



\*Correspondence author: dcsinucol@unal.edu.co

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## Fungicidal activity of volatile organic compounds from *Paenibacillus* bacteria against *Colletotrichum gloeosporioides*

### Abstract

Some bacteria release volatile organic compounds (VOCs) that can influence the growth of other microorganisms including some pathogens. Identifying bacteria with antifungal activity makes it possible to use such bacteria in the development of biocontrol agents. Thus, the present study focuses on screening VOCs released by eight isolates from *Paenibacillus* genus, collected at Old Providence and Santa Catalina coral reef (Colombian Caribbean Sea), with antifungal activity against phytopathogenic fungi *Colletotrichum gloeosporioides* 26B. The VOCs from *Paenibacillus* sp (PNM-50) showed inhibition rates higher than 50% in the mycelial fungi growth accompanied by macroscopic morphological changes and a reduction in conidiation. In order to identify the VOCs responsible for this antifungal bioactivity, Headspace-Solid Phase Microextraction (HS-SPME) from the bacterial culture was conducted, followed by Gas Chromatography Mass Spectrometry (GC-MS). The chromatographic results revealed a high abundance of VOCs released just by culture media. Once, the difference between VOCs emitted by culture media and bacteria was established, it was possible to make a putative identification of 2-furanmethanol, phenylacetone nitrile, and 2,4-dimethylpentanol as possible VOCs responsible for the antifungal activity.

**Keywords:** marine bacteria; antifungal activity; HS-SPME; GCMS.

## Actividad fungicida de compuestos orgánicos volátiles de la bacteria *Paenibacillus* contra *Colletotrichum gloeosporioides*

### Resumen

Algunas bacterias liberan compuestos orgánicos volátiles (COVs) que pueden influir en el crecimiento de otros microorganismos incluyendo algunos patógenos. La identificación de bacterias con actividad antifúngica hace posible el uso de tales bacterias en el desarrollo de agentes de biocontrol. Así pues, en este estudio, se realizó un examen dirigido exclusivamente a los COVs emitidos por ocho aislamientos bacterianos del género *Paenibacillus*, recolectados en el arrecife de coral de Providencia y Santa Catalina (Mar Caribe colombiano), con actividad antifúngica contra el hongo fitopatógeno *Colletotrichum gloeosporioides* 26B. Los COVs del aislamiento *Paenibacillus* sp (PNM-50) mostraron tasas de inhibición superiores al 50% en el crecimiento micelial del hongo, acompañado de cambios morfológicos macroscópicos y una reducción en la conidiación. Para identificar los COVs responsables de esta bioactividad antifúngica, se realizó microextracción en fase sólida del espacio de cabeza (HS-SPME) del cultivo de las bacterias y posterior análisis por cromatografía de gases acoplada a espectrometría de masas (GC-MS). Los resultados cromatográficos revelaron gran abundancia de COVs emitidos por los medios de cultivo. Una vez que se estableció la diferencia entre los COVs emitidos por el medio de cultivo y las bacterias, fue posible identificar tentativamente 2-furanmetanol, fenilacetone nitrilo y 2,4-dimetilpentanol como COVs posiblemente responsables de la actividad antifúngica.

**Palabras clave:** bacterias marinas; actividad antifúngica; HS-SPME; GCMS.

## Atividade fungicida de compostos orgânicos voláteis da bactéria *Paenibacillus* contra *Colletotrichum gloeosporioides*

### Resumo

Algunas bacterias liberan compuestos orgánicos volátiles (COV) que podem influenciar o crescimento de outros microorganismos, incluindo alguns patógenos. A identificação de bactérias com atividade antifúngica, possibilita seu uso no desenvolvimento de agentes de biocontrole. Neste estudo, foi realizada uma triagem focada nos COV liberados por oito isolados bacterianos do gênero *Paenibacillus*, coletados nos recifes de coral Old Providence e Santa Catalina (mar do Caribe colombiano), com atividade antifúngica contra fungos fitopatogênicos *Colletotrichum gloeosporioides* 26B. Os COV de *Paenibacillus* sp (PNM-50) apresentaram taxas de inibição superiores à 50% sobre o crescimento de fungos miceliais, acompanhadas de alterações morfológicas macroscópicas e redução da conidição. Para identificar os COV responsáveis por essa bioatividade antifúngica, foi conduzida uma Microextração de Fase Sólida Headspace (HS-SPME) da cultura bacteriana e, em seguida, foi analisada por Cromatografia Gasosa associada à Espectrometria de Massa (GCMS). Os resultados cromatográficos revelaram uma alta abundância de COVs liberados apenas pelos meios de cultura. Uma vez estabelecida a diferença entre os COV emitidos pelos meios de cultura e bactérias, foi possível fazer uma identificação parcial de 2-furanmetanol, fenilacetone nitrila e 2,4-dimetilpentanol como possíveis COV responsáveis pela atividade antifúngica.

**Palavras-chave:** bactérias marinhas; atividade antifúngica; HS-SPME; GCMS.

## Introduction

Marine microorganisms are a novel source of biologically active compounds which can influence the growth of pathogens [1]. Recently, metabolic profiles of selected strains of marine bacteria from the Colombian Caribbean Sea have been conducted in order to identify bacteria extracts with antibacterial effect, quorum quenching, and antifungal activity [2], with the aim of using them as phytopathogenic biocontrol agents.

Volatile Organic Compounds (VOCs) from bacteria have been studied as responsible of fungal-bacteria interactions [3, 4] acting as infochemicals in interactions among microorganisms [5] and promoting plant growth [6]. Fungi and bacteria can establish volatile mediated molecular communication that can be used to modulate the antagonistic answer of bacteria against the negative effect of fungi of a pathogenic nature, avoiding spore germination, and thus reducing growth, even decreasing pathogenicity [3, 7].

VOCs have great relevance for their applications as controllers, which takes advantage of the inter- and intra-specific communication between microorganisms through secondary metabolites, thus making it a promising environmentally friendly crop protection strategy. As a result, the use of VOCs could be a possible substitute for hazardous chemical pesticides and fertilizer.

Hundreds of VOCs released from bacteria have been identified [8, 9]. Such VOCs profile can vary depending on several factors, such as the culture media, microorganism growth stage, temperature, and even the used volatile extraction methodology [3]. Indeed, it is necessary to identify them in the specific conditions and their biological activity to get a well-defined combination of several VOCs, that could influence behaviour in fungi with accuracy.

According to current knowledge, VOCs are generally produced by catabolic pathways and belong to different chemical classes of organic compounds [8]. Acetamide, trimethylamine, 3-methylamine, 3-methyl-2-pentanone, benzaldehyde, *N,N*-dimethyloctylamine, benzothiazole, phenylacetaldehyde, cyclohexanol, nonanal, dimethyl trisulfide among others have been identified and described as VOCs released by several strains with activity against other organism such as fungi [9].

In the present work, our goal is to identify some VOCs, released by marine bacteria which are able to control the yam phytopathogen *Colletotrichum gloeosporioides* 26B. In particular, we use eight bacterial isolates obtained from marine environments, collected at coral reefs of Providencia and Santa Catalina (Colombia). To identify the antifungal activity of released VOCs against the pathogenic fungi *Colletotrichum gloeosporioides* 26B, we use a petri dishes methodology avoiding direct bacteria-fungi contact to determine mycelia growth inhibition (%) and we also evaluated the stimulation of spore germination. To evaluate only the VOCs' activity, a headspace test was designed, and HS-SPME and GCMS was used for VOCs extraction and putative identification.

## Materials and methods

### Yam Phytopathogen *Colletotrichum gloeosporioides* 26B

The phytopathogenic fungus *Colletotrichum gloeosporioides* 26B, obtained from yam plants with classical symptoms of anthracnose, was provided by "Grupo de Estudio en Ñame" from the Biotechnology Institute of the Universidad Nacional de Colombia (IBUN). The phytopathogen fungus was reactivated (by inoculating the yam tuber), fungus was grown in potato dextrose agar (PDA), and once its maximum growth was

reached in a 90 x 15 mm Petri dish (at 25 °C for four days), the spores were scraped. Those spores were suspended using 25 mL of the solution of 0.85% NaCl + 0.1% sterile Tween® 80, then it was filtered and collected in 50 mL falcon tube. Using 10 µL of the solution, the count of conidia was performed using the Neubauer chamber (2.9 x 10<sup>6</sup> conidia/mL). Besides, 10 µL of this solution was inoculated on the sterile yam slides. After 3 days of growth, conidia of the pathogen that grew on the yam were taken and inoculated in PDA. For long-term storage, small portions of an actively growing colony were brought into fungi preservation medium stored at -80 °C (Luria-Bertani LB solution fortified with glycerol 80%). A second suspension for short-term maintenance was stored at -20 °C (Luria-Bertani LB solution fortified with glycerol 20%).

For the inoculation of the pathogen, 100 µL of suspension stored at -20 °C was grown onto PDA, then the pathogen was gently scraped off the surface by hand with 5-10 mL of 0.85% NaCl + 0.1% sterile Tween® 80 solution to detach conidia. Finally, the resulting spore suspension was passed through sterile cotton to remove the mycelial fragments, obtaining a conidia solution of *Colletotrichum gloeosporioides* 26B.

## Marine bacteria

Eight bacterial isolates (Table 1) were selected from a collection obtained from the coral reef of Old Providence and Santa Catalina islands (Colombian Caribbean Sea). For this study, we selected *Paenibacillus* strains because this genus was proposed as potentially effective biocontrol and plant growth promoting agent in previous research [10-12].

All the strains were registered in the collection of microorganisms of the IBUN and were preserved in cryovials at -80 °C, each vial containing 1 mL of the suspension resulting from scraping with 10 mL of solution of Luria Bertani broth modified with glycerol 85%.

## Screening of VOCs antifungal activity

To evaluate the antifungal activity of VOCs released by the eight marine bacteria, both organisms (respective bacteria + fungi) were grown in separate petri dishes, then they were put together avoiding direct contact between both microorganisms (Figure 1), as follows: Bacterial strains were grown in Muller Hilton (MH) Agar plate by striatum in a Petri box (90 x 15 mm). Over a second Petri dish, the pathogen *Colletotrichum gloeosporioides* 26B was inoculated in PDA, using 10 µL of a solution of conidia (2.9 x 10<sup>6</sup> conidia/mL). The box containing the bacteria was placed on top and inverted over the one that kept the pathogen fungus *Colletotrichum gloeosporioides* 26B (Figure 1). The boxes were sealed with Parafilm™ tape to prevent the escape of VOCs and incubated at 28 °C for 10 days, because according to our experimental results, this is the necessary time for the fungus to reach its maximum growth in a 90 x 15 mm Petri dish. Growth inhibition of pathogen caused by bacterial VOCs were measured at the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day of inoculation. Plates with *C. gloeosporioides* cultured on PDA in the absence of the bacterial strain were used as negative control. Each assay was performed by triplicate with three independent cultures for each strain.

The diameter of each fungal colony was measured on the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day after inoculation. The fungus size is measurable after three days of growth and on the tenth day it covers the entire of Petri dish area. Three measurements of length per box were made each day using photography. The ImageJ 1.x Java [13] public image processing program was used to carry out the measurement. The inhibition percentage against the phytopathogenic fungus was calculated using Eq. (1).

$$\% \text{ inhibition} = \left(1 - \frac{\text{td}}{\text{cd}}\right) \times 100 \quad (1)$$

Here, td is the diameter of the fungal colony in the presence of the bacterial isolate and cd is the diameter of the fungal colony of negative control.

**Table 1.** Selected bacteria for antifungal activity screening.

Code	Taxonomic identification of each strain <sup>a</sup> (% Similarity)	Source	Morphology	Gram
PNM24	<i>Paenibacillus elgii</i> NBRC 100335 (99.9%)	Mangrove bark	rod-shaped	Positive
PNM32	<i>Paenibacillus ehimensis</i> IB-739 (99.8%)	Coral sand-pinnacle	rod-shaped	Variable
PNM34	<i>Paenibacillus ehimensis</i> HSCC-594 (100%)	<i>Niphates digitalis</i>	rod-shaped	Positive
PNM50	<i>Paenibacillus ehimensis</i> NBRC15659 (100%)	<i>Codium</i> (algae) sp.	coccobacillus	Variable
PNM54	<i>Paenibacillus ehimensis</i> NBRC15657 (99.9%)	<i>Codium</i> (algae) sp.	rod-shaped	Variable
PNM63	<i>Paenibacillus ehimensis</i> NPUST1 (99.9%)	Unknow	coccobacillus	Positive
PNM65	<i>Paenibacillus ehimensis</i> NBRC15659 (100%)	<i>Niphates digitalis</i>	rod-shaped	Positive
PNM68	<i>Paenibacillus ehimensis</i> NBRC15657 (99.9%)	<i>Niphates digitalis</i>	rod-shaped	Positive

<sup>a</sup>Determined by 16S rRNA gene sequencing in previous studies in our research group (unpublished data).

The *Paenibacillus* isolates, with inhibitory activity against *Colletotrichum gloeosporioides* 26B higher than 50%, were also tested on Nutritive Agar (NA) (yeast extract 3 g/L, beef extract 3 g/L, peptone 5 g/L, and NaCl 5 g/L), International Streptomyces Project (ISP2) (yeast extract 4 g/L, malt extract 10 g/L, and dextrose 4 g/L); Mueller Hinton (MH) (beef extract 2 g/L, hydrolyzated casein 17,5 g/L, and starch 1,5 g/L) and Luria Bertani (LB) (yeast extract 5 g/L, NaCl 10 g/L, and tryptone 10 g/L) media following the same procedure.

In addition, the effect of the VOCs on the conidia number was measured on the 10<sup>th</sup> day, following the methodology of Perez *et al.* [14]. In brief,

5 mL of solution, 0.85% NaCl, and 0.1% Tween® 80, were added over a fungus culture previously exposed to VOCs in order to obtain the conidia suspension. Using a Neubauer chamber the number of conidia (10 µL of suspension) were counted. The same procedure was carried out without exposure to volatiles from bacterial isolates, serving as negative control.

## Identification of VOCs by HS-SPME-GC-MS

In 100 mL glass vials, containing 20 mL of culture medium, 100 µL of each strain was inoculated in LB culture media, with an optical density OD<sub>600</sub> of 0.456 ± 0.005 (Elisa absorbance reader, IFS).

The systems were sealed with air-tight Teflon septum and an aluminium cap and were kept in incubation at 28 °C for 10 days.

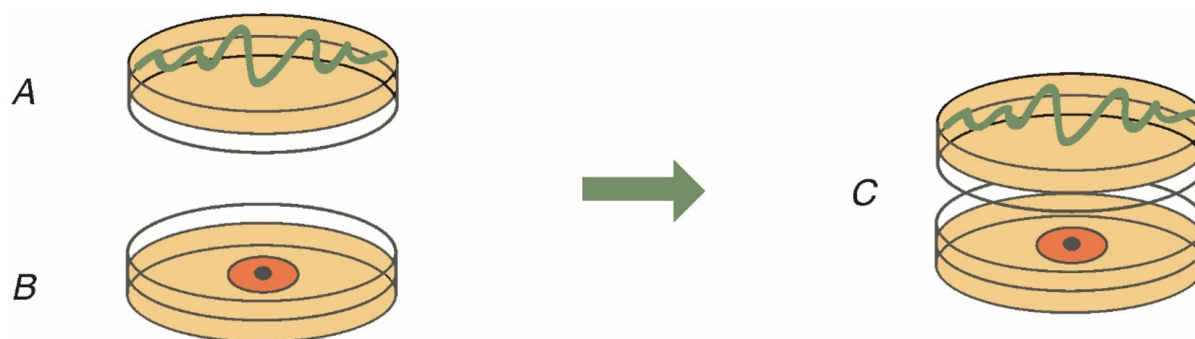
VOCs were collected on a divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS, 50/30 µm, Supelco®, Bellefonte, PA, USA) for 15 min on the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day of inoculation, i.e. at the same points of time as the inhibition measurement described above. SPME fiber, HS volume, and time conditions were selected according to previous studies (data not shown here).

SPME samples were analysed using a GC-MS-QP2010S from Shimadzu (Kyoto, Japan) and a capillary column SLB®-5ms-28471-U (Sigma-Aldrich) (30 m × 0.25 mm, df 0.25 µm). Helium was used as carrier gas with a flow rate of 1.20 mL/min. Manual injection for 5 min was done in splitless mode (1 min), the injection port and transfer line temperature were set at 250 °C. The column oven was programmed from 50 °C; increasing to 190 °C at 6 °C/min, then 12 °C/min to 230 °C and held at that temperature for 5 min. Mass spectrometry analyses were done in full-scan mode using an electron energy of 70 eV and the *m/z* range was 35-350 with a scanning speed of 0.50 scans/s.

VOCs were putatively identified by comparison of their retention indexes (RI) calculated by using the headspace of a mixture of alkanes (C<sub>6</sub>-C<sub>26</sub>) with those informed by the literature or standards. The signals from control culture media must be subtracted of each TIC to be analysed. The obtained mass spectra were compared to those present in the libraries NIST/EPA/NIH (National Institute of Standards and Technology) and VOC databases available online (<http://bioinformatics.charite.de/mvdoc>) [15].

## Statistical analysis

A two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were performed with GraphPad Prims 7® software (GraphPad Software, San Diego, CA, USA). A P value < 0.05 was considered significant (95%).



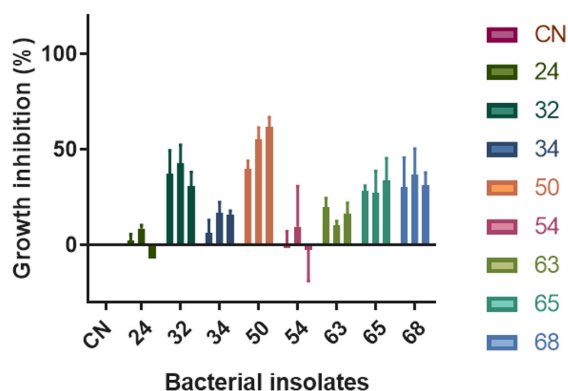
**Figure 1.** Experimental design to test the antifungal activity of bacterial VOCs. **A).** Bacterial strain growth in Muller Hinton (MH). **B).** Phytopathogen *C. gloeosporioides* 26B growth on PDA medium. **C).** Both Petri dishes were sealed with Parafilm™ tape facing each other to obtain a double-plate chamber.

## Results and discussion

Yam (*Dioscorea* sp.) is an important crop in tropical and subtropical regions around the globe [16]. In Colombia there are 340,000 hectares of it, with a production of 85,000 tons per year. This crop is affected by different fungi, including *Fusarium oxysporum*, which has been associated with dry rot in yam tubers, and the fungus *Colletotrichum gloeosporioides*, which causes Yam anthracnoses [16]. In this context, we are looking for bacteria that are capable of controlling *C. gloeosporioides* and we focus on *Paenibacillus* species due to their potential in the development of biocontrollers.

Bacteria of the genus *Paenibacillus* comprise bacterial species that have been reported as symbionts of plants such as corn, squash and rice, and it has been identified that they can promote their growth [17, 18]. They are widely distributed around the globe. In fact, they have been isolated from soils, plant roots, polar regions, marine environments, and even deserts. This genus of bacteria has been characterized as producer of enzymes such as amylases, cellulases, hemicellulases, lipases, pectinases, oxygenases, dehydrogenases among others, which may have applications for detergents, foods, textiles, etc [17]. Antimicrobial compounds have been isolated from bacteria of these genera. In this context, we selected eight marine bacteria of genus *Paenibacillus* to evaluate their capability to release VOCs with antifungal activity.

To assess the effects of VOCs released by each bacterium on the growth of yam fungi pathogen *Colletotrichum gloeosporioides* 26B, both microorganisms were cultured at the same space avoiding their direct contact, as is shown in Figure 1. VOCs produced by four marine bacteria denoted as PNM-32, 50, 65, and 68, inhibited the mycelia growth of *Colletotrichum gloeosporioides* 26B as significantly different from the control ( $P < 0.05$ ). Figure 2 shows the % inhibition presented by each bacterial isolate. Overall, growth inhibition rates over pathogenic fungi increase with incubation time.



**Figure 2.** Antifungal activity screening. The radial mycelium growth of *Colletotrichum gloeosporioides* 26B was determined on the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day of inoculation and the percent inhibition compared to the control growth was calculated. All strains were evaluated in three independent replicates. Error bars indicate  $\pm$  SD.

Since the VOCs released by PNM 50 show the highest mycelia inhibition rate ( $61.5 \pm 5.3\%$  after 10<sup>th</sup> day of inoculation), PNM 50 was selected for chemical study. PNM 50 was identified as *Paenibacillus ehimensis* NBRC15659 (100%) by 16S rRNA gene sequencing (unpublished data).

The impact of culture media on bacterial metabolic profiles is well known [19]. To establish the effect of culture media on VOCs composition (qualitative and quantitatively) and their antifungal activity, we studied the impact of four different culture media (MH, NA, ISP2, and LB) on mycelia growth inhibition (Table 2).

**Table 2.** Antagonistic effect of volatile organic compounds from *Paenibacillus* PNM50 against *C. gloeosporioides* 26B on four media on the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day of co-cultivation.

Media	Growth inhibition (%)			% Conidial reduction 10 <sup>th</sup> day
	3 <sup>rd</sup> day	6 <sup>th</sup> day	10 <sup>th</sup> day	
(NA)	29.6 $\pm$ 6.5	31.5 $\pm$ 7.4	27 $\pm$ 12	92.1
(MH)	36 $\pm$ 12	27 $\pm$ 18	37.8 $\pm$ 9.1	94.4
(ISP2)	41.8 $\pm$ 4.6	35.0 $\pm$ 5.0	28.3 $\pm$ 9.8	95.2
(LB)	40.8 $\pm$ 3.0	39.3 $\pm$ 4.5	40.8 $\pm$ 3.4	96.5

n = 3,  $\pm$  SD.

In all used media mycelia growth inhibition of phytopathogenic fungi by VOCs was observed (Table 2), as well as morphological changes on *C. gloeosporioides* 26B, such as changes in mycelium colour. The highest inhibition percentage was obtained when PNM-50 was grown over ISP-2 and LB on the 3<sup>rd</sup> day of incubation.

There was a greater inhibition of the phytopathogen when the culture medium (LB) is rich in yeast hydrolysate, which suggests that the presence of this component would contribute with the content of vitamins, amino acids, and growth factors, thus favouring the production of VOCs in bacterial isolates. Finally, the LB medium was selected for VOCs analysis.

Our results allow to point out that a culture medium serves in the first instance as external nutrient source to promote bacterial growth. As a result, greater biomass is generated, and therefore, a greater amount of active metabolite is synthesized. Secondly, some of the medium compounds could be involved in the production of active metabolite, specifically in this study yeast hydrolysate could play that role. Furthermore, there is a complex effect by interacting the culture medium- bacterial- fungal volatile emissions, as reported an ongoing dialog between the pathogen *Verticillium longisporum* and its antagonist *Paenibacillus polymyxa* [12].

In addition, the production of fungi conidia in four tested media was measured on the 10<sup>th</sup> day obtaining a conidial reduction of 92-97% in all media. This result would be associated with the reduction in the size of phytopathogen fungal colony in all the examined cases, indicating that at a lower mycelial mass, there is a lower number of asexual spores. The conidiation reduction further suggests that the exposure to VOCs emitted by bacteria influences the reproductive processes of phytopathogen and, therefore, the probability of infection would be reduced.

The next step was to identify the compounds released by PNM-50. HS-SPME and GC-MS were used for extraction and analysis of VOCs from the bacterial isolate *Paenibacillus* PNM-50 grown on LB media. The VOCs were analysed on the 3<sup>rd</sup>, 6<sup>th</sup>, and 10<sup>th</sup> day (Table 3).

To the best of our knowledge, this is the first report of VOCs produced by the culture media. For the identification of VOCs produced by bacterial isolates, the signal of compounds observed from the control culture media must be subtracted from the signal from the bacterial cultures in order to avoid misidentification of bacteria-produced compounds. In this way, PNM-50 exhibits only four compounds as true bacterial VOCs: 2-furanmethanol, phenylacetoneitrile, undecanoic acid, and 2,4-dimethylpentanol. We also identified the compounds coming from the culture media. Amongst these, benzotiazol, was reported in previous studies [4, 20-22] as constituent of the VOCs produced by bacteria. Furthermore, it has been tested for its antifungal activity showing mycelial growth reduction in *Fusarium oxysporum* [22] and *S. sclerotiorum* [20]. However, in our study we demonstrate that this compound is also released by LB media. In this sense, it is not necessarily a true bacterial VOC, which emphasizes the importance of TICs subtraction, which is not commonly done at literature thus far.

Phenylacetoneitrile has been reported in the volatile microbial database (mVOC) as a metabolic product of *Pseudomonas aeruginosa* [23].

**Table 3.** Identified Volatile Organic Compounds from *Paenibacillus* PNM50 grown on LB media

Putative identified Compounds	Experimental			Area %		
	Rt (min)	RI DB-5	m/z base peak	3 <sup>rd</sup> day	6 <sup>th</sup> day	10 <sup>th</sup> day
2-furanmethanol	11.581	1110	39	20	nd	nd
Phenylacetonitrile	12.278	1138	117	3	5	6
undecanoic acid	20.842	1497	60	1	nd	nd
2,4-dimethyl pentanol	22.977	1597	44	4	nd	nd

Rt= retention time. RI= retention index for the bacterial headspace volatiles were calculated using the headspace of a mixture of alkanes (C<sub>6</sub>-C<sub>26</sub>). Area % = relative peak area of the total ion chromatogram (TIC) of the peak corresponds to the abundance of each compound after subtraction of COVs from culture media. nd= non-detected. Identification was done by retention index comparison with those from literature and mass spectra comparison with those from NIST data base.

This aromatic compound has a nitrile moiety, which is a functional group widely distributed among fungicides such as benomyl (methyl-1-(butylcarbamoyl)-benzimidazol-2-yl-carbamate) and chlorothalonil (2,4,5,6-tetrachlorobenzene-1,3-dicarbonitrile). Faced with possible positive results of the use of phenylacetonitrile as a compound with antifungal activity against *Colletotrichum gloeosporioides* 26B, it would be necessary to study its mechanism of action and the convenience of its use in the field of sustainable agriculture, since this compound poses a toxicity hazard (R22, R24, R26).

## Conclusions

Our findings reveal that VOCs mediated interactions between bacteria and fungi occur in four out of eight *Paenibacillus* strains tested here, which are active as fungi controller in an in vitro test. The strain PNM-50 (*Paenibacillus ehimensis* NBRC15659) is demonstrated to be the most active one against *Colletotrichum gloeosporioides* 26B based on a significant reduction in the radial growth and in the stimulation of spore germination.

Our results were consistent with previous studies suggesting that the VOCs emitted by bacteria depend on the growth medium [24]. In addition, it becomes clear this field of volatile compounds needs to be studied much more thoroughly: In past research several reported bacterial VOCs actually turned out to be by-products of the culture media sterilization process, but were falsely classified as bacterial metabolic products. It is important to analyse not only the head space of cultured bacteria but also those of culture media for reference (blank).

The compounds tentatively identified at headspace of PNM-50 cultured on LB media were 2-furanmethanol, phenylacetonitrile, undecanoic acid, and 2,4-dimethyl pentanol, the quantities of which depend on the incubation period.

VOCs from *Paenibacillus* could be used as pesticides and fertilizers, being an open-field to provide new sustainable and eco-friendly alternatives to conventional agents. Among our putatively identified compounds only phenylacetonitrile was previously reported to exhibit antifungal activity, whereas the other three are new candidates. Future research must therefore identify the assigned putative compounds by using standards to compare the chromatography peaks and by carrying out agrochemical safety tests before implementation in field applications.

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