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Volatile Organic Compounds from a Lichen-Associated Bacterium, *Paenibacillus etheri*, Interact with Plant-Parasitic Cyst Nematodes

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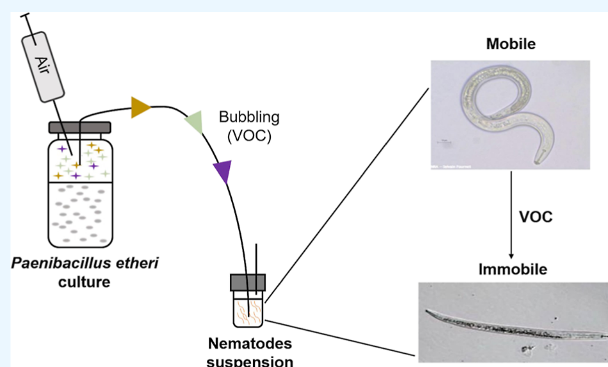
ABSTRACT: Healthy food is one of the major challenges to develop in this century. Plant-parasitic nematodes cause significant damage to many crops worldwide and till now, the use of chemical nematicides is the main means to control their populations. These chemical products must be replaced by more environmental-friendly control methods. Biocontrol methods seem to be one promising option, and the number of biopesticides derived from living organisms has increased in the last decades. To develop new plant protection products, we have decided to combine our skills in natural products chemistry and nematology and to focus on the lichen microecosystem as underexploited ecological niches of microorganisms. We present herein the potential of lichen-associated bacterial suspensions from *Paenibacillus etheri* as nematicides against the beet cyst nematode *Heterodera schachtii* and the potato cyst nematode *Globodera pallida*, in particular the effects of volatile organic compounds (VOCs) produced by the bacteria. A solid phase micro-extraction method associated to gas chromatography−mass spectrometry analysis of 14 day cultures was used to analyze these VOCs in order to identify the main produced compounds (isoamyl acetate and 2-phenylethyl acetate) and to evaluate them on the nematodes.



1. INTRODUCTION

Changes in agricultural practices, such as monoculture cropping, intensive tillage, and the use of synthetic fertilizers and pesticides, have led to a decline in soil structure and an increase in soil-borne plant diseases.[1] Plant pests and diseases are major factors leading to the destruction of food crops across the globe and have been considered historically as a public health and society matter apart from economic and environmental issues in agriculture. Around, 20−40% loss in global agricultural productivity is owed to insects, diseases, and weeds.[2] However, healthy food is one of the major challenges to develop in this century. To produce good and enough food for each inhabitant living in this planet, pest control is crucial, and the chemical products must be replaced by more sustainable management practices. Biocontrol agents and products seem to be one promising option, and the number of biopesticides derived from microorganisms has increased in the last decades. Biological control comprises natural rivals or disease-suppressive bacterial and fungal species, their genes or metabolites, to offset the pathogen population with a consequent improvement in plant health.[3] Plant-parasitic nematodes (PPNs) are one of the major constraints to crop production and especially in high-value vegetable and fruit crops. They can cause significant economic yield loss estimated to be more than US$100 billion annually.[4] The cyst nematodes (*Heterodera* and *Globodera* sp.) are among the most damaging PPNs based on scientific and economic importance throughout the world.[5] Control of those species is very difficult due to their ability to survive for prolonged periods in the soil as a cyst in the absence of their host plant. To control their population, chemical soil fumigants have been used for more than a century, and they remain nowadays the standard practice in many crops. As limitations of chemical soil fumigants are becoming more apparent, there is an urgent need to find new alternatives that are safer for the soil ecosystem and the environment.

Microorganisms produce a great variety of secondary metabolites including antibiotics, toxins, pigments, and others. Curiously, small-molecular-mass metabolites such as volatile organic compounds (VOCs) were, for a long time, overlooked. Research conducted in the last decades demonstrated that

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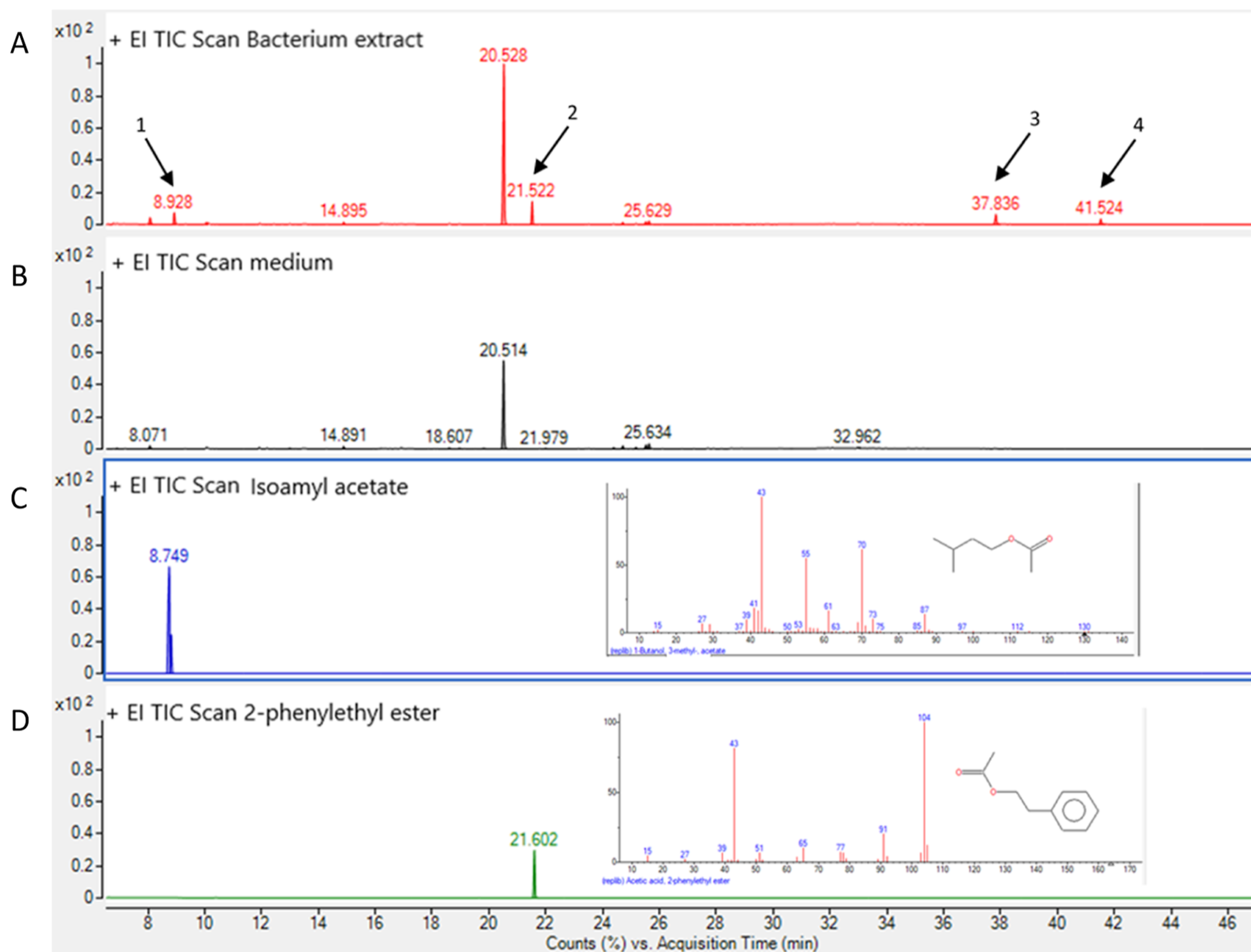

Figure 1. GC−MS TICs using EI ionization of (A) fermentation broth of *P. etheri*; (B) GYM Streptomyces medium; (C) IAA standard; (D) 2-PEA standard. Peaks: (**1**) IAA, (**2**) 2-PEA, (**3**) and (**4**) unidentified aliphatic compounds. Peaks (**1**) and (**2**) were identified after comparison of their mass spectrum with those of NIST 17 and after injection of the analytical standards.

bacteria produce a large set of VOCs including hydrocarbons, ketones, alcohols, sulfur- and nitrogen-containing compounds, terpenes, and others[6,7] with a limited knowledge about their biological and ecological function[8] except for their role as chemical mediators at long and short distances in intra- and interspecific interactions[9,10] VOCs are a broad group of lipophilic compounds with low molecular weight (100−500 Da), high vapor pressure, and low boiling point. Due to their physico-chemical properties, VOCs can easily diffuse through gas- and water-filled pores and can therefore have a wide effective range in soil.[11]

Few studies focused on managing PPNs with bacteria,[12] but *Paenibacillus* strains were described as having a deleterious effect on PPNs.[13−15] *Paenibacillus* is a cosmopolitan and ubiquitously occurring bacterial genus, which is predominantly isolated from the rhizosphere. Although it occurs naturally in soil and marine sediments, plant-associated habitats such as the rhizosphere and roots of crop plants are its preferred environments. The genus *Paenibacillus* has an enormous potential in biotechnology as a source of novel bioactive compounds, and this potential has only partially been exploited.[16,17] For instance, *Paenibacillus polymyxa* is an agriculturally important microbe already reported as an efficient biocontrol agent and biofertilizer.[18,19] *Paenibacillus* can influence plant growth and health directly by the production of phytohormones by providing nutrients, by fixing nitrogen, and/or by the suppression of deleterious micro-organisms through antagonistic functions.[20] Furthermore, *Paenibacillus* species can also be harbored in lichens,[21,22] a symbiotic association between a fungal (mycobiont) and an algal (photobiont) partner and where a rich microflora occurs. We previously isolated *Paenibacillus etheri* from the lichen *Rhizocarpon geographicum*, and it appeared that the strain represented 62.5% of total bacterial isolates.[23] Looking closer, on the lichen surface, we have also observed microscopically a bacteriophage nematode. Indeed, the existence of various invertebrates inhabiting lichens such as arthropods or nematodes is well established,[24,25] while within different genera sheltered, *Paenibacillus* was identified as "protectors" in the lichen holobiont.[26,27] Considering these insights, we asked ourselves if the abundance of this bacterium was correlated with the presence of such nematodes and investigated further this natural symbiotic system for novel biotechnological applications.

The objectives of the present study were to investigate the potential in vitro nematicidal activity of a lichen-associated bacterium's metabolome, *P. etheri*'s, and, considering the importance of microbial VOCs (mVOCs), to assess the

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Table 1. Effect of Different *P. etheri* Samples on Motility of Juveniles of *H. schachtii*

	after 6 h of contact		after 24 h of contact		
	proportion of mobile juveniles	proportion of living juveniles	proportion of mobile juveniles	proportion of living juveniles	
H_2O (control)	0.97	0.87	0.95	0.86	no effect
medium without *P. etheri* (control)	0.98	0.89	0.96	0.86	no effect
broth fermentation	0.02	0.98	0.00	0.87	nematostatic effect
supernatant	0.09	0.97	0.02	0.91	nematostatic effect
crude extract of medium without *P. etheri* (control)	0.96	0.79	0.93	0.86	no effect
crude extract	0.14	0.60	0	0.31	nematicidal effect
freeze-dried crude extract	0.97	0.93	0.95	0.77	no effect

mVOCs' activity against two nematode species of agronomical interest: the sugar-beet cyst nematode *Heterodera schachtii* and the potato cyst nematode *Globodera pallida*. The bioassays were conducted at 6 and 24 h with different *P. etheri* culture extracts using 12-microwell plates for the evaluation of direct contact or using a process specifically developed here to evaluate mVOCs by gaseous contact.

2. RESULTS AND DISCUSSION

2.1. Samples and mVOC Analysis. The liquid growth medium was selected with the aim of the best growth of *P. etheri* based on the monitoring of the optical density at 620 nm. This medium was derived from GYM Streptomyces agar medium, and $CaCO_3$ (2 g L^{-1}) was replaced by $NaHCO_3$ (2 g L^{-1}). After some optimizations, especially to compare the exponential phase (7 days) and the stationary phase (14 days), the best parameters for the production of active compounds of *P. etheri* were a culture at 25 °C and 120 rpm during 14 days. At the end of the culture, various samples were obtained: broth fermentation, supernatant obtained after removal of bacterial cells by centrifugation, organic crude extract after ethyl acetate extraction of supernatant, freeze-dried crude extract, and mVOCs contained in the headspace of culture bottles. Before developing the mVOCs approach, we exploited high-performance liquid chromatography-diode-array detection liquid fractions, but profiles were too complex and the direct injection of the headspace of microbial cultures was not operable. Consequently, we adopted an approach for mVOC qualitative analysis by using a static solid-phase micro-extraction method (SPME) fiber in order to trap most of the analytes present even at a trace level in a short period of time to provide a true representation of the studied system. The advantage of SPME fibers is that the volatiles are enriched on the fibers, and no solvent is needed. The extraction efficiency with this method depends on both the natures of the extracted compounds and the fiber, meaning that even if it can highlight the presence of active compounds, quantitation will only give best orders of magnitude. Considering that the goal of volatilomic profiling is to analyze as many metabolites as possible, the use of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers appeared to be the most suited to increase the number of analytes that can be trapped on the fiber because it can allow the capture of VOCs in a wide range of polarity and molecular weight.[28] Then, the SPME/GC−MS analysis of mVOCs produced by *P. etheri* was assessed by using an intermediate non-polar HP-5MS GC capillary column, while the polar DBWAX_UI column was inefficient. The culture medium is itself a source of volatiles, particularly as the autoclaving process forms several volatiles. It is therefore essential to run blank analyses on the medium alone to distinguish the volatiles of the medium from those of the bacteria. The comparison of GC−MS total ion chromatograms (TIC) from broth fermentation and culture medium highlighted the presence of four additional peaks, from which two were identified after National Institute of Standards and Technologies (NIST) mass spectral matching as isoamyl acetate (IAA) (**1**) and 2-phenylethyl acetate (2-PEA) (**2**). Moreover, the subsequent injection of two commercially available analytical standards confirmed these identifications (Figure 1). Even if the major peak (at Rt 20.5 min) comes from the culture medium, compounds (**1**) and (**2**) appeared relatively more abundant than the other peaks (**3**) and (**4**) attributed to unidentified aliphatic compounds. Note that IAA (**1**) has been frequently reported as mVOCs from microorganisms[7] and also described as being produced by another species of *Paenibacillus*.[29] To our knowledge, it is the first report of mVOC profile of *P. etheri*, a known pollutant degrader.[30]

2.2. Nematicidal Bioactivity. While the three controls (water, medium without *P. etheri*, and crude extract of culture medium without *P. etheri*) have no effect on nematodes, broth fermentation and supernatant from *P. etheri* cultures strongly inhibited motility of *H. schachtii* juveniles (from 0.91 to 1.00) after 6 and 24 h of direct contact (Table 1). This effect was reversible as juveniles were noted to be alive in the subsequent active passage test; those samples have a clear nematostatic activity. This nematostatic effect was also exhibited by the organic crude extract obtained after ethyl acetate extraction of the supernatant. In this case, an additional nematicidal activity was observed: the mortality rate was higher (up to 0.69 at 24 h—Table 1). Interestingly, a freeze-drying treatment on the supernatant led to the loss of these activities on juvenile nematodes (Table 1). The additional effect of the organic crude extract might be due to the result of a massive dose-effect, and it cannot be excluded that this effect is related to a higher concentration compared to that of the supernatant and its lower concentration showing only a nematostatic effect.[31]

With no effect observed with the freeze-dried crude extract in contrast to the other samples, we hypothesize that mVOCs may cause the in vitro activity against nematodes. Indeed, solvent extraction is the method most commonly used for the isolation of natural products. Nevertheless, when solvent extraction is used, it remains unclear whether a given compound is volatile enough to be released into the air and thus absent from the final crude extract. Since there is no standard procedure for testing the effects of mVOCs on plant

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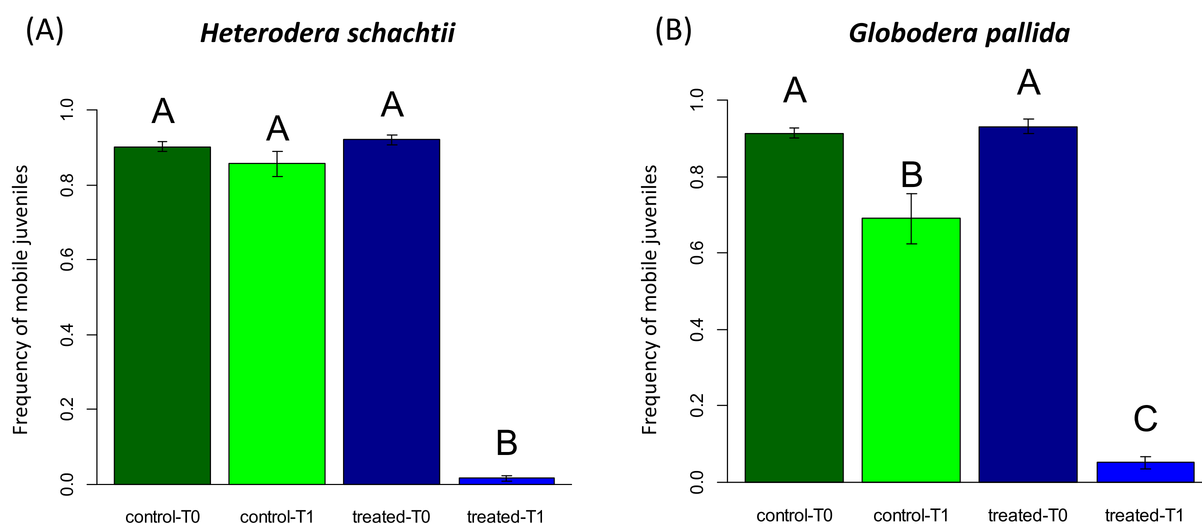

Figure 2. Bioassays of *P. etheri*'s mVOCs against (A) *H. schachtii* and (B) *G. pallida*. Frequency of mobile juveniles (means ± se) for each modality: control-T0 = GYM Streptomyces-modified medium before bubbling; control-T1 = GYM Streptomyces-modified medium after bubbling; treated-T0 = *P. etheri* broth medium before bubbling; treated-T1 = *P. etheri* broth medium after bubbling. Mobility of juveniles was observed after 10 min. Letters represent significant differences between modalities (pairwise comparisons of means, α = 0.05).

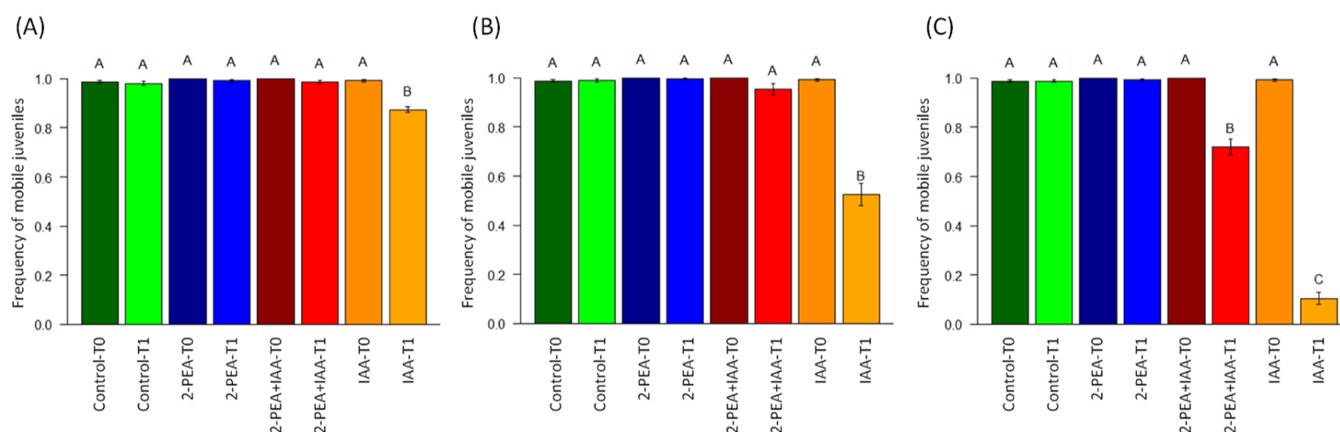

Figure 3. Bioassays of IAA and 2-PEA separately or in mixture (v/v; 1:1) against *H. schachtii* after different incubation times: (A) 5, (B) 15, and (C) 30 min. Frequency of mobile juveniles (means ± se) for each modality (control, 2-PEA, 2-PEA + IAA, and IAA) before (T0) after (T1) bubbling.

parasitic nematodes, we developed a bioassay to test these mVOCs using gas contact by bubbling them on the two cyst nematodes. Motility assay results from mVOCs are reported in Figure 2.

Regarding the *H. schachtii* experiment, the modality effect on the frequency of mobile juveniles was highly significant (χ^2 = 139.04, d*f* = 3, and *P* < 0.0001). The proportion of mobile juveniles was high before bubbling (0.90 for control-T0 and 0.92 for treated-T0), and mVOCs produced by *P. etheri* reduced significantly the proportion of mobile juveniles (0.02 for treated-T1). Bubbling had no effect on the motility of *H. schachtii* juveniles as control-T1 and control-T0 were not significantly different (Figure 2A).

Regarding the *G. pallida* experiment, the modality effect on the frequency of mobile juveniles was also highly significant (χ^2 = 207.54, d*f* = 3, and *P* < 0.0001). The proportion of mobile juveniles was high before bubbling (0.91 for control-T0 and 0.93 for treated-T0), and mVOCs produced by *P. etheri* reduced significantly the proportion of mobile juveniles (0.05 for treated-T1). The comparison of means showed that *G. pallida* juveniles were slightly impacted by bubbling as control-T1 (0.69) was significantly lower than control-T0. However, as treated-T1 was significantly lower than control-T1, there was also a strong mVOC effect on this nematode species (Figure 2B).

In comparison with the assays performed with the supernatant and the organic extract, the effects observed on the nematodes with the VOCs were extremely fast (a few minutes compared to a few hours). This suggests that the concentrations of the active compounds in the gaseous phase are much higher than in the aqueous solution, meaning they are likely strongly hydrophobic.

Interestingly, mVOCs produced a significant effect on the cyst nematodes belonging to two different genera used in this study. This ubiquitous effect could be attributed to their hydrophobic and uncharged nature which allow them to easily penetrate cell membranes and subsequently induce perturbations such as increased permeability and leakage of intracellular components.[10]

IAA (**1**) and 2-PEA (**2**), the two compounds characterized as being produced by *P. etheri*, were then evaluated, separately or in mixture (v/v; 1:1), in order to try to correlate their

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presence to the observed nematicidal effect. As these compounds have very low water solubilities, they were tested in their gaseous forms. To do so, they were placed in a water bath set to maintain the contents of the flask at 90 °C for 2 h in sealed flasks and then cooled at ambient temperature. The headspaces were bubbled in the same way as for the fermentation broth. While 2-PEA appeared not to be efficient alone, when mixed with IAA, a deleterious effect appeared at 30 min (Figure 3C). This nematicidal effect is likely assigned to IAA as the effect of IAA on *H. schachtii* is already noticeable after 5 min (Figure 3A). The modality effect on the frequency of mobile juveniles was significant after 5 (χ^2 = 51.49, d*f* = 7, and $P < 0.0001$), 15 (χ^2 = 164.87, d*f* = 7, and $P < 0.0001$), and 30 min (χ^2 = 308.78, d*f* = 7, and $P < 0.0001$). The comparison of means showed that the frequency of mobile juveniles was significantly lower after than before bubbling. Moreover, this compound (IAA) showed a clear time-dependent effect as the proportion of mobile juveniles for the modality IAA-T1 (after bubbling) decreased from 0.87 after 5 min (Figure 3A) to 0.53 after 15 min (Figure 3B) and to 0.10 after 30 min (Figure 3C).

In order to evaluate the concentrations of these two standards, GC−MS analysis of 2-PEA and IAA was assessed as described for the mVOCs produced by *P. etheri*. Calibration curves of the reference compounds IAA and 2-PEA were used to estimate their concentrations. IAA was estimated in VOC extract at 1.46 nmoles (0.19 μg) and 2-PEA at 6.40 nmoles (1.05 μg), while the amount of pure standards adsorbed on SPME in the conditions of the nematicidal assay was 1.26 μmoles (164 μg) for IAA alone, 0.86 μmoles (141 μg) for 2-PEA, and 0.62 μmoles (81 μg) and 0.68 μmoles (112 μg), respectively, in a mixture of ratio 1:1 (IAA/2-PEA) (v/v). We can observe in the mixture of the two standards that the effect is much lower than with IAA alone, indicating that there is a concentration effect (the amount of IAA submitted to the nematodes is twofold higher in the experiment alone than in the mixture). It is noteworthy that the concentrations of pure standards injected by using this methodology were much higher than those injected from the headspaces of *P. etheri*'s cultures. We can thus hypothesize that even if IAA displayed a nematicidal activity, its activity in vivo might be drastically enhanced by other *P. etheri*'s metabolites, even if not identified by our analytical method. Indeed, the use of different polymer coatings of the SPME fiber, different analytical conditions, for example, column, gradient..., might permit detection and identification of other mVOCs.

Microorganisms emit complex blends of mVOCs with specific concentrations and ratios, but in many cases, in vitro experiments under nutrient-rich conditions fail to reproduce, with a discrete VOCs mixture exposure to the tested organisms, the genuine effect occurring in natural conditions.[32,33] Regarding the scarcity of peaks encountered, some of the VOCs might not be detected by the analytical methods used. Indeed, microorganisms produce also, for instance, several volatile inorganic compounds with molecular masses less than 45 g mol^{-1} such as hydrogen sulfide, molecular hydrogen, nitric oxide, nitrogen dioxide, carbon monoxide, carbon dioxide, ammonia.[34−36] Moreover, in sealed cultivation systems, microbial respiratory CO_2, can accumulate high levels in the headspace.[37] These compounds can cross biological membranes and could have synergistic effects with the other VOCs such as IAA.

Over the four new compounds detected from the headspace of *P. etheri*'s cultures, two of them could not be strictly characterized using NIST library. The only indication given was that they belong to terpenes. Only a relatively minor fraction of terpenoid metabolites has been identified in prokaryotes.[38] Terpenes represent a structurally diverse group of bacterial volatiles[39] and they might be involved in defense mechanisms, for example to repel nematode predators.[9]

3. CONCLUSIONS

Lichens are known to be populated with bacteria, fungi, and proto- and metazoan organisms. In order to cope with this competitive environment, lichen-associated micro-organisms can release a large number of metabolites, which can modify the performance of co-habitants from the neighborhood.[40,41] In this context, we have evaluated crude extracts and mVOCs derived from the culture of a bacterium associated to a crustose lichen, which can be inhabited by nematodes. It is known that mVOCs could effectively act as the first signals or chemical "weapons" to reach a target organism.[42] We have thus demonstrated the nematicidal effect of *P. etheri* in the form of crude extract against *H. schachtii* and the nematostatic effect of mVOCs against *H. schachtii* and *G. pallida*. The ability to inhibit the motility of nematodes by one of the constituents of this mixture, IAA, is reported for the first time. However, our results suggest that the nematicidal effect in vivo might be due to a blend of volatiles. These pieces of evidence open many new doors[43] for the application of either volatile-emitting strains such as *P. etheri* or pure compounds and represent promising alternatives to currently used pesticides.

4. MATERIALS AND METHODS

4.1. Chemicals. Solvents of analytical standard grade were purchased from Merck_Sigma Aldrich (St Quentin Fallavier, France). Malt extract, yeast extract, dextrose, and $CaCO_3$ were acquired from Sigma-Aldrich (Saint Quentin Fallavier, France), IAA and $NaHCO_3$ from ThermoScientific Acros (Illkirch, France), and 2-PEA from Thermo Fisher (Kandel, Germany).

4.2. Bacteria and Nematodes. *P. etheri* was isolated from the lichen *R. geographicum*[44] and stored at −80 °C. The strain was grown on GYM Streptomyces agar (15 g L^{-1}) medium containing dextrose (4 g L^{-1}), yeast extract (4 g L^{-1}), malt extract (10 g L^{-1}), and $CaCO_3$ (2 g L^{-1}) for 48 h at 25 °C. A full plate of *P. etheri* was rinsed with 10 mL of a 0.8% NaCl solution and then the suspension was transferred to 1000 mL of liquid GYM medium [$CaCO_3$ was replaced by $NaHCO_3$ (2 g L^{-1})] in a 1 L glass bottle hermetically sealed with a septum and incubated for 14 days on a rotary shaker (120 rpm) at 25 °C.

Nematode populations used in this study were maintained in a collection at the IGEPP laboratory and regularly multiplied on sugar-beet cv. Ardan for the AM2 population of *H. schachtii* and on potato cv. Désirée for the Chavornay population of *G. pallida*. To assess the nematicidal activity of *P. etheri* and its VOCs, hatching of second-stage juveniles (J2) was performed in water for *H. schachtii* and in potato root exudates for *G. pallida*..

4.3. Sample Preparation. After 14 days of culturing, 2 L of *P. etheri*'s fermentation broth were centrifuged for 15 min at 4 °C and 3500 rpm. The supernatant obtained was split: 100 mL was kept for further experiments on cyst nematodes, 1 L was frozen for further freeze-drying, and the final 900 mL was

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used for a liquid/liquid partition repeated twice with 450 mL of ethyl acetate. After freeze-drying of 1 L of supernatant, 411 mg was obtained. Moreover, after evaporation of organic solvents, an organic crude extract (m = 83 mg) was finally obtained.

4.4. mVOC Extraction by SPME. To analyze the VOC profiles produced by the bacterium, cultures were prepared as described above. After 14 days of culture, the collection of volatiles was performed using a static headspace system. A SPME stable flex fiber of 75 mm DVB/C-WR/PDMS (Agilent) was inserted into the headspace of one bottle through the septum and incubated further for 30 min at 40 °C under agitation (120 rpm). The VOCs from 1 L modified GYM Streptomyces broth were used as controls.

4.5. mVOCs and Chemical Standards Analysis by GC–MS. A Hewlett Packard 7820AGC/5975MS (Agilent Technologies, USA) equipped with a HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column was used to separate and identify the VOCs. The carrier gas was helium with a flow rate of 1 mL/min in splitless mode. The SPME fiber was then directly inserted into the injector of the gas chromatograph and desorbed at 260 °C for 4 min. The following oven temperature protocol was used: 40 °C for 5 min and 40–250 °C at a rate of 5 °C/min held at 250 °C for 5 min. The mass spectrometer was operated at 70 eV and 260 °C in the electron impact (EI) ionization mode with a full scan from 50 to 250 m/z. Identification of VOCs was based on a comparison with mass spectra from a library NIST 17 and confirmed with the injection of commercially available standards IAA and 2-PEA. A series of standard solutions of IAA and 2-PEA dissolved in methanol were injected with the concentration range 0.197–6.29 and 0.734–23.5 mM, respectively. The correlation coefficients of the calibration curves were 0.999 and 0.9975, respectively.

4.6. Nematicidal Activity Assay. Two bioassays were conducted to determine the effects of different *P. etheri* samples on the motility of juveniles by direct (broth fermentation, supernatants, and crude extracts) or gaseous contact (mVOCs and chemical standards). By direct contact, each treatment was done on *H. schachtii* and replicated 6 times on a 12-well plate, each well containing approximately 30 juveniles (i.e., 250 μL of the nematode suspension). Culture medium or water was used as the control (six replicates). Observations were made after 6 and 24 h of contact.

4.6.1. Broth Fermentation and Supernatant. In the six treatment wells, 1 mL of broth fermentation or supernatant was added to the 250 μL suspension of nematodes. The controls consisted in adding 1 mL of GYM Streptomyces-modified medium.

4.6.2. Crude Extract. In the six treatment wells, 1 mL of an aqueous solution of freeze-dried extract at 26 mg mL^{-1} or of organic extract at 10 mg mL^{-1} was added to the 250 μL suspension of nematodes. The controls consisted in adding 1 mL of water.

After 6 or 24 h of contact, the numbers of mobile and immobile juveniles were counted, and the six wells of each treatment were pooled and rinsed onto a 10 μm sieve. Each pool was then submitted to active passage through an absorbent paper with 45 μm mesh (TORK, France Fournitures, France). After 18 h of active passage, living juveniles, that is, those able to pass through the paper, were transferred into counting cells and counted.

4.6.3. Microbial Volatile Organic Compounds. Gaseous contact experiments were realized by using a 14 day-old culture of *P. etheri* performed in 1 L culturing bottles with a 100 mL head space and sealed by a septum. To ensure that culturing bottles were in surpression, 10 mL of air was injected through the septum using a syringe prior to the experiment. Thereafter, culturing bottles were connected with a cannula to a 500 μL quartz-cell, sealed with a septum, and maintained at the atmospheric pressure by a syringe needle pricked through the septum and containing a suspension of nematodes. Then, 40 mL of air was injected using a syringe into the culturing bottle head space, bubbling in the nematode suspension contained in the quartz cell. After bubbling, the syringe needle was immediately removed and the potential VOC effect was observed under a stereo microscope using a transillumination unit. Indeed, after 10 min, the phenotype of each juvenile contained in the suspension was assessed as mobile or immobile. The VOCs of the culture medium contained in the bottle's headspace were used as the control. For each modality, treated or control, the status of juveniles was assessed before (T0) and after (T1) bubbling. For each nematode species, *H. schachtii* and *G. pallida*, experiments were repeated 6 times, and for each replicate, the number of juveniles placed into the quartz cell ranged from 28 to 51 for *H. schachtii* and 33 to 58 for *G. pallida*.

4.6.4. Chemical Standards. Gaseous contact experiments of IAA and 2-PEA were also realized. To ensure pure compound experiments, 1 mL of each standard was placed into 100 mL glass bottles and sealed by a septum. For the compound mixture experiment, 500 μL of each pure standard was placed into a 100 mL glass bottle and sealed by a septum. To transform the standards from their initial liquid form to the gaseous form, the bottles were placed in a water bath set to maintain the contents of the flasks at 90 °C for 2 h and then cooled at ambient temperature. The evaluation of standards' effect was then assessed after 5, 15, and 30 min in the same conditions as above for mVOCs.

4.7. Statistical Analysis. Statistical analyses were carried out using the appropriate packages of the statistical software R, version 4.1.1 (R Core Team, 2021).[45] Regarding the mVOC experiment, the effect of modality (control-T0, control-T1, treated-T0, and treated-T1) on the frequency of mobile juveniles was tested for each nematode species through a Wald chi-square test on a generalized linear mixed model (function "glmer") with binomial error and a logit link function. The same model was used for the chemical standard experiment to test the effect of modality (Control-T0, Control-T1, 2-PEA-T0, 2-PEA-T1, 2-PEA + IAA-T0, 2-PEA + IAA-T1, IAA-T0, and IAA-T1) at different times (5, 15, and 30 min). In both cases, the replicate effect was nested in the modality effect and introduced as a random factor in the model. Normality and homogeneity of variances of residuals were checked with the Shapiro–Wilk and the Levene tests, respectively. As significant effects were detected, pairwise comparisons of mean values were computed using an analysis of contrasts (function "emmeans", α = 0.05).

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Notes

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ABBREVIATIONS

GC−MS:gas chromatography coupled to mass spectrometry
mVOCs:microbial volatile organic compounds
SPME:solid phase microextraction
TIC:total ion chromatogram

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