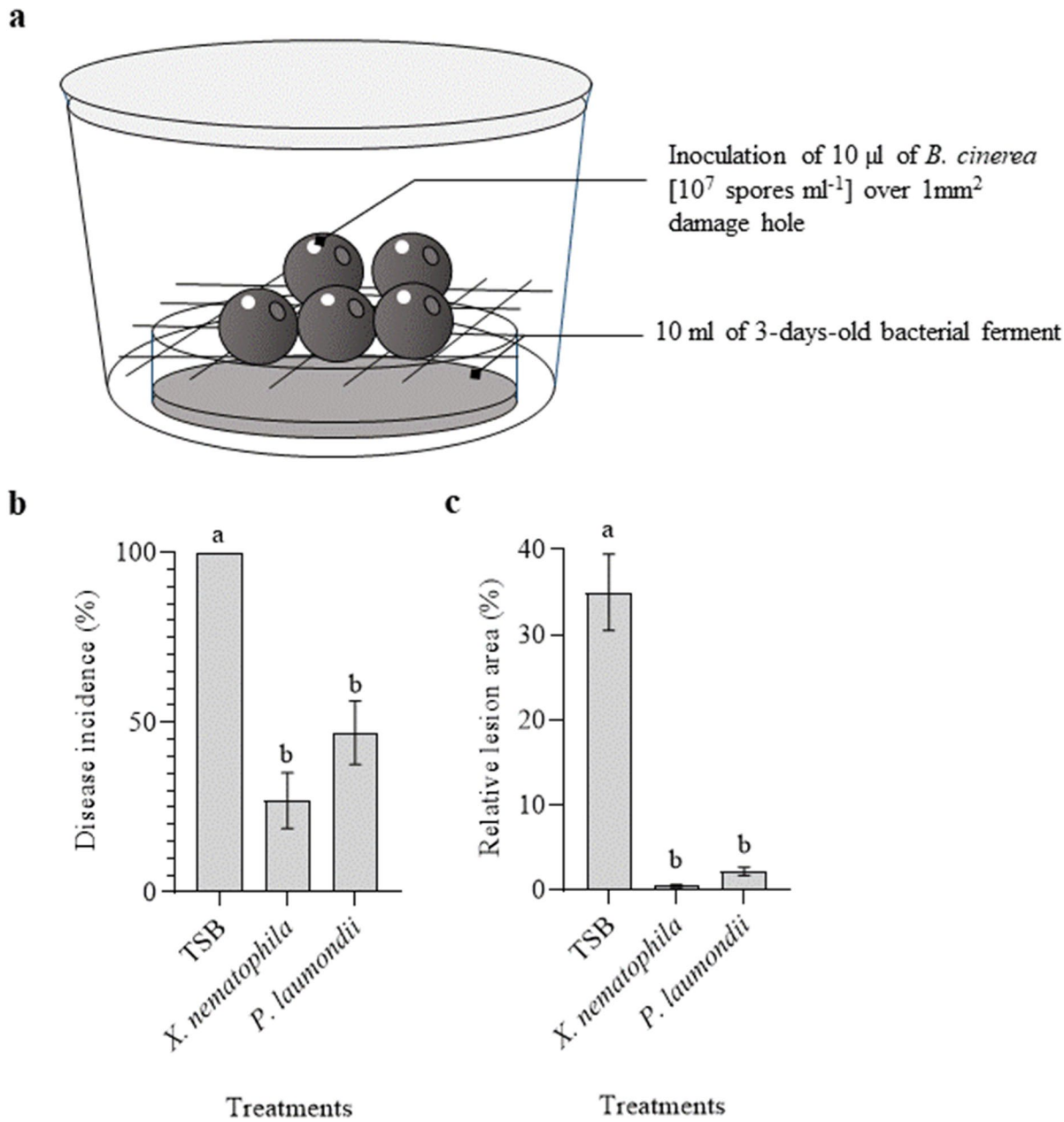


Fig. 2 In vivo antifungal effect of VOCs emitted by three-day-old *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* TSB ferments against *Botrytis cinerea* bunch rot on grapes four days after infection. **a** Schematic diagram showing the methodological approach for testing antifungal 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).

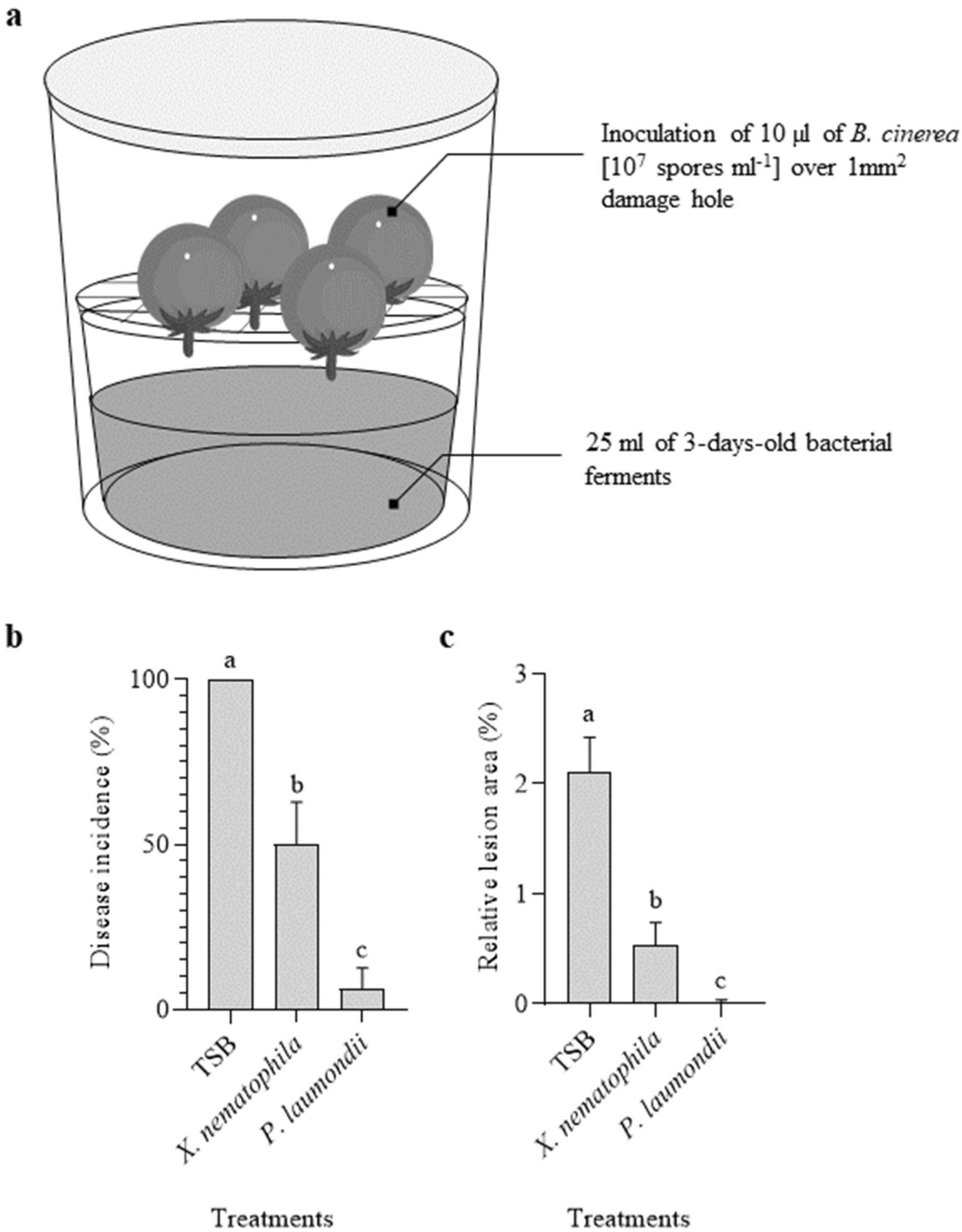
Efficacy of VOCs emitted by bacteria in controlling pathogen infection in grapes and tomatoes

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 *Xenorhabdu*s 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

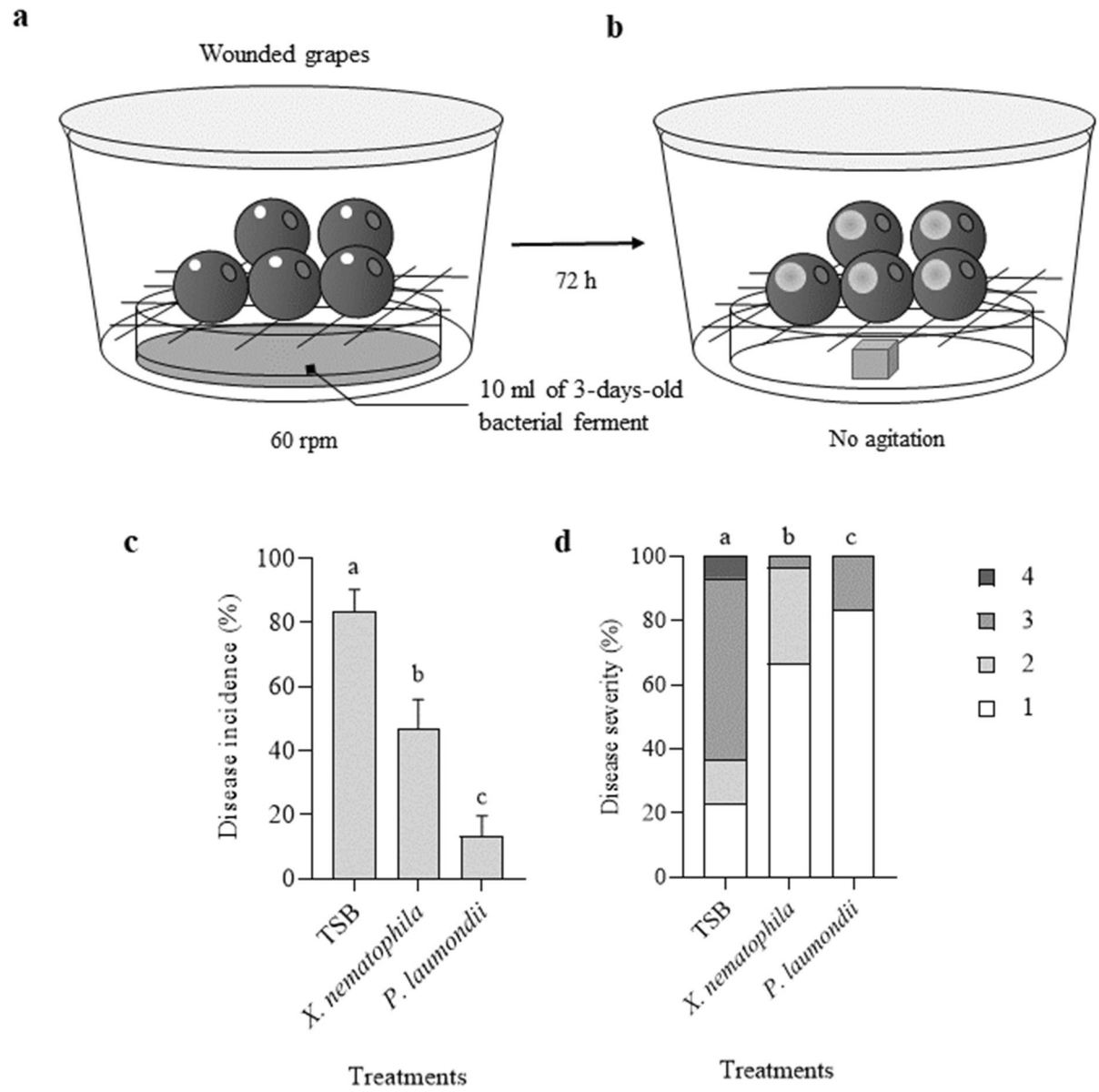


Fig. 4 In vivo antifungal preventive effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* bunch rot on wounded grapes. **a** Schematic diagram showing the methodological approach for testing antifungal preventive activity of the 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.*

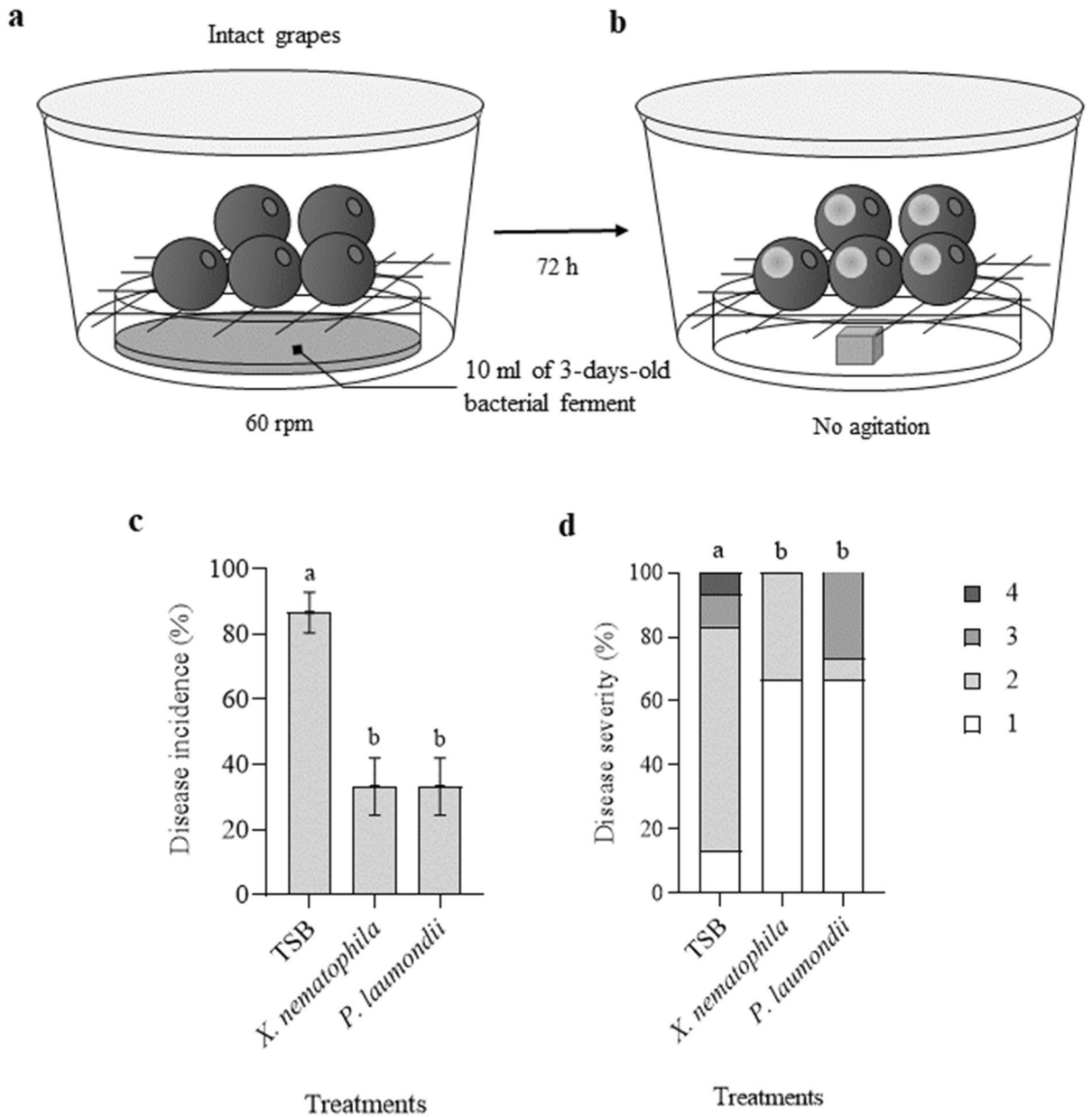


Fig. 5 In vivo antifungal preventive effect of VOCs emitted by *Xenorhabdus nematophila* and *Photorhabdus laumondii* subsp. *laumondii* against *Botrytis cinerea* bunch rot on intact grapes. **a** Schematic diagram showing the methodological approach for testing antifungal protective activity of the 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 post-harvest 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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Ignacio Vicente Díez is a PhD student from the University of La Rioja at the Instituto de las Ciencias de la Vid y del Vino, Spain. He has experience in the use of entomopathogenic bacteria from the genera *Photorhabdus* and *Xenorhabdus* against various pests and diseases associated with grapevine. This study is part of his PhD dissertation.

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.

Victoria Pastor is an associate professor at the University Jaime I in Castellón, Spain. Her research focuses on plant immunity, exploring the molecular and biochemical mechanisms that drive induced resistance and, more specifically,

priming defenses against pests and pathogens. By enhancing plant defenses through self-boosting mechanisms, her work aims to prepare plants for improved resilience against pests and diseases.

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.