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Carla de Sant'Anna Freitas

DECIPHERING THE MOLECULAR MECHANISMS INVOLVED IN THE ANTAGONISM TO THE PHYTOPATOGENIC FUNGUS *Thielaviopsis ethacetica* BY BACTERIAL VOLATILE ORGANIC COMPOUNDS

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Orientadora: Profa. Dra. Juliana Velasco de Castro Oliveira

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Arthur Schopenhauer

RESUMO

Um dos aspectos cruciais da agricultura moderna é reduzir o uso de fertilizantes químicos e pesticidas que, além do alto custo, têm um impacto negativo na saúde humana e no meio ambiente, causando, por exemplo, degradação do solo e contaminação dos recursos hídricos. Devido ao crescimento da população e a necessidade de uma agricultura mais sustentável, são necessários investimentos na área de biotecnologia agrícola, onde as bactérias promotoras de crescimento vegetal (BPCVs) certamente desempenharão um papel importante na revolução da agricultura nas próximas décadas. Muitas das interações BPCV-planta são baseadas na relação direta ou muito próxima do microrganismo com a planta, mas, recentemente, interações à distância através de compostos orgânicos voláteis (COVs) emergiram como um novo mecanismo pelo qual os microrganismos podem promover o crescimento vegetal e inibir fitopatógenos. Devido à sua natureza gasosa, ocorre uma distribuição eficiente dos voláteis através da porosidade do solo, permitindo que alcancem longas distâncias. Isso melhora a eficácia da ação antagônica nos microrganismos alvo, tornando os COVs excelentes candidatos para o biocontrole. Assim, esse estudo teve como objetivo principal avaliar o efeito dos COVs bacterianos no antagonismo ao fungo fitopatogênico *Thielaviopsis ethacetica*, agente etiológico da podridão abacaxi na cana-de-açúcar. Identificamos 3 *Pseudomonas* que foram capazes de inibir, *in vitro*, até 80% do crescimento micelial de *T. ethacetica*. Usando uma abordagem de metabolômica por GC/MS, verificamos que essas bactérias produziram, no total, 62 COVs, e os testes de validação funcional revelaram compostos que inibiram significativamente o crescimento e até mesmo levaram à morte de *T. ethacetica*. A análise transcriptômica por RNA-seq da resposta do fungo aos COVs indicou que esses metabólitos regularam negativamente os genes relacionados ao metabolismo central dos fungos, como aqueles envolvidos no metabolismo dos carboidratos. Curiosamente, os genes relacionados à resposta ao dano ao DNA foram regulados positivamente, e a análise de micro-FTIR corroborou nossa hipótese de que os COVs desencadearam dano ao DNA. A análise de microscopia eletrônica mostrou mudanças morfológicas críticas em micélios tratados com COVs. Juntos, esses resultados indicam que os COVs suprimiram o crescimento dos fungos e causaram a morte celular. Este estudo representa a primeira demonstração dos mecanismos moleculares envolvidos no antagonismo de fitopatógenos da cana-de-açúcar por COVs bacterianos e reforça que esses compostos podem ser uma alternativa sustentável para uso no biocontrole em cana-de-açúcar e outras culturas.

Palavras-chave: *Thielaviopsis ethacetica*; podridão abacaxi; controle biológico; RNA-seq; HS-SPME/GC-MS; volatiloma; microscopia eletrônica; micro-FTIR.

ABSTRACT

One of the crucial aspects of modern agriculture is to reduce the use of chemical fertilizers and pesticides, which, in addition to their high cost, have a negative impact on human health and the environment, causing, for example, soil degradation and contamination of water resources. Therefore, investments in agricultural biotechnology are needed, where plant growth promoting bacteria (PGPBs) will certainly play an important role in the agricultural revolution in the coming decades, due to population growth and the need for more sustainable agriculture. Many of the PGPB-plant interactions are based on the direct or very close relationship of the microorganism with the plant, but recently, distance interactions through volatile organic compounds (VOCs) have emerged as a new mechanism by which microorganisms can promote plant growth and inhibit phytopathogens. Due to their gaseous nature, there is an efficient distribution of volatiles through the porosity of the soil, allowing them to reach long distances. This improves the effectiveness of antagonistic action on target microorganisms, making VOCs excellent candidates for biocontrol. Thus, this study aimed to evaluate the effect of bacterial VOCs on antagonism to the phytopathogenic fungus *Thielaviopsis ethacetica*, the causal agent of pineapple sett rot disease in sugarcane. In this study, we identified 3 *Pseudomonas* that were able to inhibit, in vitro, up to 80% of mycelial growth of the phytopathogenic fungus *T. ethacetica*. Using a metabolomics approach by GC/MS, we found that these bacteria produced 62 different VOCs, and further functional validation revealed compounds that inhibited growth and even led to death of the *T. ethacetica*. Transcriptomic analysis by RNA-seq of the fungal response to VOCs indicated that these metabolites downregulated genes related to fungal central metabolism, such as those involved in carbohydrate metabolism. Interestingly, genes related to the DNA damage response were upregulated, and micro-FTIR analysis corroborated our hypothesis that VOCs triggered DNA damage. Electron microscopy analysis showed critical morphological changes in mycelia treated with VOCs. Altogether, these results indicated that VOCs hampered fungal growth and could lead to cell death. This study represents the first demonstration of the molecular mechanisms involved in the antagonism of sugarcane phytopathogens by VOCs and reinforces that VOCs can be a sustainable alternative for use in crop pathogen biocontrol.

Keywords: *Thielaviopsis ethacetica;* pineapple sett rot disease; biological control; RNA-seq; HS-SPME/GC-MS; volatilome; electron microscopy; micro-FTIR.

SUMÁRIO

CAPÍTULO 1 – INTRODUÇÃO GERAL

Historicamente, a agricultura brasileira é uma das principais bases de nossa economia. Neste cenário, a cana-de-açúcar foi a primeira iniciativa agrícola colonial de larga escala, representando a base econômica do governo português após a descoberta do Brasil em 1500 (Luz, 1963). Atualmente, o Brasil é o maior produtor mundial desta cultura, com área total projetada em 8,42 milhões de hectares e produtividade estimada em 628 milhões de toneladas para 2021/2022 (CONAB, 2021).

Assim como outras grandes culturas, a cana-de-açúcar é constantemente afetada por doenças e pragas que impactam na sua produtividade. A podridão abacaxi é uma das doenças economicamente mais importantes (Hewajulige e Wijesundera, 2014), e tornou-se particularmente importante após a mecanização, pois o corte do colmo pelas lâminas provoca lesões que facilitam a entrada do fungo *Thielaviopsis ethacetica* (Teleomorfo: *Ceratocystis ethacetica*). Um dos sintomas iniciais da doença é o aspecto encharcado nas pontas dos toletes (Borges *et al.*, 2019) e, à medida que a infecção avança, o tecido interno fica vermelho/preto, resultante da produção de esporos. Durante esse processo, esses tecidos fermentam e produzem um odor característico de abacaxi, devido à liberação de acetato de etila que é a toxina responsável por inibir mudas ou toletes (Hewajulige e Wijesundera, 2014; Borges *et al.*, 2019). A doença retarda severamente a germinação dos toletes, o desenvolvimento e o vigor das mudas e, às vezes, é necessário fazer o replantio. Estudos anteriores mostraram que a podridão abacaxi pode diminuir a produtividade da cana-de-açúcar em 31% a 42% (Chapola *et al.*, 2014; Pandey *et al.*, 2014), o que significa um impacto negativo expressivo na economia brasileira, uma vez que o setor sucroenergético corresponde a 2% do produto interno bruto brasileiro (PIB) (Neves *et al.*, 2010).

O fungo ascomiceto *T. ethacetica* é um fitopatógeno de distribuição mundial que infecta uma ampla gama de hospedeiros causando doenças economicamente importantes em outras culturas agronômicas, como abacaxi, coco, palma, banana, sorgo, cacau e milho (Hewajulige e Wijesundera, 2014). No Brasil, foi relatado pela primeira vez em cana-de-açúcar em 1893 por Went como *Thielaviopsis ethaceticus* Went (Wismer e Bailey, 1989), no entanto, devido à sua semelhança morfológica foi

por muito tempo descrito como *Thielaviopsis paradoxa* (De Seynes) Höhn (Do Nascimento *et al.*, 2020). A doença da podridão abacaxi tem ocorrência mundial e já foi relatada nos Estados Unidos, Índia, África do Sul, Filipinas, China, Haiti, República Dominicana, Cuba, México e Colômbia (Hewajulige e Wijesundera, 2014; Borges *et al.*, 2019; Viswanathan e Malathi, 2019).

Usualmente, o tratamento utilizado para reduzir os impactos causados pela doença é por meio de variedades de crescimento rápido e tratamento químico com fungicidas (Ferreira *et al.*, 2008; Borges *et al.*, 2019; Viswanathan e Malathi, 2019). Muitos estudos relatam a eficiência da estrobilurina, fenilpiridilamina e fungicidas à base de triazol no controle da podridão abacaxi (Raid, 1990; Ferreira *et al.*, 2008; Cantarel *et al.*, 2009; Bhuiyan *et al.*, 2014; Chapola *et al.*, 2014), no entanto, esses produtos químicos têm um alto custo. Além disso, e talvez mais importante, um dos aspectos cruciais para a agricultura moderna é reduzir o uso de fertilizantes químicos e pesticidas, que geram impactos negativos na saúde humana (Chen *et al.*, 2017; Alori e Babalola, 2018) e no meio ambiente, prejudicando a saúde e a fertilidade do solo (Cerqueira Leite *et al.*, 2009; de Souza *et al.*, 2015; Santoyo *et al.*, 2016), contaminação dos recursos hídricos (Francis *et al.*, 2010) e poluição do ar (Bhattacharyya e Jha, 2012).

Para isso, investimentos em biotecnologia agrícola são necessários para obter uma agricultura mais sustentável e as Bactérias Promotoras de Crescimento Vegetal (BPCVs) desempenharão um papel fundamental na revolução da agricultura. As BPCVs são essenciais para a fertilidade do solo e podem aumentar o crescimento vegetal e melhorar seu desempenho contra estresses bióticos e abióticos por meio de uma ampla variedade de mecanismos, incluindo *(i)* síntese de hormônios vegetais, *(ii)* fixação de nitrogênio, *(iii)* aumentando a disponibilidade de fosfato inorgânico no solo, *(iv)* facilitando a absorção de ferro pelas plantas através da produção de sideróforos, *(iv)* controlando a produção de etileno, *(v)* protegendo as plantas de patógenos, *(vi)* atuando como agentes de biorremediação, entre outros (Francis *et al.*, 2010; Bhattacharyya and Jha, 2012; de Souza *et al.*, 2015; Santoyo *et al.*, 2016). Todas essas características permitem o uso de BPCVs para melhorar o rendimento das culturas, diminuir a aplicação de fertilizantes químicos e reduzir os custos de produção (Ramakrishna *et al*., 2019; Murgese *et al*., 2020).

Dentre os diversos mecanismos que BPCVs possuem para promover o crescimento e a saúde das plantas, destacamos a produção de compostos orgânicos voláteis (COVs), que são pequenas moléculas de natureza lipofílica (<300 Da), derivadas de uma ampla gama de vias biossintéticas, e com baixo ponto de ebulição e alta pressão de vapor (Schulz e Dickschat, 2007; Effmert *et al.*, 2012; Lemfack *et al.*, 2014; Silva Dias *et al.*, 2021). Os COVs são uma mistura de metabólitos voláteis que podem ser produzidos por todos os microrganismos vivos, compostos por uma heterogeneidade de classes químicas que incluem álcoois, tioálcoois, hidrocarbonetos, cetonas, tioésteres, ciclohexanos, compostos heterocíclicos, fenóis e derivados de benzeno, entre outros (Tilocca *et al.*, 2020).

Atualmente, os COVs relatados como sendo produzidos por microrganismos estão organizados no banco de dados mVOC, que reúne informações baseadas na literatura (Lemfack *et al.*, 2014, 2018). Estão registrados aproximadamente 2.000 COVs produzidos por cerca de 600 fungos e 300 bactérias, sendo grande parte (250) pertencentes ao filo Proteobacteria, principalmente à classe das Gammaproteobacteria (150) (Lemfack *et al.*, 2020). O gênero mais investigado quanto à emissão de COVs é *Pseudomonas*, respondendo por um terço de todas Gammaproteobacteria listadas no mVOC. Quanto à origem dessas bactérias, 32% foram isoladas da rizosfera e solo (Lemfack *et al.*, 2020). No entanto, embora esses registros sejam substanciais, muitos mais compostos devem ser identificados no futuro, uma vez que, apenas 10^3 microrganismos de 10^{16} que se espera que existam na terra, e apenas 6 filos bacterianos de um total de 26 foram investigados até agora quanto à emissão de voláteis (Farré-Armengol *et al.*, 2016; Lemfack *et al.*, 2020; Piechulla *et al.*, 2020).

Apesar de diversos estudos estarem sendo realizados, é importante considerar que as condições no laboratório e na natureza são bastante diferentes e, consequentemente, os perfis de COVs obtidos de bactérias nestas duas condições são provavelmente distintos (Piechulla *et al.*, 2020). Uma vez que não é possível investigar todas as condições de crescimento desses microrganismos e as variações que ocorreriam naturalmente, o potencial da biossíntese de compostos de uma dada bactéria permanece desconhecido (Lemfack *et al*. 2020). Diante disso, é muito importante um delineamento experimental que contemple diferentes condições para que os resultados possam se aproximar do que seria em condições naturais. Ademais,

embora os experimentos em laboratório sejam apenas uma fatia diante de toda complexidade envolvida nas interações entre os microrganismos e ambiente, os resultados nos permitem identificar microrganismos e/ou moléculas com potencial para serem utilizados como alternativa aos agroquímicos.

Os COVs são moléculas importantes e amplamente utilizadas na indústria de alimentos (Hung *et al*. 2015). Também têm sido utilizados como indicadores de contaminantes e poluentes com consequências potenciais para a saúde humana (Korpi *et al*. 2009) bem como na medicina, agricultura e aplicações biotecnológicas (Elmassry e Farag, 2020; Kusstatscher *et al*. 2020; Piechulla *et al.*, 2020). Existe uma demanda atual por novos compostos bioativos para diferentes aplicações, uma vez que muitos compostos utilizados anteriormente não podem mais serem usados devido a fenômenos de resistência (no caso de antibióticos, por exemplo), às novas regulamentações de segurança ambiental (fungicidas, herbicidas, inseticidas, nematicidas, etc.). Neste cenário, a bioprospecção de COVs produzidos por microrganismos pode resultar novas moléculas com bioatividade específica que atendam aos requisitos exigidos (Lemfack *et al*. 2020; Piechulla *et a*l., 2020)

Recentemente, os COVs bacterianos têm recebido atenção como importantes moléculas sinalizadoras para o controle de fitopatógenos e promoção do crescimento vegetal (Kai *et al.*, 2009; Syed-ab-rahman *et al.*, 2019; Piechulla *et al.*, 2020). Devido à sua natureza gasosa, ocorre uma distribuição eficiente dos voláteis através da porosidade do solo, permitindo que alcancem longas distâncias. Isso melhora a eficácia da ação antagônica nos microrganismos alvo, tornando estas moléculas excelentes candidatas para o biocontrole (Wenke *et al.*, 2010; Peñuelas *et al.*, 2014; Massalha *et al.*, 2017; Schulz-Bohm *et al.*, 2018; Sharifi e Ryu, 2018; Schenkel *et al.*, 2019).

Embora seja um campo crescente de estudos, até o presente, pouco se sabe sobre os mecanismos moleculares associados ao antagonismo de fitopatógenos por COVs. Explorar técnicas que ajudem a elucidar esses mecanismos é essencial para o desenvolvimento de estratégias para o uso dessas moléculas como compostos bioativos para o controle de patógenos de plantas. Nesse cenário, a transcriptômica é uma ferramenta que pode clarificar essas interações de modo a identificar os mecanismos de ação pelos quais os COVs inibem o fitopatógeno.

O transcriptoma é o conjunto de transcritos em uma célula, tanto em termos qualitativos quanto quantitativo (Nagalakshmi *et al.*, 2010) e a transcriptômica permite a análise dos transcritos em uma determinada condição, por exemplo, exposto ou não aos COVs. As técnicas mais utilizadas nos estudos de transcriptômica são: RT-qPCR; qPCR *array*, microarranjos e RNA-seq (Mutz *et al.*, 2013). Para identificar os genes alvos nesse antagonismo, nós utilizamos o RNA-seq, que é uma técnica de biologia molecular que tem se mostrado o principal método para estudar a expressão gênica e identificar novas espécies de RNA, uma vez que revela diretamente a identidade da sequência, o que é crucial para a análise de genes desconhecidos e novas isoformas de transcrição (Nagalakshmi *et al.*, 2010).

Até onde sabemos, este estudo é pioneiro em investigar os mecanismos moleculares envolvidos na interação entre COVs e *T. ethacetica*. Neste trabalho, realizamos um *screening* a partir de nossa coleção de BPCV, para identificar novos isolados bacterianos para o antagonismo ao *T. ethacetica*. A vantagem do nosso banco de microrganismos é que as bactérias foram principalmente isoladas de áreas altamente produtivas de cana-de-açúcar. Assim, é possível supor que a comunidade nativa das bactérias ali presente teve um papel na saúde da cultura e, além disso, não se sabe se esta comunidade também estava naturalmente impedindo o estabelecimento de microrganismos patogênicos.

Para melhor compreensão, esta tese foi estruturada em três capítulos, sendo o **CAPÍTULO 1** uma introdução geral sobre o assunto abordado e objetivos gerais e específicos propostos; o **CAPÍTULO 2** apresenta o artigo publicado pelo grupo de pesquisa da doutoranda contendo os principais resultados da pesquisa realizada durante o doutoramento e o **CAPÍTULO 3** as conclusões gerais e considerações finais com base nos dados obtidos. Os resultados deste estudo podem ajudar a projetar melhores estratégias para desenvolver um produto de biocontrole como uma alternativa *eco-friendly* aos agroquímicos.

Na seção de anexos, encontra-se o termo da comissão de bioética (**ANEXO I**), a declaração de direitos autorais (**ANEXO II**) e a autorização da Editora para o uso do artigo nesta tese (**ANEXO III**).

OBJETIVOS

Objetivo geral

O objetivo principal desse trabalho foi identificar voláteis bacterianos com atividade antagonista ao fungo fitopatogênico *T. ethacetica* e elucidar os mecanismos moleculares associados a esse antagonismo.

Objetivos específicos

(i) Prospectar bactérias capazes de inibir o crescimento de *T. ethacetica*;

(ii) Avaliar o efeito de diferentes meios de cultivo bacteriano utilizados nos isolados selecionados em (i) na inibição do crescimento de *T. ethacetica*;

(iii) Avaliar diferentes doses de inóculo em (*i*) na inibição do crescimento de *T. ethacetica*;

(iv) Identificar em nível de gênero os isolados mais promissores selecionados em (*i*);

(v) Identificar os COVs produzidos pelos isolados

(vi) Identificar quais os COVs identificados em (*v*) são responsáveis pela inibição do crescimento de *T. ethacetica*;

(vii) Identificar os mecanismos moleculares envolvidos na inibição do crescimento do *T. ethacetica*;

(viii) Identificar as alterações morfológicas causadas no *T. ethacetica* pelos COVs.

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CAPÍTULO 2 - BACTERIAL VOLATILE ORGANIC COMPOUNDS INDUCE ADVERSE ULTRASTRUCTURAL CHANGES AND DNA DAMAGE TO THE SUGARCANE PATHOGENIC FUNGUS *Thielaviopsis ethacetica*

Os resultados obtidos nessa pesquisa foram publicados na revista Environmental Microbiology (fator de impacto 5,491), em janeiro de 2022, com o título "Bacterial volatile organic compounds induce adverse ultrastructural changes and DNA damage to the sugarcane pathogenic fungus *Thielaviopsis ethacetica*" (doi:10.1111/1462-2920.15876).

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Bacterial volatile organic compounds induce adverse ultrastructural changes and DNA damage to the sugarcane pathogenic fungus *Thielaviopsis ethacetica*

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Summary

Due to an increasing demand for sustainable agricultural practices, the adoption of microbial volatile organic compounds (VOCs) as antagonists against phytopathogens has emerged as an eco-friendly alternative to the use of agrochemicals. Here, we identified three *Pseudomonas* strains that were able to inhibit, in vitro, up to 80% of mycelial growth of the phytopathogenic fungus *Thielaviopsis ethacetica*, the causal agent of pineapple sett rot disease in sugarcane. Using GC/MS, we found that these bacteria produced 62 different VOCs, and further functional validation revealed compounds with high antagonistic activity to *T. ethacetica*. Transcriptomic analysis of the fungal response to VOCs indicated that these metabolites downregulated genes related to fungal central metabolism, such as those involved in carbohydrate metabolism. Interestingly, genes related to the DNA damage response were upregulated, and micro-FTIR analysis corroborated our hypothesis that VOCs triggered DNA damage. Electron microscopy analysis showed critical morphological changes in mycelia treated with VOCs. Altogether, these results indicated that VOCs hampered fungal growth and could lead to cell death. This study represents the first demonstration of the molecular mechanisms involved in the antagonism of sugarcane phytopathogens by VOCs and reinforces that VOCs can be a sustainable alternative for use in phytopathogen biocontrol.

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Introduction

The ascomycete fungus *Thielaviopsis ethacetica* (Teleomorph: *Ceratocystis ethacetica*) is a pathogen that is distributed worldwide and infects a wide range of hosts, causing economically important diseases in several agronomic crops, such as pineapple, coconut, palm, banana, sorghum, cocoa, corn and sugarcane (Hewajulige and Wijesundera, 2014). In Brazil, the fungus is the etiological agent of pineapple sett rot disease in sugarcane (*Saccharum* spp.) (Borges *et al*., 2019). Pineapple sett rot disease has a worldwide occurrence and has already been reported in the United States, South Africa, Philippines, China, Haiti, Dominican Republic, Cuba, Mexico and Colombia (Hewajulige and Wijesundera, 2014; Borges *et al*., 2019).

INTRODUCTION

The ascomycete fungus *Thielaviopsis ethacetica* (Teleomorph: *Ceratocystis ethacetica*) is a pathogen that is distributed worldwide and infects a wide range of hosts, causing economically important diseases in several agronomic crops, such as pineapple, coconut, palm, banana, sorghum, cocoa, corn and sugarcane (Hewajulige and Wijesundera, 2014). In Brazil, the fungus is the etiological agent of pineapple sett rot disease in sugarcane (*Saccharum* spp) (Borges *et al.*, 2019). Pineapple sett rot disease has a worldwide occurrence and has already been reported in the United States, South Africa, Philippines, China, Haiti, Dominican Republic, Cuba, Mexico and Colombia (Hewajulige and Wijesundera, 2014; Borges *et al.*, 2019).

Brazil is the world's largest producer of sugarcane, with a total area projected at 8.42 million hectares and an estimated productivity of 628 million tons for 2021/2022 (CONAB, 2021), making pineapple sett rot one of the most economically important diseases affecting sugarcane (Hewajulige and Wijesundera, 2014). It has become particularly important after harvest mechanization, since cutting of the sugarcane culm by blades causes injuries that facilitate fungal entry. The disease severely slows the germination of settings, seedling development and vigor, and sometimes it is necessary to replant sugarcane. It has been shown that pineapple sett rot can decrease the sugarcane yield by up to 42% (Pandey *et al.*, 2014; Borges *et al.*, 2019), causing a negative impact on the Brazilian economy, since the sugarcane production chain represented 2.4% of the agricultural Brazilian gross domestic product in 2020 (Kalaki *et al.*, 2016).

The treatment used to reduce the impacts caused by the disease typically consists of fast-growing varieties and chemical treatment with fungicides (Bhuiyan *et al.*, 2014; Borges *et al.*, 2019). Many studies have reported the efficiency of phenylpyridylamine and triazole-based fungicides in the control of pineapple sett rot (Bhuiyan *et al.*, 2014; Brandi *et al.*, 2018); however, these chemicals have a high cost, and continuous application may compromise their effectiveness due to the emergence of resistance that reduces disease control (Lucas *et al.*, 2015). In addition, and maybe more importantly, one of the crucial aspects for modern agriculture is to reduce the use of chemical fertilizers and pesticides, which generate negative impacts on human health and in the environment, harming soil health and fertility and causing water resource contamination and air pollution (de Souza *et al.*, 2015; Carvalho, 2017).

Therefore, investments in agricultural biotechnology are necessary, and plant growth-promoting bacteria (PGPB) play a fundamental role in revolutionizing agriculture. PGPBs have several mechanisms that promote plant growth and health (Ryu *et al.*, 2020; Silva Dias *et al.*, 2021); among them, we highlight the production of volatile organic compounds (VOCs), which are small molecules of a lipophilic nature derived from a wide range of biosynthetic pathways and have a low boiling point and high vapor pressure (Lemfack *et al.*, 2014).

VOCs are a blend of volatile metabolites that may be produced by all living microorganisms and are composed of heterogeneous chemical classes that include alcohols, ketones, thioesters, phenols and benzene derivatives (Tilocca *et al.*, 2020). Currently, it is possible to find the reported microbial VOCs through the *mVOC database*, which gathers information based on the literature for known compounds (Lemfack *et al.*, 2014, 2018). Approximately 2000 VOCs produced by approximately 300 bacteria and 600 fungi are registered in the database. Although these records are substantial, many more compounds may be identified in the future, given that only 10^3 microorganisms of 10^{16} that are expected to exist on Earth have been investigated thus far (Piechulla *et al.*, 2020).

Recently, bacterial VOCs have received increased attention as important signaling molecules for the biocontrol of phytopathogens and the promotion of plant growth. Due to its gaseous nature, an efficient distribution of volatiles through soil porosity may take place, allowing them to reach long distances. This phenomenon improves the effectiveness of the antagonistic action on target microorganisms, making VOCs excellent candidates for biocontrol (Peñuelas *et al.*, 2014; Schenkel *et al.*, 2019).

In this study, we identified 3 *Pseudomonas* emitters of relevant volatile signals that were able to inhibit the mycelial growth of *T. ethacetica* by up to 80%. We found that these bacterial isolates produced 62 VOCs, and further functional validation assays revealed that 2-ethyl,1-hexanol, 2-nonanol, 2-nonanone and 2-tridecanone inhibited *T. ethacetica* growth up to 70%. The different responses of this phytopathogenic fungus to VOCs highlight the incredible complexity of the mechanisms involved in these associations, and transcriptomic analysis revealed that exposure to bacterial VOCs downregulated genes related to fungal central metabolism, such as those involved in carbohydrate metabolism. Interestingly, genes related to the DNA damage response were upregulated in response to VOCs, a discovery that is

supported by a micro-FTIR analysis that also indicated DNA damage. Ultrastructural analysis of the pathogen by transmission and scanning electron microscopy showed morphological changes in cells, which may correspond to cell death. To the best of our knowledge, this study is a prime report of the molecular mechanisms involved in the interaction between VOCs and *T. ethacetica*. It also provides new information about the *Pseudomonas* mode of action in antagonism to phytopathogenic fungi. Hence, we envision our findings as relevant building blocks for the design of better strategies to prospect a biocontrol product as an eco-friendly alternative to agrochemicals.

EXPERIMENTAL PROCEDURES

Microorganisms and growth conditions

The phytopathogenic fungus *T. ethacetica* (received as *Ceratocystis paradoxa* MMBF 01/00) was kindly provided by the Phytopathological Mycology Laboratory of the Biological Institute of São Paulo. It was grown on potato dextrose agar (PDA) for antagonistic assays and on MEX medium (modified from de Paula *et al.* 2019 [30 $g.l^{-1}$ malt extract; 1 $g.l^{-1}$ peptone]) for VOC assays. The evaluated bacteria are part of the Brazilian Biorenewables National Laboratory – LNBR/CNPEM Microorganism Collection (Campinas, SP) and were previously isolated from the soil and roots of highly productive sugarcane-producing regions in Brazil. All bacteria were routinely grown on Luria-Bertani (LB) (Sambrook *et al*., 1989) medium at 28°C.

Phylogenetic analysis of *Thielaviopsis ethacetica*

Sequences of the ribosomal internal transcribed spacer (ITS) were recovered from the assembled transcriptome (see below) with ITSx v.1.1.3 (Bengtsson-Palme *et al.*, 2013). The coding sequences for translation elongation factor 1-alpha (TEF1-α) were identified using BLASTn v.2.7.1+ (Altschul *et al.*, 1990). Additional sequences of ITS and TEF1-α were obtained from Borges *et al*. (2019) for *Ceratocystis fimbriata* (outgroup) and several species in the genus *Thielaviopsis.* Sequences of the markers (noncoding: ITS1, 5.8S, ITS2; coding: TEF1-α) were individually aligned with MAFFT v.7.397 (Katoh and Standley, 2013). A supermatrix was generated with FASconCAT v.1.11 (Kück and Meusemann, 2010), and phylogenetic inference was carried out in IQ-TREE v.2 (Nguyen *et al.*, 2015) with partitions, employing the GRT + I + G model

for TEF1-α and GTR+G for ITS and 1000 bootstrap replicates. The final tree was edited on iTOL v.6 (Letunic and Bork, 2007).

In vitro antagonistic activity assays

For the initial screening, the effect of VOCs produced by 70 bacteria from different genera was evaluated in 2-compartment Petri dishes. *T. ethacetica* was grown in a Petri dish containing PDA medium and incubated in a growth chamber at 28°C until sporulation (approximately 5 days). One hundred microliters of a fresh bacterial suspension (~5x10^8 CFU) was spread in one compartment of the plate containing LB medium, and 20 µl of the spore suspension adjusted for 10^6 spores.ml^{-1} was dripped in the other compartment containing PDA. The plates were sealed with Parafilm® and incubated at 28°C for 2 days. Control plates were prepared without bacterial inoculum. This first screening was performed without replicates, and the diameter of the mycelial growth was measured after the incubation period. Afterwards, triplicate tests using the same methodology were carried out with the 10 isolates that had the greatest impact on the growth of *T. ethacetica*. The top 3 isolates were selected for the following tests.

The effects of 3 different culture media on the growth inhibition of *T. ethacetica* were evaluated: DYGS (Scheidt *et al.*, 2020), LB (both rich media), and ANGLE medium agar (Blom *et al.*, 2011). Three independent experiments were carried out following the methodology described above with 3 replicates. The effect of VOCs produced by different concentrations of the bacterial isolates was also determined. For this assay, 3 concentrations of bacterial inoculum were evaluated: 5x10^7 CFU, 2.5x 10^8 CFU and 1.25x 10^9 CFU; in the first 2, the bacterial inoculum was dripped on the plate, and in the last, it was spread. Three independent experiments were carried out with 3 replicates.

Statistical data analysis

Data obtained were analyzed with one-way ANOVA to determine if there were significant differences. The means and standard deviations were compared by Tukey's test using GraphPad Prism v.8.0.2 software (San Diego, USA). P values less than 0.05 were considered statistically significant.

Molecular identification of the selected bacterial isolates

The isolates were grown in liquid LB medium at 200 rpm and incubated at 28°C for 16 h. The cells were harvested, and genomic DNA was extracted using the phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) method described by Gomes *et al*. (2000). Amplification of the 16S DNA region was performed by PCR using the *Taq* DNA Polymerase enzyme (Invitrogen, Carlsbad, USA) and the oligonucleotides 20F (5 'GAGTTTGATCCTGGCTCAG 3') and 1500R (5 'GTTACCTTGTTACGACTT 3').

The amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. The purified 16S rRNA amplicons were sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit on a 3500 xL Genetic Analyzer (Applied Biosystems, California, USA). The following oligonucleotides were used for sequencing: 20F, 1500R, 520F (5´ CAGCAGCCGCGGTAATAC 3´), 520R (5´ GTATTACCGCGGCTGCTG 3´), 920F (5´ AAACTCAAATGAATTGACGG 3´) and 920R (5´ CCGTCAATTCATTTGAGTTT 3´) (Yukphan *et al.*, 2004). The contigs were assembled with Geneious v8.1.9 (Kearse *et al.*, 2012), and the sequences obtained were compared with the sequences available in the EzBioCloud database (Yoon *et al.*, 2017). The 16S rRNA gene sequences were deposited in NCBI Genbank under accession number OK274033-OK274035.

Bacterial VOCs identification by HS-SPME/GC-MS

Volatilome analysis of the antagonists was performed in the 3 tested media at the Metabolomics Facility (Labmet - https://lnbr.cnpem.br/instalacoes/metabolomica/) located at LNBR/CNPEM (Campinas, SP). Twenty microliters of bacterial suspension (10^8 CFU) of the selected isolates were inoculated in 20-ml flasks with a silicone septum and screw cap (Agilent Technologies, Santa Clara, USA) containing 5 ml of solid culture medium and grown for 72 h at 30°C in quintuplicate. For control samples, a flask containing only culture medium without bacterial inoculum was used. To extract the volatiles, a SPME fiber of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS Supelco®, Sigma Aldrich, St. Louis, USA) was inserted into the headspace of the vials and adsorbed at 50°C for 30 min. GC-MS analysis was performed using a 7890A gas chromatograph (Agilent Technologies) coupled to a

Pegasus® HT TOFMS mass spectrometer (LECO Corporation, St. Joseph, USA). Helium was used as the carrier gas and flowed at 1 $ml.min^{-1}$ through a DB5 apolar column (30 m x 0.25 mm x 0.25 µm) (Agilent Technologies). The SPME fiber was inserted into the injector and desorbed at 250°C for 5 min. The temperature of the column was set as follows: 30°C for 2 min, followed by a ramp of 20°C/min up to 300°C and holding for 2 min; a final ramp of 20°C/min up to 330°C, with a final hold for 30 s. The mass spectrometer was operated in electron ionization mode with collision ionizing energy at -70 eV, a source temperature of 250°C with continuous scanning of m/z in the range from 20-450, and an acquisition rate of 25 spectra per second. As an internal normalizer, 1 µl of cis-3-hexenyl acetate (1 $µg.µl^{-1}$), a green leaf volatile (Frost *et al.*, 2008; Farag *et al.*, 2017), was added to each flask.

Bacterial volatilome analysis

The data obtained in the GC-MS runs were analyzed using Leco-ChromaTOF v.5.088 software. Manual peak-to-peak curing was performed using the public library NIST v.17 Mass Spectrum Library and an in-house library built by the Metabolomics Facility of LNBR/CNPEM (Campinas, SP) comparing the fragmentation profile and retention time of the generated data with those of the libraries. The data were filtered by removing VOCs that were not present in at least 4 biological replicates and with an area at least twice as large in the samples as in the control. The peak areas were normalized by the cis-3-hexenyl marker. Statistical analysis was performed using the MetaboAnalyst 4.0 online tool (Chong *et al.*, 2019), log-transforming the data. Dendrograms (hierarchical clustering), principal component analysis (PCA) and heatmaps were performed to visually compare VOC profiles. Venn diagrams were made using the online tool from Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Functional validation of the identified bacterial VOCs

Thirteen compounds were selected for functional validation by using synthetic chemicals from Sigma-Aldrich Chemical Co. (Table S6). Ten microliters of a spore suspension of *T. ethacetica* at $1.3x10^6$ $spores.ml^{-1}$ was inoculated in vials containing 3 ml of liquid MEX medium. The vials were closed and incubated in an orbital shaker at

28°C and 200 rpm for 2 days. After this time, the mycelia from 3 vials were collected (13.000 g for 15 min at 4°C) to evaluate the dry weight before VOC treatment. Then, the synthetic compound (Table S6) was added at concentrations of 50 mM, 5 mM, 0.5 mM and 0.05 mM, and the vials were incubated in an orbital shaker at 28°C and 200 rpm for 2 more days. As controls, the fungus was grown without synthetic VOCs and the fungus with ethanol (15 µl), which was the solvent used for dilution of the compounds. The experiments were performed in triplicate, and the evaluations consisted of determining the dry weight of the mycelium. The weight of the mycelia collected before inoculation of the synthetic compounds were subtracted from these values.

Transcriptomic analysis experimental design

The effect of bacterial VOCs on *T. ethacetica* gene expression was analyzed by RNA-seq. The antagonistic bacterial isolate FBJ P5B2 was selected for this assay performed in 2-compartment Petri dishes and in 4 biological replicates. One hundred microliters of the bacterial suspension at a concentration of $5x10^8$ CFU.ml^{-1} was spread in one compartment containing LB medium, and 5 Eppendorf tubes (1.5 ml) containing liquid MEX medium with $2x10^5$ spores of *T. ethacetica* (final volume of 200 µl) were placed in the other compartment. For the control condition, the bacterial compartment was inoculated only with LB medium. The plates were sealed with Parafilm® and incubated at 28°C for 2 days. Then, mycelia were harvested by centrifugation at 13.000 g for 15 min at 4°C and stored at -80°C until RNA extraction.

RNA extraction and transcriptome sequencing (RNA-seq)

Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA) using the Qubit RNA HS Assay Kit, and RNA integrity was assessed using the Agilent RNA 6000 Nano kit on a 2100 Bioanalyzer (Agilent Technologies). Eight libraries (4 biological replicates per condition) were prepared using the TruSeq Stranded mRNA Sample Prep LS Kit (Illumina, San Diego, USA) according to the manufacturer's protocol, and the library quality was checked on the 2100 Bioanalyzer using the Agilent DNA 1000 Kit according to the manufacturer's protocol. Libraries were quantified using

a KAPA qPCR MasterMix kit (Roche, Basel, Switzerland) on the Viia7 Real-Time PCR System (Thermo Fischer Scientific), pooled at equimolar concentrations, and sequenced on an Illumina® HiSeq 2500 (2x 200 bp) available at the LNBR/CNPEM NGS facility. The RNA-seq data and gene annotation for *T. ethacetica* have been deposited in the NCBI Gene Expression Omnibus under GEO Series accession number GSE158776.

RNA-seq data analysis

A raw read quality check was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads and adapter sequences were removed using Trimmomatic v.036 with parameters of SLIDINGWINDOW:4:15 and MINLEN:50 (Bolger *et al.*, 2014). Filtered reads were mapped to the genome of *T. ethacetica* (GCA_001599055) available at the GenBank database using Hisat2 v.2.0.4 with the --rna-strandness RF parameter (Kim *et al.*, 2019). A GTF annotation file for the reference genome was automatically generated by BRAKER v. 1.9 (Hoff *et al.*, 2019) using the aligned reads as evidence. Transcript expression was estimated using the featureCounts command from the R package Rsubread v. 1.16.1 (Liao *et al.*, 2019) with BAM and GTF files generated in the previous steps as inputs. DESeq2 v. 1.6.3 was used to perform the differential expression analysis. Transcripts with an adjusted p-value <0.05 and log2-fold-change >1 or <-1 were considered differentially expressed. Gene Ontology (GO) terms were associated with the transcripts using the online tool PANNZER2 (Törönen *et al.*, 2018). Based on this GO annotation, the enrichment analysis of differentially expressed categories was performed using a hypergeometric test with the BiNGO tool (Maere *et al.*, 2005) using the whole transcriptome as a reference and a FDR ≤0.05 as the cutoff. Dotplots were generated in R using the clusterProfile package (Yu *et al.*, 2012).

RT-qPCR analysis of selected genes

Ten differentially expressed genes (DEGs), including virulence genes, CAZymes and other genes of interest, were selected for validation by RT-qPCR. Extracted RNA was treated with a TURBO DNA-free kit (Applied Biosystems), and SuperScript™ II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA

according to the manufacturer's instructions. Primers were designed with Primer3 (v. 0.4.0) (Koressaar and Remm, 2007), and the sequences are listed in Table S7. The relative standard curve method was used (Borin *et al.*, 2017). Reactions were performed in a final volume of 10 µl, with 5.0 µl of SYB Select Master Mix (Applied Biosystems), 2 pmol of each primer and 1.0 µl of cDNA or points of the standard curve. RT-qPCR reactions were carried out in triplicate as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Myosin regulatory light chain, casein kinase and mitochondrial genes, which showed lower coefficients of variation in the RNA-seq analyses, were used as internal standards for data normalization, which was done using the average of the 3 genes. Primer pairs with amplification efficiencies ≥90% and ≤110 and correlation coefficients (R^2) ≥0.99 were considered for analysis. Melting curves were generated and analyzed to confirm the specificity of each amplification reaction. Amplification, detection, and data analysis were performed using a Viia 7 Real Time PCR System and QuantStudio Real-Time PCR software v1.3 (Applied Biosystems).

Fourier transform infrared microspectroscopy (micro-FTIR) for the evaluation of *T. ethacetica* DNA integrity

For the micro-FTIR analysis, *T. ethacetica* was grown with the synthetic compounds 2-nonanol and 2-tridecanone. Vials with a silicone septum and screw cap containing 3 ml of MEX medium were inoculated with 10 µl of the spore suspension at $1.5x10^6$ spores.ml^{-1}, after which the synthetic compound was added at a final concentration of 0.5 mM and the vials were closed and incubated at 28°C and 200 rpm. Fungal growth without VOCs served as controls. Mycelia were collected after 2 days of growth using a toothpick. Hyphae were fixed overnight at 4°C with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2-7.4) with calcium chloride (3 mM). Next, the specimens were washed 3 times in sodium cacodylate buffer and dehydrated through a graded ethanol series (15%, 30%, 50%, 70%, 90% and 100%, 15 min in each concentration). The samples were then mounted on gold substrates of 5 x 5 mm and air-dried at room temperature.

Single-point micro-FTIR absorbance measurements, in reflection geometry, were carried out using an FTIR spectromicroscope (Cary 620, Agilent Technologies). The choice for reflectance mode FTIR was based on the samples that were deposited

on Au/Si substrates. As conventionally practiced in FTIR analysis, the measurements were produced by interferometry from a Michelson interferometer using a Globar source as the illumination source and a Mercury-Cadmium-Telluride (MCT) detector. In this setup, the IR light passes first through the interferometer and then is directed to the microscope, where it is focused on the sample surface using an objective lens. We used a 15´ objective lens that produced an ~700 ´ 700 μm^2 spot size on the sample surface. This objective lens collected the reflected light in a confocal arrangement and sent it to the MCT detector. Therefore, FTIR spectra were obtained by computing the FTs of the acquired interferograms. In our measurements, the spectral resolution was 16 $cm^{-1,}$ and the covered spectral range spanned from 6001.4 to 393.3 cm^{-1}. Each spectrum contained 728 points using a zero filling factor of 2 in the FT processing. All presented micro-FTIR absorbance spectra corresponded to the average of 64 single spectra. All spectra were normalized to the spectrum of a clean Au surface taken as the reference background.

Morphological characterization of *T. ethacetica* after VOC exposure

Changes in the external morphology and ultrastructure of *T. ethacetica* after exposure to the synthetic compounds 2-nonanol and 2-tridecanone at a final concentration of 0.5 mM were examined using scanning and transmission electron microscopy (SEM and TEM, respectively). *T. ethacetica* was grown with the synthetic compounds as performed for the FTIR analysis. As controls, fungal growth without VOCs and with ethanol, the solvent used for dilution of the compounds, were used. Fungi were collected after 2 days of growth using a toothpick to preserve the integrity of the mycelia.

For SEM analysis, fixation of the mycelia was performed with the standard protocol of 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and 3 mM $CaCl_2$ for 24 h at 4°C. Then, the samples were washed in the same buffer and resuspended in postfixation buffer (1% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4, 3 mM $CaCl_2$ and 0.8% potassium ferrocyanide) for 1 hour. The samples were then dehydrated in 100% alcohol dried at a critical point using exchanges of liquid CO_2, mounted on aluminum stubs and metallized in sputtering with a thin gold film. The samples were visualized using a QUANTA 650 FEG Scientific Scanning Electron Microscope

(Thermo Fisher) operating at an accelerating voltage of 5 kV at the Nanotechnology National Laboratory (LNNano) at CNPEM.

For the TEM analysis, the samples were fixed as described above for SEM analysis. After fixation, the samples were washed 3 times in 0.1 M sodium cacodylate and 3 mM $CaCl_2$ buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate, 3 mM $CaCl_2$ and 0.8% potassium ferrocyanide for 1 h. After washing, the samples were mounted in 1.5% agarose and *en bloc* stained with 2% uranyl acetate overnight at 4°C. Next, the blocks were dehydrated in ethanol and acetone at room temperature. The dehydrated cells were infiltrated in Epon resin. Blocks were placed in a vacuum oven at 60°C to be polymerized for 72 h. Ultrathin sections were cut with a Leica Ultracut microtome, stained with 2% uranyl acetate and Reynold's lead citrate and examined under a ZEISS Leo 906 transmission electron microscope at an accelerating voltage of 120 kV at Nanotechnology National Laboratory (LNNano) at CNPEM.

RESULTS AND DISCUSSION

Fungal isolate MMBF 01/00 belongs to the species *Thielaviopsis ethacetica*

Although the sugarcane pineapple sett rot has been known to be caused by *Ceratocystis paradoxa*, species reassignment of this etiological agent to *Thielaviopsis ethacetica* was recently performed (Borges *et al*., 2019). Thus, to ensure the species placement of the isolate used in our experiments, a phylogenetic analysis was performed. The complete full length of TEF1-α and rRNA sequence was recovered from a *de novo* assembly of the transcriptomic data. The rRNA sequence encompass the small subunit ribosomal RNA gene; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 sequence; and large subunit ribosomal RNA gene. A high read coverage was obtained for both rRNA and TEF1-α transcripts (mean coverage of 54,032 and 25,769 respectively). The phylogenetic tree of the concatenated sequences, including the sequences of closely related species from a previous publication (Borges *et al*. 2019), showed that our strain belonged to *T. ethacetica* (Fig. 1).

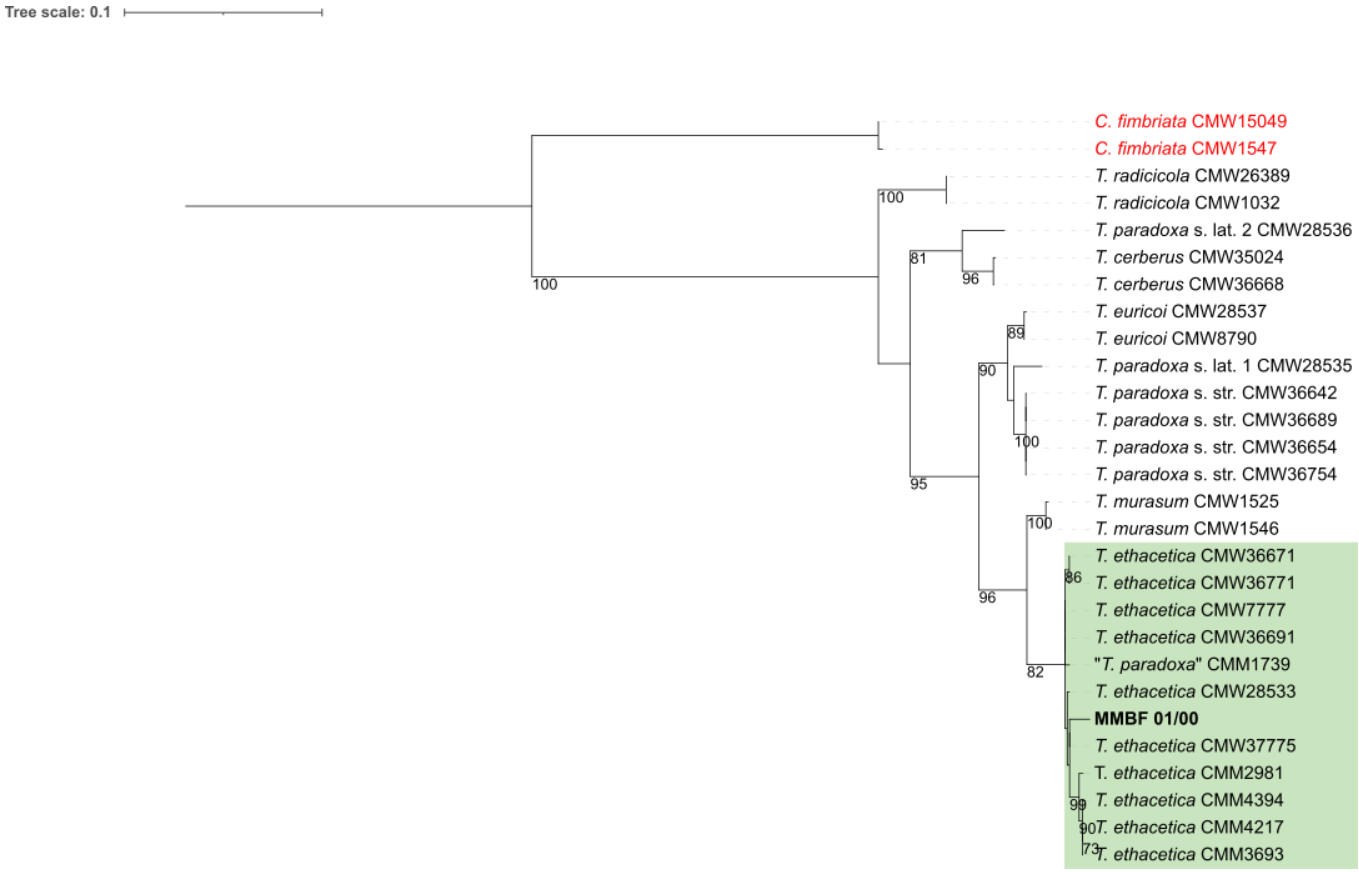


Fig. 1. Maximum likelihood phylogenetic tree of *Thielaviopsis ethacetica* strains, including the strain used in the present study (highlighted in bold), based on rRNA (internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2; and large subunit ribosomal RNA gene) and TEF1-α. Support values are based on 1000 bootstrap replicates (values above 70 are shown). *Ceratocystis fimbriata* (red label) was used as the outgroup species. The scale bar represents substitutions of 0.1 per site. The tree was visualized using iTol v6 software (Letunic and Bork, 2007).

Selected bacteria produce volatiles with antifungal activity on *T. ethacetica*

Aiming to identify VOC-producing bacteria with inhibitory activity against the growth of the fungus *T. ethacetica,* an initial screening of 70 bacterial isolates was performed. From this set, the top 10 most promising bacteria were selected for triplicate tests by quantitative analysis measuring the diameter of the mycelium (Fig. 2A). The best 3 isolates were selected for further experiments. The antagonistic bacteria FBJ P5B2 and ITA P2F2 showed a growth inhibition of *T. ethacetica* of approximately 80% (Fig. 2A), followed by BNG P1D2 at 70% compared with the control (without the bacterial inoculum).

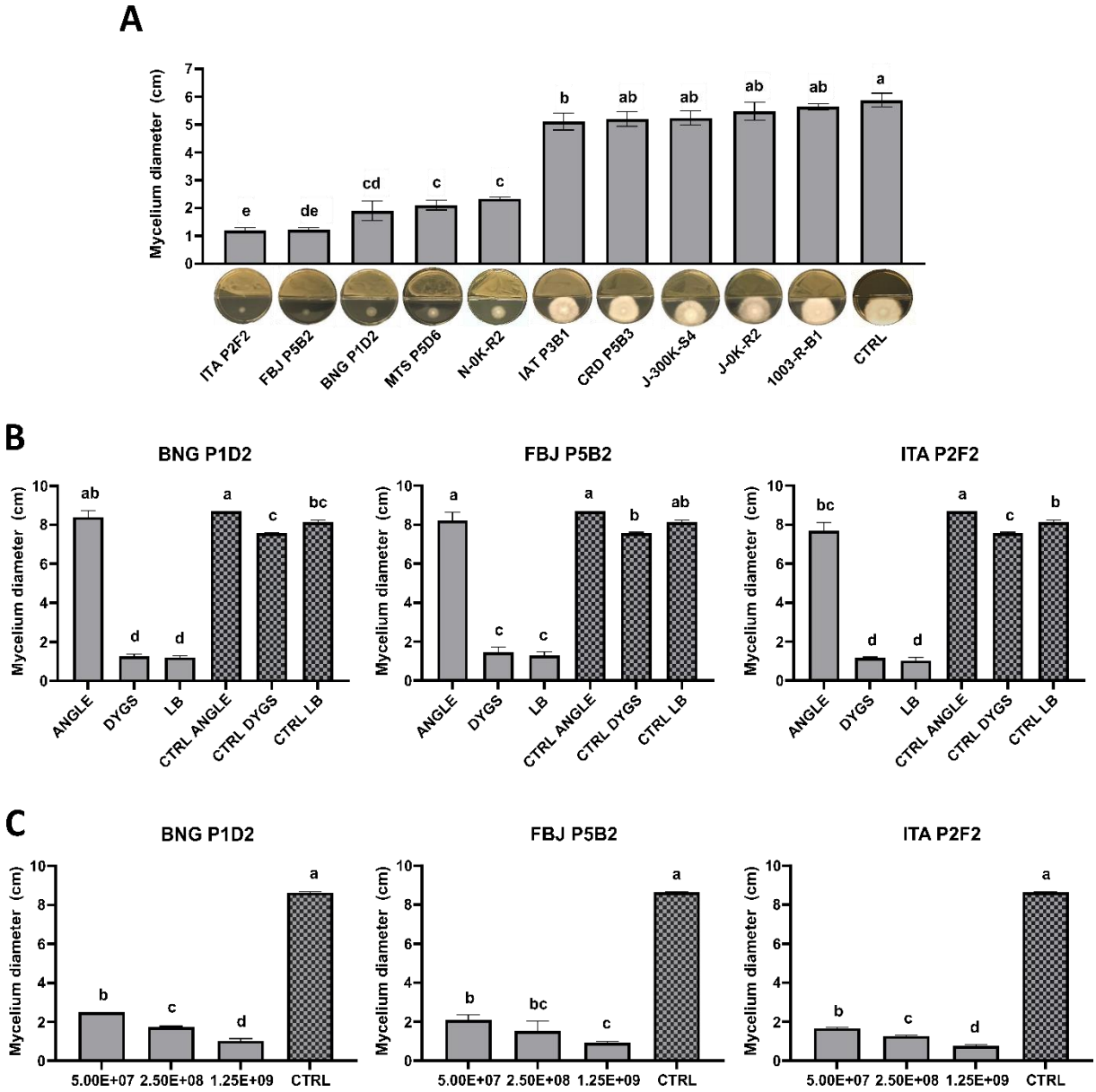


Fig. 2. Effect of bacterial VOCs on *T. ethacetica* growth inhibition after 2 days of cocultivation. (A) Inhibitory effect of bacterial VOCs from 10 selected isolates on the growth of *T. ethacetica* mycelium. (B) Inhibitory effect of bacterial VOCs in 3 different culture media: ANGLE, DYGS and LB. (C) Inhibitory effect of bacterial VOCs in different concentrations of the bacterial inoculum: $5x10^7$ CFU.ml^{-1}, $2.5x10^8$ CFU.ml^{-1} and $1.25x10^9$ CFU.ml^{-1}. Control (CTRL) corresponds to *T. ethacetica* without the bacterial inoculum. Different letters indicate significant differences between treatments. Standard errors are indicated by vertical lines (Tukey's test, p-value <0.0001).

Some conditions, such as culture media, temperature, pH, interactions with other organisms, stage of growth, and others, affect the composition of the volatilome

(Kai *et al.*, 2009; Weisskopf *et al.*, 2021). To assess differences in the VOC emission profile and to evaluate whether this could affect the antagonistic effect against *T. ethacetica*, the selected bacteria were tested for their ability to emit antifungal VOCs in 3 culture media with different nutritional compositions (Fig. 2B). In the DYGS and LB nutrient-rich media, the 3 antagonists inhibited *T. ethacetica* growth up to 80%. However, in the nutrient-poor ANGLE medium, only the ITA P2F2 isolate showed slight antifungal activity of approximately 12%, as shown in Fig. 2B. These results indicated a predisposition for greater antifungal activity when antagonistic bacteria were cultivated in nutrient-rich media. The nutrient-poor ANGLE medium limited the growth of antagonist bacteria, thus compromising the ability to produce VOCs and consequently decreasing the antagonist effect of the isolates.

In screening (Fig. 2A) and media (Fig. 2B) experiments, an inoculum concentration of $5x10^8$ $CFU.ml^{-1}$ was spread on the plate, and to evaluate a possible dose-response effect, different concentrations were tested (Fig. 2C). We observed a correlation between the inhibition rate and the inoculum concentration; the greater the bacterial concentration, the greater was the inhibitory effect, as shown in Fig. 2C. The 3 isolates were able to inhibit phytopathogen mycelial growth by up to 80%. Taken together, these results demonstrated that the culture medium nutrients and bacterial inoculum concentration were determining factors in the bacterial volatilome.

Selected antagonistic bacteria belong to the *Pseudomonas* genus

To further investigate the *T. ethacetica* antagonist VOC-producing bacterial isolates, we aimed to assign their identification. It was performed by comparison to the closest type strain using the EzBioCloud database. The 3 selected bacterial isolates were assigned to the *Pseudomonas* genus, probably to different species. The bacterial isolated BNG P1D2 exhibited the highest similarity to *Pseudomonas moorei* RW10(T) (99.10% sequence identity), followed by *P. batumici* UCM B-321(T)YY (98.90%) and *P. umsongensis* DSM 16611(T) (98.77%). The sequence of isolate FBJ P5B2 was 98.73% similar to that of *P. soli* F-279,208(T), followed by *P. batumici* UCM B-321(T) (98.56%) and *P. moorei* RW10(T) (98.54%). The 16S rRNA gene sequence of ITA P2F2 was 99.93% similar to that of *P. kribbensis* 46-2(T), followed by *P. glycinae* MS586(T) (99.93%) and *P. atacamensis* M7D1(T) (99.73%).

Pseudomonas are Gram-negative bacteria that belong to the Proteobacteria phylum and Gammaproteobacteria class, and they are widely reported in the literature for producing VOCs with biocontrol activity and the ability to promote plant growth (Hernández-León *et al.*, 2015). This genus comprises one-third of all bacteria listed in the mVOC 2.0 database (Lemfack *et al.*, 2020). Several works have reported the *in vitro* action of VOCs produced by *Pseudomonas* in the antagonism of phytopathogenic fungi such as *Botrytis cinerea* (Di Francesco *et al.*, 2015), *Fusarium* sp. (Ossowicki *et al.*, 2017), *Sclerotinia sclerotiorum* (Vespermann *et al.*, 2007) and *Verticillium dahliae* (Vespermann *et al.*, 2007; Ossowicki *et al.*, 2017), as well as *in vivo* biocontrol of *Monilinia fructicola* and *M. fructigena* others (Aiello *et al.*, 2019). Our data reinforces the biotechnological potential of *Pseudomonas*.

Volatilomes are species-specific and differ according to nutrient availability for bacterial growth

The volatilome profile of the *Pseudomonas* bacterial strains was analyzed by HS-SPME/GC-MS. Although we have identified a total of 91 compounds, we considered only the 62 VOCs found in mVOCs 2.0 (Table S1). In total, 39, 35 and 42 VOCs could be detected in the volatilomes of BNG P1D2, FBJ P5B2 and ITA P2F2, respectively. These compounds comprise several chemical classes, including alcohols, ketones, benzenoids, alkenes, and others (Table S1). The approach used herein allowed us to identify a greater number of compounds produced by our isolates compared with the literature data, which shows how VOC identification can vary between studies and between bacterial species (Hernández-León *et al.*, 2015).

Hierarchical clustering analysis (HCA) and PCA were used to show the discrimination among the volatilomes of the bacterial isolates in the evaluated media (Fig. 3A-C, Table S2). As expected, the replicates were grouped together, demonstrating data robustness. The volatilome profiles of all antagonists were fairly different, but BNG P1D2 and FBJ P5B2 were more similar between each other than the volatilome of ITA P2F2 in all media evaluated.

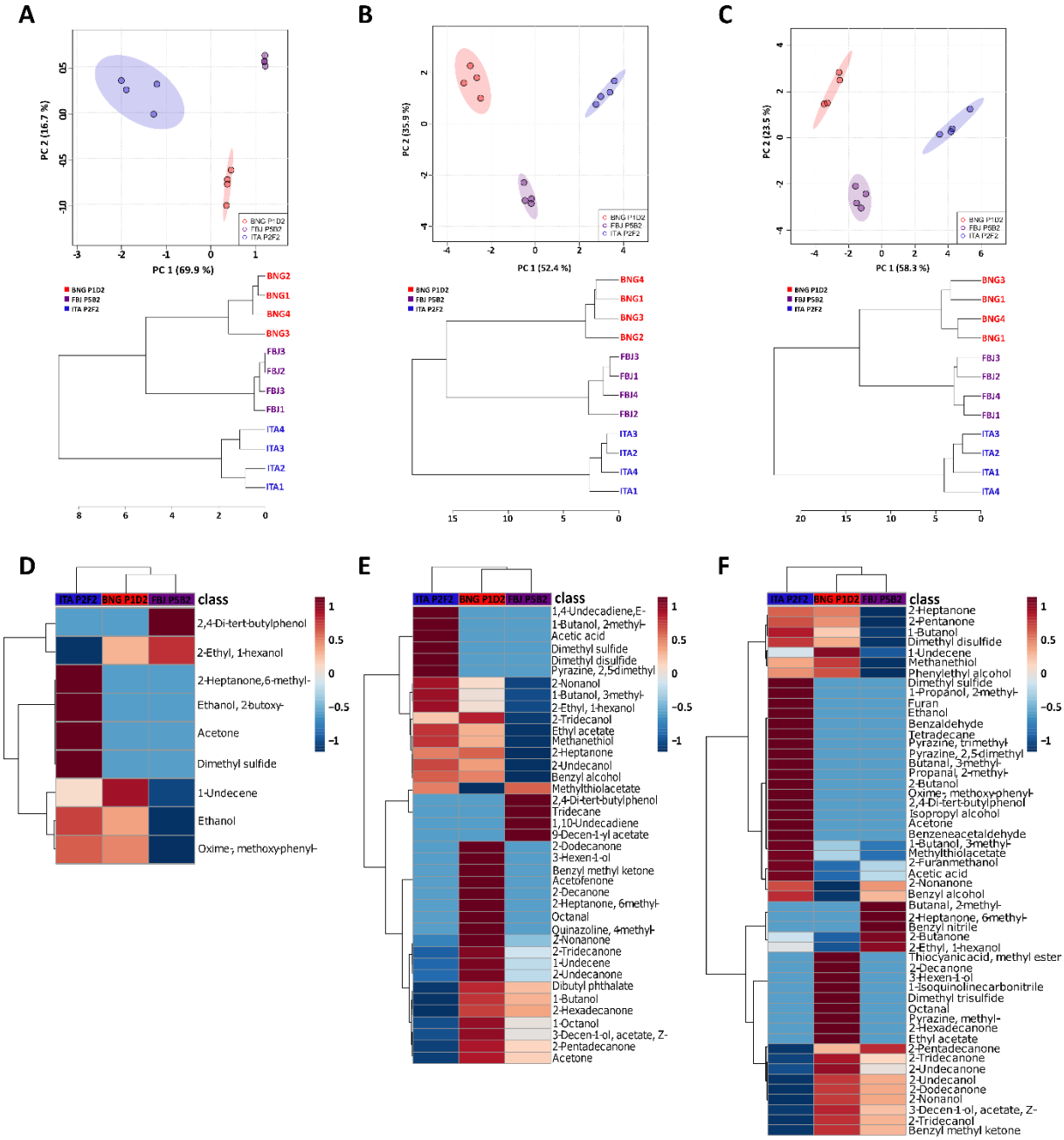


Fig. 3. Analysis of the volatilomes produced by the bacterial isolates BNG P1D2, FBJ P5B2 and ITA P2F2 in ANGLE, DYGS and LB culture media. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) based on the Euclidean distance of the compounds identified in the volatilome of the grown isolates in (A) ANGLE, (B) DYGS, and (C) LB. Heatmap of the compounds identified in the volatilome of the isolates grown in (D) ANGLE, (E) DYGS, and (F) LB. Columns represent each bacterial isolate. Rows represent the different VOCs evaluated in at least 4 replicates. The color code indicates the abundance of each compound (the values refer to the average peak areas normalized by cis-3-Hexenyl acetate: blue, low abundance; red, high abundance).

In addition, a heatmap was generated to show the variations in abundance of volatile compounds produced by the antagonist bacteria in the 3 media. In this analysis, clear differences in VOC profiles were observed. For instance, in nutrient-rich LB and DYGS media (Fig. 3D-F), a greater number of VOCs were identified: 52 and 39, respectively. In the nutrient-poor ANGLE medium (Fig. 3D), only 9 compounds were identified. Regarding the VOCs that were produced in specific culture media, LB was prominent, with 16 compounds produced exclusively in that medium, followed by DYGS with 9 and ANGLE with only one compound, reinforcing that the culture medium is critical for the composition of the volatilome of antagonists, as previously shown in the media assay results (Fig. 2B) and as reported previously (Gotor-Vila *et al.*, 2017; Huang *et al.*, 2018). It is worth to mention that the production of these compounds can be different during the interaction with other microorganisms (Ebadzadsahrai *et al*., 2020; Sánchez-Fernández *et al*., 2016).

The largest number of compounds was identified in the volatilome of ITA P2F2, with 42 compounds, of which 15 were exclusively produced by this isolate (Fig. S1). Furthermore, BNG P1D2 produced 39 VOCs (9 were exclusive), and FBJ P5B2 produced 35 VOCs (5 exclusive) (Fig. S1). Interestingly, from the 21 compounds commonly produced by the 3 isolates (Fig. S1), some have already been validated in literature for biocontrol activity such as 2-ethyl,1-hexanol that was previously reported to be bioactive against *P. capsici* (Syed-ab-rahman *et al.*, 2019) and *C. acutatum* (Che *et al.*, 2017); 1-undecene that was shown to inhibit the growth *of Pleutorus eryngii*, *P. ostreatus* (Lo Cantore *et al.*, 2015) and *P. infestans* (Hunziker *et al.*, 2015), and 2-nonanone that was validated as a bioactive compound against *F. oxysporum* (Wu *et al.*, 2019) and *S. sclerotium* (Giorgio *et al.*, 2015).

In addition to the VOCs, hydrogen cyanide (HCN), a volatile inorganic compound with great importance as an antimicrobial agent, was also identified in the volatilome produced by the isolates BNG P1D2 and FBJ P5B2 grown in DYGS and LB media (data not shown). Although it is not an organic compound, HCN is also a metabolite of interest since it has been reported in several studies as a key compound in biocontrol activity produced by many *Pseudomonas* species (Blom *et al.*, 2011; Ossowicki *et al.*, 2017; Anand *et al.*, 2020).

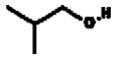
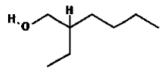
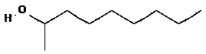
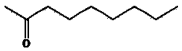
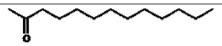
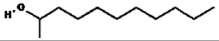
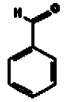
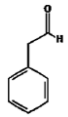
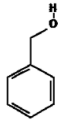
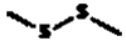
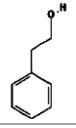
Different chemical class compounds have high antagonistic activity on *T. ethacetica*

To further investigate the volatile-mediated antifungal activity, 13 compounds of several chemical classes (Table 1) at 4 concentrations (50 mM, 5 mM, 0.5 mM and 0.05 mM) were evaluated. Some compounds at several concentrations showed negative values after the weight of the mycelia collected before inoculation of the synthetic compounds were subtracted (see Experimental Procedures). We believe that this occurred because these samples had cell lysis, and consequent released the cellular content into de liquid medium (not recovery by centrifugation at 13.000 g for 15 min at 4°C). Transmission microscopy analyzes showed membrane lysis and cell death in the samples treated with synthetic volatile, which confirms our hypothesis (see below).

The compounds 2-nonanone, 2-ethyl,1-hexanol, 2-nonanol, 2-tridecanone, dimethyl trisulfide, benzeneacetaldehyde, benzaldehyde, 2-undecanol, dimethyl disulfide and 1-undecene showed the greatest antagonistic activity at a concentration of 5 mM (Table 1). We obtained remarkable results when compared to data from the literature, especially when comparing the quantities of the compound used. For example, Fernando *et al.* (2005) showed complete inhibition of the fungus *S. sclerotiorum* using 100 µl and 150 µl of the pure compounds dimethyl trisulfide and 2-ethyl,1-hexanol, while we observed fungus death using 15 µl (final concentration of 5 mM) of the same compounds. We also focused on evaluating compounds with antagonistic activity even at low concentrations, causing fungal death at a concentration of 0.5 mM, such as 2-nonanol, 2-nonanone, 2-undecanol, and dimethyl trisulfide.

In general, we expected to observe greater antagonism when larger concentrations of each compound were used, similar to the findings of in assays using antagonist bacteria. This effect was observed in most compounds. However, interestingly, we observed that 2-ethyl, 1-hexanol, 2-nonanol, 2-nonanone and 2-tridecanone had a better inhibitory effect at an intermediate final concentration of 5 mM. To the best of our knowledge, there is no report about the antifungal activity of 2-tridecanone, but antibacterial properties have already been reported against *Ralstonia solanacearum* (Raza *et al.*, 2016a). In addition, 2-nonanol has not yet been reported to be bioactive against phytopathogenic fungi; however, Cheng *et al*. (2017) reported its nematicidal activity against *Meloidogyne incognita*.

Table 1: Inhibitory effects of different chemical classes and concentrations of synthetic VOCs on *T. ethacetica* mycelial growth.

						Mycelium weight after VOCs treatment in different concentrations[a]				
Volatile compound	**Class[b]**	**CAS no.**	**Molecular formula**	**Structural formula**	**Molecular weight**	**50 mM**	**5 mM**	**0.5 mM**	**0.05 mM**	**CTRL[c]**
1-propanol, 2-methyl	Alcohols	78-83-1	$C_4H_{10}O$		74.123	4.16± 1.72	3.13±0.97	5.34±1.52	4.25±1.02	5.32±0.76
1-undecene	Alkenes	821-95-4	$C_{11}H_{22}$		154.297	-1.80±0.31	-0.88±0.03	1.56±0.60	3.37±0.74	5.32±0.76
2-ethyl, 1-hexanol	Alcohols	104-76-7	$C_8H_{18}O$		130.231	-0.90±0.12	-4.38±0.29	1.55±0.56	1.60±0.32	5.32±0.76
2-nonanol	Alcohols	628-99-9	$C_9H_{20}O$		144.258	-2.66±0.88	-4.09±0.26	-1.68±0.64	0.21±0.12	5.32±0.76
2-nonanone	Ketones	821-55-6	$C_9H_{18}O$		142.242	-0.79± 0.10	-4.49±0.75	-1.73±0.31	0.93±0.30	5.32±0.76
2-tridecanone	Ketones	593-08-8	$C_{13}H_{26}O$		198.35	-4.54±0.28	-4.09±0.15	-1.72±0.42	1.23±0.45	5.32±0.76
2-undecanol	Alcohols	1653-30-1	$C_{11}H_{24}O$		172.312	-3.35±0.56	-2.20±0.91	-1.89±0.55	2.10±0.32	5.32±0.76
Benzaldehyde	Benzenoids	100-52-7	C_7H_6O		106.124	-2.94±0.35	-2.25±0.13	1.43±0.13	3.55±0.22	5.32±0.76
Benzeneacetaldehyde	Benzenoids	122-78-1	C_8H_8O		120.151	-3.32±0.62	-2.55±0.11	2.03±0.03	3.51±0.23	5.32±0.76
Benzyl alcohol	Benzenoids	100-51-6	C_7H_8O		108.14	-2.98±0.72	5.20±0.63	4.05±0.28	3.67±0.70	5.32±0.76
Dimethyl disulfide	Sulfides	624-92-0	$C_2H_6S_2$		94.19	-2.05±0.32	-1.15±0.47	4.75±1.18	5.77±0.26	5.32±0.76
Dimethyl trisulfide	Sulfides	3658-80-8	$C_2H_6S_3$		126.25	-	-2.94±0.94	-1.50±0.36	2.95±.050	5.32±0.76
Phenylethyl alcohol	Benzenoids	60-12-8	$C_8H_{10}O$		122.167	-3.14±0.20	0.86±0.25	4± 1.13	6.74±0.88	5.32±0.76

- Means that the compound has not been tested at this concentration.
[a]Average of mycelium dry weight values normalized by the "zero point" collected before the VOCs inoculation ± the standard deviation.
[b]According to first chemical classification provided by the mVOC 2.0 database (Lemfack *et al.*, 2018).
[c]Corresponds to *T. ethacetica* without synthetic compound. - Means that the compound has not been tested at this concentration.

Aiming to investigate the possible synergism between the VOCs, we tested artificial mixtures of synthetic compounds (2-nonanone, 2-tridecanone, 2-ethyl,1-hexanol, 1-undecene and benzaldehyde); however, a significant synergistic effect could not be observed (File S1). Although further studies are required to evaluate the effectiveness of these compounds *in vivo*, our results showed that the compounds identified in this study possessed high antagonistic activity even at low concentrations. It is important to point out that the technologies used to evaluate microbial volatiles may not measure the actual concentration of a given emitted compound in nature, and when we consider the cell-cell signaling in nature (Schmidt *et al*., 2016; Weisskopf *et al*., 2021), the concentrations used in this study may be higher. Thus, it is difficult to set the proper ranges of concentrations that are representative of the natural conditions when using pure compounds for biological activities. But we have shown here compounds that present potential for the control of *T. ethacetica*.

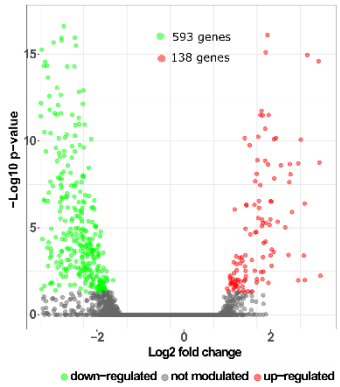
It is also crucial to emphasize that, although the ecological function of VOCs has not yet been fully unraveled, the compounds evaluated in the present study may also have effects on plant metabolism. For example, Fincheira *et al*. (2016) reported the ability of 2-nonanone and 2-tridecanone to stimulate seed germination of *Lactuca sativa*. Moreover, some compounds showed ambiguous effects according to the plant studied and VOC concentration. Dimethyl disulfide promoted tomato and *Nicotiana attenuata* growth (Meldau *et al*., 2013), but it also caused growth inhibition of *Arabidopsis thaliana* (Kai and Piechulla, 2009). In addition, postharvest studies showed that benzaldehyde can cause toxic effects in grape, but this phytotoxicity was not observed in apples (Calvo *et al*. 2020). These reports show the different responses that these VOCs can trigger on plants and reinforce the need of deep studies to understand how these compounds can act on the target as well as non-target organisms.

VOCs affect critical metabolic pathways of *T. ethacetica*

To further investigate the mechanisms involved in the inhibition of *T. ethacetica* growth and the alterations caused by bacterial VOCs in the gene expression of this important phytopathogen, RNA-seq analysis was performed. Transcriptome sequencing of *T. ethacetica* grown under contrasting conditions (exposed and not exposed to volatiles produced by FBJ P5B2), resulted in a total of 8 samples, yielding

68 Gb and 171 million paired-end reads. First, we annotated the genome of *T. ethacetica* JCM 6961, and from the 6843 annotated genes, 677 were differentially expressed (Fig. 4A), with 138 upregulated and 539 downregulated when the fungus was exposed to VOCs (Table S3). Ten genes from the RNA-seq data were selected for further validation by qPCR, and the results showed that the expression patterns of the selected genes were highly correlated (Fig. S2).

A



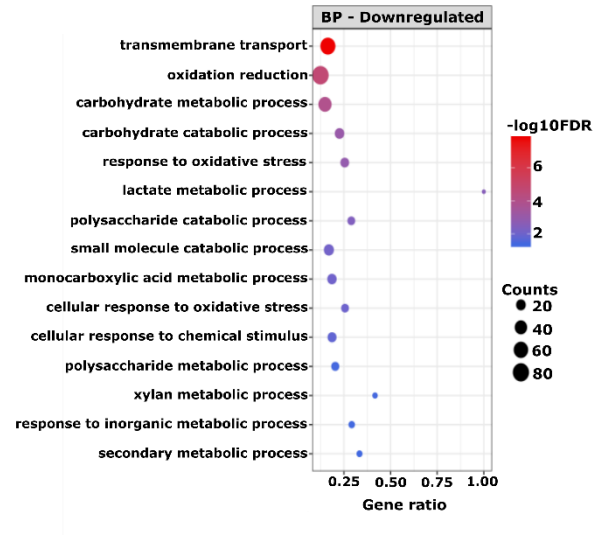
B

Genes of interest

Gene ID	Gene name	Description[1]	log_2FC^*
g4789.t1	npp1	necrosis inducing protein	-6,590623138
g793.t1	ste3	pheromone A receptor	-5,586778132
g5841.t1	katE	catalase	-3,630585059
g4397.t1	sod2	superoxide dismutase, Fe-Mn family [EC:1.15.1.1]	-3,390458186
g6550.t1	sod2	superoxide dismutase, Fe-Mn family [EC:1.15.1.1]	-3,178857519
g3293.t1	map3k9, mlk1	mitogen-activated protein kinase kinase kinase 9 [EC:2.7.11.25]	-1,480338739
g6888.t1	hypT, qseD	hypochlorite-specific transcription factor HypT	5,651648537

[1] The KEGG Orthology And Links Annotation (KOALA) and PFAM was used to description.
*The color gradient shows the gene expression values. Dark blue color have the smallest values of log_2FC and red color have the biggest values of log_2FC.

C



D

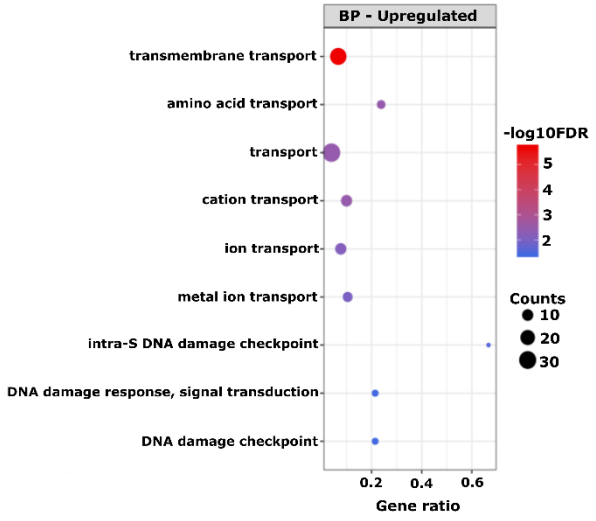


Fig. 4. Global transcriptomic responses of *T. ethacetica* to VOCs. (A) Volcano plot of DEGs between *T. ethacetica* exposed and not exposed to VOCs produced by FBJ P5B2. The X- and y-axis represent log2-fold-change differences between the compared samples, and statistical significance is expressed as the negative log10 of adjusted p-values. The significantly downregulated and upregulated genes are indicated by green and red dots, respectively, while nonregulated genes are shown as gray dots. (B) Genes of interest that were differentially expressed in *T. ethacetica*. (C) Dot plot of the GO term enrichment analysis with the downregulated GO terms (FDR <0.05) and (D) upregulated GO terms (FDR <0.05) for biological processes. The size of the dot is based on the gene count enriched in the pathway, and the color of the dot shows the pathway enrichment significance.

Differentially expressed genes related to fungal virulence or fungal growth and development were identified (Fig. 4B). Most of these genes were downregulated in the presence of VOCs, such as *npp1*, which refers to a group of proteins identified by the

presence of a common necrosis-inducing *Phytophthora* protein (NPP1) domain (Fellbrich *et al.*, 2000; Zhou *et al.*, 2012) named NLPs (Nep1-like proteins). NLPs can act as cytolytic toxins inducing cell death or noncytotoxic proteins that can trigger immune response in plants (Seidl and Van Den Ackerveken, 2019). Many species encoding NLP genes are plant pathogens, and although their function is not clear, there is evidence that they can act as positive virulence factors in plant–pathogen interactions (Zhou *et al.*, 2012; Seidl and Van Den Ackerveken, 2019).

Genes encoding catalase (*katE*) and superoxide dismutase (*sod*) were also downregulated. These proteins are antioxidant enzymes involved in the protection of cells against damage by reactive oxygen species (ROS), a defense mechanism of plants against the invasion of pathogens (Garbeva *et al.*, 2014; Raza *et al.*, 2016a, 2016b;). However, it is known that VOCs trigger an intracellular accumulation of ROS, which, in excess, disturbs the redox state of the hyphae by disrupting the detoxification mechanisms (Vázquez *et al.*, 2019). The downregulation of these genes is indicative that the activity of these enzymes may have been reduced and consequently hampered oxidative stress regulation. In this scenario, a decrease by almost 60% in superoxide dismutase and catalase was observed by Raza *et al.* (2016b) when the pathogen *R. solanacearum* was exposed to VOCs produced by *Bacillus amyloliquefaciens* and *P. fluorescens* WR-1 (Raza *et al.*, 2016c).

Still related to ROS, we observed an upregulation of the hypochlorite-responsive transcription factor (*hypT*). It is activated through the oxidation of methionine residues and regulates the expression of several genes in response to hypochlorite (HOCl), a ROS with strong antimicrobial activity and oxidizing potential (Drazic *et al.*, 2013).

We also found that a gene encoding MAP kinase (*map3k9, mlk1*) was downregulated. In fungi, MAP kinase pathways are key mediators of signaling, involved in cell physiology, morphogenesis, virulence, stress response, mating, and others (Takano *et al.*, 2000; Kojima *et al.*, 2004; González-Rubio *et al.*, 2019). Curiously, we observed a downregulation of a gene encoding the pheromone receptor (*ste3*) involved in the MAP kinase cascade related to the pheromone response pathway described in *S. cerevisiae* (Kronstad *et al.*, 1998; Zhao *et al.*, 2007). In ascomycetes, *ste3* activates a signaling cascade during mating, leading to chemotropic growth toward the mating partner and it is related to the regulation of conidial germination in a cell density-dependent manner (Vitale *et al.* 2019).Therefore, this study is important to provide information about the transcriptome of this pathogen

in response to antagonism by VOCs, and no studies to date have analyzed the function of these genes in *T. ethacetica*.

In general, phytopathogenic fungi secrete several enzymes to degrade plant cell walls, which are related to virulence (Lyu *et al.*, 2015; Ma *et al.*, 2019), and to use monosaccharides derived from this hydrolytic action as source of energy and nutrients (Toth and Birch, 2005; Lagaert *et al.*, 2009). The enzymes related to the modeling and degradation of cell wall components are named carbohydrate-active enzymes (CAZymes). In our study, 59 genes encoding CAZymes were found in the transcriptome analysis, most of which were downregulated (51 genes), and 31 proteins had a predicted signal peptide, indicating an extracellular secretion (Table S4). Representative genes of the 3 GH families of important enzymes that work for the degradation of plant cell wall were downregulated in the presence of VOCs: GH3 (beta-glucosidase), GH10 (endo-1,4-beta-xylanase) and GH7 (endo-β-1,4-glucanase).

Interestingly, GH16 and GH 17 were upregulated and these CAZymes were already reported as important to cell morphogenesis and integrity (Millet *et al*., 2018; Ribeiro *et al*., 2019). In addition, we also identified one AA9 upregulated, and the function of this enzyme is, in general, associated with cell wall breakdown. We have analyzed this sequence more carefully, and it showed a high identify (58.25%) with the AA9 of *Collariella virescens* (*Cv*AA9A) (Simmons *et al*., 2017). Curiously, this enzyme was active on mixed-linkage glucan, and β-glucans is an essential component of cell wall, in which GH16 and GH17 act (Latgé, 2010). We can hypothesize that the *T. ethacetica* AA9 (and others closest related), even with a not yet described role in the integrity of fungal cell wall, could be acting in synergy with GH16 and GH17 as an attempt by the fungus to repair the cellular damage caused by VOCs (we will discuss it in more detail further).

Additionally, a gene ontology (GO) term enrichment analysis of these differentially expressed genes was performed. A total of 59 gene categories were enriched between the control and VOC-treated samples, of which 28 were downregulated and 31 were upregulated (Table S5). The biological process (BP) analysis revealed a general downregulation of genes related to transmembrane transport, carbohydrate metabolism, response to oxidative stress, cellular response to chemical stimulus and secondary metabolism (Fig. 4C), most of which were crucial for fungal growth. The metabolism of carbohydrates, part of the primary metabolism, is responsible for the synthesis of essential compounds for fungal survival, whereas

transmembrane transport has a regulatory function of the movement of substances into the cell and is directly related to the response to oxidative stress (Davis *et al.*, 2010; Sailer *et al.*, 2018).

To our knowledge, however, no studies have investigated the global transcriptomic responses of phytopathogens to VOCs. Thus, we correlated our results to proteomics studies that showed changes in the regulation of proteins related to metabolism, transmembrane transport, and response to oxidative stress after exposure to VOCs. In accordance with our findings, Raza *et al.* (2016a) reported changes in the expression of *R. solanacearum* proteins involved in antioxidant activity and carbohydrate metabolism that were downregulated after exposure to VOCs produced by *P. fluorescens.* Studies have revealed that VOCs from *S. cerevisiae* decrease the fluidity of the membrane and increase the permeability of ions, generating an accumulation of harmful levels of reactive oxygen species and causing cell disruption in *P. citricarpa* (Fialho *et al.*, 2014). The effect of sulfur-containing VOCs has been reported by Chinchilla *et al.* (2019) when dimethyl disulfide, dimethyl trisulfide and S-methyl methane thiosulfonate induced specific changes in the proteome of *P. infestans*, such as in the translational machinery or perturbation of many important steps of sulfur metabolism, including the redox balance, suggesting multitarget modes of action of different VOCs.

The interaction between the antagonist bacteria and *T. ethacetica* was also characterized by an upregulation of genes mostly encoding proteins involved in transmembrane transport, ion transport and, more intriguingly, DNA damage pathways (Fig. 4D), a protective mechanism that guarantees maintenance of the integrity of the genome, that can occur spontaneously or be induced by external factors (Malavazi *et al.*, 2006; Vítor *et al.*, 2020). We identified 3 genes (*mre11*, *rad50* and *nbs1*) that form the MRN complex, highly conserved from bacteria to humans (Lavin, 2007), which play an crucial role in double-strand DNA (DSB) repair (D'Amours and Jackson, 2002; Uziel *et al.*, 2003), homologous recombination and nonhomologous DNA end-joining (Haber, 1998), correcting erroneous insertions, deletions, and the misincorporation of bases during DNA replication and recombination to prevent mutations (Kunkel and Erie, 2005; Li, 2008). The upregulation of these genes was validated by RT-qPCR (Fig. S2).We also observed that ion transport classes were enriched in the transcriptome, and it is important to highlight that metal ions play a critical role in stabilizing conformations, especially in the double helix structure of DNA (Cardin, 2019). Metal

ions also play an important role in the MRN complex (Lee and Paull, 2005; Nicolette *et al.*, 2010). Wiltzius *et al.* (2005) reported significant DSB repair defects in yeast with mutation of the zinc-coordinating cysteine residues of Rad50. Hopfner *et al.* (2002) showed that zinc is essential to the Rad50 hook conformation and, consequently, to the functionality of the MRN complex.

VOCs cause DNA damage in *T. ethacetica*

To validate our hypothesis and investigate DNA damage using Fourier transform infrared spectroscopy (FTIR) spectra, we selected the most representative compounds in our analysis of 2 important chemical groups, the alcohols that were represented by 2-nonanol and the ketones by 2-tridecanone. An ethanol treated sample, and a control sample of the fungus growing under normal conditions without exposure to volatiles was also analyzed. The mean absorbance spectra (1800 – 725 cm^{-1}) along with their secondary derivatives in the spectral range of nucleic acids (1280-1000 cm^{-1}) of all samples are shown in Fig. 5A and B, respectively.

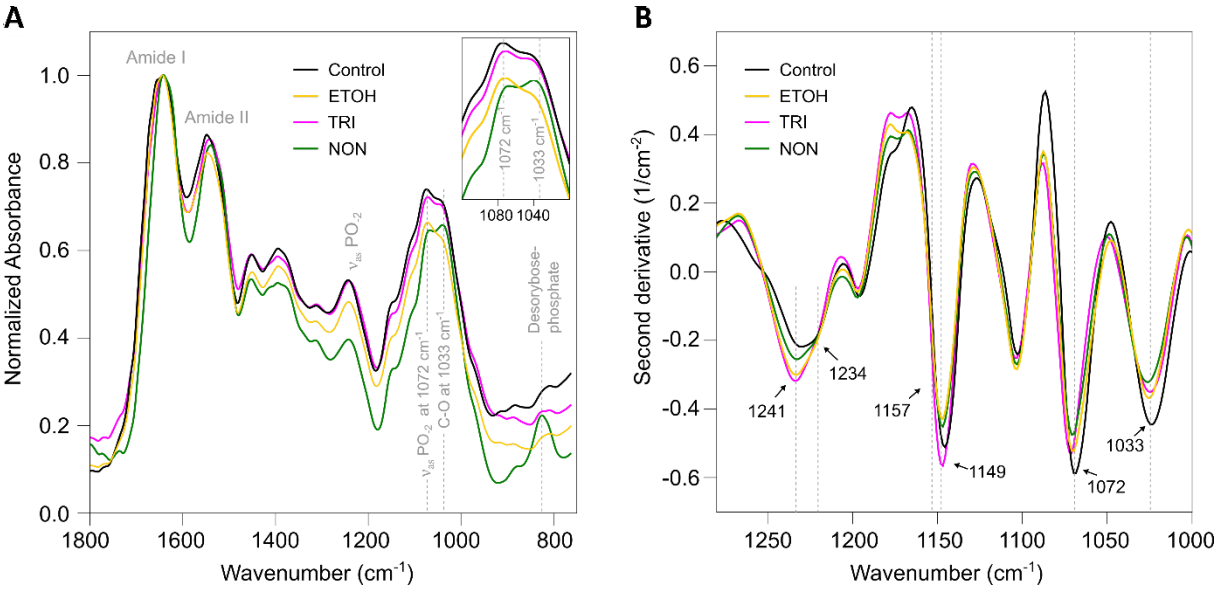


Fig. 5. Absorbance spectra collected from control, ETOH-, TRI- and NON-treated hyphae (A) along with their second derivative (B) in the spectral range of nucleic acids (1280-1000 cm^{-1}). The measurements in (A) are normalized to 1 to facilitate comparison. Control: *T. ethacetica* grown without synthetic compound. ETOH: *T. ethacetica* grown with ethanol TRI: *T. ethacetica* grown with 2-tridecanone. NON: *T. ethacetica* grown with 2-nonanol.

Spectra collected from control hyphae, TRI- and NON-treated hyphae (Fig. 5A) highlighted variations in the IR absorbance as a function of the treatment applied to the hyphae. Ethanol (ETOH) was also analyzed, as well as a control sample of the fungus growing under normal conditions without exposure to volatiles. First, we have analyzed the relative high IR signal observed for all types of hyphae regarding the protein absorption bands assigned to amides I and II at 1643 cm^{-1} and 1542 cm^{-1}, respectively. The absorption band centered at 1643 cm^{-1} was mainly associated with stretching vibrations of the C=O bond of the amide, and the absorption bands at 1542 cm^{-1} were attributed to bending vibrations of the N-H bond and C-N stretching vibrations of the peptide backbone. Although amides I and II are sensitive to the secondary structure content of proteins, amide I is particularly sensitive to the secondary structure based on different hydrogen-bonding environments for α-helix, β-sheet, turn, and unordered conformations (Miller *et al*., 2013). Thus, Fig. 5A shows that there was no modification on the secondary structure of *T. ethacetica* proteins.

Overall, the main spectral differences for the hyphae occurred over the DNA backbone region (1280–1000 cm^{-1}). The inset in Fig. 5A shows the region from 1100 to 1000 cm^{-1} related to the $\upsilon_{sym}(PO_2^-)$ (1072 cm^{-1}) and the stretching of the C-O band (1033 cm^{-1}), both of which are associated with DNA. In the hyphae treated with 2-nonanol, a clear inversion of strength can be noticed between the 2 absorption bands, which is associated with multiple DNA breaks; therefore, it is strongly indicative of DNA cleavage and chromatin fragmentation (Sailer *et al*., 1996). Furthermore, analysis of the second derivative (Fig. 5B) indicated variations in the stretching bands PO_2^- and C-O and a decrease in the intensity of the bands in the control compared with the hyphae treated with 2-nonanol, confirming a DNA deformation mechanism (Sailer *et al*., 1996). Curiously, we can notice the same pattern (but as a smaller intensity difference) on the samples treated with 2-tridecanone and ethanol, which could suggest minor modifications in DNA also in these samples. Although ethanol did not significantly inhibit the growth of *T. ethacetica* (data no shown), it was already reported the importance of DNA damage repair in yeast under ethanol treatment, even at concentrations that do not inhibit its growth (Ristow *et al.*, 1995; Pais *et al.,* 2013).

Additionally, other prominent band was assigned as the anti-symmetric phosphate stretching vibration ($\upsilon_{asym}(PO_2^-)$ = 1241 cm^{-1}) (Fig. 5B), and it presented a redshift of 7 cm^{-1} (from 1241 to 1234 cm^{-1}) in relation to the control, indicating local

conformational changes in the A and B forms of the DNA (Lipiec *et al.*, 2014; Whelan *et al.*, 2014; Han *et al.*, 2018).

In addition to the aforementioned changes, 2-nonanol-treated hyphae also presented strong absorbance at 825 cm^{-1} (Fig. 5A), a band associated with deoxyribose-phosphate, again evidencing deformations associated with the structural properties of the DNA molecules (de los Santos, 1999; Han *et al.*, 2018). Therefore, this analysis corroborated our hypothesis of DNA damage triggered by VOCs. There are few reports in the literature regarding the relationship between VOCs and DNA damage. Alpha *et al.* (2015) observed that VOCs produced by the fungus *M. albus*, in addition to affecting growth and pathogenicity, also cause cell death and induce DNA damage in *E. coli* pathogenic strains.

VOCs affect the morphology and ultrastructure of *T. ethacetica*

To better understand the mode of action of the VOCs in the cell structures of the pathogen, we investigated morphological and ultrastructural changes caused by the exposure of *T. ethacetica* to volatiles. To achieve this goal, the same compounds from the FTIR analysis were used. In the SEM analysis, we observed several protuberances on the hyphal surface treated with 2-nonanol (Fig. 6A); however, our analysis did not allow us to ensure whether the volatiles adhered to the cell wall or whether some hyphal deformity was caused by the action of VOCs or even extracellular vesicles. In contrast, hyphae treated with 2-tridecanone (Fig. 6B) appeared to be smoother than the other samples, including mycelia treated with ethanol and untreated mycelia (Fig. 6C and D).

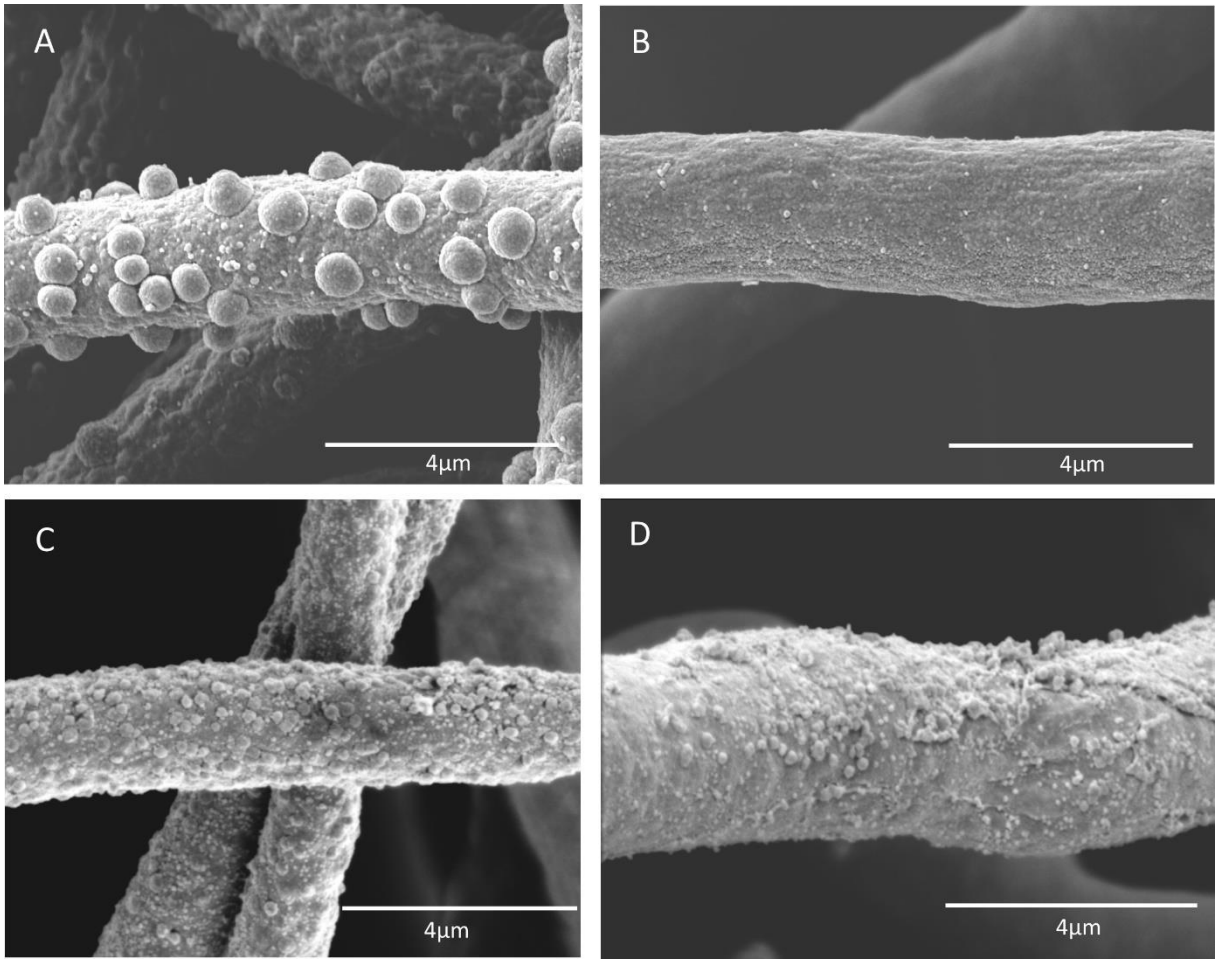


Fig. 6. Representative scanning electron micrographs of the mycelium of *T. ethacetica* treated with 2-nonanol (A), 2-tridecanone (B), ethanol (C) or untreated (D).

Examination of the effect of VOCs on the ultrastructure by TEM revealed that control sample micrographs showed a homogeneous cytoplasm, the presence of a large number of mitochondria, normal vacuoles, a regular-shaped endoplasmic reticulum, close contact between the cytoplasmic membrane and cell wall, and an intact nucleus (Fig. 7A-C). Around the hyphae in the control treatment, we observed something similar to mucilage that was not observed when the fungus was exposed to VOCs (Fig. 7G-L). Overall, the ultrastructure of the fungi revealed that exposure to volatiles caused changes in the cell wall (granulation), detachment of the cytoplasmic membrane from the cell wall, collapse of the vacuoles and cytoplasm, formation of vesicles, a decrease of mitochondria number, vacuolization, destruction of organelles and internal cell darkening, which were probably caused by the accumulation of electron-dense material inside the cells, resulting in abnormal fungal cell growth or cell death. Curiously, although ethanol did not significantly inhibit the growth of *T.*

ethacetica (data no shown), we also noted some ultrastructural changes indicative of cellular damage (Fig. 7D-F) but to a reduced extent compared with the other compounds.

Similar alterations to those found in our study in the structures of the cell wall and plasma membrane have been reported. Disorganization and destruction of the organelles and, eventually, cell death of *C. acutatum* caused by exposure to (E)-hex-2-enal was demonstrated by Arroyo *et al.* (2007). Distorted and collapsed hyphae and irreversibly damaged cell membranes and organelles were observed by Park *et al.* (2009) after exposure of *Trichophyton mentagrophytes* to terpenes, eugenol, nerolidol and α-terpineol. Li *et al*. (2012) reported an increase in vesiculation and strong retraction of the plasma membrane in hyphae of *B. cinerea* exposed to *Streptomyces globisporus* volatiles. In addition, Zhang *et al.* (2019) observed a loss of cellular contents of *C. fimbriata* caused by VOCs produced by *Pseudomonas.* It is worth to mention that membrane along with the cell wall are also primary targets of many fungicides (Jampilek , 2016; Mazu *et al*., 2016).

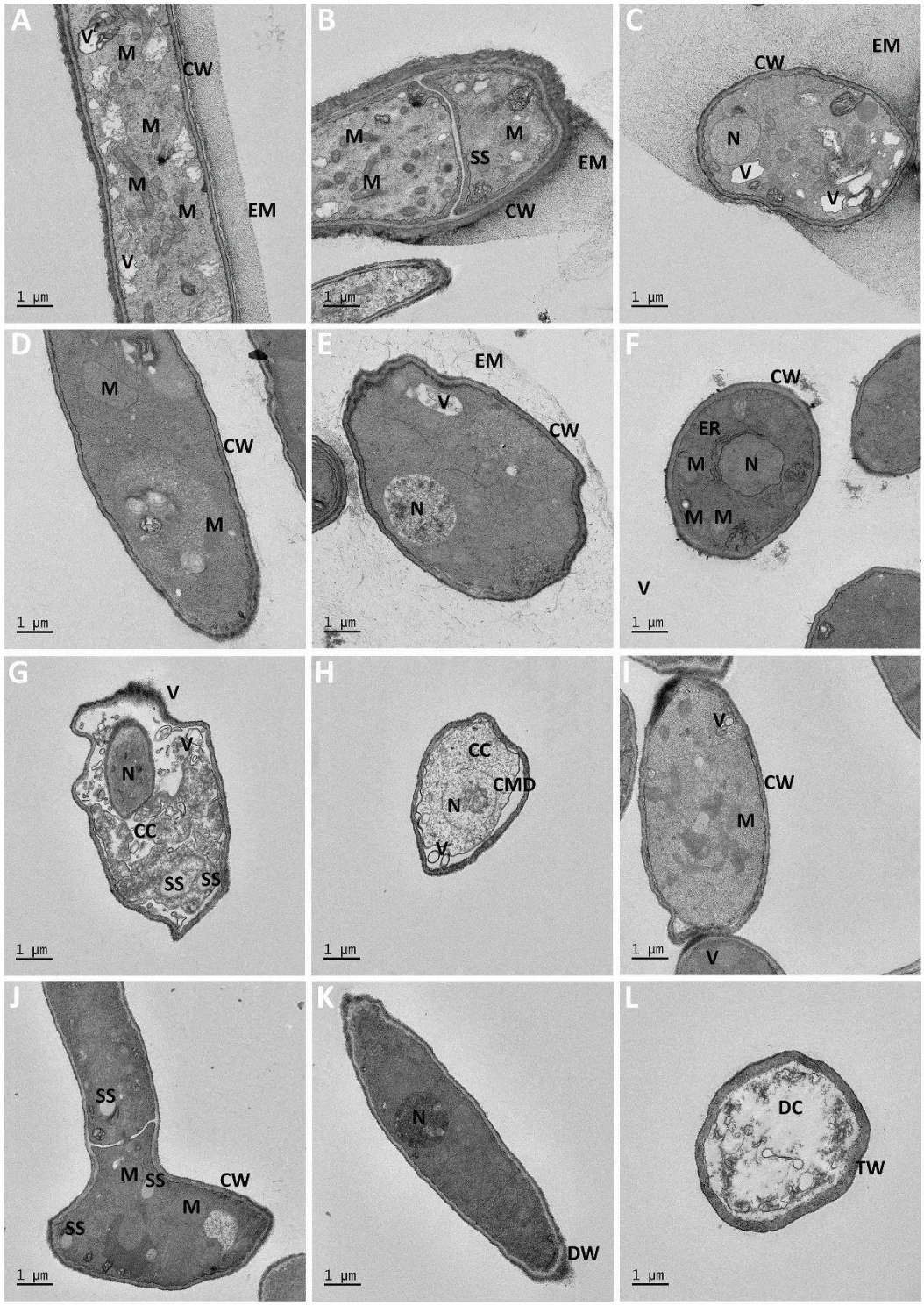


Fig. 7. Representative transmission electron micrographs of the untreated mycelium of *T. ethacetica* (A, B, C) treated with ethanol (D, E, F), 2-nonanol (G, H, I) and 2-tridecanone (J, K, L). C = cytoplasm, CC = collapsed cytoplasm, CMD = cytoplasmatic membrane detachment, CW = cell wall, DC = dead cell, DW = deformed cell wall, EM = external mucilage, ER = endoplasmic reticulum, M = mitochondria, N = nucleus, SS = storage structure, TW = thickened cell wall, V = vacuole.

Interestingly, we observed chromatin condensation in the nucleus in the micrographs of hyphae treated with 2-nonanol (Fig. 7H), which may be correlated with activation of the DNA damage response (Burgess *et al*., 2014). We can correlate this observation with RNA-seq data revealing that VOCs activated the DNA repair mechanism in the fungus, and FTIR analysis, reinforcing our hypothesis that the action of VOCs may be to cause DNA damage. Similar to our results, Zhang and Sun (2018) reported that cyclic lipopeptides (CLPs) of the fengycin family produced by *Bacillus subtilis* could induce chromatin condensation, which activates DNA damage response in *M. grisea*, leading to cell death.

There were differences in the morphological changes of *T. ethacetica* according to the compound treatment, and 2-tridecanone apparently caused less damage to hyphae than 2-nonanol, since more preserved structures such as mitochondria and storage structures which may be lipid accumulation were observed (Fig. 7J). He *et al*. (2018) reported lipid accumulation in sclerotia and hypha during sclerotial morphogenesis of *Morchella importuna.* In yeasts there are several reports about lipid droplets as ergosterol (Bard *et al*., 1993; Lv *et al*., 2016; Hu *et al*., 2017), which is the main component of fungal membranes (Alcazar-Fuoli and Mellado, 2013). Although the composition of these storage structures in *T. ethacetica* is unclear, the absence of them were correlated with the growth inhibition.

However, we also observed the presence of dead cells (Fig. 7L) in smaller amounts than with 2-nonanol as well as cell wall thickening. Park *et al*. (2009) also observed this alteration in the cell wall when *T. mentagrophytes* were exposed to a citral compound. Comparing the chemical classes, an additional difference observed between the action of alcohols and ketones was the invagination of the cell membrane observed in hyphae treated with 2-nonanol, which seemed to cause more damage to the fungus.

Taken together, SEM analysis showed some unprecedented alterations in the external cell wall, and TEM analysis revealed that VOCs caused severe damage to the cellular ultrastructure of *T. ethacetica*. This analysis also indicated that 2-tridecanone and 2-nonanol controlled pathogen growth through different mechanisms that might involve cell wall, membrane, and DNA damage.

CONCLUDING REMARKS

We investigated VOCs produced by bacteria isolated from very healthy and productive regions of sugarcane that could be natural potential fungal biological control agents. We found that *Pseudomonas* strains produced VOCs that could inhibit fungal growth by 80%. Different strategies, such as different culture media and amounts of inocula when using antagonist bacteria, influenced the antifungal activity of VOCs. In total, 62 compounds were identified in the volatilomes of these bacteria, and volatiles such as 2-nonanol, 2-nonanone and 2-tridecanone significantly suppressed mycelial growth of *T. ethacetica* even at very low concentrations.

One of the most significant findings of this study was the identification of several VOCs-downregulated pathways essential to fungal growth using RNA-seq. Moreover, an upregulation of DNA repair was also observed, and micro-FTIR analysis revealed spectral differences in the hyphae treated with 2-nonanol that indicated DNA damage. Further studies are required to evaluate the true impact of VOCs on phytopathogens, but our data show that DNA damage could be associated with the growth inhibition caused by these signaling molecules. Electron microscopy revealed morphological changes in hyphae and chromatin condensation that may have led to cell death. Based on the analysis of the pathogen's ultrastructure, we also observed that different chemical groups of VOCs (alcohols and ketones) might have different modes of action in the inhibition of mycelial growth.

Although there is a need to further investigate the effect of VOCs *in vivo*, the results obtained herein provide important insights to elucidate the modes of action of volatiles in phytopathogenic fungi. These findings also contribute to the development of biological strategies to control phytopathogenic fungi, to increase agricultural productivity and to reduce dependence on agrochemicals and fertilizers.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

ORIGINALITY-SIGNIFICANCE STATEMENT

Volatile organic compounds (VOCs) are gaseous signaling molecules that differently from other molecules that microorganisms produce (such as antibiotics), VOCs can act far away from the point of production, making them ideal info-chemicals for mediating short- and long-distance microorganism interactions (microorganism-microorganism and microorganism-plant). Despite the biotechnological potential of these molecules, it is not clear how microbial VOC affect other organisms. This study demonstrates the molecular mechanisms involved in the antagonism of sugarcane phytopathogens by bacterial VOCs. Our analysis revealed several downregulated metabolic pathways essential to fungal growth and also uncovered that these gaseous compounds lead to fungal DNA damage and crucial ultrastructural changes. This study provides new information about the *Pseudomonas* mode of action in antagonism to phytopathogenic fungi and is also the first report of the antifungal activity of compound 2-tridecanone.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:
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Fig. S1. Venn diagram of VOCs produced by the bacterial antagonists BNG P1D2, FBJ P5B2 and ITA P2F2.

Fig. S2. Validation of *T. ethacetica* data RNA-seq data by RT-qPCR.

Table S1. VOCs produced by the bacterial antagonists BNG P1D2, FBJ P5B2 and ITA P2F2 cultured in three different media (ANGLE, DYGS and LB) identified by HS/SPME-GC/MS.

Table S2. Scores of the produced metabolites in principal component analysis (PCA) in three culture media.

Table S3. Differently expressed genes of *T. ethacetica* transcriptome.

Table S4. Differentially expressed CAZymes in the *T. ethacetica* transcriptome.

Table S5. Gene ontology enrichment analysis of *T. ethacetica*.

Table S6. VOCs selected for functional validation by using synthetic compounds.

Table S7. Primers used in RT-qPCR assays.

File S1. Artificial VOC mixtures assay.

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CONCLUSÕES GERAIS E CONSIDERAÇÕES FINAIS

Como principais conclusões, é possível destacar que:

- Três isolados apresentaram capacidade de inibição do crescimento do *T. ethacetica, in vitro,* superior a 80%;
- Os isolados de interesse pertencem ao gênero *Pseudomonas* e provavelmente a diferentes espécies;
- A inibição ocorreu de forma significativa em 2 dos 3 meios de cultura testados;
- A inibição e, em alguns casos, a morte do fungo ocorreu mesmo quando foram utilizadas baixas concentrações de COVs;
- As análises de metabolômica nos permitiram identificar 62 compostos produzidos pelos 3 isolados bacterianos;
- Os compostos identificados pertencem a diversas classes químicas como álcoois, cetonas, aldeídos, alcanos e alcenos;
- O meio de cultura é crucial para a composição do volatiloma da bactéria, e o volatiloma é espécie-específico;
- As análises de RNA-seq mostraram que os COVs atuam no metabolismo de carboidratos do fungo, na resposta ao estresse oxidativo e no transporte transmembrana, entre outros;
- As análises de RNA-seq e micro-FTIR mostraram que os COVs causam danos ao DNA do fitopatógeno;
- As análises de microscopia eletrônica revelaram alterações morfológicas nas hifas, que podem culminar na morte celular;
- As análises de TEM revelaram que diferentes grupos químicos de COVs (álcoois e cetonas) podem ter diferentes modos de ação na inibição do crescimento micelial.

A população global está em constante crescimento e para atender essa demanda de alimento e energia para essa população é necessário maximizar a produtividade e o rendimento das culturas, minimizando os custos e os impactos ambientais, tornando a agricultura mais sustentável. O objetivo principal desse

trabalho foi utilizar a biotecnologia para auxiliar no desenvolvimento de estratégias que reduzam o uso de insumos químicos e favoreçam o uso de microrganismos que possam contribuir para uma agricultura com menor uso de agroquímicos.

Os resultados obtidos aqui mostram o grande potencial dos COVs produzidos pelos isolados da nossa coleção de serem utilizadas no controle de fitopatógenos. Nossas análises utilizaram diferentes abordagens e técnicas que permitiram, além de identificar esses compostos e validar sua atividade antagonista, elucidar os modos de ação pelos quais se dá o antagonismo ao fungo fitopatogênico. Nós pudemos observar, por exemplo, utilizando técnicas de microscopia e micro-FTIR que voláteis de classes químicas diferentes inibem o crescimento do fungo através de modos de ação diferentes.

Embora haja a necessidade de investigar o efeito dos COVs *in vivo*, esses resultados contribuem para o desenvolvimento de estratégias biológicas de controle de fungos fitopatogênicos. No futuro, como uma meta de longo prazo, buscamos desenvolver produtos para biocontrole como uma abordagem sustentável para reduzir a incidência de doenças na cana-de-açúcar. Dessa forma, espera-se substituir ou reduzir a dependência suprimentos químicos, aumentar a produtividade agrícola, além de reduzir os custos de produção e melhorar a longevidade dessa cultura no Brasil e em outros países produtores.

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ANEXO I

Uso exclusivo da CIBio:

Número de projeto / processo: B-2019-01

Formulário de encaminhamento de projetos de pesquisa sem OGMs, para análise da CIBio - CNPEM

1. Título do projeto: Identificação e uso de voláteis bacterianos contra patógenos de cana-de-açúcar e promoção do crescimento vegetal.

2. Pesquisador responsável: Juliana Velasco de Castro Oliveira

3. Experimentador: Carla de Santa´anna Freitas, Aline Tieppo Nogueira Mulato, Luciane Fender Coerini, Bruno Henrique Silva Dias, Octavio Augusto Costa Almeida, Guilherme Masiero Fontanetti, Natália Oliveira de Araujo

Nível do treinamento do experimentador: | GMF |-Iniciação científica, | BHSD, NOA |-mestrado, |CSF, LFC, OACA |-doutorado, | |-doutorado direto, | |-pós-doutorado, | ATNM |-nível técnico, | |-outro, especifique:_____

4. Unidade operativa: [ ] LNLS [ ] LNNano [X] CTBE [ ] LNBio

5. Classe de risco dos organismos: [ ] Risco I [X] Risco II [ ] Risco III [ ] Risco IV

6. O projeto é confidencial? [X] não [ ] sim

7. O título do projeto pode constar em lista aberta no CNPEM? [ ] não [X] sim

8. Qual é o objetivo do projeto? Identificar bactérias capazes de inibir o crescimento de patógenos da cana-de-açúcar, bem como promover o crescimento das plantas através da emissão de compostos orgânuicos voláteis.

9. Quais organismos serão manipulados? *Bipolaris sacchari*, *Fusarium moniliforme*, além de bactérias isoladas do solo dos gêneros *Acinetobacter*, *Burkholderia*, *Klebsiella* e *Enterobacter*.

10. No caso da aquisição do material, organismos ou derivado é necessário solicitar autorização de alguma agência regulamentadora/fiscalizadora? Não

11. No caso do transporte do material, organismos ou derivados é necessário solicitar autorização de alguma agência regulamentadora/fiscalizadora?

12. Algum dos materiais, organismos ou derivados apresentam riscos (patogenicidade, infectividade, toxicidade) para manipulador, comunidade ou meio ambiente? Cite estes riscos classificados por material, organismo ou derivados. Na lista do ministério consta especificamente a espécie do *Fusarium moniliforme*, mas o *Bipolaris* está genérico (ssp), e estamos trabalhando com o *Bipolaris sacchari*. Ambos os fungos são patógenos de planta, mas em casos específicos já foi descrito infectando humanos (imunidade baixa, lesão cutânea, e após tratamento dc câncer). O mesmo é válido para as bactérias. Todas as foram isoladas de solo de cultura de cana-de-açúcar, com as quais os agricultores têm contato direto, e embora o gênero esteja dentro da classificação como NB2, não devem conferir doenças a manipuladores sadios.

13. Descreva brevemente os procedimentos e técnicas experimentais. As bactérias e/ou fungos são crescidos em meio solido e líquido (no máximo 10mL), e são cultivados em placa bipartidas (fungo *versus* fungo, bactérias *versus* bactéria, fungo *versus* bactéria) para avaliar a inibição de crescimento de patógenos bem como promoção do crescimento vegetal (placa bactéria *versus* planta). Em alguns casos estas cepas serão crescidas em falcon de 50mL (volume máximo de 10mL de meio), peletadas por centrifugação e lisadas para isolamento do DNA. O DNA purificado será amplificado por PCR e sequenciado. Elas também serão crescidas em frasco de 5mL para extração de compostos orgânicos voláteis para análise em GC-MS (3mL de meio), ou ensaios de inibição de crescimento em meio líquido (máximo 5mL de meio).

14. Descreva as medidas de biossegurança para operador, comunidade e meio ambiente:
- Procedimentos de descarte/limpeza do laboratório: os resíduos de todas as culturas, tubos e demais vidrarias utilizados no cultivo das bactérias e fungos, ou que entraram em contato com estes microrganismos, são devidamente esterilizados por autoclavagem, de acordo com as práticas microbiológicas exigidas para Nível de Biossegurança 2. Os resíduos somente são descartados no lixo de descarte biológico do CNPEM após a autoclavagem dos mesmos (realizado no próprio NB2). Rotores e tubos de centrífuga também são descontaminados com hipoclorito de sódio e etanol 70%, caso haja vazamento de soluções contendo microrganismo. As bancadas do laboratório, assim como o chão, também são frequentemente desinfetadas.

- - Procedimento de armazenamento de bactérias e fungos: As cepas de bactérias serão armazenadas no freezer -80°C ou geladeiras (que ficam dentro do NB2), devidamente acondicionados e identificados pelo nome, volume, data e pessoa responsável pelo microrganismo.
- Os principais EPIs obrigatórios são avental, luvas descartáveis e óculos de proteção (este último só será utilizado em experimentos onde ocorra a formação de aerossois).
- As pessoas que irão trabalhar nesta área não apresentam nenhuma desordem do sistema imune, nem utilizam medicamentos para induzir a imunossupressão (por exemplo, necessárias em casos de tratamento de doenças auto-imunes ou em transplantes de órgãos).

15. Descreva a estrutura laboratorial necessária para o desenvolvimento do projeto e sua localização no CNPEM. De modo a atender todas as especificações necessárias para manipulação de microrganismos classe de risco 2, as atividades serão realizadas no NB2 do CTBE. Ele possui uma pia, para lavar as mãos antes e depois da manipulação dos microrganismos, mesmo com o uso obrigatório de luvas descartáveis. Além disso, também possui um fluxo de segurança biológica (Filtro HEPA H14 de acordo com EN 1822: eficiência MPPS de 99.995% e DOP de 99.999%) e uma autoclave especial, itens obrigatórios segundo a resolução especifica do CTNBio. O acesso e uso deste laboratório é controlado, limitado a pessoas que necessitam trabalhar neste espaço. A sala é sempre trancada e só pessoas que receberam treinamento especial tem acesso a mesma. As janelas da sala também foram modificadas de forma a impedir sua abertura. O fluxo contém cesto de lixo para descarte de OGMs e outros microrganismos NB2, que são autoclavados previamente ao descarte. Nenhum microrganismo vivo ou ativo é retirado desta sala. As câmaras de fluxo laminar onde os organismos são manipulados vivos são esterilizadas com etanol 70%; e após esta limpeza, submetidas a irradiação UV por 20 min (isso é realizado antes e após o uso).

16. Será necessário solicitar aprovação, credenciamento ou vistoria de alguma agência regulamentadora/fiscalizadora antes de iniciar o projeto no CNPEM? | | Sim | X | Não

No caso de responder "sim" , quais?

17. Com relação aos cuidados preventivos associados a manipulação dos materiais, organismos e derivados, será necessária alguma avaliação médica periódica para experimentadores? | X | Não | | Sim. Que tipo de avaliação? (Ex: consulta com médico, exames laboratoriais etc...) Qual periodicidade? Onde será realizada esta avaliação?

18. Com relação aos cuidados preventivos associados a manipulação dos materiais, organismos e derivados, será necessária alguma vacinação preventiva para experimentadores? | X | Não | | Sim. Qual periodicidade? Onde será realizada esta vacinação?

19. No caso de uma eventual contaminação com materiais, organismos e derivados, descreva medidas emergenciais para tratamento de pessoas envolvidas, descontaminação de equipamentos, instalações e meio ambiente.

19.1 Contaminação Pessoal

- Remover vestimenta contaminada e lavar vigorosamente a área exposta com água e sabão, por pelo menos 2 minutos;
- Em caso de contaminação nos olhos, utilizar o lavador de olhos portátil que fica embaixo da pia do laboratório (preenchido com soro fisiológico) ou o lavador de olhos próximo a sala NB2;
- Contatar imediatamente a segurança do CNPEM no ramal 1253 ou 1092, e obter cuidado médico apropriado se necessário.

19.2 Contaminação fora da cabine de segurança biológica

- Verificar se houve contaminação pessoal e tomar medidas acima caso tenha ocorrido;
- Evacuar a sala onde ocorreu o acidente e identificar na porta da sala a ocorrência do mesmo;
- Em caso da possibilidade de formação de aerossol, sair da sala e aguardar 30 minutos para efetuar a limpeza;
- Durante toda a limpeza, utilizar luvas (resistentes), avental e proteção facial (máscara/óculos), proteger os calçados com pro-pé descartável;
- Cobrir completamente a área de derramamento com material absorvente (toalhas de papel, compressas de gaze, panos de limpeza) e aplicar solução de hipoclorito concentrado. Após 30 minutos, deve ser iniciado o procedimento de limpeza;
- Se o volume derramado for grande, pode ser utilizado material absorvente para absorver o líquido (vermiculita);

- Se o derramamento contiver vidro quebrado ou outros objetos, esses devem ser descartados sem contato manual direto. Devem ser removidos através de meios mecânicos (vassoura e uma pá de lixo ou pinças). Os resíduos deverão ser descartados em recipiente apropriado para material com risco biológico e à prova de perfurações (caixa Descarpak) e os materiais utilizados desinfectados;
- Ao final, limpar o local do derramamento com água a fim de remover produtos químicos nocivos ou odores. Descartar material envolvido na limpeza (luvas, máscara, papeis, gazes, vermiculita, etc) no lixo autoclavável.

19.3 Contaminação de centrífuga e estufa
- Verificar se houve contaminação de seu avental/luva. Use luvas (resistentes), avental e proteção facial;
- Antes de se iniciar a descontaminação da centrifuga/estufa, o equipamento deverá permanecer fechado durante pelo menos 30 minutos a fim de permitir que as gotas "assentem". Absorver a maior parte do líquido antes da limpeza;
- Utilizar a solução de desinfecção concentrada e, se necessário, remover o rotor para limpeza (ou prateleiras da estufa);
- Limpar o local do derramamento com água a fim de remover produtos químicos nocivos ou odores. Descartar material envolvido na limpeza (luvas, mascara, papeis, gazes, vermiculita, etc) no lixo autoclavável.

19.4 Contaminação no interior da cabine (fluxo) de segurança
- Abaixar o vidro da cabine e aguardar 5 minutos para depurar o aerossol. Abrir novamente a cabine e deixar o fluxo de ar estabilizar por mais 5 minutos.
- Para descontaminação use luvas (resistentes) e avental; com o fluxo funcionando, cobrir a área de derramamento completamente com material absorvente (toalhas de papel, compressas de gaze, panos de limpeza) e aplicar solução de hipoclorito concentrado. Apenas após 30 minutos, deverá ser iniciado o procedimento de limpeza;
-Se o derramado for grande, pode ser usado material absorvente granulado (vermiculita) para absorver o líquido;
- Se o derramamento tiver vidro quebrado ou outros objetos, esses devem ser descartados sem contato manual direto, com auxílio de pinças, pás de lixo, etc;
- Descontaminar acessórios, vidro e o interior da cabine de segurança;
- Certifique-se que não houve vazamento para debaixo da chapa da cabine, ou por trás. Se tiver ocorrido, esta área deverá ser descontaminada (da mesma maneira como descrito acima) após a limpeza da superfície;
- Após desinfecção, limpar o local do derramamento com água a fim de remover produtos químicos nocivos ou odores. Descartar material envolvido na limpeza (luvas, mascara, papeis, gazes, vermiculita, etc) no lixo autoclavável.

Em todos os casos recomenda-se acionar o coordenador de biossegurança ou técnico responsável para auxiliar na tomada de providências. Todo "lixo" gerado na limpeza é posteriormente autoclavado.

20. No caso de órgãos, células primárias, fluidos, sangue, derivados de origem humana, é feita alguma pré-triagem para descartar eventuais amostras contaminadas com microrganismos patogênicos (Ex. HIV, hepatite, HTLV entre outros), antes de transportar amostras ao CNPEM? ( )Sim (X) Não. Explique:

_____

_____

21. Projetos que façam uso de organismos ou genes associados ao patrimônio genético brasileiro precisam de cadastro na plataforma SISGEN (www. sisgen.gov.br). É de total responsabilidade do pesquisador responsável esse cadastramento e cumprimento da legislação. O projeto envolve manipulação, transferência, modificação, armazenamento, coleta de Organismos e derivados relativos ao patrimônio genético brasileiro? (X) SIM, ( ) Não. No caso de responder sim, mencionar a seguir quais os códigos de acesso do cadastro no SISGEN:_____

O pesquisador principal tem conhecimento de que conforme a RDC 50 de 21/02/2002 da Anvisa, é responsável por determinar a classificação de riscos de seu projeto, assim como determinar EPIs e medidas de segurança necessárias para prevenir a contaminação de experimentadores, equipamentos, instalações, terceiros e meio ambiente. O pesquisador responsável também precisará providenciar rotina para realização de exames médicos e laboratoriais para sua equipe, bem como vacinações quando aplicável. Todos os experimentadores envolvidos devem ser supervisionados pelo pesquisador principal, que é o responsável pelo treinamento de biossegurança adequado às suas necessidades para a manipulação, armazenamento, descarte e transporte dos

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organismos, atendendo a legislação e normativas preconizadas pela, Anvisa e outros órgãos e/ou agências regulamentadoras e fiscalizadoras.

Assinatura eletrônica do pesquisador responsável:

Juliana Velasco de Castro Oliveira

A CIBio analisou este projeto em reunião realizada no dia: 11/4/2019 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências.

comentários da CIBio:

Presidente da CIBio CNPEM
Marcio Chaim Bajgelman

Membro da CIBio CNPEM
Juliana Velasco de Castro Oliveira

Membro da CIBio CNPEM
Celso Eduardo Benedetti

Membro da CIBio CNPEM
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Membro da CIBio CNPEM
Juliana Conceição Teodoro

Membro da CIBio CNPEM
Douglas Galante

Membro da CIBio CNPEM
Diego Stefani Teodoro Martinez

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Campinas, 15 de fevereiro de 2022.

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