UNIVERSIDADE DE CAXIAS DO SUL
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

INSTITUTO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

Identificação de espécies de *Colletotrichum* associadas com videiras e atividade antifúngica de monoterpenos sobre *Colletotrichum* spp. e *Saccharomyces cerevisiae*

FERNANDO JOEL SCARIOT

CAXIAS DO SUL

2021

FERNANDO JOEL SCARIOT

Identificação de espécies de *Colletotrichum* associadas com videiras e atividade antifúngica de monoterpenos sobre *Colletotrichum* spp. e *Saccharomyces cerevisiae*

Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando a obtenção de grau de Doutor em Biotecnologia.

Orientador: Prof. Dr. Sergio Echeverrigaray
Coorientadora: Profa. Dra. Ana Paula Longaray Delamare

CAXIAS DO SUL

2021

Dados Internacionais de Catalogação na Publicação (CIP)
Universidade de Caxias do Sul
Sistema de Bibliotecas UCS - Processamento Técnico

S285i Scariot, Fernando Joel

Identificação de espécies de *Colletotrichum* associadas com videiras e atividade antifúngica de monoterpenos sobre *Colletotrichum* spp. e *Saccharomyces cerevisiae* [recurso eletrônico] / Fernando Joel Scariot. – 2021.

Dados eletrônicos.

Tese (Doutorado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Biotecnologia, 2021.

Orientação: Sergio Echeverrigaray.

Coorientação: Ana Paula Longaray Delamare

Modo de acesso: World Wide Web Disponível em: https://repositorio.ucs.br

1. Colletotrichum. 2. Fungos fitopatogênicos. 3. Videira - Controle biológico. 4. Uva - Cultivo. I. Echeverrigaray, Sergio, orient. II. Delamare, Ana Paula Longaray, coorient. III. Título.

CDU 2. ed.: 582.28

Catalogação na fonte elaborada pela(o) bibliotecária(o)
Márcia Servi Gonçalves - CRB 10/1500

Identificação de espécies de *Colletotrichum* associadas com videiras e atividade antifúngica de monoterpenos sobre *Colletotrichum* spp. e *Saccharomyces cerevisiae*

Fernando Joel Scariot

Tese submetida a banca examinadora designada pela coordenação do Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutor em Biotecnologia.

Tese aprovada em 29 de outubro de 2021.

Orientador: Prof. Dr. Sergio Echeverrigaray Laguna
Universidade de Caxias do Sul

Co-orientadora: Profa. Dra. Ana Paula Longaray Delamare
Universidade de Caxias do Sul

Prof. Dr. Francisco Carrau
Universidad de la Republica

Profa. Dra. Louise Larissa May de Mio
Universidade Federal do Paraná

Profa. Dra. Mirian Salvador
Universidade de Caxias do Sul

AGRADECIMENTOS

Aos meus pais Ernestina e Dorvalino, e às minhas irmãs Andressa e Catiane pelo carinho e apoio.

Aos professores Dr. Sergio Echeverrigaray e Dra. Ana Paula Longaray Delamare, que acreditaram na minha capacidade desde a época da graduação, quando eu entrei no laboratório, e que aceitaram me orientar nesta etapa.

Aos colegas do Laboratório de Microbiologia Aplicada, que de alguma forma auxiliaram na realização do trabalho, seja de forma direta na bancada ou dando sugestões pertinentes.

Gostaria de agradecer especialmente aos alunos de graduação Mariliza Sallete Pansera e Lucino Foresti, que me auxiliaram de forma direta durante o doutorado.

Aos colegas do Laboratório de Biotecnologia de Plantas e o Laboratório de Fitopatologia pelo auxílio na obtenção dos isolados de *Colletotrichum*.

Às professoras que fizeram parte da minha banca de acompanhamento, Dra Mariana Roesch Ely e Dra Mirian Salvador, pelas correções e sugestões realizadas.

Aos professores do Programa de Pós Graduação em Biotecnologia, que de forma direta contribuíram na minha formação intelectual.

Aos funcionários, pelo auxílio prestado.

À Universidade de Caxias do Sul pela oportunidade de realização deste trabalho.

Ao CAPES pela concessão da bolsa.

A todos que de alguma forma contribuíram para que este trabalho fosse concluído.

ÍNDICE

LISTA DE TABELAS

CAPITULO 1

CAPITULO 2

LISTA DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

NOMENCLATURAS

AIF	fator indutor de apoptose
AnnV	Annexina V-FITC
ATP	adenosina trifosfato
CFDA	*5-carboxyfluorescein diacetate*
CFU	unidade formadora de colônia
DHE	*dihydroethidium*
$DiOC_6$	*3,3'-dihexyloxacarbo-cyanine iodide*
DMSO	dimetilsulfoxido
DNA	ácido desoxirribonucleico
ERO	espécies reativas de oxigênio
FSC	*forward scatter*
GAPDH	gliceraldeído-3-fosfato desidrogenese
ITS	*Internal transcribed spacer*
LSU	*Large subunit ribossomal RNA*
MFC	concentração fungicida mínima
MIC	concentração inibitória mínima
PCR	*polymerase chain reaction*
PI	iodeto de propídio
RNA	ácido ribonucleico
ROS	espécies reativas de oxigênio
SSC	*side scatter*
WT	*wild type*

RESUMO

A podridão da uva madura é uma das principais doenças que ocorrem nos vinhedos do sul do Brasil, entretanto, existe pouca informação sobre as espécies de fungos responsáveis pela doença. Os óleos essenciais e seus compostos majoritários, os monoterpenos, são considerados alternativas para o controle de doenças fúngicas, como a podridão da uva madura causada por espécies de *Colletotrichum*. Entretanto, o modo de ação desses compostos ainda precisa ser elucidado. O trabalho teve como objetivo o isolamento e identificação de espécies de *Colletotrichum* oriundas de videiras com sintomas de podridão da uva madura, assim como a avaliação da atividade fungicida e determinação do modo de ação de monoterpenos sobre os isolados de *Colletotrichum* e a levedura modelo *Saccharomyces cerevisiae*. Um total de 61 isolados foram obtidos a partir de uvas com sintomas de podridão da uva madura, os isolados foram identificados por sequenciamento e análise filogenética de GAPDH, TUB2, LSU e ITS. Os dados mostraram a presença de seis espécies de *Colletotrichum*, *C. viniferum* (23), *C. fructicola* (22), *C. kahawae* (6), *C. nymphaeae* (7), *C. limitticola* (1) e *C. karstii* (2). Com destaque para *C. limitticola* e *C. karstii* que pela primeira vez foram associados com a podridão da uva madura. Então, a atividade fungicida de 20 monoterpenos foi testada sobre isolados de *Colletotrichum* e sobre *S. cerevisiae*. Observou-se que os monoterpenos oxigenados apresentaram maior atividade antifúngica em comparação com os outros grupos. Os monoterpenos citral e geraniol apresentaram as maiores atividade sobre *Colletotrichum* e *S. cerevisiae*. O modo de ação destes monoterpenos em *Colletotrichum* foi associado com perda da integridade da membrana celular, acúmulo intracelular de espécies reativas de oxigênio, embora sem evidências de perda do potencial da membrana mitocondrial. Complementarmente, em *S. cerevisiae* foi observado que o tratamento com citral e geraniol causou perda na integridade da membrana e parede celular, acumulo de espécies reativas de oxigênio, e evidências de apoptose metacaspase dependente, em que o mutante *Δyca1* apresentou maior resistência a ambos monoterpenos. Esse estudo contribui para uma melhor compreensão das espécies de *Colletotrichum* associadas com a podridão da uva madura em videiras, além disso, apresenta alternativas para o controle desse fungo através da utilização de monoterpenos.

ABSTRACT

Ripe grape rot is one of the main diseases that occur in vineyards in southern Brazil, however, there is little information about the species of fungi responsible for the disease. Essential oils and their major compounds, monoterpenes, are considered alternatives for the control of fungal diseases, such as the grape ripe rot caused by *Colletotrichum* species. However, the mode of action of the monoterpenes has yet to be elucidated. The study aimed at the isolation and identification of *Colletotrichum* species from grapevines with symptoms of grape ripe rot, as well as the evaluation of fungicidal activity and mode of action of monoterpenes on isolates of *Colletotrichum* and the model yeast *Saccharomyces cerevisiae*. A total of 61 isolates were isolated from grapes with symptoms of grape ripe rot, the samples were identified by sequencing and phylogenetic analysis of GAPDH, TUB2, LSU, and ITS. Data showed the presence of six *Colletotrichum* species, *C. viniferum* (23), *C. fructicola* (22), *C. kahawae* (6), *C. nymphaeae* (7), *C. limitticola* (1) e *C. karstii* (2). Highlighting *C. limitticola* and *C. karstii*, which by the first time were associated with the grape ripe rot. Then, the fungicidal activity of 20 monoterpenes was evaluated on *Colletotrichum* isolates and *S. cerevisiae*. It was observed that the oxygenated monoterpenes showed greater antifungal activity compared to the other groups. Citral and geraniol monoterpenes showed the highest activity on *Colletotrichum* and *S. cerevisiae*. The mode of action of these monoterpenes on *Colletotrichum* was associated with a loss of cell membrane integrity, intracellular reactive oxygen species accumulation, although without evidence of loss of mitochondrial membrane potential. In addition, in *S. cerevisiae* was observed that citral and geraniol treatments caused loss of cell membrane and cell wall integrity, intracellular reactive oxygen species accumulation, and metacaspase-dependent apoptosis evidence, in which the *Δyca1* mutant showed greater resistance to both monoterpenes. This study contributes to a better understanding of *Colletotrichum* species associated with grape ripe rot, moreover, shows alternatives for this fungus control through monoterpenes utilization.

1 INTRODUÇÃO

Um dos gêneros de fungos fitopatogênicos que causam prejuízos a um amplo espectro de plantas cultivadas é o *Colletotrichum*. Considerado um dos dez fungos mais importantes em termos agronômicos, virtualmente qualquer planta cultivada é afetada por ao menos uma espécie do gênero *Colletotrichum*. Em vinhedos, fungos do gênero *Colletotrichum* causam uma doença chamada de podridão da uva madura, que afeta os frutos próximo a etapa de colheita, tornando as estratégias de controle limitadas.

A utilização constante de fungicidas químicos para o controle de doenças fúngicas tem sido uma importante ferramenta na agricultura moderna, garantindo estabilidade de produção e aumentando a produtividade. Entretanto, atualmente, devido ao uso excessivo de fungicidas químicos, associado a limitada disponibilidade de moléculas, estão ocorrendo constantes problemas de resistência dos fungos fitopatogênicos a esses fungicidas. Neste contexto, torna-se relevante a procura por novos compostos com atividade fungicida.

Visando a busca por novas moléculas com atividade fungicida, a utilização de óleos essenciais produzidos a partir de plantas é uma importante fonte para a obtenção de moléculas com atividade fungicida. Entretanto, óleos essenciais geralmente apresentam ampla variação em suas composições, que dependem das condições de cultivo das plantas utilizadas na produção do óleo essencial, além de variações de quimiotipos das mesmas. Portanto, a avaliação de compostos de forma isolada se torna interessante, permitindo a especificação do modo de ação de cada composto e evitando efeitos sinérgicos de difícil explicação.

Os óleos essenciais apresentam como seus compostos majoritários os terpenos, e de forma mais específicas os terpenos pertencentes ao grupo dos monoterpenos, portanto a avaliação de monoterpenos se torna um interessante ponto de partida na busca por moléculas com atividade fungicida.

A avaliação da atividade fungicida de compostos é importante, entretanto compreender como esses compostos levam o fungo a morte se torna fundamental para as estratégias de

controle e manejo de resistência, além disso permite estimar efeitos adversos em organismos não alvos.

Este trabalho buscou uma avaliação das espécies de *Colletotrichum* associadas com a podridão da uva madura em videiras, assim como a utilização de monoterpenos para o controle destes fungos e finalmente, a levedura modelo *Saccharomyces cerevisiae* foi utilizada para definir como os monoterpenos podem lever o fungo a morte.

2 REVISÃO BIBLIOGRÁFICA

2.1 Fungos do gênero *Colletotrichum*

Os membros do gênero *Colletotrichum* representam um grupo de fungos fitopatogênicos que podem infectar um amplo grupo de plantas, sendo o principal fungo patogênico responsável por causar principalmente uma doença chamada antracnose, uma doença fúngica com potencial de devastar diversas culturas e causar enormes perdas econômicas na maior parte das regiões agrícolas do mundo (Cannon *et al*., 2012). Além da antracnose, o *Colletotrichum* pode causar algumas podridões, como a podridão da uva madura em videira (Figura 1). De acordo com Dean *et al*., (2012), *Colletotrichum* é classificado como um dos fungos fitopatogênicos com maior potencial de causar perdas econômicas no mundo. O gênero *Colletotrichum* engloba uma ampla quantidade de espécies, que são responsáveis por doenças em um amplo espectro de plantas, incluindo frutíferas, plantas de lavoura e olerícolas (Cannon et al, 2012).

Figura 1. Evolução da podridão da uva madura em bagas de uva (Fonte: The American Phytopathological Society).

A maior parte das espécies de *Colletotrichum* podem sobreviver no solo de forma saprofítica sobre fragmentos mortos das plantas hospedeiras, sendo disperso através da chuva, permitindo a liberação dos conídios, que eventualmente entrarão em contato com a planta hospedeira, reiniciando o ciclo de infecção (Figura 2) (de Silva *et al*., 2017).

O processo de penetração e colonização dos tecidos das plantas hospedeiras por *Colletotrichum* spp. geralmente inicia-se com a germinação do conídio e a formação de uma estrutura especializada, chamada apressório, que facilita o processo de entrada do fungo a partir da cutícula e das células da epiderme da planta hospedeira (Wharton & Schilder, 2008). Entretanto, em raros casos, ocorre a penetração através de estômatos ou feridas, sem a formação do apressório ser reportada (Latunde-Dada *et al*., 1996). As estruturas na superfície da planta hospedeira, como a espessura da cutícula e da epiderme, além da presença de estômatos e tricomas, podem influenciar no processo inicial de infecção (Serrano *et al*., 2014).

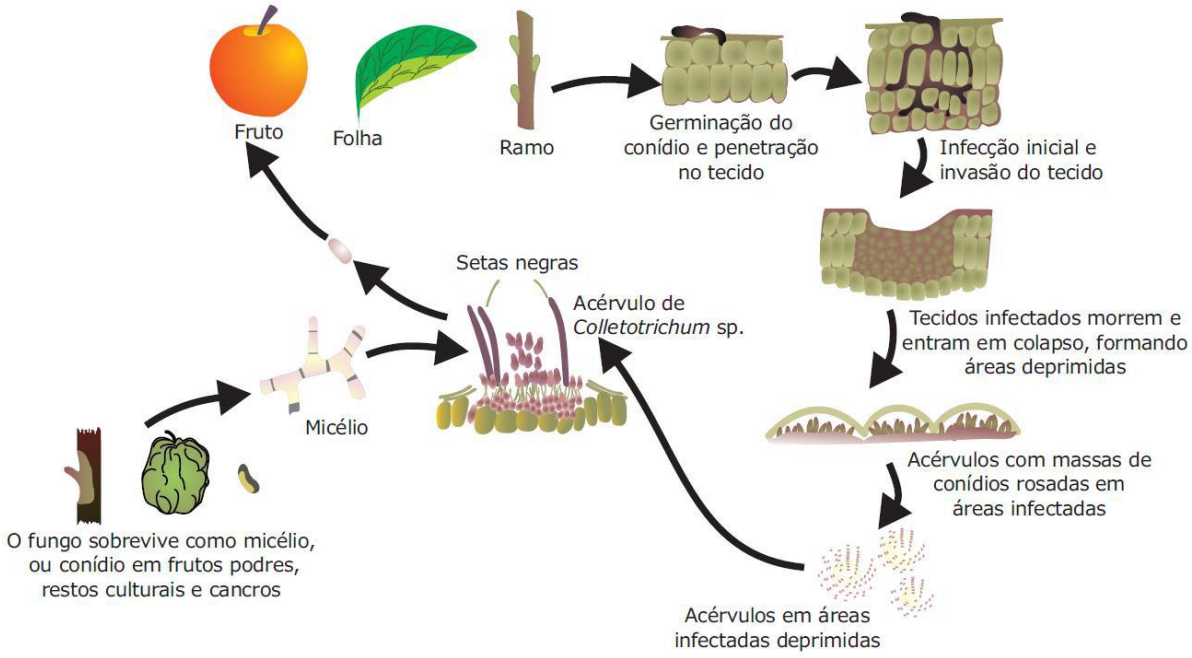


Figura 2. Ciclo de vida de espécies de *Colletotrichum* (Adaptado de De Silva *et al*., 2017).

Após o processo de infecção inicial, as estratégias de colonização variam dependendo da espécie de *Colletotrichum*, podendo ser hemibiotróficas ou necrotróficas (Bailey *et al*., 1992; De Silva *et al*., 2017). Nos estágios iniciais da infecção hemibiotrófica ocorre uma fase biotrófica assintomática, em que as hifas primárias invadem as células epidérmicas e são formadas vesículas no interior das células da epiderme e do mesófilo. Neste estágio as células

hospedeiras ainda estão vivas e a planta não apresenta nenhum sintoma. Então, segue-se para uma fase necrotrófica, em que finas hifas secundárias começam a crescer no interior das células infectadas e começam a secretar enzimas que degradam a parede celular, e finalmente acabam por matar a célula hospedeira (O'Connell *et al*., 2012; Gan *et al*., 2013). No processo de infecção necrotrófico, o fungo cresce abaixo da cutícula, penetrando entre as paredes celulares das células epidérmicas e iniciando a entrada nas células do mesófilo e consequente destruição do tecido da planta hospedeira (Peres *et al*., 2005).

2.2 Sistemática do gênero *Colletotrichum*

Colletotrichum foi inicialmente reportado por Tode (1790), sendo denominado *Vermicularia*, e foi posteriormente renomeado como *Colletotrichum* (Corda, 1837) na ordem Melanconiales; classe Coelomycetes; subdivisão Deuteromycotina. As espécies de *Colletotrichum* apresentam uma fase imperfeita ou assexual com o estágio teleomórfico *Glomerella* (Sutton, 1992).

Após a descrição inicial do *Colletotrichum*, durante várias décadas, centenas de novas espécies de *Colletotrichum* foram descritas e então von Arx (1957) estudou a taxonomia deste gênero de forma cuidadosa e reduziu o número de espécies de várias centenas para apenas 11 espécies aceitas. Posteriormente, Sutton (1992) aumentou o número de espécies de *Colletotrichum* aceitas para 39, entretanto, segundo o autor, a posição taxonômica de algumas espécies ainda continuava obscura. Até esse ponto, os métodos disponíveis para o processo de classificação dos fungos se baseavam em características morfológicas e culturais.

As primeiras aplicações de dados de sequenciamento de DNA para auxiliar a classificação de espécies de *Colletotrichum* foram publicadas por Mills *et al*., (1992) e Sreenivasaprasad *et al*. (1992), que identificaram variações em sequencias da região ITS1 do nrDNA em seis espécies de *Colletotrichum*, e também detectaram polimorfismos na mesma

região quando isolados de *C. gloeosporioides* originados de diferentes plantas hospedeiras eram comparados entre si. Posteriormente, Sherriff *et al*., (1994) apresentaram as primeiras árvores filogenéticas para *Colletotrichum* utilizando sequencias de ITS2 e LSU (*Large subunit ribossomal RNA*) oriundas de 27 linhagens de *Colletotrichum*, indicando que as mesmas pertenciam a 13 espécies diferentes. Neste estudo foi mostrado, que nem todas as linhagens classificadas por características morfológicas eram corretamente identificadas.

Os primeiros trabalhos de análise filogenética de espécies de *Colletotrichum* utilizando *multilocus* foram publicados por Talhinhas *et al*., (2002), um estudo com isolados de *C. acutatum* oriundos de lúpulo utilizando sequencias de ITS, TUB2 e HIS4, e Vinnere *et al*., (2002) utilizando sequencias de ITS, TUB2 e mtSSU em um estudo de espécies associadas com *Rhododendron*. A partir de então, com a redução dos custos de sequenciamento as análises filogenéticas utilizando *multilocus* se tornaram a norma, com sequencias geradas a partir de loci como actina (ACT), calmodulina (CAL), quitina sintase I (CHS-1), DNA liase (APN2), superóxido dismutase (SOD2), gliceraldeído-3-fosfato desidrogenese (GAPDH), RNA polimerase II (RPB1) e fator de elongação 1-α (EF1α) (Cannon *et al*., 2012). Análises filogenéticas de genes utilizados em estudos multilocus em espécies do clado *C. acutatum* indicaram que os dois melhores marcadores foram TUB2 e GAPDH, que permitiram o posicionamento correto de todos os 29 subclados testados (Damm *et al*., 2012).

Posteriormente, Bhunjun *et al*. (2021) utilizaram as sequencias previamente depositadas dos marcadores ACT, CHS-1, GAPDH, ITS e TUB2 e definiram a presença de 248 espécies de *Colletotrichum*, ditribuidas em 14 complexos e 12 espécies sem associação (Figura 3). Os autores concluíram ainda que nenhum dos marcadores testados apresenta capacidade de separar as diferentes espécies de *Colletotrichum* quando utilizados individualmente.

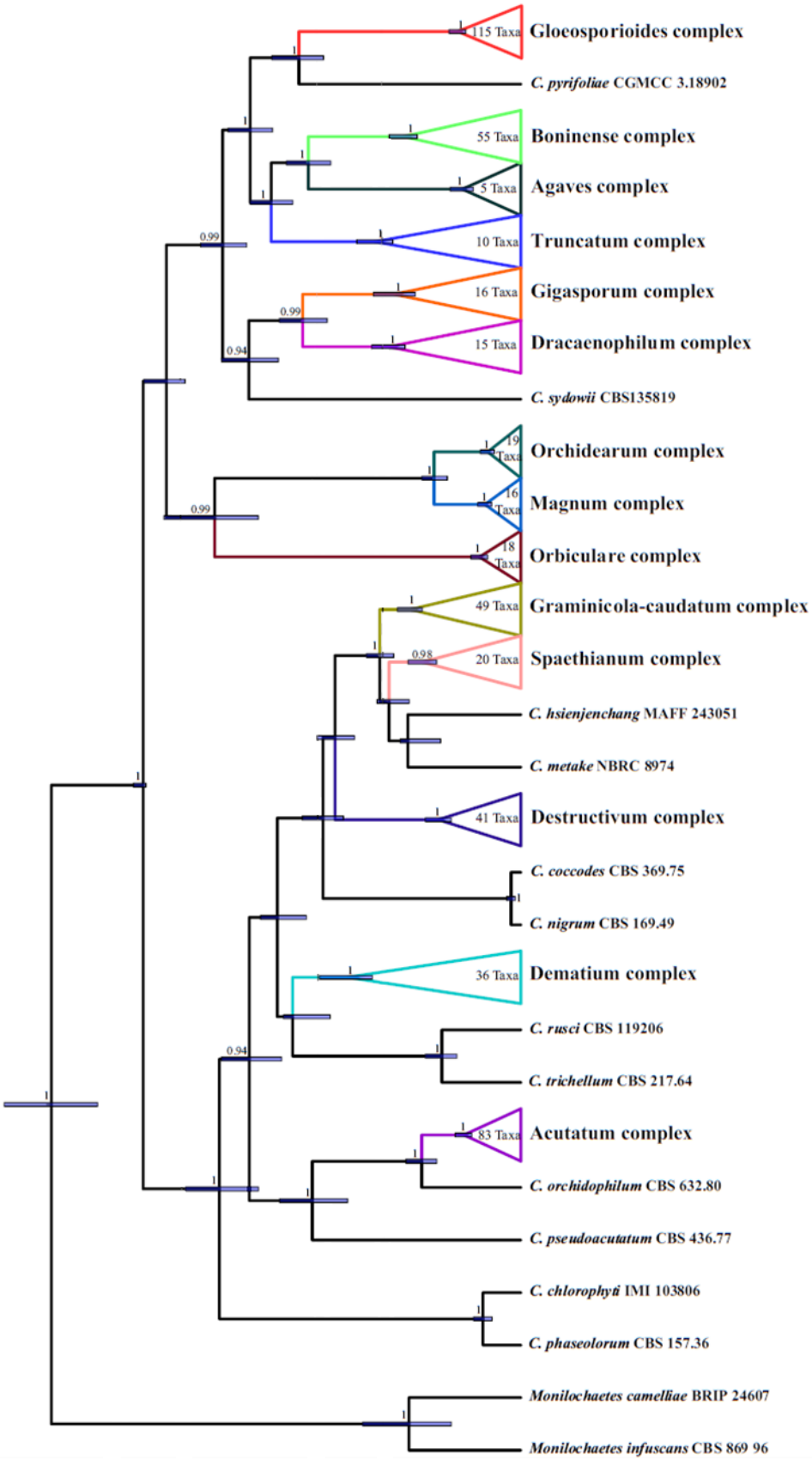


Figura 3. Árvore filogenética de espécies de *Colletotrichum*, montrando a mascroestrutura da distribuição dos diferentes complexos. Dados derivados das sequencias de ACT, CHS-1, GAPDH, ITS e TUB2 (Bhunjun *et al*., 2021).

2.3 Controle de *Colletotrichum*

O controle de doenças causadas por *Colletotrichum* normalmente envolve a utilização de uma, ou a combinação das seguintes estratégias: utilização de variedades resistentes, controle cultural, controle químico ou controle biológico. A aplicabilidade das estratégias de controle depende principalmente da cultura a ser utilizada, bem como o estágio fenológico da mesma (Wharton & Diéguez-Uribeondo, 2004).

2.3.1 Cultivares resistentes

A resistência a doença é provavelmente o aspecto mais relevante no controle de doenças na agricultura, embora não seja amplamente utilizada em pomares, devido principalmente, ao longo tempo requerido para os sistemas de melhoramento nestas culturas. Na maior parte das interações entre planta-patógeno, o processo de resistência envolve a ativação de sistemas de resposta do hospedeiro, que previne ou retarda o processo de infecção do fungo, o que pode ser condicionado por um único gene ou um conjunto de genes para a resposta a infeção fúngica (Kumar *et al*., 2020).

A utilização de plantas resistentes a *Colletotrichum* parece ser uma opção lógica e eficiente para o controle da doença. Entretanto, deixando de lado os custos associados com o replantio de pomares e estabelecimento de novas cultivares com resistência ou ao menos tolerância a doença, a maior parte dos produtores tendem a selecionar as cultivares baseado em outros fatores que não resistência ao fungo (Wharton & Diéguez-Uribeondo, 2004).

2.3.2 Controle cultural

O controle cultural normalmente se refere a práticas agrícolas para o controle de doenças utilizando táticas que evitam a utilização de agentes fitossanitários, através da manipulação dos períodos de cultivo, poda e colheita ou evitando condições para o surgimento da doença. Em frutas, o controle cultural de *Colletotrichum* envolve a poda adequada das frutíferas, a remoção

de frutos mumificados do inteior do pomar e todo o processo de pós-colheita, uma vez que o fungo causa diversos problemas neste período. Isso envolve a manipulação cuidadosa dos frutos, de forma a evitar injúrias mecânicas e temperaturas extremas, que aumentam a predisposição do surgimento da doença nos frutos (Wharton & Diéguez-Uribeondo, 2004).

2.3.3 Controle químico

A utilização de métodos de controle químico é amplamente utilizada em frutiferas, devido aos custos de produção quando comparados com outras estratégias e a eficiência dos agroquímicos no controle fitossanitário, que em geral se torna superior quando comparado com outras estratégias de controle (Phoulivong, 2011).

O desenvolvimento de antracnose em frutos tem sido controlado tradicionalmente pela aplicação de fungicidas sintéticos, como os pertencentes aos grupos MBCs (*Methyl Benzimidazole Carbamates*), ditiocarbamatos e inibidores da biossíntese de ergosterol (Gopinath *et al*., 2006; Moral *et al*., 2018, Moreira *et al*., 2019). Embora o tratamento com os fungicidas possa reduzir de forma relevante a incidência e severidade da doença, a erradicação do patógeno não pode ser atingida. Portanto, se as aplicações forem interrompidas e houver condições adequadas, a doença irá voltar a ocorrer. Aplicações de forma preventiva, assim como uma rotatividade entre os fungicidas de diferentes classes são práticas recomendadas (Adaskaveg & Förster, 2000).

2.3.4 Controle alternativo

Além das formas convencionais, o controle de *Colletotrichum* pode ser realizado com a utilização de extratos naturais (Vilaplana *et al*., 2018), microrganismos antagonistas (Campos-Martínez *et al*., 2016) ou utilização de indutores de resistência (Ramos-Guerrero *et al*., 2018). Vilaplana *et al*. (2018) mostraram a eficiência no controle de *Colletotrichum musae* em bananas com a utilização de óleo essencial de tomilho. As estratégias de contole alternativo auxiliam no

controle da doença, apesar de ainda apresentarem utilização limitada, espera-se que no futuro ocorra um aumento na demanda das mesmas, seguindo uma tendência de redução na utilização de agroquímicos (Han *et al*., 2021).

2.4 *Colletotrichum* em videiras

Em videiras a presença de *Colletotrichum* está relacionado principalmente com a doença denominada podridão da uva madura, que afeta a cultura no final do ciclo de produção causando perdas e reduzindo a qualidade do produto final (Bragança *et al*., 2016).

As espécies de *Colletotrichum* associadas a videiras apresentam variações dependendo da região em que a mesma foi isolada. Por exemplo, Baroncelli *et al*., (2014) encontraram a presença de *C. godetiae* em amostras de videiras do Reino Unido, enquanto estudos realizados na China encontraram a presença de *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. hebeiense* e *C. viniferum* (Peng *et al*., 2013; Yan *et al*., 2015). Amostras isoladas em vinhedos do Brasil, e identificadas a partir de sequencias da região ITS, mostraram principalmente a presença das espécies *C. viniferum* e *C. fructicola* (Echeverrigaray *et al*., 2019).

2.5 Óleos essenciais e terpenos

Óleos essenciais são misturas complexas de compostos voláteis biossintetizados e extraídos a partir de plantas aromáticas. De forma geral, apresentam odor acentuado e são normalmente obtidos a partir de hidrodestilação do material vegetal. A composição de óleos essenciais depende de diversos fatores, como as características genéticas da planta, características edafoclimáticas e a estratégia de extração.

Os componentes mais abundantes nos óleos essenciais são os terpenos, amplo grupo de moléculas biossintetizadas pelo metabolismo secundário das plantas, eles apresentam alta diversidade de estruturas químicas. Apesar dos terpenos não participarem do metabolismo primário das plantas, eles desempenham diversas funções, sendo as principais de proteção da

planta contra fitopatógenos e herbivorismo, além de contribuir para a atração de polinizadores (Bakkali *et al*., 2008). Os terpenos são utilizados para diversas aplicações, incluindo seu uso como produtos agrícolas, aromatizantes e farmacêuticos (Vermaas *et al*., 2018).

Terpenos são sintetizados a partir da condensação de unidades de isopreno (C_5H_8). A fórmula base de todos os terpenos é $(C_5H_8)_n$, sendo n o número de unidades de isopreno ligadas em cadeia ou arranjadas em forma de anéis. Terpenos podem existir como hidrocarbonetos ou podem possuir outros grupos associados como hidroxilas, carbonilas, cetonas e aldeídos. Após as alterações químicas dos terpenos, os compostos formados são denominados terpenóides (Gao & Singh, 1998).

Os terpenos são classificados quanto ao número de unidades de isoprenos. Os monoterpenos (C_{10}) e sesquiterpenos (C_{15}) são os terpenos mais abundantes na natureza, porém hemiterpenos (C_5), diterpenos (C_{20}), triterpenos (C_{30}) e tetraterpenos (C_{40}) também podem ser encontrados (Bakkali *et al*., 2008). Alguns exemplos de estruturas de terpenos estão apresentadas na figura 4.

Os terpenos apresentam importantes atividades biológicas, incluindo efeitos antibacterianos (Lang & Buchbauer, 2012), antifúngicos (Devkatte *et al*., 2005; Irkin & Korukluoglu, 2009), antiviral (Schnitzler *et al*., 2007), nematicida (Echeverrigaray *et al*., 2010) e antiprotozoário (Saeidnia & Gohari, 2012).

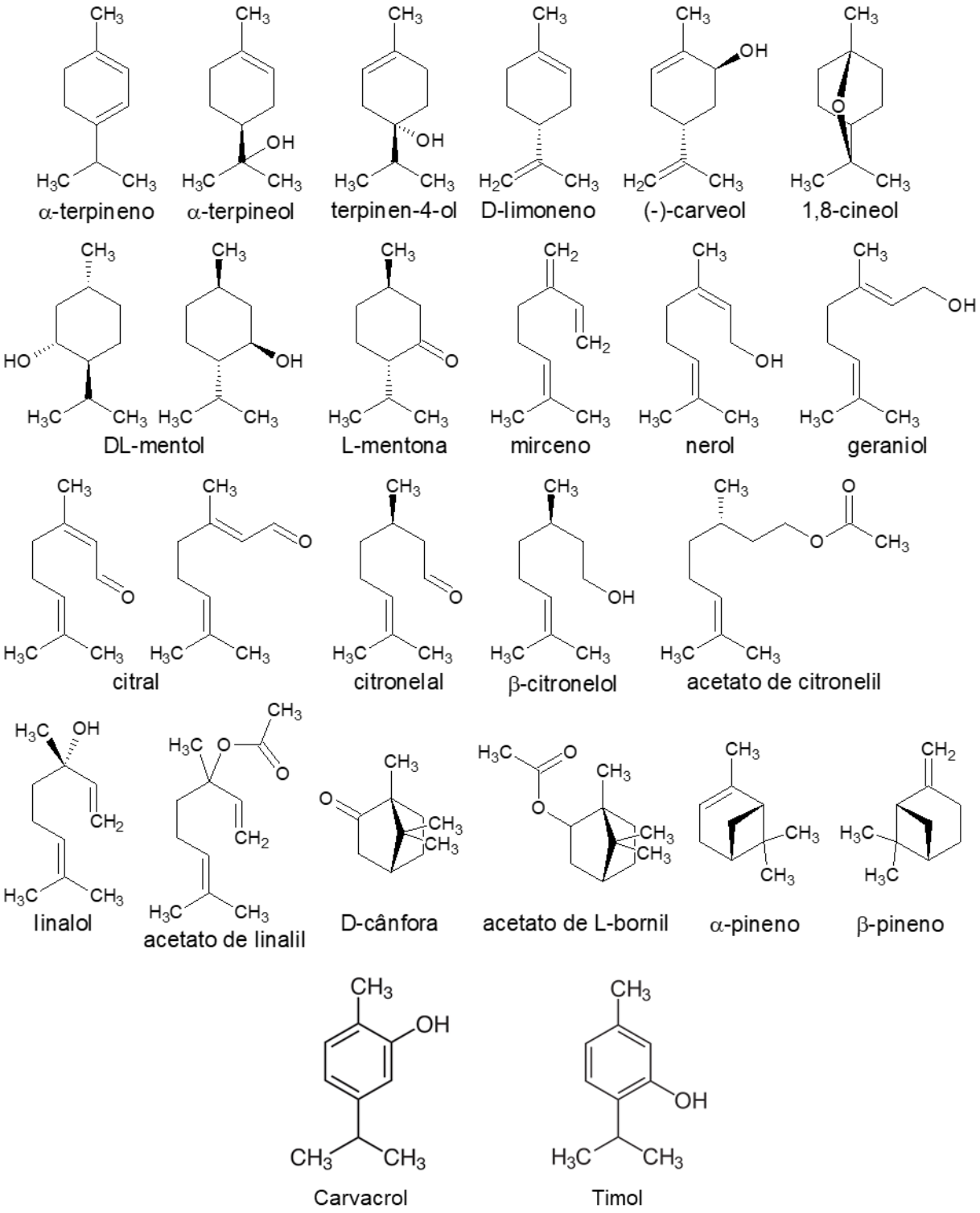


Figura 4. Estrutruras de alguns terpenos.

2.5.1 Citral

Citral é a mistura de dois terpenóides geranial e neral, sendo encontrado principalmente em frutas cítricas, capim limão e verbena. Ele é amplamente utilizado como aditivo alimentício

e aromatizante, apresentando reconhecida atividade antimicrobiana contra bactérias e fungos (Kumari *et al*., 2019; Sun *et al*., 2019).

A atividade fungicida do citral foi observada sobre alguns fungos filamentosos, sobre *Meganoporthe grisea* a atividade fungicida do citral foi atribuída principalmente ao seu ataque sobre a parede celular e membrana citoplasmática (Li *et al*., 2014), seguido pela sua interferência no metabolismo energético, na síntese proteica e na atividade enzimática (Li *et al*., 2015).

O efeito inibitório do citral sobre o fungo *Penicillium digitatum* está envolvido com alterações morfológicas nas mitocôndrias, inibindo o metabolismo respiratório e reduzindo a atividade de enzimas do ciclo de Krebs (Zheng *et al*., 2015). A expressão de proteínas relacionadas à obtenção de energia e regulação da concentração intracelular de ERO (espécies reativas de oxigênio) foram amplamente alteradas após o tratamento com citral em *P. digitatum*. Esse efeito no metabolismo energético causado pelo citral se mostrou diretamente relacionado com um desequilíbrio na atividade dos complexos da cadeia transportadora de elétrons, em que os autores observaram uma redução na atividade dos complexos I e IV após 60 minutos de tratamento com citral. As alterações na mitocôndria e interferências nos complexos mitocondriais levam a uma redução na síntese de ATP e acúmulo de ERO (OuYang *et al*., 2018). Além disso, alterações nos níveis de expressão de enzimas envolvidas na biossíntese de ergosterol foram observadas devido o tratamento de citral sobre *P. digitatum*, incluindo ERG7, ERG11, ERG6, ERG3 e ERG5, o que resultou em uma redução na concentração de ergosterol e acúmulo do intermediário lanosterol (OuYang *et al*., 2016).

O efeito fungicida do citral sobre o fungo *Alternaria alternata* foi relacionado com sua capacidade de causar danos estruturais irreversíveis aos esporos, além de causar distúrbios no balanço oxidativo da célula e na integridade celular. O monoterpeno foi capaz de afetar a síntese de duas micotoxinas produzidas pelo fungo, alternariol e monometil éter alterariol (Wang *et al*., 2019).

Em *Cryptococcus neoformans*, o citral foi capaz de afetar a membrana citoplasmática, e em nível intracelular causou inibição na biossíntese de ergosterol, alteração no efluxo de K^+, acúmulo intracelular de ERO e peroxidação lipídica (Kumari *et al*., 2019).

O efeito do citral sobre a levedura *Candida tropicalis* apresentou ligação direta com alterações na membrana citoplasmática, sem evidências da interação entre o citral e o ergosterol, entretanto após o tratamento com citral a levedura apresentou elevação na expressão da enzima ERG11 (lanosterol 14α-demetilase), que está diretamente envolvida na síntese de ergosterol na levedura, além de ser um alvo terapêutico dos fungicidas pertencentes ao grupo azol (Chatrath *et al*., 2019).

2.5.2 Carvacrol

O carvacrol apresentou efeitos fungicidas sobre diversos isolados da levedura *Cryptococcus neoformans*, a forma de ação não foi completamente elucidada, entretanto, os autores atribuíram o efeito fungicida do terpeno a sua ligação ao ergosterol, podendo assim causar um desbalanço no fluxo dos íons Ca^{+2} e K^+ (Nóbrega *et al*., 2016).

Zhou *et al*., (2018) investigando o efeito do carvacrol sobre o fungo *Rhizopus stolonifer* mostraram que o tratamento com o monoterpeno causou um aumento na permeabilidade da membrana citoplasmática e consequente liberação de componentes celulares, como ácidos nucleicos, aminoácidos e proteínas, para o espaço extracelular. Além disso, observou-se ainda uma redução no conteúdo de ergosterol presente no fungo.

O efeito do carvacrol em diferentes isolados do gênero *Candida* mostraram um efeito parecido, com uma perda na integridade da membrana citoplasmática e afetando a via de biossíntese do ergosterol (Ahmad *et al*., 2011).

2.5.3 Citronelal

O efeito fungicida do citronelal sobre a levedura *Candida albicans* foi atribuído a interferências na homeostase da membrana citoplasmática, com redução nos níveis de ergosterol e adicionalmente um aumento nas espécies reativas, levando a danos oxidativos. O monoterpeno foi capaz ainda de inibir a interação entre células e pseudohifas formadas pela levedura, evitando a formação de biofilmes (Trindade *et al*., 2015; Singh *et al*., 2016). O acúmulo de ERO em *C. albicans*, devido ao tratamento com citronelal, foi posteriormente explicado por alterações no potencial da membrana mitocondrial e redução na expressão das enzimas *SOD2* (superóxido dismutase 2) e *GPX2* (glutationa peroxidase 2) (Saibabu *et al*., 2017).

A atividade fungicida do citronelal foi observada em outros fungos, como o *P. digitatum* que apresentou drástica redução na germinação de esporos após o tratamento com citronelal por seis horas. O modo de ação do citronelal foi observado nos esporos e constatou-se uma permeabilidade dos esporos ao corante iodeto de propídio, assim como a liberação do conteúdo citoplasmático dos esporos, indicando a perda de integridade da membrana citoplasmática dos mesmos (Wu *et al*., 2016).

2.5.4 Citronelol

O citronelol é um monoterpeno alcoólico encontrado principalmente em óleos essenciais de plantas do gênero *Cymbopogon*, possuindo atividades farmacológicas e antimicrobianas (Santos *et al*., 2019).

O efeito do citronelol sobre o fungo *Trichophyton rubrum* inclui uma redução no desenvolvimento micelial, alterando a morfologia das hifas formadas, além de causar liberação de conteúdo intracelular. O monoterpeno também inibiu a germinação dos conídios. Em relação ao mecanismo de ação do citronelol sobre o fungo, apenas foi observada uma redução na

concentração de ergosterol nas células tratadas com o monoterpeno, sugerindo uma inibição na biossíntese de ergosterol (Pereira *et al*., 2015).

O citronelol apresenta capacidade fungicida sobre *C. albicans*, afetando a capacidade de formação de biofilme da levedura e inibindo a liberação extracelular de proteases e fosfolipases, importantes fatores de virulência atribuídos à levedura. A exposição de *C. albicans* ao monoterpeno modulou a expressão de genes envolvidos na síntese de ergosterol, reduzindo sua expressão e consequentemente reduzindo os níveis de ergosterol presente na membrana citoplasmática (Sharma *et al*., 2020). O citronelol ainda levou a interferências na dinâmica do ciclo celular de *C. albicans*, levando as células a ficarem acumuladas na fase S do ciclo celular (Zore *et al*., 2011).

2.5.5 Geraniol

O geraniol é um monoterpeno alcoólico acíclico isolado do óleo essencial de diversas plantas aromáticas, incluindo *Cinnamomum tenuipilum* e *Valeriana officinalis*. Ele é amplamente utilizado como fragrância, além de apresentar aspectos farmacológicos relevantes, como atividade antioxidante, antitumoral e antimicrobiana (Lei *et al*., 2019).

Leveduras do gênero *Candida* tratadas com geraniol apresentaram alterações morfológicas, incluindo rupturas na parede celular, e redução na formação de pseudohifas e clamidoconídios (Leite *et al*., 2014). Outro efeito observado foi uma redução significativa nos níveis de ergosterol nas células tratadas com geraniol, além de uma diminuição no efluxo de prótons da ATPase, associado com a perda de potencial de membrana mitocondrial (Sharma *et al*., 2016; Singh *et al*., 2018). Por fim o geraniol mostrou potencial na modulação da bomba de efluxo CaCdr1p (responsável pela resistência a diversas drogas) através da ligação do geraniol ao sítio ativo da mesma (Singh *et al*., 2018).

2.5.6 Limoneno

O limoneno apresentou atividade fungicida sobre isolados da levedura *Candida albicans*, tanto em sua forma planctônica quanto em biofilmes, através de análise proteômica constatou-se que, em resposta ao tratamento com limoneno as leveduras elevaram a expressão de proteínas envolvidas com a síntese de glucanos da parede celular, estresse oxidativo, estresse nucleolar e proteínas envolvidas no reparo ao DNA. O tratamento com limoneno levou as leveduras a fragmentação do DNA e exposição de fosfatidilserina, indicando presença de apoptose, além disso houve parada do ciclo celular na fase G1 (Thakre *et al*., 2018).

O efeito do limoneno em fungos filamentosos como *Sclerotinia sclerotiorum* evolveu alterações morfológicas nas hifas, inibição na biossíntese de ergosterol, redução na atividade das enzimas malato desidrogenase e succinato desidrogenase, além da acidificação do meio extracelular (Ma *et al*., 2015).

2.5.7 Timol

O timol é um monoterpeno fenólico obtido a partir de plantas como o orégano e alecrim, ele é sintetizado a partir do *p*-cimeno. O timol apresenta diversos efeitos biológicos, incluindo sua atividade antioxidante, bactericida e fungicida (Marchese et al, 2016).

O efeito do timol sobre esporos do fungo *Aspergillus flavus* envolve a uma elevação na geração de ERO e óxido nítrico, que parece ocorrer de forma sequencial com um acúmulo inicial de ERO e subsequente acúmulo de óxido nítrico (Shen *et al*., 2016). Enquanto isso, o efeito do timol sobre *Fusarium graminearum* envolve danos na membrana citoplasmática originados principalmente pela peroxidação lipídica e distúrbios na biossíntese de ergosterol (Gao *et al*., 2016). Efeitos similares foram encontrados em *Alternaria alternata* após o tratamento com timol, em que foi observado além de danos na membrana citoplasmática, alterações na parede celular e consequente liberação de conteúdo citoplasmático (Perina *et al*., 2015). Zhang *et al*., (2018) avaliando o efeito do tratamento com timol sobre o fungo *Fusarium*

oxysporum observaram que o timol induz um aumento no acúmulo de ERO e destruição da parede celular e membrana citoplasmática.

Em leveduras do gênero *Candida* o modo de ação do timol parece estar envolvido com sua ligação ao ergosterol presente na membrana citoplasmática (De Castro *et al*., 2015).

2.5.8 α-terpineol

O efeito do α-terpineol sobre o fungo *Penicillium digitatum* envolve alterações na síntese da parede celular, levando a disrupção da mesma, além disso, foram observadas evidências de danos na membrana citoplasmática, com liberação de conteúdo intracelular e elevação da condutividade extracelular (Jing *et al*., 2015).

Em *Candida albicans* o efeito do tratamento com α-terpineol levou a perda da integridade de membrana citoplasmática, além de interferir no processo de formação de biofilme (Ramage *et al*., 2012).

2.6 *Saccharomyces cerevisiae* como organismo modelo

A utilização de organismos modelo é uma marca da comunidade científica. Os organismos modelos são utilizados devido alguns fatores, eles podem auxiliar a superar empecilhos éticos e experimentais, eles permitem o desenvolvimento de métodos analíticos padronizados e por último eles permitem uma representação de fenômenos e processos biológicos que podem ser aplicados para uma classe mais ampla de organismos vivos (Karathia *et al*., 2011).

A levedura *S. cerevisiae* vem sendo utilizada como organismo modelo para o estudo de diferentes processos biológicos como estudos de envelhecimento (Prada-Luengo *et al*., 2020), regulação da expressão gênica (Kaczmarek Michaels *et al*., 2020), sinalização (Takayama *et al*., 2019), ciclo celular (Matellán & Monje-Casas, 2020), metabolismo (Dai *et al*., 2018), apoptose (Bonomelli *et al*., 2020) e várias outras aplicações.

Devido a sua facilidade de manutenção e elaboração de ensaios, *S. cerevisiae* tem sido utilizada também no *screening* de novas moléculas com potencial atividade fungicida. Diversos trabalhos exemplificam este potencial, mostrando a eficiência de *S. cerevisiae* para o *screening* de compostos para o controle de outros fungos (Tani *et al*., 2012; Tebbets *et al*., 2012), ou na procura por moléculas com modos de ação específicos (Sussman *et al*., 2004; Krysan & Didone, 2008; Ishikawa & Lisiecki, 2020).

3 OBJETIVOS

3.1 Objetivo geral

Isolar e identificar espécies de *Colletotrichum* a partir de videiras com sintomas de podridão da uva madura, assim como avaliar a atividade antifúngica e determinar o modo de ação de monoterpenos sobre isolados de *Colletotrichum* e a levedura modelo *S. cerevisiae*.

3.2 Objetivos específicos

- Isolar, identificar e caracterizar fungos do gênero *Colletotrichum* a partir de frutos de videiras com sintomas de podridão da uva madura.

- Verificar a patogenicidade das diferentes espécies de *Colletotrichum* isoladas sobre frutos de videiras.

- Avaliar a atividade fungicida de diferentes monoterpenos sobre os isolados de *Colletotrichum* e *S. cerevisiae*.

- Determinar o modo de ação dos monoterpenos com maior potencial fungicida sobre *Colletotrichum* spp. e *S. cerevisiae*.

4 RESULTADOS E DISCUSSÃO

Os resultados desta tese serão apresentados na forma de capítulos, onde cada capítulo engloba um artigo.

- *Colletotrichum* species causing grape ripe rot disease in *Vitis labrusca* and *V. vinifera* varieties in the highlands of southern Brazil

- Activity of monoterpenoids on the in vitro growth of two *Colletotrichum* species and the mode of action on *C. acutatum*

- Antifungal Activity of Monoterpenes Against the Model Yeast *Saccharomyces cerevisiae*

- Citral and geraniol induce necrotic and apoptotic cell death on *Saccharomyces cerevisiae*

4.1 CAPÍTULO 1

Colletotrichum species causing grape ripe rot disease in _Vitis labrusca_ and _V. vinifera_ varieties in the highlands of southern Brazil

Sergio Echeverrigaray, Fernando J. Scariot, Gabrielli Fontanella, Francesco Favaron, Luca Sella, Murilo C. Santos, Joséli Schwambach, Carine Pedrotti, Ana Paula L. Delamare

Artigo publicado na revista *Plant Pathology*.

Received: 23 March 2020 | Accepted: 24 June 2020
DOI: 10.1111/ppa.13240

ORIGINAL ARTICLE

Plant Pathology WILEY

Colletotrichum species causing grape ripe rot disease in *Vitis labrusca* and *V. vinifera* varieties in the highlands of southern Brazil

Sergio Echeverrigaray[1] | Fernando J. Scariot[1] | Gabrielli Fontanella[1] | Francesco Favaron[2] | Luca Sella[2] | Murilo C. Santos[3] | Joséli Schwambach[4] | Carine Pedrotti[4] | Ana Paula L. Delamare[1]

[1]Laboratory of Enology and Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil
[2]Territorio e Sistemi Agro-Forestali (TeSAF), Univesità di Padova, Padova, Italy
[3]Laboratory of Phytopathology, University of Caxias do Sul, Caxias do Sul, Brazil
[4]Laboratory of Plant Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil

Correspondence
S. Echeverrigaray, Laboratory of Enology and Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul 95070-560, RS, Brazil.
Email: selaguna@ucs.br

Funding information
Brazilian National Council; CNPq, Grant/Award Number: 304906/2017-4; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Abstract

Ripe rot is one of the most important bunch diseases of grapes in the highlands of southern Brazil and a matter of concern for winegrowers. Sixty-one strains isolated from berries of *Vitis labrusca* and *V. vinifera* varieties with symptoms were classified by phylogenetic analysis of the sequences of β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, D1/D2 domain of 28S rDNA, and rDNA internal transcribed spacer. They were also characterized by morphology, and their pathogenicity was evaluated. The combined molecular data allowed identification of six *Colletotrichum* species: *C. fructicola*, *C. kahawae* and *C. viniferum* (gloeosporioides complex), *C. limitticola*, *C. nymphaeae* (acutatum complex), and *C. karstii* (boninense complex). This is the first report of *C. karstii* and *C. limitticola* associated with the ripe rot of grapes. Morphological characteristics varied within and among species, confirming their separation at the complex level. Pathogenicity tests on *V. vinifera* berries showed that the most prevalent species, *C. viniferum* (37.8%) and *C. fructicola* (36.1%), were more virulent than the less prevalent species *C. limitticola* and *C. karstii*. Our findings indicate that there is a high diversity of *Colletotrichum* species associated with ripe rot disease of grapes in Brazil. There were no clear differences in the distribution of *Colletotrichum* species between *V. labrusca* and *V. vinifera* varieties. The determination of fungal species responsible for grape ripe rot in Brazilian vineyards may contribute to further epidemiological studies and the development of more efficient prophylactic methods for ripe rot management.

Colletotrichum species causing grape ripe rot disease in _Vitis labrusca_ and _V. vinifera_ varieties in the highlands of Southern Brazil

S. Echeverrigaray [a*], F. J. Scariot [a], G. Fontanella [a], F. Favaron [b], L. Sella [b], M. C. Santos [c], J. Schwambach [d], C. Pedrotti [d], A. P. L. Delamare [a]

[a] Laboratory of Enology and Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul 95070-560, RS, Brazil.

[b] Territorio e Sistemi Agro-Forestali (TeSAF), Univesità di Padova, Padova 35020, Italy.

[c] Laboratory of Phytopatology, University of Caxias do Sul, Caxias do Sul 95070-560, RS, Brazil.

[d] Laboratory of Plant Biotechnology, University of Caxias do Sul, Caxias do Sul 95070-560, RS, Brazil.

* E-mail address: selaguna@ucs.br (S. Echeverrigaray)

Abstract: Ripe-rot is one of the most important bunch diseases of grapes in the highlands of Southern Brazil, and a matter of concern for winegrowers. Sixty-one strains isolated from symptomatic berries of *Vitis labrusca* and *V. vinifera* varieties were classified by phylogenetic analysis of β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, D1/D2 domain of 28S rDNA, and rDNA internal transcribed spacer sequences, characterized by morphology, and their pathogenicity evaluated. The combined molecular data allowed identification of six *Colletotrichum* species: *C. viniferum, C. fructicola, C. kahawae* (Gloeosporioides complex), *C. nymphaeae, C. limitticola* (Acutatum complex), and *C. karstii* (Boninense complex). This is the first report of *C. limitticola* and *C. karstii* associated with the ripe-rot of grapes. Morphological characteristics varied within and among species, confirming their separation at the complex level. Pathogenicity tests made on *V. vinifera* berries showed that the most prevalent species *C. viniferum* (37.8%) and *C. fructicola* (36.1%) were more virulent than *C. limitticola* and *C.*

karstii, the less prevalent species. The present results providence evidence of the high diversity of *Colletotrichum* spp. associated with ripe rot disease of grapes in the most important viticultural Brazilian region, and non-clear differences on the distribution of *Colletotrichum* species between *V. labrusca* and *V. vinifera* varieties. The determination of fungal species responsible for grapes ripe-rot in Brazilian vineyards may contribute to further epidemiological studies and the development of more efficient prophylactic methods for ripe-rot management.

Keywords: *Vitis*, grapevine, multilocus phylogeny, virulence, *Glomerella*

Introduction

The highlands of Southern Brazil, known as "Serra Gaucha" region (29°10′ S/51°32′ W), is responsible for more than 80% of Brazilian grape production. Official data showed that, in 2019, this region produced and processed (wine and juice) 614 thousand tons of grapes, of which 88% were *Vitis labrusca* L. and hybrid varieties (ex. Niagara, Isabella, Ives, among others), and 12% *Vitis vinifera* L., mainly Merlot, Chardonnay, Moscato, Cabernet Sauvignon. With a mean altitude of 750 to 890 m, this region has a subtropical climate with a mean annual temperature of 17.2 °C, and precipitation of 1,736 mm per year. The high temperature and precipitation during vintage are responsible for a high incidence of bunch diseases such as Botrytis rot, sour rot, bitter rot, and ripe rot (Almança et al., 2015; Wang et al., 2015).

Ripe rot is one of the most important diseases of grapes in Southern Brazil. It was first reported in the late nineteen century in São Paulo and in 1960's in Southern Brazil, becoming a matter of concern of Brazilian grape growers since the beginning of 2000's (Garrido and Sônego 2004). Ripe rot disease of grapes is associated with *Glomerella cingulata* (Stonemam) Spauld & Schrenk, or *Colletotrichum* sp., the teleomorph and the anamorph of the same fungus, respectively.

Colletotrichum is a large genus with more than 200 species, most of which affect crop

plant species causing leaves, stalks and fruit lesions currently known as anthracnose (Cannon et al. 2012; Marin-Felix et al.. 2017; De Silva et al.. 2017). These fungi have hemibiotrophic behavior with a biotrophic phase that progresses to a necrotrophic phase under appropriate host and environmental conditions (De Silva et al. 2017). The necrotrophic phase involves the alkalization of host tissues by ammonia secretion, and the production of several cell wall degrading enzymes, including, pectinases, cutinases, cellulases and peptidases (Joshi 2018). The ammonia and enzymes secretion varied among and within *Colletotrichum* species, affecting host specificity and pathogenesis (De Silva et al. 2017; Joshi 2018).

Grapes are susceptible to infection by *Colletotrichum* anytime from flowering to veraison, but it is during ripening that ripe rot disease becomes evident. Infected berries develop circular reddish-brown or rosy regions with the sunken surface. The lesions enlarge in concentric zones that can reach the whole berry. More or less abundant orange conidia are produced in rotten berries and spread via rain splash to other fruits. In later stages, the mummified fruits collapse (Cannon et a., 2012; De Silva et al., 2017). Important losses can occur when frequent rains, high humidity, and warm temperatures occur near the harvesting time (Wang et al. 2015).

The classical identification of *Colletotrichum* species is based on colony morphology, shape and size of conidia, appressoria, physiological traits, host plant, among other characteristics (Cannon et al. 2012). However, although important, these characters are variable, can lead to misclassification, and cannot differentiate many species within a *Colletotrichum* complex. Thus, molecular data, especially multigene sequencing have been used for the more precise classification of *Colletotrichum* species (De Silva et al. 2017).

Based on morphological and molecular data, several species of *Colletotrichum* have been associated with ripe rot disease of grapes in different viticultural regions worldwide, including *Colletotrichum gloeosporioides* Penz., *C. fruticola* Prihastuti, Cai and Hyde, *C. viniferum* Peng, Cai, Hyde and Ying, *C. acutatum* Simmonds, *C. capsici* Syd. And Syd., *C. aenigma* Weir and Johnst., among others (Whitelaw-Weckert et al. 2007; Greer et al. 2011; Stankova et al. 2011;

Sawant et al. 2012; Peng et al. 2013; Yan et a. 2015). The prevalence of these species varied according to the region. For example, while in China the most prevalent species are *C. viniferum* and *C. fructicola* (Peng et al. 2013, in India, *C. gloeosporioides* represent about 60% of the isolated from ripe rot grapes (Chowdappa et al. 2009), and in Australia, the most prevalent species is *C. acutatum* (Whitelaw-Weckert et al. 2007). In a preliminary survey of *Colletotrichum* species in Southern Brazil, we identified six species based on ITS sequencing, including representatives of the Gloeosprioides, Acutatum and Boninese clades (Echeverrigaray et al., 2019). Although similar, these species showed important differences in their host-specificity, pathogenic potential and fungicides resistance (Peng et al. 2013; Hei et al. 2019), which points to the need for classification at the species level to establish appropriate prophylactic and control strategies.

In order to contribute to epidemiologic studies and disease management, the present study aimed to identify the *Colletotrichum* species causing ripe rot disease of grapes in the most important Brazilian viticultural region, including molecular classification based on four DNA sequences, morphological characterization, and virulence evaluation.

Material and methods

Sampling and isolation of *Colletotrichum*

Grape berries with typical symptoms of ripe rot were collected from vineyards in different counties of the highlands of Southern Brazil (Table 1). Berry skin tissues of approximately 5mm in diameter were collected, disinfected with 1% hypochlorite for 1 min, washed with sterile distilled water, and dried with sterilized towel paper. The samples were plated on PDA (potato-dextrose-agar) with gentamicin (50 mg/L) and incubated at 28 °C for 4 d. Single-spore cultures were isolated and stored at -80°C in PDB (potato-dextrose-broth) with 25% glycerol.

Cultural, conidial and appressoria characterization

Radial mycelial growth was recorded daily (two perpendicular measurements) for 7 d on PDA cultures at 28 °C with a 16 h photoperiod, initiated from 5 mm diam mycelium plugs. Colony appearance and conidial production were evaluated on the seventh day. Conidia were recovered after 7 d on PDA plate by scraping the surface of the culture with water, followed by filtration. Conidial shape and size were microscopically evaluated at 400X magnification using a Nikon E200 microscope coupled with a CCD camera. Conidial and mycelial appressoria were evaluated as proposed by Liu et. (2016).

Pathogenicity assay

Conidial suspensions were obtained from 14 days old cultures on PDA. Conidia were harvest by adding 5 mL of sterilized water and gently scraping the surface of the fungal culture. The suspension was filtered and adjusted to 10^6 conidia/mL.

For pathogenicity assay healthy berries of *V. vinifera* cv. Italia were surface disinfected with 70% ethanol for 30 seconds, hypochlorite solution (1%) for 5 min, and washed three times with sterile water. Disinfected grapes were puncture wounded with a sterilized needle, placed in boxes and inoculated with 5 µL suspension of conidia (10^6 conidia/mL). Control berries were inoculated with 5 µL of sterilized water. Control and fungal inoculated samples (5 replications) were incubated at 28 °C with 90% humidity, and the diameter of the lesions was evaluated after 7 d. Fungi were isolated from the lesions and sequenced (ITS) to confirm the Kock postulate.

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from fungal mycelia according to the method of Tapia-Tussell et al. (2006). DNA concentrations were determined by absorbance at 260 nm.

PCR amplificons of the ITS1-5.8S-ITS2 region, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) intron, the D1/D2 domain of the large subunit (LSU) of 28S rDNA, and the TUB2 (β-tubulin) gene of fungal isolates, were obtained using the primers described by White et al. (1990), Templeton et al. (1992), O'Donnell et al. (1997), and Glass & Donaldson

(1995), respectively. PCR reaction mixtures (25 μL) contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, each dNTPs at a concentration of 0.2 mM, 1.25 U of Taq polymerase Platinum (Invitroten®), each primer at a concentration of 0.2 mM and 80 – 100 ng of fungal DNA template. PCR amplifications were performed in a Mastercycler (Eppendorf ®) equipment using an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C (ITS and TUB2) or 59 °C (GADPH), and 1.5 min at 72 °C, and a final extension of 5 min at 72 °C. The amplifications were confirmed in 2% agarose gels with TBE (Tris-Borate-EDTA), stained with GelRed (Uniscience), and visualized under UV light.

Amplicons were purified with EXOI/SAP (ThermoFisher®) following manufacture instructions. Purified amplicons (50 – 60 ng) were sequenced using Big Dye Terminator V.3.1 sequencing kit (ThermoFisher®) and analyzed with a 3500 Genetic Analyzer (ThermoFisher®). Data were collected by Data Collection software (ThermoFisher®). The PCR products were sequenced in both directions, and deposited in GenBank (Table S1).

Molecular classification and phylogenetic analysis

The DNA sequences were compared with those deposited in GenBank using the BLAST similarity test. Alignment of the ITS, LSU, GADPH and TUB2 sequences of the isolates, references of *Colletotrichum* species obtained from GenBank, and *Monilochaetes infuscans* Halst. ex. Harter (out-group) was performed with CLUSTAL X. MEGA X was used to build a distance tree with the Maximum Likelihood algorithm. Statistical confidence of groups was evaluated by bootstrap (1000 replicates).

Statistical analysis

Morphological and pathogenicity quantitative data were analyzed by ANOVA and means compared by the Tukey method.

Results

Grapes with classical symptoms of ripe rot disease were collected from commercial vineyards in the highlands of Southern Brazil, "Serra Gaucha" region, during the 2017 and 2018 vintages. *Vitis labrusca* and *V. vinifera* varieties are grown in this region, consequently samples were obtained from 39 vineyards of *V. labrusca* (Isabella, Ives, Concord, and Niagara), and 27 of *V. vinifera* (Cabernet Sauvignon, Moscato Bianco, Malvasia de Candia, Cabernet Franc, Merlot, and Trebbiano). From these 66 samples, we obtained 61 isolates of *Colletotrichum* (one per sample) giving a 92.4% efficiency. A summary of the isolates and their source are present in Table 1.

Table 1- *Colletotrichum* isolates from the "Serra Gaucha" Region, Brazil.

Code	Grape Variety	Geographic Origin (County)	Code	Grape Variety	Geographic Origin (County)
CI001	Isabella	Bento Gonçalves	CA035	Concord	Garibaldi
CA002	Niagara Rosa	Bento Gonçalves	CI036	Isabella	Monte Belo do Sul
CI003	Isabella	Bento Gonçalves	CI037	Isabella	Bento Gonçalves
CA004	Moscato hybrid	Bento Gonçalves	CI039	Isabella	Caxias do Sul
CI005	Isabella	Bento Gonçalves	CI040	Isabella	Pinto Bandeira
CI006	Isabella	Veranópolis	CA041	Cabernet Franc	Pinto Bandeira
CA007	Trebbiano	Veranópolis	CI042	Isabella	Pinto Bandeira
CI008	Isabella	Monte Belo do Sul	CA043	Moscato hybrid	Pinto Bandeira
CI009	Isabella	Monte Belo do Sul	CA044	Malvasia	Pinto Bandeira
CA010	Trebbiano	Monte Belo do Sul	A001-18	Isabella	Caxias do Sul
CI011	Isabella	Bento Gonçalves	A002-18	Moscato Branco	Farroupilha
CI012	Isabella	Bento Gonçalves	A21-17	Merlot	Bento Gonçalves
CI013	Isabella	Cotiporã	44-17	Ives	Antonio Prado
CI014	Isabella	Bento Gonçalves	A056-17	Isabella	Veranópolis
CI015	Isabella	Bento Gonçalves	A031-18MF	Merlot	Nova Pádua
CA016	Niagara Rosa	Bento Gonçalves	A031-18M9	Ives	Nova Pádua
CA017	Niagara Branca	Bento Gonçalves	LMF18-1	Lorena	Flores da Cunha
CA018	Malvasia	Bento Gonçalves	LMF18-2	Lorena	Farroupilha
CI019	Isabella	Bento Gonçalves	LMF18-3	Lorena	Ipê
CI020	Isabella	Bento Gonçalves	LMF18-4	Moscato EMBRAPA	Pinto Bandeira
CI021	Isabella	Bento Gonçalves	LMF18-6	Lorena	Farroupilha
CI022	Isabella	Bento Gonçalves	LMF18-7	Moscato EMBRAPA	Pinto Bandeira
CI023	Isabella	Pinto Bandeira	LMF18-8	Moscato	Pinto Bandeira
CA024	Cabernet Sauvignon	Pinto Bandeira	LMF18-10	Merlot	Flores da Cunha
CI025	Isabella	Veranópolis	LMF18-12	Merlot	Caxias do Sul
CI026	Isabella	Veranópolis	LMF18-14	Merlot	Caxias do Sul
CI027	Isabella	Veranópolis	LMF18-16	Cabernet Sauvignon	Caxias do Sul
CI028	Isabella	Cotiporã	LMF18-17	Cabernet. Sauvignon	Caxias do Sul
CI029	Isabella	São Valentim do Sul			
CI030	Isabella	São Valentim do Sul			
CI031	Isabella	São Valentim do Sul			
CI032	Isabella	Cotiporã			
CI033	Isabella	Bento Gonçalves			
CI034	Isabella	Garibaldi			

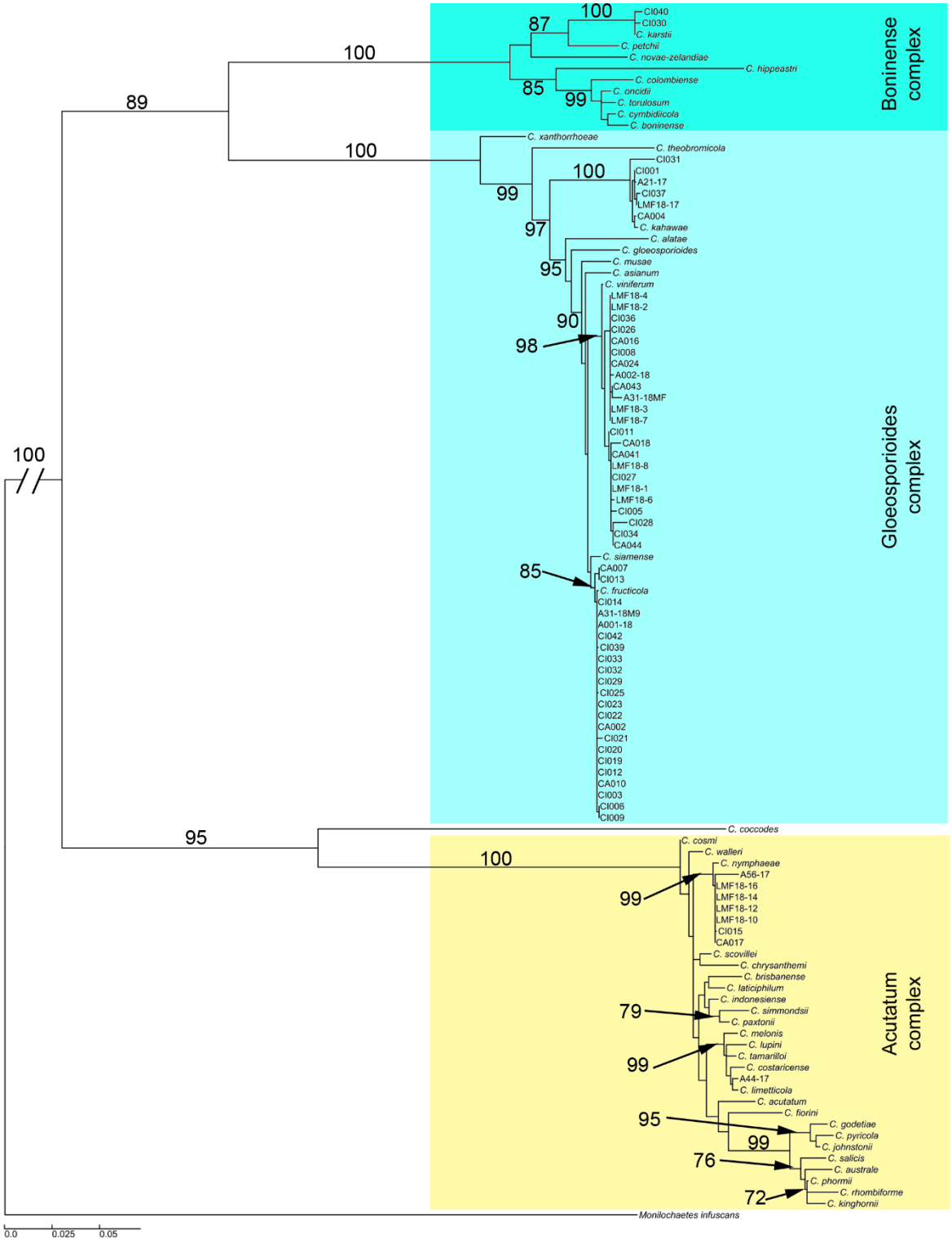


Figure 1 Dendrogram generated from Maximum Likelihood algorithm based on ITS, GAPDH, TUB2 and LSU combined sequences. Parsimony bootstrap support values greater than 75% are indicated. The tree is rooted with *Monilochaetes infuscans*.

Isolates were classified by PCR amplification and sequencing of four conserved regions (TUB2, ITS, LSU, and GAPDH). The GenBank accession number of the isolates and references strains are present as supplementary material. The TUB2, ITS, LSU and GAPDH amplicons yielded 521, 532 bp, 258 bp, and 539 bp sequences comparable with those deposited in GenBank. Variable nucleotides represented 53.7%, 21.6%, 84.5% and 12.05%, respectively. Considering the most informative Parsimony informative variations varied between TUB2 that showed 44%, and LSU with 8.9%. Singletons, random nucleotide variation not associated with the Parsimony separations, variated from 10.7% (TUB2) to 3.1% (LSU), indicating that TUB2 sequences are more informative to separate different isolates within a *Colletotrichum* species.

As can be observed in Fig 1, the maximum parsimony analysis based on the alignment of ITS, TUB2, GAPDH, and LSU gene sequences, allowed to separate the isolates in six groups with high bootstrap values. Each group was associated with reference species, allowing classification of the isolates into five well-defined species: *C. viniferum, C. fructicola, C. hahawae, C. kastii, C. nymphaeae,* and an isolate that closely related with *C. limentticola,* and other species like *C. melonis, C. costaricense, C. lupini, and C. tamarilloi* (Fig. 1). The most prevalent species were *C. viniferum* with 23 isolates (37.8%), and *C. fructicola* with 22 isolates (36.1%). These species, and the six isolates classified as *C. kahawae,* belongs to the Gloeosporioides latus sensus clade or complex, representing 83.6% of the isolates. Moreover, eight isolates were classified within the Acutatum complex (13.11%): seven closely related with C. *nymphaeae,* and one not well defined, but associated with *C. limetticola*. The last two isolates (3.3%) were classified as *C. karstii,* a species of the Boninense complex.

Representative isolates of the species identified by molecular classification were selected for further morphological evaluation. As can be observed in Fig. 2 and Table 2, *C. viniferum* and *C. fructicola* isolates showed very similar characteristics white to pale grey colonies with a light yellow bottom with grey sectors. Moreover, both species produced cylindrical conidia with length/width relation of 2.8 to 3.1, brown to dark brown ovoid to

cylindrical conidial and mycelial appressoria. These high similarities explain the difficulty to differentiate these species based just on morphological data. However, *C. kahawae* showed longer and narrower conidia and exhibited lower growth that the other species of the Gloeosporioides clade.

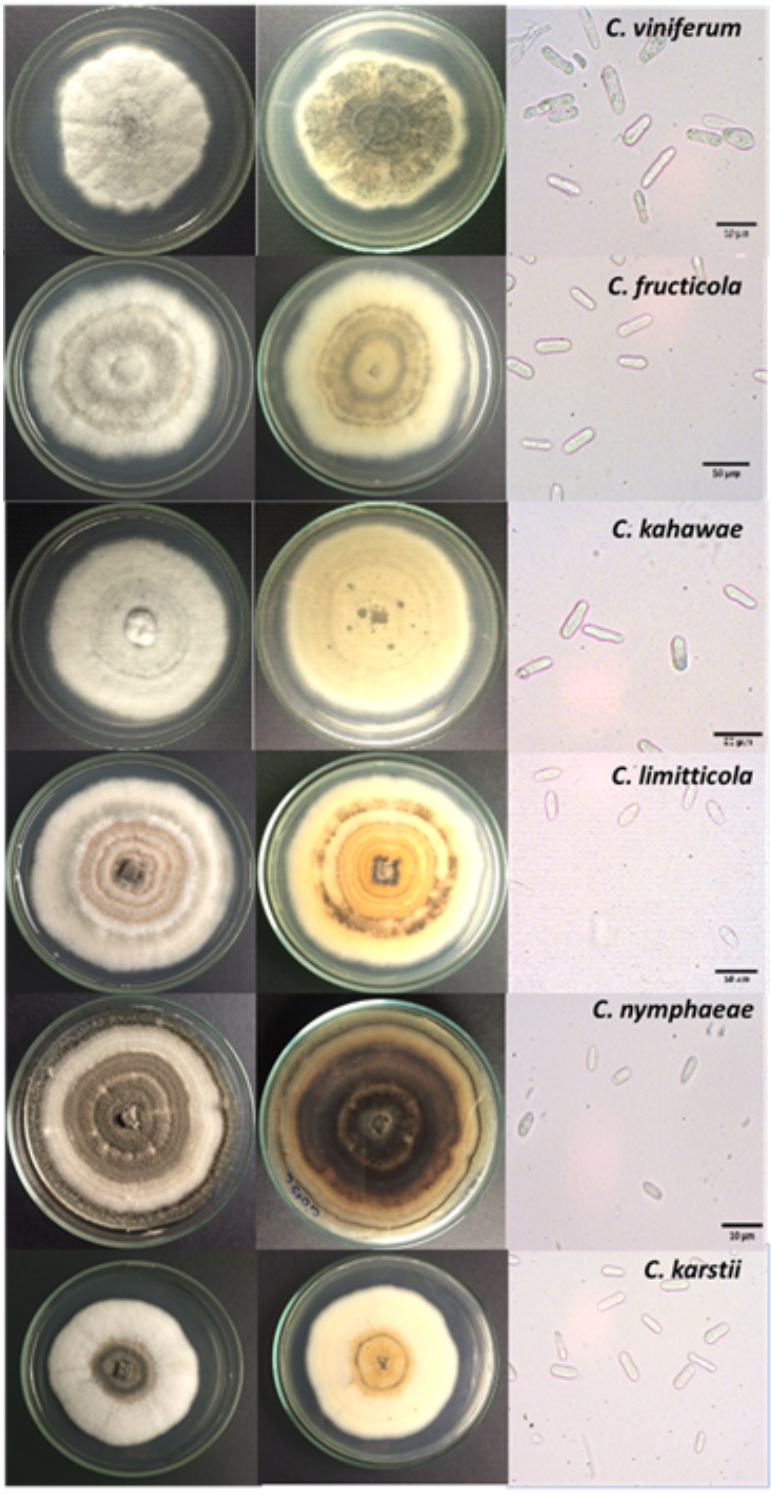


Figure 2 Colony morphology and conidial shape of *Colletotrichum* species isolated from ripe rot disease of grapes in Southern Brazil.

The two Acutatum complex species exhibited different colonial morphology, while the isolate of *C. limetticola* showed a typical light yellow colony with well-defined orange concentric rings, *C. nymphaeae* isolates resembled the Gloeosporiodes complex species. However, both Acutatum species exhibited larger conidia, with a length/width relation of 2.7, and a lower growth rate (Fig. 2; Table 3). Conversely, the two isolates of *C. kastii* (Boninense clade) showed characteristic light yellow colonies with an orange center, lower growth rate, and wider conidia (length/width relation of 2.0).

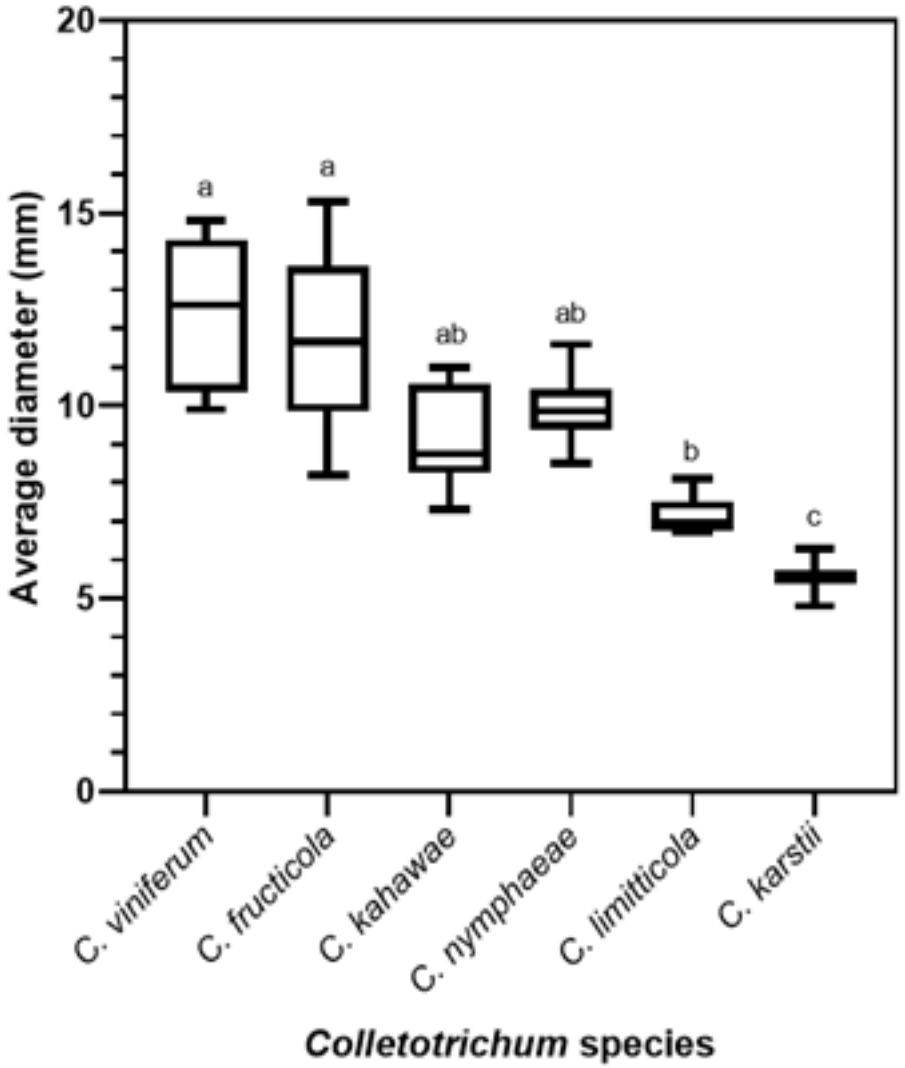


Figure 3 Pathogenicity test on berries of *Vitis vinifera* var. Itália. The results are the average lesion diameter of *Colletotrichum viniferum* (n=10), *C. fructicola* (n=10), *C. kahawae* (n= 4), *C. nymphaeae* (n=5), *C. limitticola* (n=1) and *C. karstii* (n=2) isolates, all of them with 10 replications.

Table 2 – Morphological characteristics of *Colletotrichum* species isolated from ripe rot of grape in the "Serra Gaucha" region, Brazil.

	Gloeosporioides complex			**Acutatum complex**		**Boninense complex**
Species	***C. viniferum***	***C. fructicola***	***C. kahawae***	***C. limentticola***	***C. nymphaeae***	***C. karstii***
N° Isolates evaluated	12	13	4	1	6	2
Colony morphology	White to pale grey, reverse light yellow with grey sectors	White to pale grey with concentric grey sectors, reverse light yellow with concentric yellow to orange sectors	White to pale grey, reverse yellow	Light yellow with concentric orange sectors, orange conidia evident at the center of the colony, reverse pale yellow with concentric orange sectors	Dark grey colonies, reverse dark brown	White to pale grey, reverse pale yellow with orange center
Growth rate (mm/day)	7.21 ± 1.70	8.05 ± 1.85	5.72 ± 2.38	6.09 ± 0.45	4.71 ± 1.15	4.91 ± .61
Conidia length (µm)	15.72 ± 2.45 b	16.12 ± 2.07 ab	16.59 ± 2.05 a	13.23 ± 1.74 c	10.71 ± 1.4 d	15.28 ± 1.99 b
Conidia width (µm)	5.58 ± 1.13 bc	5.16 ± 1.14 c	6.12 ± 1.25 b	6.19 ± 1.03 b	4.96 ± 0.83 c	7.64 ± 1.38 a
Conidia shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Cylindrical
Conidial appressoria	Dark brown, ovoid to cylindrical	Dark brown, ovoid or slightly irregular	Dark brown, spherical or ovoid	Dark brown, globular	Brown, globular	Dark brown, ovoid, irregular
Mycelial appressoria	Brown, ovoid, irregular	Dark brown, ovoid, irregular	Dark brown, cylindrical or irregular	Dark brown, ovoid, irregular	Brown, ovoids	dark brown, ovoid, irregular

The pathogenicity of representatives of the species isolated from grapes with classical ripe rot symptoms was evaluated by the measure of the lesion diameter on *Vitis vinifera* var. Itália berries. As can be observed in Fig. 3, the isolates of the six species of *Colletotrichum* obtained from grapes were virulent. However, the isolates of *C. viniferum* and *C. fructicola* exhibited higher virulence compared with those of *C. limitticola* and *C. karstii*. The isolates of *C. kahawae* and *C. nymphaeae* showed intermediary values, indicating mildly virulence. Non-significant differences were observed within each species, indicating that virulence is highly species-specific, and *C. viniferum* and *C. fructicola* are the most virulent species. Fungi were isolated from the lesions and their identity confirmed by ITS-sequencing, fulfilling the Kock's postulate.

Discussion

Since its first report in the highlands of Southern Brazil at the beginning of 2000's (Garrido and Sônego 2004), ripe rot disease of grapes has become the most important and devastating bunch disease. Becoming evident just during the ripening period, the products for treatments are limited and the efficiency reduced during rainy and hot years. Ripe rot disease is caused by *Colletotrichum* species vary greatly on their virulence and fungicides sensibility (Peng et al. 2013; Gang et al., 2015; Hei et al. 2019).

In the present survey, six species of *Colletotrichum* associated with ripe rot disease of grapes in the "Serra Gaucha" region were identified by DNA sequencing of regions of GAPDH, ITS, LDU and TUB2: *C. viniferum*, *C. fructicola*, *C. kahawae*, *C. nymphaeae*, *C. limitticola*, and *C. karstii*. The results partially confirmed the species reported in a first survey of Colletotrichum species associated with ripe rot disease of grapes in Southern Brazil, except for *C. limitticola* that was previously classified as *C. lupini* (Echeverrigaray et al., 2019). The species of the Gloeosporioides complex were the most prevalent, particularly *C. viniferum*, and *C. fructicola.* Species of this complex associated with ripe rot disease of grapes has been reported in other viticultural regions of the world like Korea (Oo and Oh, 2017), China (Peng et al., 2013; Yan et al., 2015; Lei et al., 2016), United States (Daykin and Milholland, 1984), India (Chowdappa et al., 2009; Sawant et al., 2012), and Australia (Greer et al., 2011). Since its first report (Peng et al., 2013), a high prevalence of *C. viniferum* has also been detected in vineyards of other Chinese regions (Yan et al., 2015; Lei et al., 2016), and Korea (Oo and Oh, 2017). *Colletotrichum fructicola*, the second most prevalent species identified in Southern Brazil, was previously reported on grapes collected in Guizhou and Yunnan provinces of China (Peng et al., 2013), and Korea (Lim et al., 2019). In Brazil, *C. fructicola* and *C. nymphaeae* have been isolated from grapevine leaves with anthracnose symptoms (Guginski-Piva et al., 2018). Moreover, *C. kahawae*, that represented 9.8% of the isolates, has been reported just in Korean vineyards (Oo and Oh, 2017).

Grape ripe rot disease-associated species of the acutatum complex have been reported in Korea (Hong et al., 2008), Japan (Watauchi et al., 2018), Australia (Greer et al., 2011), England (Baroncelli et al., 2014), and India (Chowdappa et al., 2009). Except form Australia, where they represent the most prevalent group, species of the acutatum complex varied from 10 to 12% of isolates, with representatives of *C. acutatum, C. nymphaeae, C. meloni*, among others. These prevalences are similar to those reported in the present survey where species of the Acutatum complex, identified as *C. nymphaeae* and *C. limetticola*, represented 13.8%. It is important to emphasize that species of the acutatum complex (*C. nymphaeaem C. meloni, C. abscissum,* and *C. paranaense*) were associated to anthracnose disease of several tropical and subtropical fruit species (Bragança et al., 2016), indicating the high incidence of these species on Brazilian ecosystems.

Two isolates were classified as *C. karstii*, a species that belongs to the Boninense complex. This is the first report of *C. karstii* or any other species of the Boninense complex associated with grape rot disease. However, this species has been reported in Brazil associated with apple (Velho et al., 2013), mango (Lima et al., 2013), pitaya (Nascimento et al., 2019), chilli (Saini et al., 2016) anthracnose, among other tropical plants (Cannon et al., 2012).

Although other species of *Colletotrichum* associated with ripe rot disease of grapes such as *C. citri, C. cliviae, C. hebeiense, C. aenigma, C. capsica*, and *C. godetiae,* were not found in Southern Brazil, *Colletotrichum* species diversity in the "Serra Gaucha" region was higher than that reported in other winegrowing regions of the world (Chowdappa et al., 2009; Greer et al., 2011; Sawant et al., 2012; Peng et al., 2013; Yan et al., 2015; Lei et al., 2016; Oo and Oh, 2017). The high incidence and diversity of Colletotrichum in grapes may be associated to the particular climatic conditions of the "Serra Gaucha" region characterized by high precipitations (120 – 150 mm/mo) and temperatures (25 – 30 °C) during ripening, the amplitude of the ripening and harvesting season, that considering both *V. labrusca* and *V. vinifera* varieties, extends from December to April, and the diversity of fruits plants cultivated in the area (apple,

persimmon, peach, plum, chilli, avocado, among others) which can be responsible for cross-infection (Whitelaw-Weckert et al 2007; Peng et al. 2013).

It is important to emphasize that *C. viniferum*, *C. fructicola*, *C. kahawae*, and *C. nymphaeae*, where equally distributed in both *V. labrusca* and *V. vinifera* varieties, while the two isolates of *C. karstii* were obtained from *V. labrusca* (Isabella). In this sense, Shiraishi et al. (2007) showed that the resistance or susceptibility to *C. acutatum* is independent of the species (*V. labrusca*, *V. vinifera*) but varies between varieties.

The *Colletotrichum* species isolated differed on several morphological and growing characteristics, like colony color, growth rate, and conidial morphology, and they resemble those previously reported by Whitelaw-Weckert et al. (2007). Peng et al. (2013), Bragança et al. (2016), Oo and Oh (2017), Dos Santos et al. (2018), Guginski-Piva et al. (2018), among others. However, the variation in morphological characteristics is not enough to clearly classify *Colletotrichum* isolates at the species level, and confirm the need for molecular data for the proper classification of *Colletotrichum* species, particularly within the *lato sensu* complexes (Cannon et al., 2012).

The virulence of the six species towards *V. vinifera* cv. Italia showed significant interspecies differences. Among the *Colletotrichum* species evaluated, the representatives of *C. viniferum* and *C. fructicola* were the most virulent, followed by those classified as *C. kahawae* and *C. nymphaeae*. The isolates of *C. limitticola* and *C. karstii* showed lower virulence, a fact that can explain their low frequency in ripe rot disease of grapes in Southern Brazil. Interspecific variation of virulence among *Colletotrichum* species have been reported by several authors (Whitelaw-Weckert et al., 2007; Yan et al., 2015, among others). However, in contrast to the results obtained by Yan et al. (2015), non-significant differences were observed in virulence within species, particularly *C. viniferum* and *C. fructicola* isolates.

In conclusion, based on molecular classification and morphological data, six species of *Colletotrichum* were identified as causal agents of ripe rot disease of grapes in the most

important viticultural Brazilian region. These species belong to three different complexes (Gloeosporioides, Acutatum, and Boninense clades), with a high prevalence of the *C. viniferum* and *C. fructicola*. Although all the species exhibited virulence and were able to develop typical ripe rot disease on *V. vinifera* var. Italia, those that belonged to the Gloeosporioides clade showed higher virulence. Conversely, the species with lower frequency (*C. limitticola* and *C. kartii*) exhibited lower virulence. Compared with other viticultural regions, the highlands of Southern Brazil exhibited a higher number of species of *Colletotrichum* associated with ripe rot disease of grapes.

Acknowledgements

This study was supported by the Brazilian National Council for Scientific and Technological Development - CNPq (Process: 304906/2017-4), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Finance Code 001).

Data Availability Statement

The data that support the findings of this study are available in GenBank. The accession codes of all the sequences are listed in Table S1 (supporting information).

References

Almança MAK, Lerin S, Cavalcanti FR. 2015. Doenças da videira. *Informe Agropecuário* **36**, 7-12.

Baroncelli R, Sreenivasprasad S, Lane CR, Thon MR, Sukno SA, 2014. First report of *Colletotrichum acutatum* sensu lato (*Colletotrichum godetiae*) causing anthracnose on grapevine (*Vitis vinifera*) in the United Kingdom. *New Disease Reports* **29**, 26.

Bragança CAD, Damm U, Baroncelli R, Massola Junior NS, Crous PW, 2016. Species of *Colletotrichum acutatum* complex associated with anthracnose disease of fruits in

Brazil. *Fungal Biology* **120**, 547-561.

Cannon PF, Damm U, Johnston PR, Weir BS, 2012. *Colletotrichum*- current status and future directions. *Studies in Mycology* **73**, 181-213.

Chowdappa P, Reddy GS, Kumar A, Rao BM, Rawal RD, 2009. Morphological and molecular characterization of *Colletotrichum* spp. causing anthracnose disease of grapes. *The Asian and Australasian Journal of Plant Science and Biotechnology* **3**, 71-77.

Daykin ME, Milholland RD, 1984. Ripe rot of muscadine grape caused by *Colletotrichum gloeosporioides* and its control. *Phytopathology* **74**, 710–714.

De Silva AGDD, Crous PW, Ades PK, Hyde KD, Taylor P, 2017. Life styles of *Colletotrichum* species and implications for plant biosecurity. *Fungal Biology Reviews* **31**, 155-168.

Dos Santos RF, Guillardi MC, Amorim L, Massola N, Sposito MB 2018. Aetiology of anthracnose on grapevine shoots in Brazil. *Plant Pathology* **67**, 692-706.

Echeverrigaray S, Delamare APL, Fontanella G, Favaron F, Stella L, Scariot FJ, 2019. *Colletotrichum* species associated to ripe rot disease of grapes in the "Serra Gaucha" region of Southern Brazil. *BIO Web of Conferences*. **12**, 01008.

Gang GH, Cho HJ, Kim HS, Kwack YB, Kwak YS, 2015, Analysis of fungicide sensitivity and geneti diversity among Colletotrichum species in sweet persimmon. *Plant Pathology Journal* **3**, 115-122.

Garrido LR, Sônego OR, 2004. Available at: http://ainfo.cnptia.embrapa.br/digital/bitstream/CNPUV/8130/1/cir052.pdf

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**, 1323-1330.

Greer LA, Harper JDI, Savocchia S, Samuelian SK, Steel CC, 2011. Ripe rot of south-eastern Australian wine grapes is caused by two species of *Colletotrichum*: *C. acutatum* and *C.*

gloeosporioides with differences in infection and fungicide sensitivity. ***Australian Journal of Grape and Wine Research*** **17**, 123–128.

Guginski -Piva CA, Bogo A, Gomes BR, Menon JK, Nodari RO, Welter LJ, 2018. Morphological and molecular characterization of *Colletotrichum nymphaeae* and *C. fructicola* associated with anthracnose symptoms of grape in Santa Catarina State, southern Brazil. *Journal of Plant Disease and Protection* **125**, 405-413.

Hong SK, Kim WG, Yun HK, Choi KJ, 2008. Morphological variations, genetic diversity and pathogenicity of *Colletotrichum* species causing grape ripe rot in Korea. *Plant Pathology Journal* **24**, 269–278.

Joshi R, 2018. A Review on *Colletotrichum* spp. Virulence mechanism against host plant defensive factors. *Journal of Medicinal Plants Studies* **6**, 64-67

Hei L, Li X, Gao Y, Li B, Mu W, Liu F, 2019. Characterization and fungicide sensitivity of *Colletotrichum* spp. from different hosts in Shandong, China. *Plant Disease* 103, 34-43.

Lei Y, Tang XB, Jayawardena RS, Yan JY, Wang XD, Liu M, Chen T, Liu XM, Wang JC, Chen QX, 2016. Identification and characterization of Colletotrichum species causing grape ripe rot in southern China. *Mycosphere* 7, 1177-1191.

Lim YS, Hassan O, Chang T, 2019. First report of anthracnose of Shine muscat caused by *Colletotrichum fructicola* in Korea. *Mycobiology* DOI: 10.1080/12298093.2019.1697190

Lima NB, Marques MW, Michereff SJ, Morais MA, Barbosa MAG, Câmara MPS, 2013. First report of mango anthracnose caused by *Colletotrichum karstii* in Brazil. *Plant Disease* 97, 1248.

Liu F, Tang G, Zheng X, Li Y, Sun X, Qi X, Zhou Y, Xu J, Chen H, Chang X, Zhang S, Gong G, 2016. Molecular and phenotypic characterization of Colletotrichum

species associated with anthracnose disease in peppers from Sichuan Province, China. *Science Reports* 32761.

Marin-Felix Y, Groenewald JZ, Cai L, Chen Q, Marincowitz S, 2017. Genera of phytopathogenic fungi: GOPHY 1. *Studies in Mycology* **86**, 99-216.

Nascimento MB, Bellé C, Azambuja RM, Maich SLP, Neves CG, Souza-Junior IT, Jacobsen CRF, Barros DR (2019) First report of *Colletotrichum karstii* causing anthracnose spot in pitaya (*Hylocereus undatus*) in Brazil. *Plant Disease* **103**, 2137.

O'Donnell K, Cigelnik E, 1997. Two divergent intra genomic DNA rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologus. *Molecular Phylogenetics and Evolution* **7**, 103-116.

Oo MM, Oh SK, 2017. Identification and characterization of new record of grape ripe rot disease caused by *Colletotrichum viniferum* in Korea. *Mycobiology* **45**, 421-425.

Peng LJ, Sun T, Yang YL, Cai L, Hyde KD, Bahkali AH, Liu ZY, 2013. *Colletotrichum* species on grape in Guizhou and Yunnan provinces, China. *Mycoscience* **54**, 29-41.

Saini TJ, Gupta SG, Char BR, Zehr UB, Anandalakshmi R, 2016. First report of chilli anthracnose caused by *Colletotrichum karstii* in India. *New Disease Reports* **34**, 6.

Sawant IS, Narkar SP, Shetty DS, Upadvay A, Sawant SD, 2012. Emergence of *Colletotrichum gloeosporioides* sensu lato as the dominant pathogen of anthracnose disease of grapes in India as evidenced by cultural, morphological and molecular data. *Australasian Plant Pathology* **41**, 492-504.

Shiraishi M, Koide M, Itamara H, Yamada M, Mitani N, Ueno T, Nakaune R, Nakano M, 2007. Screening for resistance to ripe rot caused by *Colletotrichum acutatum* in grape germplasm. *Vitis* **46**, 196-200.

Stankova P, Vichova J, Pokorny R, 2011. Virulence of *Colletotrichum acutatum* isolates to several host plants. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis* **59**, 161-169.

Tapia-Tussell R, Lappe P, Ulloa M, Quijano-Ramayo A, Cáceres-Farfán M, Larqué-Saavedra A, Perez-Brito D, 2006. A rapid and simple method for DNA extraction from yeasts and fungi isolated from Agave fourcroydes. *Molecular Biotechnology* **33**, 67-70.

Templeton MD, Rikkerink EH, Solon SL, Crowhurst RN, 1992. Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogen *Glomeralla cingulata. Gene* **122**, 225-230.

Velho AC, Stadnik MJ, Casanova L, Mondino P, Alaniz S, 2013. First report of *Colletotrichum karstii* causing Glomerella leaf spot on apple in Santa Catarina State, Brazil. *Plant Disease* **98**, 157.

Wang B, Li BH, Dong XL, Wang CX, Zhang ZF, 2015. Effects of temperature, wetness duration, and moisture on the conidial germination, infection, and disease incubation period of *Glomerella cingulata*. *Plant Disease* **99**, 249-256.

Watauchi K, Uchida K, Sato T, 2017. Ocurrence of ripe rot of grape (*Vitis vinifera* L.) caused by *Colletotrichum nymphaeae* (Passerini). *Japan Journal of Phytopathology* **84**, 3-7.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. editors. PCR protocols: a guide to methods and applications. San Diego (CA), Academic Press, 315-322.

Whitelaw-Weckert MA, Curtin SJ, Huang R, Steel CC, Blanchard CL, Roffey PE, 2007. Phylogenetic relationships and pathogenicity of Colletotrichum acutatum isolates from grape in subtropical Australia. *Plant Pathology* **56**, 448-463.

Yan J, Jayawardena MMRS, Luang MF, Li X, 2015. Diverse species of *Colletotrichum* associated with grapevine anthracnose in China. *Fungal Diversity* **71**, 233-246.

Table S1. *Colletotrichum* isolates, reference species, and their GenBank accession numbers.

Species	Isolate	Reference	GenBank accession number			
			ITS	GAPDH	TUB2	LSU
C. acutatum	CBS 129921	1, 2	MH865673	JQ948711	JQ950031	MH877100
C. alatae	CBS 304.67	1, 3	JX010190	JX009990	JX010383	MH870668
C. asianum	LC0038; C1315.4	3, 4	JN943089	JX010053	JX010406	JN940409
C. australe	CBS 131325	1, 2	JQ948456	JQ948787	JQ950107	MH877401
C. boninense	CBS 123755	1, 5	JQ005153	JQ005240	JQ005588	MH874855
C. brisbanense	CBS 292.67	1, 2	JQ948291	JQ948621	JQ949942	MH877698
C. chrysanthemi	CBS 126519	1, 2	JQ948272	JQ948602	JQ949923	MH875602
C. coccodes	CPOS1; CBS 127562	1, 6	GQ485588	GQ856744	GQ849444	MH876008
C. colombiense	CBS 129818	1, 5	JQ005174	JQ005261	JQ005608	MH876878
C. cosmi	CBS 853.73	1, 2	JQ948274	JQ948604	JQ949925	MH877746
C. costaricense	CBS 330.75	1, 2	JQ948180	JQ948510	JQ949831	MH877747
C. cymbidiicola	IMI 347923; CBS 128543	1, 6	JQ005166	JQ005253	JQ005600	MH876450
C. fioriniae	CBS 128555	1, 2	JQ948305	JQ948635	JQ949956	MH876456
C. fructicola	CBS 125395	1, 3	JX010172	JX009992	JX010408	MH874993
C. fructicola	CA002	This work	MN758895	MN784997	MN785058	MN758960
C. fructicola	CI003	This work	MN758896	MN784998	MN785059	MN758961
C. fructicola	CI006	This work	MN758899	MN785001	MN785062	MN758964
C. fructicola	CA007	This work	MN758900	MN785002	MN785063	MN758965
C. fructicola	CI009	This work	MN758902	MN785004	MN785065	MN758967
C. fructicola	CA010	This work	MN758903	MN785005	MN785066	MN758968
C. fructicola	CI012	This work	MN758905	MN785007	MN785068	MN758970
C. fructicola	CI013	This work	MN758906	MN785008	MN785069	MN758971
C. fructicola	CI014	This work	MN758907	MN785009	MN785070	MN758972
C. fructicola	CI019	This work	MN758912	MN785014	MN785075	MN758977
C. fructicola	CI020	This work	MN758913	MN785015	MN785076	MN758978
C. fructicola	CI021	This work	MN758914	MN785016	MN785077	MN758979
C. fructicola	CI022	This work	MN758915	MN785017	MN785078	MN758980
C. fructicola	CI023	This work	MN758916	MN785018	MN785079	MN758981
C. fructicola	CI025	This work	MN758918	MN785020	MN785081	MN758983
C. fructicola	CI029	This work	MN758922	MN785024	MN785085	MN758987
C. fructicola	CI032	This work	MN758925	MN785027	MN785088	MN758990
C. fructicola	CI033	This work	MN758926	MN785028	MN785089	MN758991
C. fructicola	CI039	This work	MN758930	MN785032	MN785093	MN758995
C. fructicola	CI042	This work	MN758933	MN785035	MN785096	MN758998
C. fructicola	A001-18	This work	MN758950	MN785052	MN785113	MN759015
C. fructicola	A031-18M9	This work	MN758953	MN785055	MN785116	MN759018
C. gloeosporioides	ICMP 17821; CBS 127555	1, 3	JX010152	JX010056	JX010445	MH876003
C. godetiae	CBS 155.25; MH875599	1, 2	JQ948410	JQ948741	JQ950061	MH875599
C. hippeastri	CBS 125376	1, 6	JQ005231	JQ005318	JQ005665	MH874999
C. indonesiense	CBS 127551	1, 2	JQ948288	JQ948618	JQ949939	MH875999
C. johnstonii	IMI 357027; CBS 128532	1, 6	JQ948443	JQ948774	JQ950094	MH876448
C. kahawae	CBS 12988; CBS 125394	1, 3	JX010236	JX009965	JX010428	MH874995
C. kahawae	CI001	This work	MN758894	MN784996	MN785057	MN758959
C. kahawae	CA004	This work	MN758897	MN784999	MN785060	MN758962
C. kahawae	CI031	This work	MN758924	MN785026	MN785087	MN758989

C. kahawae	CI037	This work	MN758929	MN785031	MN785092	MN758994
C. kahawae	LMF18-17	This work	MN758947	MN785049	MN785110	MN759012
C. kahawae	A21-17	This work	MN758948	MN785050	MN785111	MN759013
C. karstii	CI030	This work	MN758923	MN785025	MN785086	MN758988
C. karstii	CI040	This work	MN758931	MN785033	MN785094	MN758996
C. karstii	CBS 129832	1, 6	JQ005177	JQ005264	JQ005611	MH877253
C. kinghornii	CBS 198.35	1, 2	JQ948454	JQ948785	JQ950105	MH867153
C. laticiphilum	CBS 129827; CBS 112989	1, 2	JQ948290	JQ948620	JQ949941	MH874480
C. limetticola	CBS 114.14	1, 2	JQ948193	JQ948523	JQ949844	MH866150
C. lupini	A44-17	This work	MN758949	MN785051	MN785112	MN759014
C. lupini	CBS 129944	1, 2	JQ948178	JQ948508	JQ949829	MH877123
C. melonis	CBS 159.84	1, 2	JQ948194	JQ948524	JQ949845	MH877750
C. musae	CBS 116870; CBS 125356	1, 3	JX010146	JX010050	HQ596280	MH875030
C. novae zelandiae	CBS 130240	1, 6	JQ005229	JQ005316	JQ005663	MH877051
C. nymphaeae	CBS 126370	1, 2	JQ948257	JQ948587	JQ949908	MH875405
C. nymphaeae	CI015	This work	MN758908	MN785010	MN785071	MN758973
C. nymphaeae	CA017	This work	MN758910	MN785012	MN785073	MN758975
C. nymphaeae	LMF18-10	This work	MN758943	MN785045	MN785106	MN759008
C. nymphaeae	LMF18-12	This work	MN758944	MN785046	MN785107	MN759009
C. nymphaeae	LMF18-14	This work	MN758945	MN785047	MN785108	MN759010
C. nymphaeae	LMF18-16	This work	MN758946	MN785048	MN785109	MN759011
C. nymphaeae	A56-17	This work	MN758954	MN785056	MN785117	MN759019
C. oncidii	CBS 130242	1, 6	JQ005170	JQ005257	JQ005604	MH877053
C. paxtonii	CBS 502.97	1, 2	JQ948286	JQ948616	JQ949937	MH874269
C. petchii	CBS 125957	1, 6	JQ005226	JQ005313	JQ005660	MH875299
C. phormii	CBS 118194	1, 2	JQ948446	JQ948777	JQ950097	MH877757
C. pyricola	CBS 128531	1, 2	JQ948445	JQ948776	JQ950096	MH876447
C. rhombiforme	CBS 129953	1, 2	JQ948457	JQ948788	JQ950108	MH877132
C. salicis	CBS 128559	1, 2	JQ948471	JQ948802	JQ950122	MH876460
C. scovillei	CBS 126530	1, 2	JQ948268	JQ948598	JQ949919	MH875607
C. siamense	CBS 125378	1, 3	JX010278	JX010019	JX010410	MH875001
C. simmondsii	CBS 126524	1, 2	JQ948281	JQ948611	JQ949932	MH875606
C. tamarilloi	CBS 129812	1, 2	JQ948186	JQ948516	JQ949837	MH876872
C. theobromicola	CBS 124945	1, 3	JX010294	JX010006	JX010447	MH874938
C. torulosum	CBS 128544	1, 6	JQ005164	JQ005251	JQ005598	MH876451
C. viniferum	yg1; CBS 130645	1, 7	JN412804	JN412798	JN412813	MH877255
C. viniferum	CI005	This work	MN758898	MN785000	MN785061	MN758963
C. viniferum	CI008	This work	MN758901	MN785003	MN785064	MN758966
C. viniferum	CI011	This work	MN758904	MN785006	MN785067	MN758969
C. viniferum	CA016	This work	MN758909	MN785011	MN785072	MN758974
C. viniferum	CA018	This work	MN758911	MN785013	MN785074	MN758976
C. viniferum	CA024	This work	MN758917	MN785019	MN785080	MN758982
C. viniferum	CI026	This work	MN758919	MN785021	MN785082	MN758984
C. viniferum	CI027	This work	MN758920	MN785022	MN785083	MN758985
C. viniferum	CI028	This work	MN758921	MN785023	MN785084	MN758986
C. viniferum	CI034	This work	MN758927	MN785029	MN785090	MN758992
C. viniferum	CI036	This work	MN758928	MN785030	MN785091	MN758993
C. viniferum	CA041	This work	MN758932	MN785034	MN785095	MN758997

C. viniferum	CA043	This work	MN758934	MN785036	MN785097	MN758999
C. viniferum	CA044	This work	MN758935	MN785037	MN785098	MN759000
C. viniferum	LMF18-1	This work	MN758936	MN785038	MN785099	MN759001
C. viniferum	LMF18-2	This work	MN758937	MN785039	MN785100	MN759002
C. viniferum	LMF18-3	This work	MN758938	MN785040	MN785101	MN759003
C. viniferum	LMF18-4	This work	MN758939	MN785041	MN785102	MN759004
C. viniferum	LMF18-6	This work	MN758940	MN785042	MN785103	MN759005
C. viniferum	LMF18-7	This work	MN758941	MN785043	MN785104	MN759006
C. viniferum	LMF18-8	This work	MN758942	MN785044	MN785105	MN759007
C. viniferum	A002-18	This work	MN758951	MN785053	MN785114	MN759016
C. viniferum	A031-18MF	This work	MN758952	MN785054	MN785115	MN759017
C. walleri	CBS 125472	1, 2	JQ948275	JQ948605	JQ949926	MH877867
C. xanthorrhoeae	CBS 127831	1, 3	JX010261	JX009927	JX010448	MH876162

* Reference sequences: (1) Vu et al. Stud. Mycol. 92, 135-154 (2019); (2) Damm et al. Stud. Mycol. 73 (1), 37-113 (2012); (3) Weir et al. Stud. Mycol. 73 (1), 115-180 (2012); (4) Schoch et al. Proc. Natl. Acad. Sci. U.S.A. (2012); (5) Damm et al. Stud. Mycol. 73 (1), 1-36 (2012); (6) Liu et al. Mycology, 2: 248-254.

4.2 CAPÍTULO 2

Activity of monoterpenoids on the in vitro growth of two *Colletotrichum* species and the mode of action on *C. acutatum*

Fernando J. Scariot, Luciano Foresti, Ana Paula L. Delamare, Sergio Echeverrigaray

Artigo publicado na revista *Pesticide Biochemistry and Physiology*.

Pesticide Biochemistry and Physiology 170 (2020) 104698

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Activity of monoterpenoids on the *in vitro* growth of two *Colletotrichum* species and the mode of action on *C. acutatum*

Fernando J. Scariot*, Luciano Foresti, Ana Paula L. Delamare, A.P.L. Sergio Echeverrigaray

Institute of Biotechnology, University of Caxias do Sul, Rio Grande do Sul, Brazil

ARTICLE INFO

Keywords:
Terpenes
Membrane integrity
ROS
Apoptosis/necrosis

ABSTRACT

Essential oils and their main compounds, monoterpenoids, are considered as alternative control systems for phytopathogenic fungi, particularly those related to late diseases of fruits and vegetables, like anthracnose caused by *Colletotrichum* species. In this context, we studied the effect of twenty monoterpenoids on *Colletotrichum fructicola* and *C. acutatum* to elucidate their effectiveness and mechanisms of action. Thus, we analyzed mycelial growth and conidial inhibitory concentration, as well as the effect of selected monoterpenoids on membrane integrity and cell vitality, reactive oxygen species (ROS) accumulation, and mitochondrial membrane potential by flow cytometry. The results showed that oxygenated monoterpenoids (alcohols and aldehydes) exhibited higher antifungal activity than their corresponding hydrocarbons, esters, and cyclic counterparts. Indicating that OH^- and O^- radicals react with cellular components affecting fungal homeostasis. In this sense, selected monoterpenoids (citral, citronellol, geraniol, carvacrol, and thymol) inhibited conidial germination of *C. acutatum* in a dose-dependent manner. The inhibition of conidial germination is associated with a loss of membrane integrity, a decrease of cell metabolism, and a dose-dependent accumulation of ROS, which was non-directly associated with modifications on mitochondrial membrane potential. Membrane dysfunction and ROS accumulation may be responsible for the necrotic behavior induced by high monoterpenoids concentrations, and possible apoptotic response in sub dosages of these compounds.

Activity of monoterpenoids on the in vitro growth of two *Colletotrichum* species and the mode of action on *C. acutatum*

Fernando J. Scariot[1], Luciano Foresti, Ana Paula L. Delamare, Sergio Echeverrigaray.

Institute of Biotechnology, University of Caxias do Sul, Rio Grande do Sul, Brazil.

[1]Corresponding author at: Institute of Biotechnology, University of Caxias do Sul, Rio Grande do Sul, Brazil. E-mail address: ferjscariot@gmail.com (F.J. Scariot).

Abstract

Essential oils and their main compounds, monoterpenoids, are considered as alternative control systems for phytopathogenic fungi, particularly those related to late diseases of fruits and vegetables, like anthracnose caused by *Colletotrichum* species. In this context, we studied the effect of twenty monoterpenoids on *Colletotrichum fructicola* and *C. acutatum* to elucidate their effectiveness and mechanisms of action. Thus, we analyzed mycelial growth and conidial inhibitory concentration, as well as the effect of selected monoterpenoids on membrane integrity and cell vitality, reactive oxygen species (ROS) accumulation, and mitochondrial membrane potential by flow cytometry. The results showed that oxygenated monoterpenoids (alcohols and aldehydes) exhibited higher antifungal activity than their corresponding hydrocarbons, esters, and cyclic counterparts. Indicating that $HO^{\bullet}$ and $O^{\bullet}$ radicals react with cellular components affecting fungal homeostasis. In this sense, selected monoterpenoids (citral, citronellol, geraniol, carvacrol, and thymol) inhibited conidial germination of *C. acutatum* in a dose-dependent manner. The inhibition of conidial germination is associated with a loss of membrane integrity, a decrease of cell metabolism, and a dose-dependent accumulation of ROS, which was non-directly associated with modifications on mitochondrial membrane

potential. Membrane dysfunction and ROS accumulation may be responsible for the necrotic behavior induced by high monoterpenoids concentrations, and possible apoptotic response in sub dosages of these compounds.

Key-words: terpenes; membrane integrity; ROS; apoptosis/necrosis

1. Introduction

Colletotrichum species are responsible for some of the most common pre and post-harvest diseases of fruits, known as anthracnose or ripe rot diseases. When anthracnose symptoms are evidenced as dark depressed circular lesions with orange to pinky conidia masses [1], conventional or alternative control systems are not sufficient to prevent commercial losses. Thus, preventive treatments should be developed to reduce losses by *Colletotrichum* infection. These alternatives should be eco-friendly and with a low potential toxic effect on humans. Among natural products, essential oils showed insecticidal, herbicidal, fungicidal, and bactericidal properties [2-4], and had been evaluated to control fungal diseases of several fruits, including those caused by *Colletotrichum* species in avocado, mango, papaya, apple, chili pepper, grapes, among many other tropical and subtropical fruits [5-10]. These extracts are considered as GRAS (Generally Recognized as Safe) products, and can be applied close to harvest or post-harvest without risk to the consumers.

Essential oils are currently obtained by steam distillation or other systems from leaves, flowers, or other parts of higher plants [11]. These extracts are a complex mixture of several compounds, including terpenoids, phenolics, and other less representative volatile compounds [12]. In general, the most prevalent class of compounds in essential oils of aromatic plants are monoterpenoids, a class of ten carbon secondary metabolites (isoprenoids) originated from the cytoplasmic mevalonic acid or the chloroplastic methyl D-erythritol 4-phosphate (MEP) pathway and accumulated in glandular trichomes of aromatic plants [12].

The antifungal effect of essential oils is determined by their chemical composition and, in general, their main compounds are responsible for their antimicrobial activity, though synergic effects have been reported [12]. In practice, the evaluation of the effectiveness of given essential oils involves the characterization of its composition and its effect on one or several fungal species. This is laborious, as it implicates in the individual test of essential oils from different plants, which composition can variate depending on the cultivar, geographic characteristics, agricultural conditions, among other variables. Moreover, each product should be tested against different fungi, creating virtually eternal factorial tests. This dilemma can be resolved or helped by determining the action of the individual compounds present in essential oils and establishing their mechanism of action.

Studies to elucidate the mechanism of action of essential oils and monoterpenoids on phytopathogenic fungi are still limited, and their activity has been associated to the fungal cell wall and membrane disruption, inhibition of chitin synthesis, ROS accumulation, mitochondrial dysfunction, and the inhibition of some specific enzymatic activities [13-14]. For example, Zhou et al. [15] showed that carvacrol treatment on *Rhizopus stolonifer* leads to an increasing in the cytoplasmic membrane permeability and consequent cellular components liberation, with evidence of ergosterol content reduction. Similarly, citronellal treated spores of *Pennicilium digitatum* showed a loss of cytoplasmic membrane integrity [16]. It has been reported that the citral fungicidal effect on *P. digitatum* is related with mitochondrial morphological alterations and respiration inhibition [17], as well as a reduction in ATP production and increase in intracellular ROS [18]. Although different fungal species can variate on their response to a given compound, the effect of a compound on fungi should be similar, and the evaluation of monoterpenoids on *Colletotrichum* species, as a model system, should represent their effect on other species and genera.

In order to elucidate the potential antifungal activity of the most prevalent monoterpenoids on essential oils of aromatic plants, we evaluated the *in vitro* antifungal

efficiency of twenty monoterpenoids and acetylated compounds against *C. fructicola* and *C. acutatum*, two of the most important species associated with anthracnose disease on grapes, apples, and other fruits in Southern Brazil [19]. Moreover, we evaluated the mode of action of selected monoterpenoids on conidial germination and viability of *C. acutatum* using a flow cytometry approach with specific dyes for cell membrane integrity, cell vitality, ROS accumulation, and mitochondrial membrane potential.

2. Material and methods

2.1. Monoterpenoids and fungal strains

Twenty monoterpenoids (Supplementary material S1) were purchased from Acros-Organics or Sigma-Aldrich. Stock solutions (50 g/L) of monoterpenoids were prepared in a Tween 20 (1:1) and used on the experiments.

Two *Colleotrichum* species isolated from ripe rot disease of grapes in Southern Brazil were used in the experiments. *C. fructicola* CA002 (Gloeosporioides clade) was isolated from Niagara Rosa (*Vitis labrusca*) from Bento Gonçalves in 2018 and, *C. acutatum* 44/17 (Acutatum clade) was isolated from Ives (*V. labrusca*) from Antônio Prado in 2017. Both isolates were classified by the sequencing of ITS, GAPDH, TUB2, and LSU genes (GenBank numbers CA002- MN758895, MN784997, MN785058, and MN758960; and 44/17- MN758949, MN785051, MN785112, and MN759014).

2.2. In vitro evaluation of monoterpenoids antifungal activity

An initial selection of monoterpenoids against *Colletotrichum* was made on PDA (Potato dextrose agar medium, Hymedia, India) supplemented with 1000 mg/L of the

compounds from stock solutions. Petri dishes were inoculated with fungal plugs of 5.0 mm diameter, obtained from 5 days-old PDA cultures maintained at 28°C with a photoperiod of 16 h. Cultures were incubated at 28°C with a 16 h photoperiod for 7 days and the colony diameters determined and compared with the controls (with no terpenoids), and expressed in percentage mycelial growth inhibition (%). The experiments were made in triplicate.

2.3. Inhibitory concentration 50% (IC 50) and minimal inhibitory concentration (MIC) of selected monoterpenoids

The monoterpenoids that exhibited high antifungal activity in the exploratory experiments were evaluated in different concentrations to determine their IC50 and MIC values on *C. fructicola* and *C. acutatum*. Fungal plugs (5.0 mm diameter) obtained from PDA cultures of 120 h at 28°C with a photoperiod of 16 h were placed on PDA media supplemented with 0 (control), 75, 150, 300, 600, 1200, and 2400 mg/L of the monoterpenoids from stock solutions. Cultures were incubated at 28°C with a 16 h photoperiod for 168 h and the colony diameters determined and compared with the controls. Data were expressed as a percentage of mycelial growth inhibition (%). The experiments were made in triplicate. IC50 and MIC values were determined by the Probit analysis using the IBM-SPSS Statistics, version 22.

2.4. Effect of selected monoterpenoids on the conidial germination of C. acutatum

Conidial suspensions of *C. acutatum* – Acutatum clade (44/17) were obtained from 14 days old cultures on PDA (28°C with 16 h photoperiod). Conidia were harvested by adding 5.0 mL of sterilized water and gently scraping the fungal surface. The suspension was filtered and adjusted to 1 x 10^6 conidia/mL. For the germination test, 15 µL of conidial suspension were mixed in Eppendorf tubes with 150 µl of selected monoterpenoids (carvacrol, citral, citronellol,

geraniol, and thymol) in different concentrations (control, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 mg/L), and incubated in the dark at 28°C for 16 h with gentle agitation. The percentage of germinated conidia were then assessed by microscopic observation (400x). Conidia were considered germinated when the length of the germ tube equaled or exceeded conidial length. Experiments were conducted in triplicate with more than two hundred conidia per replicate.

2.5. Flow cytometry evaluation of monoterpenoids

Fungal spores were harvest from 14 days old PDA plates and purified by filtration. Spores were washed with 0.9% NaCl solution and concentration was adjusted to 1 x 10^7 spores/mL in SD medium (0.67% Yeast Nitrogen Base (Y1250, Merk), 2% glucose, pH 6.8). Cells treated with monoterpenoids in different concentrations (125, 250, and 500 mg/L) and control (untreated cells) were incubated for 9 h in an orbital shaker (150 rpm, 28°C). Cell/conidial size (SSC) and complexity (FSC), membrane integrity, cell vitality (esterase activity), ROS accumulation, and mitochondrial potential ($\Delta\psi m$) were evaluated by flow cytometry in a FACSCalibur flow cytometer (Becton-Dickinson) equipped with an argon-ion laser emitting at 488 nm. Flow cytometer data of 10,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc). All procedures followed those adopted by Scariot et al. [20].

Particle (cell size) and cell complexity were evaluated by the side scatter (SSC) and forward scatter (FSC) values. Moreover, cell membrane integrity and esterase activity were determined using the FungaLight Yeast CFDA, AM/Propidium Iodide Vitality kit (Invitrogen) that includes CFDA (5-carboxyfluorescein diacetate) to determine esterase activity, and PI (propidium iodide) to evaluate membrane integrity. Staining and flow cytometer assay followed manufacturing instructions.

Intracellular ROS accumulation was performed utilizing dihydroethidium (DHE, Sigma-Aldrich). The stock solution of DHE was prepared in DMSO (5 mg/mL). Monoterpenoids-treated or control samples were stained with a final concentration of 5 µg/mL DHE, and incubated for 30 minutes at room temperature. Samples were evaluated by flow cytometry using FL3 (488/670).

To evaluate mitochondrial membrane potential (Δψm) samples were stained with 175 nM of 3,3'-dihexyloxacarbocyanine iodide (DiOC6) for 30 minutes at 30°C in the dark. After staining, conidia were analyzed by flow cytometry using FL1 filter.

2.6. Statistical analysis

The results were statistically analyzed by analysis of variance (one-way ANOVA), Tukey's test, and Probit. The statistical analyses were performed using IBM-SPSS Statistics - version 22 software, and statistical significance was attributed to values of $P \leq 0.05$.

3. Results

The first *in vitro* evaluation of the antifungal effect of monoterpenoids against *C. fructicola* and *C. acutatum* using a fixed concentration of 0.1% (w/v) or 1000 mg/L showed (Fig. 1) that the most effective compounds were carvacrol, carveol, citral, citronellol, geraniol, and thymol, while other monoterpenoids, particularly, citronellal, menthol, and α-terpineol exhibited a significant reduction of *in vitro* mycelial growth. The most efficient compounds completely or quite completely reduce the mycelial growth of the two species of *Colletotrichum*, but significant differences of antifungal activity between *Colletotrichum* species were observed for a group of low efficient monoterpenoids (1,8-cineol, limonene, linalool, linalyl acetate, menthol, terpinene-4-ol, and α-terpinene), and

citronellal, an aldehyde with intermediary activity. For these compounds, except α-terpinene, the *C. fructicola* was more resistant than the *C. acutatum* strain.

Citronellyl acetate showed lower antifungal activities than its alcoholic counterpart citronellol. Moreover, the alkanes (camphor, terpinene, limonene, menthone, myrcene, α-pinene, and α- terpinene) showed lower activities than the oxygenated compounds (alcohol or aldehydes).

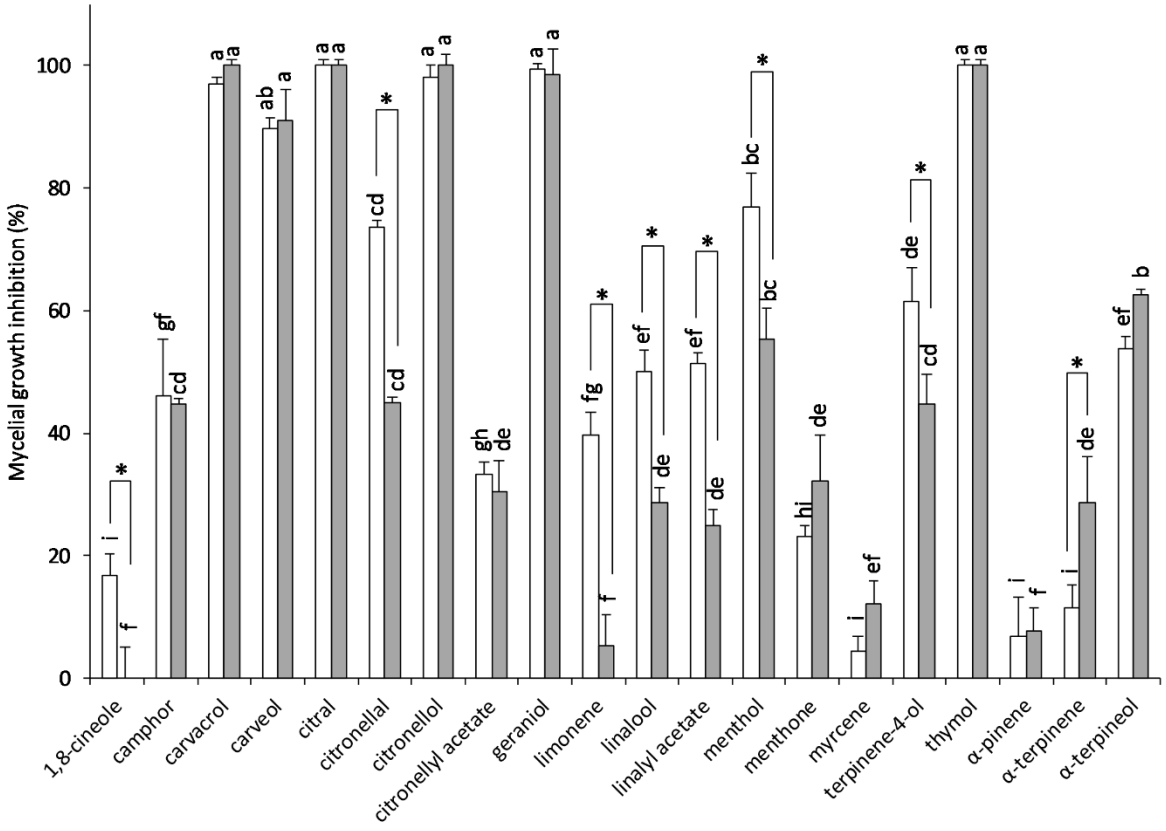


Figure 1. Relative mycelial growth inhibition of *C. fructicola* CA002 (white bars) and *C. acutatum* 44/17 (grey bars) in the presence of 1 g/L of different monoterpenes. Data are percentage values of the controls with no-terpenes. Different letters of each fungi represent significant difference of mycelial growth inhibition by the Tukey test ($p \leq 0.05$).

Considering the exploratory data, the most effective compounds against *Colletothrium* species (carvacrol, carveol, citral, citronellol, geraniol, and thymol) were further evaluated to determine their IC50 and MIC. As can be observed in Figure 2, these monoterpenoids showed

a dose-dependent inhibitory effect on the mycelial growth of *Colletotrichum*, and no significant differences were observed between the responses of the *C. fructicola* and *C. acutatum* isolates.

The highest IC50 and MIC values were those of carveol, 650 and 1350 mg/L, and 640 and 1320 mg/L, for *C. fructicola* and *C. acutatum* respectively, and the lowest values were observed for carvacrol and thymol, with an average IC50 of 100 to 140 mg/L, and MIC values of approximately 400 mg/L. Geraniol showed IC50 of 530 mg/L and MIC of 1040 and 940 mg/L for *C. fructicola* and *C. acutatum*, respectively, while the corresponding aldehyde, citral (a mixture of the geranial and neral isomers) exhibited lower average IC50 (370 mg/L) and MIC (750 mg/L) values.

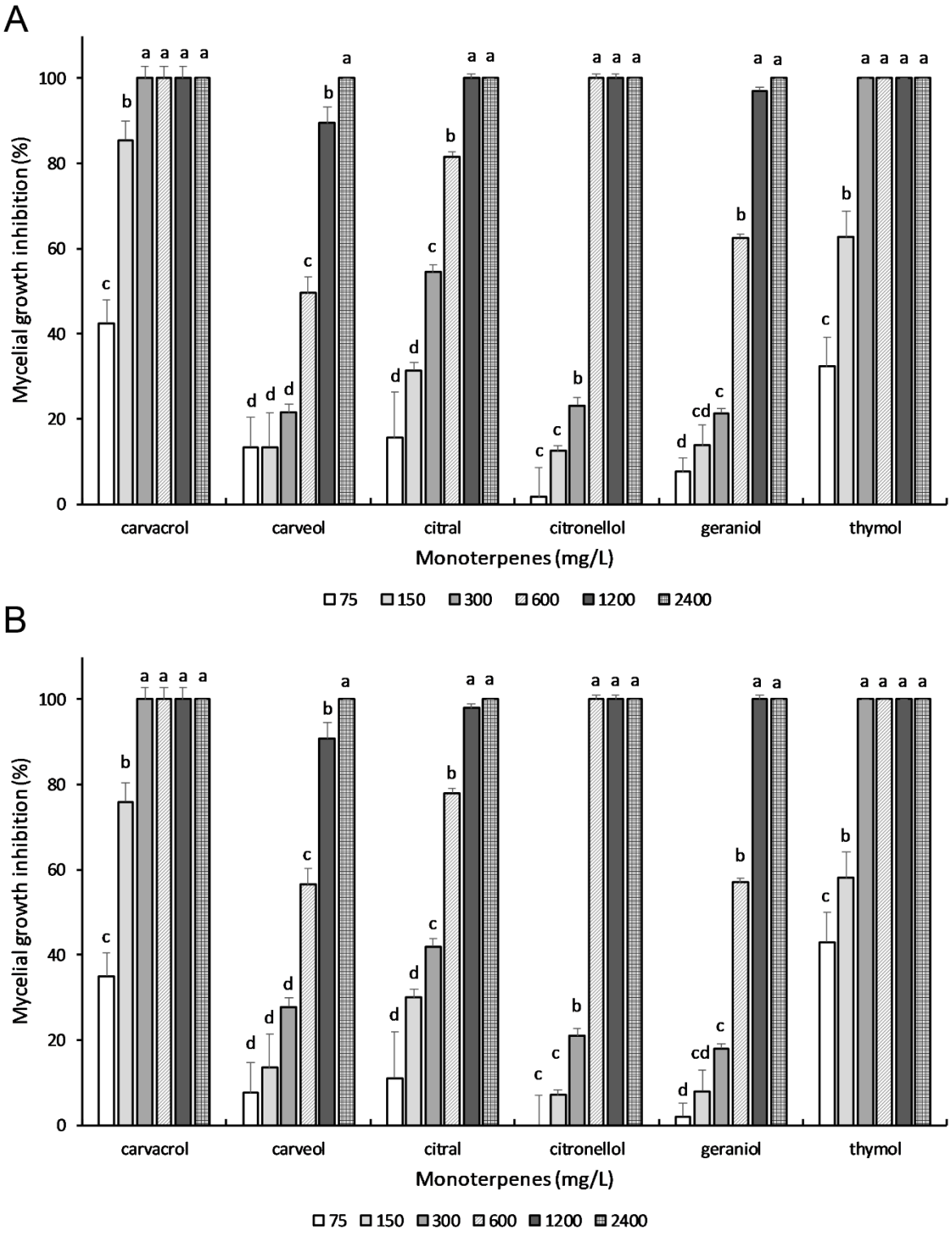


Figure 2. Dose-dependent effect of monoterpenoids on the mycelial growth inhibition of *C. fructicola* (A) and *C. acutatum* (B). Different letters correspond to significant different mean values at $p \leq 0.05$ by Tukey test.

Considering the similar responses of the *C. fructicola* and *C. acutatum* isolates to the selected monoterpenoids, the evaluation of these compounds on conidial germination was made

with the high sporulating strain of *C. acutatum* 44/17. Results (Figure 3) showed a dose-dependent effect, and all the selected monoterpenoids were able to completely inhibit conidial germination in the highest concentration (1000 mg/L) evaluated. However, the minimal concentration that completely inhibited conidial germination varied among compounds. Thymol and carvacrol were the most efficient, inhibiting ≥ 90% conidial germination at 125 mg/L. Citral and citronellol inhibited conidial germination at 250 mg/L, and geraniol at 500 mg/L.

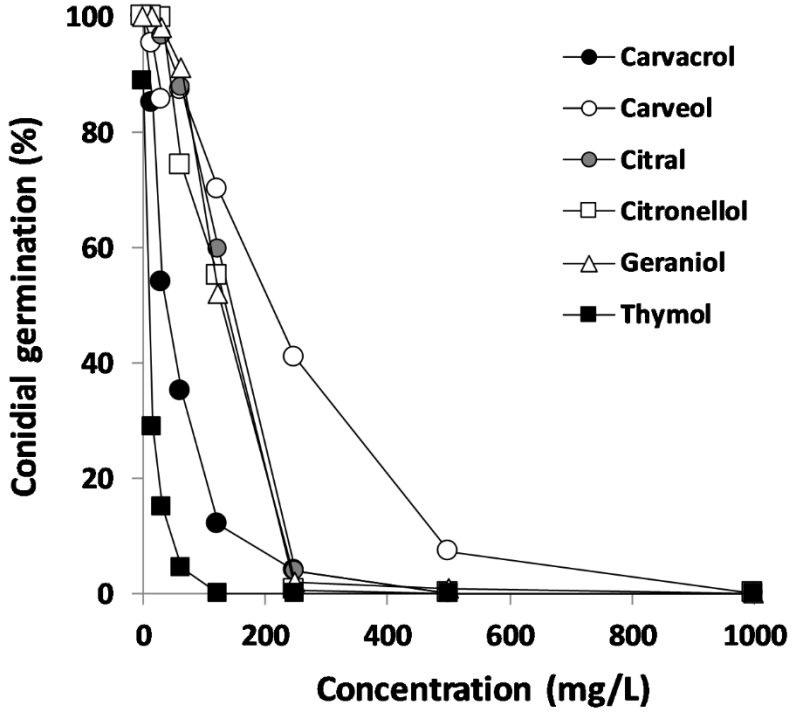


Figure 3. Conidial germination (%) of *C. acutatum* after 16 h on different concentrations of selected monoterpenoids.

To establish the ideal incubation time for flow cytometry analysis, *C. acutatum* conidia were incubated in SD medium for 0 to 16 h The ideal incubation time was 9 hours, a period when the conidia significantly increase their size (FSC) and cell complexity (SSC) parameters (Fig. 4). At longer incubation times yield hyphae and were no longer available by flow cytometry analysis.

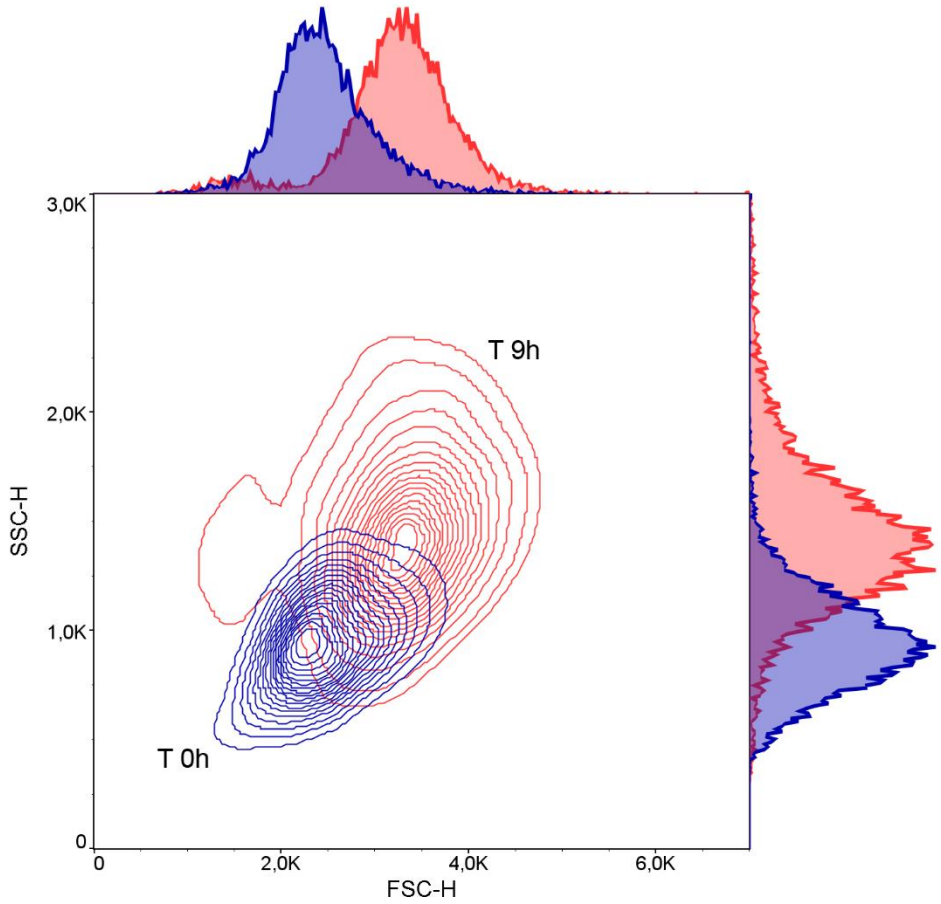


Figure 4. SSC (cell size) vs. FSC (cell complexity) of conidia (blue) and germinated conidia after 9 h on SD medium at 28°C (red).

The flow cytometry analysis of cell vitality and membrane integrity (Table 1) showed that all the monoterpenoids reduced cell membrane integrity, and this in dependence of monoterpenoids concentration. Moreover, in low terpenoids concentration (125 mg/L) conidia exhibited a reduction in cell metabolisms, as determined by a cell esterase activity (CFDA fluorescence). This effect was particularly evident in citral (Fig. 5A), citronellol, and geraniol, the lower effective monoterpenoids, where the 125 mg/L concentration was subinhibitory. At the highest concentrations, all the monoterpenoids showed similar behavior with a drastic reduction in cell membrane integrity (Supplementary material S2).

The five selected monoterpenoids evaluated increased ROS with a dose-dependent effect (Supplementary material S3), and highly correlated with cell membrane integrity. Correlation between the percentage of cell death (PI positive) and DHE fluorescence for the six

monoterpenoids was 0.95, varying between 0.95 and 0.99, for geraniol and carvacrol, respectively. In sublethal concentrations (125 and/or 250 mg/L) two populations with different ROS accumulation were evident, as exemplified by citral behavior (Fig. 5B). As can be observed in Fig 5C, the population with high ROS accumulation correspond to the lower size and less complex non-germinated conidia, indicating that monoterpenoids affect conidial germination and hyphal growth.

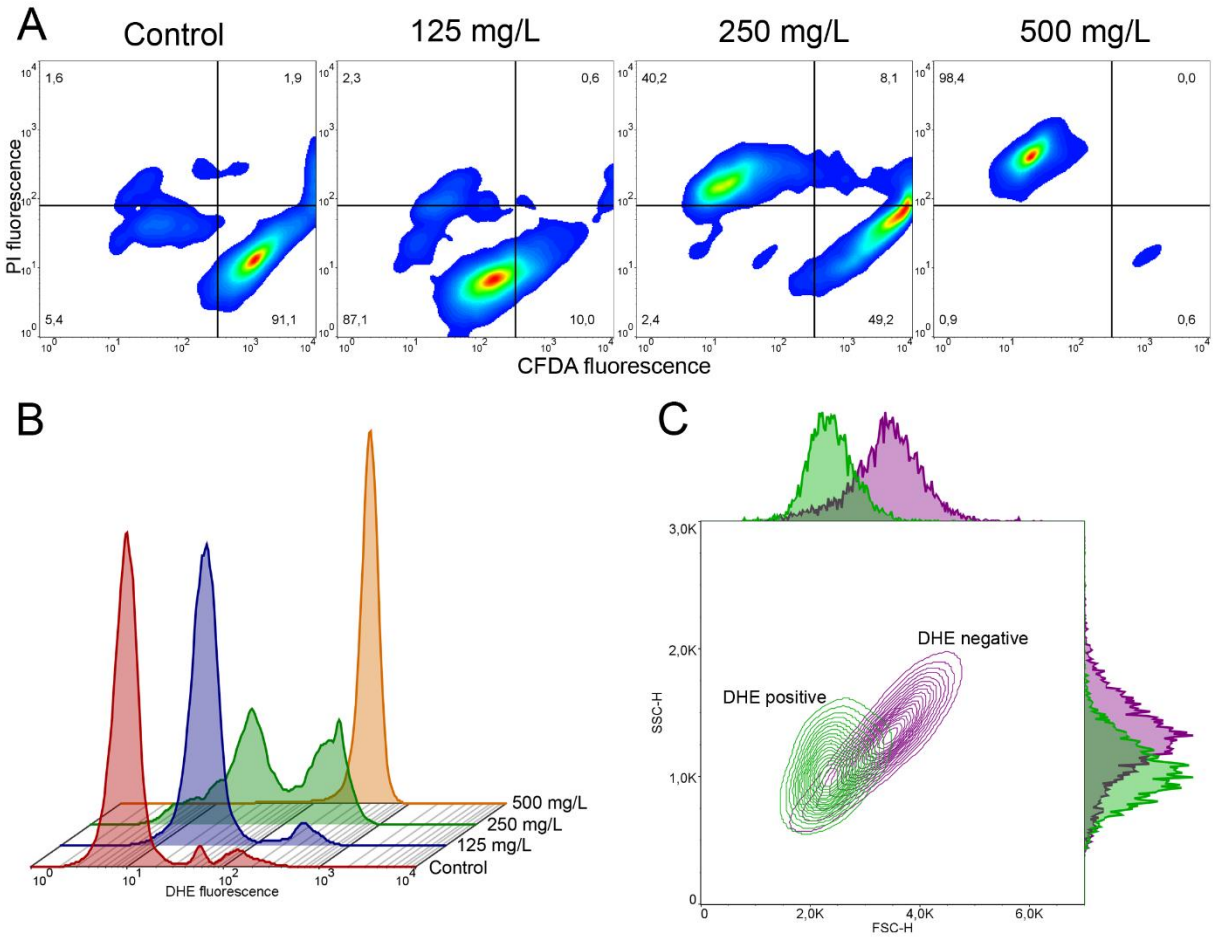


Figure 5. The effect of citral on *C. acutatum* conidia: (A) Membrane integrity and cell vitality; (B) intracellular ROS accumulation (DHE fluorescence), and (C) the relation between SSC/FSC spectra and high (green)/low (violet) DHE fluorescence on conidia treated by 250 mg/L citral for 9 h.

ROS intracellular concentration can be associated with modulation of mitochondrial activity, in this sense, mitochondrial membrane potential (Δψm) on the spores treated with different concentrations of the five monoterpenoids showed no evident relation with DHE

fluorescence. Moreover, two different behaviors were evidenced: citral (Figure 5A), citronellol, and geraniol treated conidia showing an increase in mitochondrial potential, while those treated with carvacrol and thymol exhibited a reduction on this parameter.

4. Discussion

Essential oils originated from several plant species showed important antimicrobial activity against a broad range of microorganisms, and have been proposed or used as natural and eco-friendly alternative treatment systems in medicine [12] and agriculture [14], including fungal diseases that cause pre and postharvest decays. The main constituents of most essential oils are monoterpenes or their oxygenated derivatives (monoterpenoids) whose presence and/or concentration varied among plant species and genotypes, and is affected by environmental, agricultural, and harvesting conditions [21].

Among the twenty monoterpenoids tested *in vitro* against *Colletotrichum fructicola* and *C. acutatum*, six showed high efficiency, nine exhibited intermediary effectiveness, and five were low effective. The monoterpenoids with higher efficiency were thymol, carvacrol, citral, geraniol, citronellol, and carveol. These compounds are the main components of the essential oils of thyme, oregano, lemon myrtle, lemongrass, lime, among other plants, some of which showed antifungal activity against phytopathogenic fungi, including *Colletotrichum* species [6, 14, 22- 25]. Although few data on the effect of particular terpenoids activity are available, the present data corroborate the antifungal activity of thymol and carvacrol, and essential oils with a high concentration of these compounds against phytopathogenic fungi obtained by Hong et al. [6] and Marei et al. [14]. As pointed out by Numpaque et al. [23], thymol and carvacrol could be partially metabolized by *C. acutatum*, producing several terpenoids derivatives (thymohydroquinone, thymyl acetate, carvacryl acetate, and carvacryl and thymyl methyl esters), thymol and carvacrol showed high antifungal activity against the fungi. Moreover, some of these compounds also exhibited high

antibacterial [26], and nematicidal activity [27], indicating that their effect is somewhat general, affecting basic cellular structures and/or metabolism.

Some terpenoids with low activity against *Colletotrichu*m species like limonene, 1,8-cineole, menthone, and eugenol have been reported as efficient compounds against other phytopathogenic fungi (*Alternaria solani, Aspergillus niger, Penicillium digitatum*, and *Rhizoctonia solani*), indicating that fungal sensitivity monoterpenes is somewhat species-dependent [14, 28]. However, some monoterpenoids, like thymol, carvacrol, citral, and eugenol, and essential oils with a high concentration of these compounds exhibited a broad spectrum of antifungal activity [6, 14 28-29].

In general, terpenoids with higher antifungal activity are oxygenated compounds with hydroxyl (alcohols) or carboxylic (aldehydes) radicals, indicating that these radicals and their reactiveness are responsible for the antifungal properties. This is supported by the fact that hydrocarbon monoterpenes, like α-terpinene, showed less antifungal activity than their oxygenated counterparts (α-terpineol or terpinene-4-ol, and α-terpineol), in addition, oxygenated monoterpenes showed higher antifungal activity than its cyclic form 1,8-cineol. Moreover, high antifungal monoterpenoids exhibited a dose-dependent inhibition of mycelial growth, indicating a terpene interaction with a set of target molecules on the fungus. The highest antimicrobial activity of oxygenated monoterpenoids and their dose-dependence action has been previously reported on bacteria [30].

The five selected monoterpenoids (citral, citronellol, geraniol, carvacrol, and thymol) showed a dose-dependent activity on *C. acutatum* conidial germination. The most efficient monoterpenoids on fungal growth were the most efficient on the inhibition of *C. acutatum* conidial germination, indicating that these activities are related. However, the IC50 and MIC values were somewhat lowered on mycelial growth inhibition assays, which can be explained by the contact time (16h) between the terpenoid and the fungi, and an eventual physiological adaptation of the fungi to monoterpenoids.

Flow cytometry data showed that thymol, carvacrol, citral, carvacrol, and geraniol affect cell membrane permeability, and reduce cell metabolism, indicating an interaction of monoterpenoids with cell membrane that affects cell permeability and homeostasis. These results give cytological evidence that corroborates the data obtained by several authors on other species of fungi and yeasts, which pointed out essential oils and monoterpenes action (thymol, carvacrol, linalool, among others) on the cell membrane and cell wall integrity and, the inhibition of efflux pump (revised by Nazzarro et al., [29] and Cannon et al., [31]). Moreover, these effects can be responsible for microscopic alterations observed on *Botrytis cinerae* [32]. Paz-Arraiza et al. [13] attribute the effect of monoterpenoids on cell membrane integrity to their lipophilic or hydrophobic nature that allows them to interact with membrane lipids, resulting in the modification of membrane properties and function. In this sense, RNA-Seq-transcriptomic analysis of *Fusarium oxysporum* responding to thymol showed downregulation of sphingolipid metabolism-related genes that can be responsible for modification on membrane structure [33].

Our data showed that the five more efficient monoterpenoids induced an increase on cellular ROS on *C. acutatum* spores, indicating that all of them can trigger necrotic or apoptotic cell death [34-35]. Our results showed that ROS accumulation (on the surface or within the cell) may be one of the most important effects of monoterpenoids on conidial fungal inhibition and death. This hypothesis is corroborated by the presence of two populations with different ROS accumulation in sublethal concentrations of some monoterpenoids, which correspond to the percentage of live/dead cells. Based on the gene expression of *Fusarium* under thymol treatments, Zhang et al. [33] reported that disruption of fungal membranes is related to ROS, and possible lipids-peroxidation. Considering that DHE detects intracellular superoxide [36], its high fluorescence in conidia treated with monoterpenoids corroborates with a lipid-peroxidation hypothesis. Moreover, the present data showed a different response to monoterpenoids on the mitochondrial membrane potential, indicating that the acute effect of monoterpenoids is mainly associated with cell membrane disruption and ROS accumulation,

but can be also associated to an increase of cell respiration. These results obtained with thymol corroborate those reported by Ahmad et al. [37] showed a significant reduction in ATPase activity by thyme essential oil on *Candida albicans*. Moreover, cell membrane disfunction and ROS accumulation can be responsible for the morphological variations associated to monoterpenoid treatments reported by these authors.

Taken together, our experimental results provide strong evidence that oxygenated monoterpenoids have higher antifungal activity than their hydrocarbon or esters counterparts. The monoterpenoids with higher antifungal activity against both *C. fructicola* and *C. acutatum*, were thymol, carvacrol, citral, citronellol, and geraniol Moreover, these monoterpenoids can inhibit both mycelial grow and conidial germination in a dose-dependent manner. Data obtained by the acute treatment of conidia of *C. acutatum* showed that the inhibition of conidial germination is associated with a loss of cell membrane integrity and the accumulation of ROS, which can trigger apoptotic or necrotic cell death.

Acknowledgments

This study was supported by the Brazilian National Council for Scientific and Technological Development - CNPq (Process: 304906/2017-4), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Finance Code 001). The authors acknowledge Cytogene Diagnósticos Moleculares Ltda. for access to flow cytometry equipment.

References

[1] P. Jeffries, J.C. Dodd, M.J. Jeger, R.A. Plumbley, The biology and control of *Colletotrichum* species on tropical fruit crops. Plant Pathol. 39 (1990) 343-366.

[2] M. Hamesh, A.A. Alamri, A.A. Shathan, S.A. Alrumman, M.F.M. Moustafa, Supression of phytopathogenic fungi by plant extract of some weeds and the possible mode of action. Br.

Microbiol. Res. J. 5 (2016) 1-13.

[3] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, Biological effect of essential oils – a review. Food Chem. Toxicol. 46 (2008) 446-475.

[4] H.A.E. Shaaban, A.H. El-Ghorab, T. Shibamoto. Bioactivity of essential oils and their volatile aroma components: review. J. Essent. Oil Res. 24 (2012) 203-212.

[5] M.S. Barbosa, G.H.C. Vieira, A.V. Teixeira, In vitro biological activity of propolis and essential oils on the fungus *Colletotrichum musae* isolated from banana *Musa* spp. Rev. Bras. Plantas Med. 17 (2015) 254-261.

[6] J.K. Hong, H.J. Yang, H. Jung, D.J. Yoon, M.K. Sang, Y.C. Jeun, Application of volatile antifungal plant essential oils for controlling pepper fruit anthracnose by *Colletotrichum gloeosporioides*. Plant Pathol. 31 (2015) 269-277.

[7] A. Yilmaz, E. Ermis, N. Boyraz, Investigation of in vitro and in vivo anti-fungal activities of different essential oils against postharvest apple rot diseases – *Colletotrichum gloeosporioides*, *Botrytis cinerea*, and *Penicillium expansum*. J. Food Saf. Food Qual. 67 (2016) 113-148.

[8] A. Sarkhosh, B. Schaffer, A.L.Vargas, A.J. Palmateer, P. Lopez, A. Soleyma, In vitro evaluation of eight plant essential oils for controlling *Colletotrichum*, *Brotrysphaeria*, *Fusarium* and *Phytophtora* fruit rots of avocado, mando and papaya. Plant Protect. Sci. 54 (2018) 153-162.

[9] R. Vilaplana, L. Pazmiño, S. Valencia-Chamorro, Control of anthracnose, caused by *Colletotrichum musae*, on postharvest organic banana by thyme oils. Postharvest Biol. Tec. 138 (2018) 56-63.

[10] C. Pedrotti, A.R. Marcon, A.P.L. Delamare, S. Echeverrigaray, R.T. da Silva Robeiro, J. Schwambach, Alternative control of grape rots by essential oils of two *Eucalyptus* species. J. Sci. Food Agric. 99 (2019) 6552-6561.

[11] A. Akdag, E. Ozturk, Distillation methods of essential oils. Nisan 45 (2019) 22-31.

[12] M.E. Bergman, B. Davis, M.A. Phillips, Medically useful plant terpenoids: biosynthesis, occurrence, and mechanism of action. Molecules 24 (2019) 3961; doi:10.3390/molecules24213961

[13] M. Paz-Arriaza, A. González-Coloma, M.F. Nadres, M. Berrocal-Lobo, J.A. Domínguez-Núñez, A. C. da Costa Jr., J. Navarro-Rocha, C. Calderón-Guerrero. 2018. Antifungal effect of essential oils. In Potential of essential oils, ed. Hany El-Shemy, Cairo, p.145-148.

[14] G.I.Kh. Marei, M.A.A. Rasaul, S.A.M. Abdelgaleil, Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. Pestic. Biochem. Physiol. 103 (2012) 56-61.

[15] D. Zhou, Z. Wang, M. Li, M. Xing, T. Xian, K. Tu, Carvacrol and eugenol effectively inhibit *Rhizopus stolonifer* and control postharvest soft rot decay in peaches. J. Appl. Microbiol. 124 (2018) 166-178.

[16] Y. Wu, Q. OuYang, N. Tao, Plasma membrane damage contributes to antifungal activity of citronellal against *Penicillium digitatum*. J. Food Sci. Tech. 53 (2016) 3853-3858.

[17] S. Zheng, G. Jing, X. Wang, Q. Ouyang, L. Jia, N. Tao, Citral exerts its antifungal activity against *Penicillium digitatum* by affecting the mitochondrial morphology and function. Food Chem. 178 (2015) 76-81.

[18] Q. OuYang, N. Tao, M. Zhang, A damaged oxidative phosphorylation mechanism is involved in the antifungal activity of citral against *Penicillium digitatum*. Front. Microbiol. 9 (2018) 239.

[19] R.F. Santos, M. Ciampi-Guilardi, L. Amorim, N.S. Massola Júnior, M.B. Spósito, Aetiology of anthracnose on grapevine shoots in Brazil. Plant Pathol. 67 (2018) 692-706.

[20] F.J. Scariot, L. Jahn, A.P.L. Delamare, S. Echeverrigaray, Necrotic cell death induced by dithianion on *Saccharomyces cerevisieae*. Pestic. Biochem. Physiol. 149 (2018) 137-142.

[21] A. Barra, Factors Affecting Chemical Variability of Essential Oils: a Review of Recent Developments. Nat. Prod. Commun. 4 (2009) 1147-1154.

[22] S.O. Lee, G.J. Choi, K.S. Jang, H.K. Lim, K.Y. Cho, J.C. Kim, Antifungal activity of five essential oils as fumigant against postharvest and soilborne plant pathogenic fungi. Plant Pathol. J. 23 (2007) 97-102.

[23] M.A. Numpaque, L.A. Oviedo, J.H. Gil, C.M. García, D.L. Durango, Thymol and carvacrol: biotransformation and antifungal activity against the plant pathogenic fungi Colletotrichum acutatum and Botryodiplodia theobromae. Trop.Plant Pathol. 36 (2011) 3-13.

[24] S. Kuinkel, R.D. Tiwari, S. Bhattarai, S. Antifungal activity of essential oils against Glomerella cingulate (ston,) Spauld & H. Schrenk. EJPMR 3 (2016) 233-237.

[25] V.P. Rabiri, K.S. Chudashma, V.S. Thaker, In vitro screening of 75 essential oils against Colletotrichum gloeosporioides: a causal agent of anthracnose disease of mango. Int. J. Fruit Sci. 18 (2018) 1-13.

[26] R. Kotan, S. Kordall, A. Cakir, Screening of antibacterial activities of twenty-one oxygenated monoterpenes. Z Naturforsch C J Biosci. 62 (2007) 507-13.

[27] S. Echeverrigaray, J. Zacaria, R. Beltrão Nematicidal activity of monoterpenoids against the root-knot nematode Meloidogyne incognita. Phytopathology 100 (2010) 199-203.

[28] G.I.Kh. Marei, S.A.M. Abdelgaleil. 2018. Antifungal potential and biochemical effects of monoterpenes and phenylpropenes on plant pathogenic fungi. Plant Protect. Sci. 54 (2018) 9–16

[29] F. Nazzaro, F. Fratianni, R. Coppola, V. De Feo, Essential oils and antifungal activity. Pharmaceuticals 10 (2017) 86

[30] A.C. Guimarães. L.M. Meireles. M.F. Lemos, Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules 24 (2019) 2471.

[31] P.F. Cannon, U. Damm, P.R. Johnston, B.S. Weir, *Colletotrichum*- current status and future directions. Stud. Mycol. 73 (2012) 181-213.

[32] E.M. Soylu, S. Kurt, S. Soylu, In vitro and in vivo antifungal activities of the essential oils

of various plants against tomato grey mold disease agent *Botrytis cinereal*. Int. J. Food Microbiol. 143 (2010) 183-189.

[33] M. Zhang, J. Ge, X. Yu, Transcriptome analysis reveals the mechanism of fungicidal of thymol against *Fusarium oxysporum* f. sp. *niveum*. Curr. Microbiol. 75 (2018) 410-419.

[34] P. Belenky, D. Camacho, J.J. Collins, Fungicidal Drugs Induce a Common Oxidative Damage Cellular Death Pathway. Cell Rep. 3 (2013) 350–358.

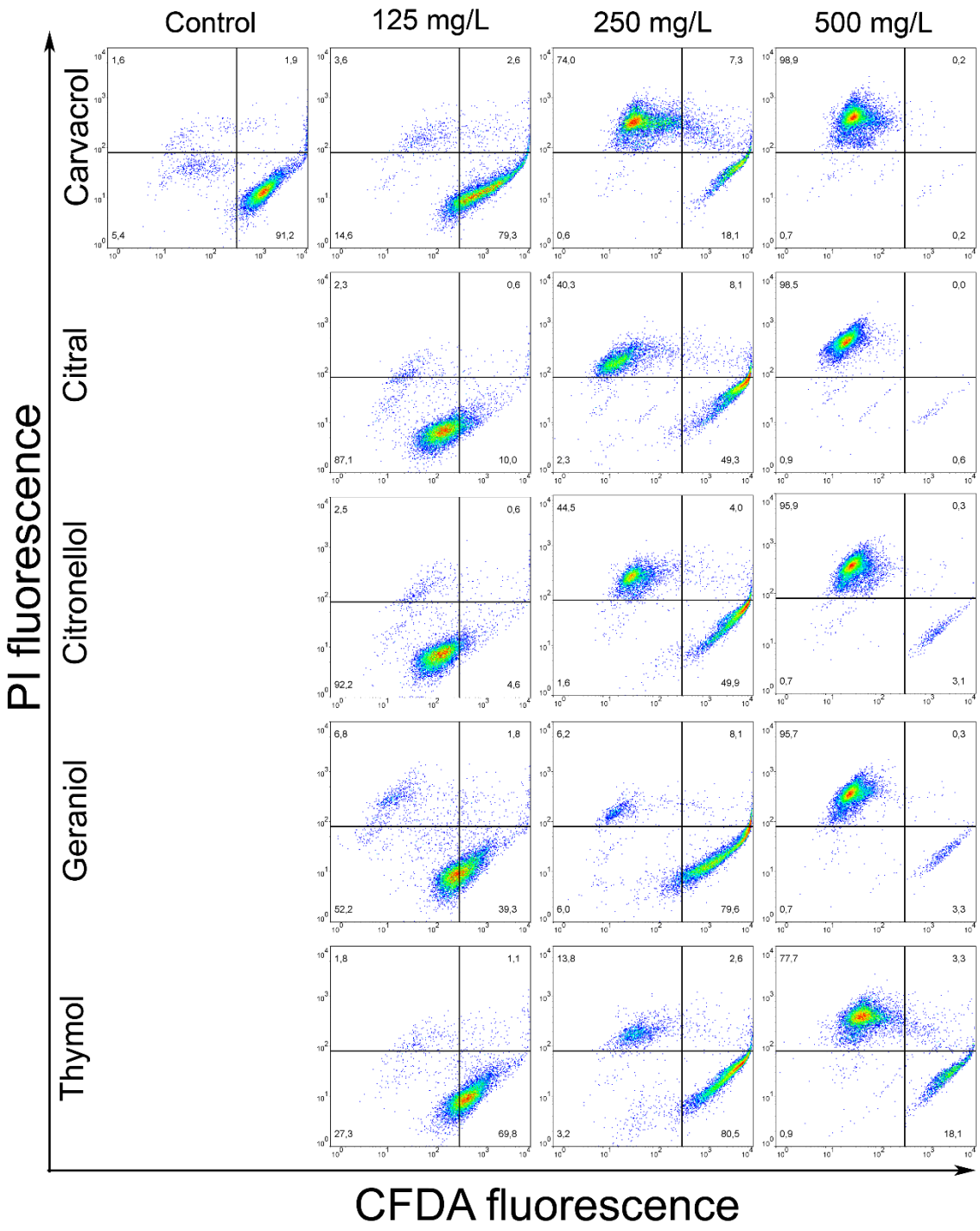
[35] M. Redza-Dutordoir, D.A. Averill-Bates, Activation of apoptosis signalling pathways by reactive oxygen species. Biochem. Biophys. Acta 1863 (2016) 2977-2992.

[36] H. Peshavariya, G. Dusting, S. Selemidis, Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. Free Radical Res. 41 (2007) 699–712.

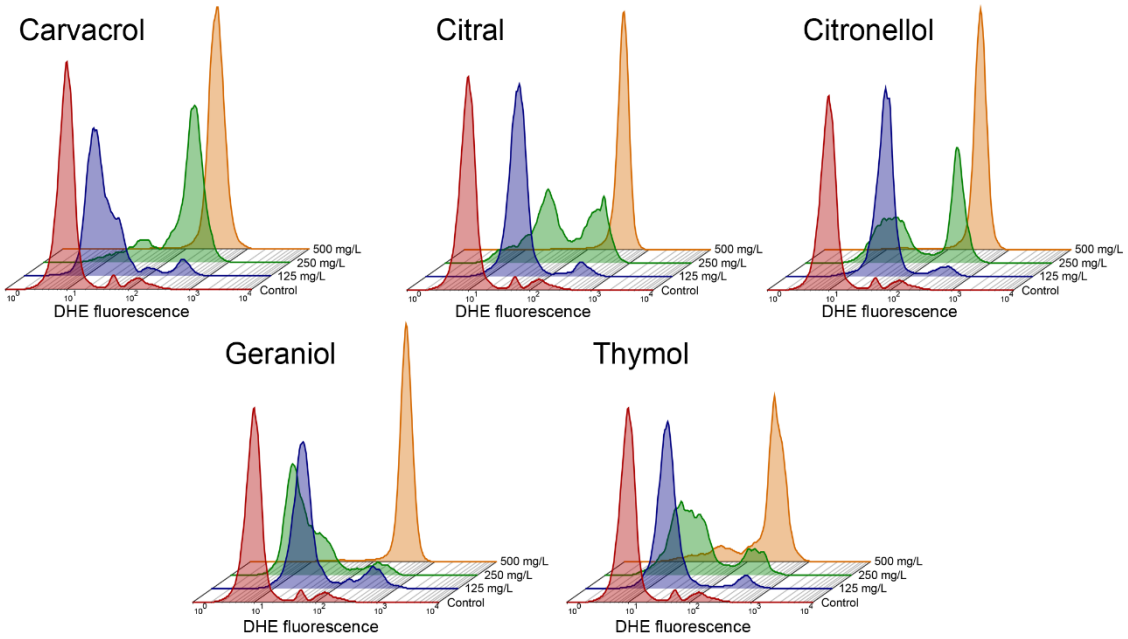
[37] A. Ahmad, A. Khan, N. Manzoor, Reversal of efflux mediated antifungal resistance underlies synergistic activity of two monoterpenes with fluconazole. Eur. J. Pharm. Sci. 48 (2013) 80–86.

Supplementary material S1. Monoterpenoids evaluated in the present study.

Monoterpenoids	Chemical group	Supplier	Cataloge number	Concentration and other specifications according to the supplier.
1,8- cineole	Cyclic	Acros-Organics	110341000	99%
camphor (L-)	Ketone	Acros-Organics	227840050	97%
carvacrol	Phenolic	Sigma-Aldrich	282197	98%
carveol (L-)	Alcohol	Sigma-Aldrich	W224707	≥ 95% of cis and trans
citral	Aldehyde	Acros-Organics	110441000	95% of cis and trans
citronellal	Aldehyde	Acros-Organics	405291000	93%
citronellol	Alcohol	Acros-Organics	110461000	95%
citronelyl acetate	Acetate	Acros-Organics	259611000	97%
geraniol	Alcohol	Sigma-Aldrich	163333	98%
limonene	Hydrocarbon	Acros-Organics	179395000	97%
linalool	Alcohol	Acros-Organics	125151000	97%
linalyl acetate	Acetate	Acros-Organics	232301000	95%
menthol (DL-)	Alcohol	Acros-Organics	125381000	99%
menthone (L-)	Ketone	Acros-Organics	203610250	85%
myrcene	Hydrocarbon	Acros-Organics	128085000	90%
terpinene-4-ol	Alcohol	Acros-Organics	360020250	97%
thymol	Phenolic	Sigma-Aldrich	16254	99%
α- pinene	Cyclic	Acros-Organics	131270050	98%
α- terpinene	Hydrocarbon	Acros-Organics	207491000	90%
α- terponeol	Alcohol	Acros-Organics	203740500	97% of alpha and gamma



Supplementary material S2. Cell membrane integrity and cell vitality of the *C. acutatum* conidia after monoterpenes treatment. A total of 10,000 cells were analyzed and the numbers within figures show the percentage of cells in each quadrant.



Supplementary material S3. Intracellular ROS accumulation (DHE fluorescence) in *C. acutatum* conidia after monoterpenes treatment. Histograms were obtained from the data of 10,000 cells.

4.3 CAPÍTULO 3

Antifungal Activity of Monoterpenes Against the Model Yeast *Saccharomyces cerevisiae*

Fernando Joel Scariot; Mariliza Salete Pansera; Ana Paula Longaray Delamare; Sergio Echeverrigaray

Artigo publicado na revista *Journal of Food Processing and Preservation*.

ORIGINAL ARTICLE

Journal of Food Processing and Preservation | Institute of Food Science + Technology | ifst | WILEY

Antifungal activity of monoterpenes against the model yeast *Saccharomyces cerevisiae*

Fernando Joel Scariot | **Mariliza Salete Pansera** | **Ana Paula Longaray Delamare** | **Sergio Echeverrigaray**

Laboratory of Enology and Applied Microbiology, University of Caxias do Sul, Rua Francisco Getúlio Vargas, 1130, Caxias do Sul, 95070-560, Brazil

Correspondence
Fernando Joel Scariot, Laboratory of Enology and Applied Microbiology, University of Caxias do Sul, R. Francisco G. Vargas 1130, 95070-560, Caxias do Sul, Rio Grande do Sul, Brazil.
Email: ferjscariot@gmail.com

Funding information
National Council for Scientific and Technological Development-CNPq, Grant/Award Number: 431538/2016-6; Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior, Grant/Award Number: 001

Abstract

The antifungal activity of 20 monoterpenes, currently found as main compounds in many essential oils, were evaluated against the model yeast *Saccharomyces cerevisiae*. Minimal inhibitory concentration (MIC), Minimal fungicidal concentration (MFC), and the time/dosage effect of selected monoterpenes were determined. The results showed that oxygenated monoterpenes exhibited higher fungistatic and fungicidal activity than hydrocarbons. Among oxygenated monoterpenes, the more effectives were citral, geraniol, citronellol, and citronellal, with MIC and MFC values between 0.64 and 3.68 mM, and 1.56 and 6.25 mM, respectively. Time response experiments showed that the selected monoterpenes rapidly reduce yeast cell viability in a time and dose-dependent manner. Moreover, the reduction of viability was associated with loss of cell membrane integrity. These results may aid in the selection of essential oils for the control of undesirable yeasts or fungi, and serve as a basis for the study of chemical structure influence on the mode of action of monoterpenes.

Novelty impact statement:

- Oxygenated monoterpenes have more fungicidal activity against *S. cerevisiae*.
- Citral, citronellol, citronellal, and geraniol were the most efficient monoterpenes.
- Monoterpenes' fungicidal activity is involved with cell membrane damages.

Antifungal Activity of Monoterpenes Against the Model Yeast *Saccharomyces cerevisiae*

Antifungal activity of monoterpenes

Fernando Joel Scariot*, Mariliza Salete Pansera, Ana Paula Longaray Delamare and Sergio Echeverrigaray

*Correspondence to: F. J. Scariot, Laboratory of Enology and Applided Microbiology, University of Caxias do Sul, R. Francisco G. Vargas 1130, 95070-560, Caxias do Sul, RS, Brazil. E-mail: ferjscariot@gmail.com

Laboratory of Enology and Applided Microbiology, University of Caxias do Sul, R. Francisco G. Vargas 1130, 95070-560, Caxias do Sul, RS, Brazil.

ABSTRACT: The antifungal activity of twenty monoterpenes, currently found as main compounds in many essential oils, were evaluated against the model yeast *Saccharomyces cerevisiae*. Minimal inhibitory concentration (MIC), Minimal fungicidal concentration (MFC), and the time/dosage effect of selected monoterpenes were determined. The results showed that oxygenated monoterpenes exhibited higher fungistatic and fungicidal activity than hydrocarbons. Among oxygenated monoterpenes, the more effectives were citral, geraniol, citronellol, and citronellal, with MIC and MFC values between 0.64 and 3.68 mM, and 1.56 and 6.25 mM, respectively. Time response experiments showed that the selected monoterpenes rapidly reduce yeast cell viability in a time and dose-dependent manner. Moreover, the reduction of viability was associated with loss of cell membrane integrity. These results may aid in the selection of essential oils for the control of undesirable yeasts or fungi, and serve as a basis for the study of chemical structure influence on the mode of action of monoterpenes.

Keywords: terpenes; antifungal activity; yeast; essential oil; cell membrane integrity.

Introduction

Plants produce a large number of molecules with potential biological activity, such as alkaloids, terpenoids, phenolic compounds, among others. Essential oils are a complex mixture of low molecular weight metabolites, isolated from vegetal material by hydrodistillation and with application in folk medicine and fragrance industries[1].

The main constituents of plant's essential oils are terpenes, particularly monoterpenes and sesquiterpenes, but also phenolic compounds, among other volatile chemical groups. Monoterpenes (C10) are hydrocarbons formed by two isoprene units and can be aliphatic or cyclic. Moreover, monoterpene derivatives or monoterpenoids can contain several functional groups, being classified as alcohols, aldehydes, ketones or esters[2].

The biological proprieties of several essential oils and their chemical constituents have been reviewed by several authors, and include cytotoxic, antimicrobial, antitumor, pro and antioxidant, stimulant, and allelopathic activities, among others[2,3-5]. Antimicrobial activity against filamentous fungi[6] positive and gram-negative bacteria[3,7], virus[8], phytonematodes[9], and yeasts[10-12] have been studied, and they highly depend on the chemical composition of the oils[2,3,13,14]. The essential oil composition is species-specific, but it is also influenced by many factors, like plant variety, geographic location, seasonal variations, agricultural practices, nutrition and stress during plant growth, extraction methods, among others[15].

Essential oils are an alternative to synthetic fungicides on the treatment of several plant diseases, particularly those known as fruit or post-harvest rots that can drastically affect fruit crop production and quality, like ripe rot (*Glomerella cingulata*) and grey rot (*Botrytis cinerea*) of grapes[16,17]. Fruits treatments with essential oils can increase the concentration of essential oil components on the fruits and their industrial by-products, and interfere with the alcoholic fermentation and organoleptic properties of wines. Moreover, several yeasts are considered spoilage organisms, and essential oils can be used in controlling microbial food contamination[18-20].

Due to the enormous variation of essential oil composition, their antifungal mechanisms remain unclear. However, several studies indicate that essential oils can be responsible for cell wall and membrane damage, mitochondrial dysfunction, reactive oxygen species accumulation, and inhibition of efflux pumps, which in turn can trigger apoptotic or necrotic cell death[21,22]. For example, Chami et al.[23] showed that the essential oils of clove and oregano induce surface yeast alterations and lysis. A similar effect was reported for turpentine oil which drastically increases extracellular pH in a concentration dependent-manner, indicating deterioration of cell membrane integrity of *S. cerevisiae*[13]. Conversely, *Mentha piperita* essential oil effect on yeast is associated with increased levels of intracellular reactive oxygen species, mitochondrial fragmentation and chromatin condensation, without modification of membrane integrity, indicative of apoptotic process[21]. Miron et al.[24] showed that geraniol and citral have an affinity for ergosterol triggering membrane destabilization on pathogenic yeasts. Moreover, these monoterpenoids induced osmotic stress with Na and K leakage, but only geraniol induced DNA damage, indicating that the radical (aldehyde or alcohol) affects the antifungal activity and mode of action of terpenoids[25].

This study aimed to evaluate the fungistatic and fungicidal effect of twenty monoterpenes, most of which were never tested before, against the model yeast *S. cerevisiae*. The monoterpenes used in the experiments were selected based on their prevalence in essential oils of aromatic plant species, and represent the different chemical groups of terpenes (hydrocarbons, alcohols, aldehydes and esters).

Experimental

Materials

Twenty monoterpenes: bornyl acetate (95%), camphor (97%), carveol (98%), 1,8-cineole (99%), citral (95%), citronellal (93%), citronellol (95%), citronellyl acetate (97%),

geraniol (96%), limonene (97%), linalool (97%), linalyl acetate (95%), menthol (99%), menthone (85%), myrcene (90%), beta-pinene (98%), rose oxide (99%), terpinen-4-ol (97%), alpha-terpinene (90%), and alpha-terpineol (97%) were purchased from Acros Organics, Geel, Belgium. Chemical structures of used monoterpenes are shown in Figure 1.

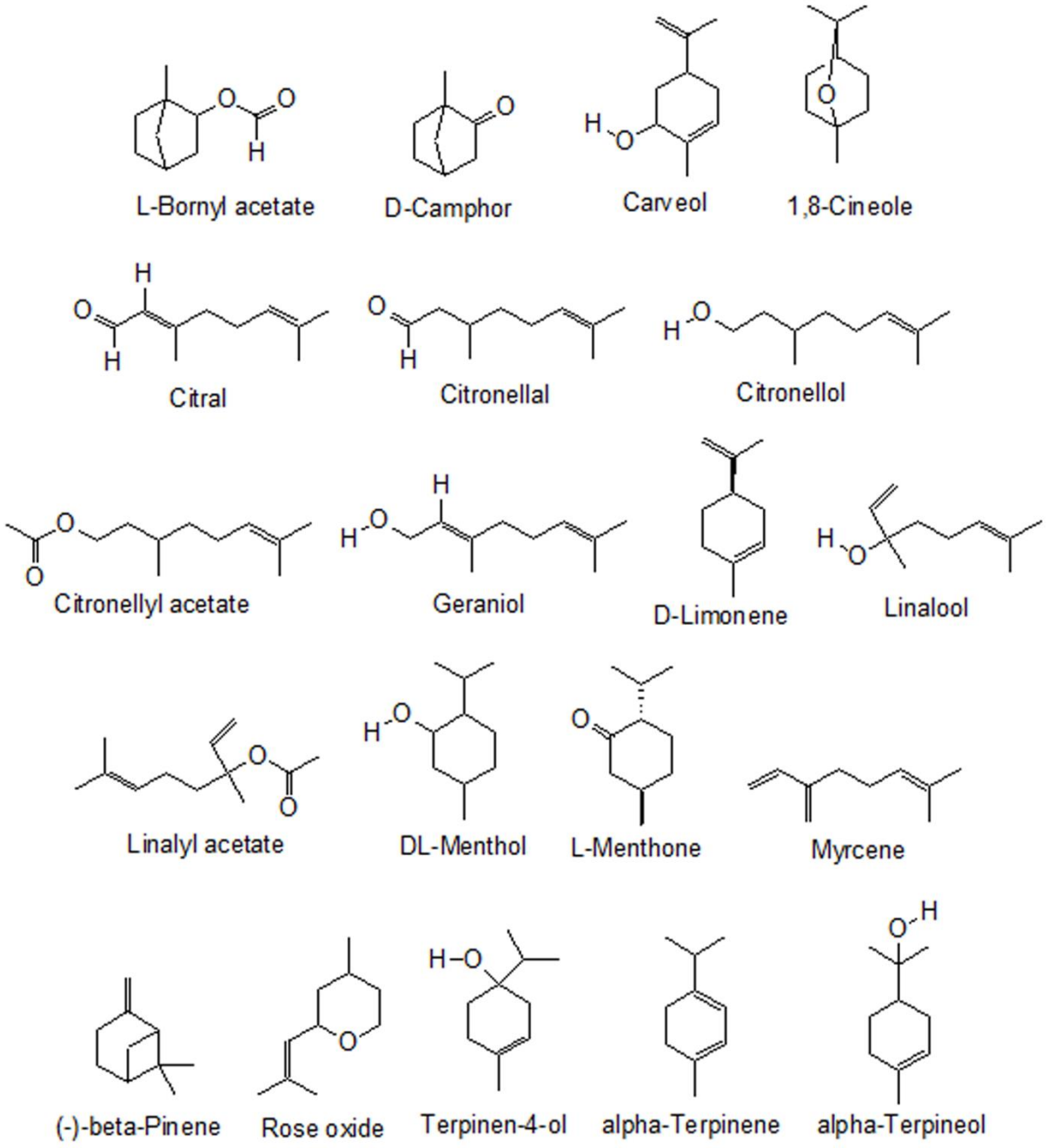


Figure 1. Chemical structure of the 20 monoterpenes used in this study.

Yeast and media

S. cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from

Euroscarf (Frankfurt, Germany). This is a model strain used for the deletion strain collection of Euroscarf and largely used in works related to stress response, fungicides activity, yeast metabolism, functional analysis, among other studies. Yeast was growth in YEPD broth (2 % yeast extract, 2 % glucose, 1 % peptone, pH 6.5) for inoculum. SD medium (0.67 % Yeast Nitrogen Base without amino acids, 2 % glucose, with 20 mg/L histidine, methionine, and uracil, and 60 mg/L leucine, pH 6.5) was used for viability assays.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC were determined in a micro-dilution assay. *S. cerevisiae* was grown overnight at 28°C in YEPD broth. Yeasts were harvested by centrifugation and washed in saline solution (NaCl 0.9 % w/v), then cells were suspended in YEPD broth to give a final concentration of 1 x 10^4 cells/mL. The assay was made in microplates with a two-fold dilution, ranging from 50 mM to 0.78 mM, of each monoterpene, including control with YEPD broth + 0.5% Tween 80. Plates were incubated at 28°C for 48 h, and yeast growth determined by absorbance using a Biochrom Asys Expert Plus microplate reader at 620 nm. MIC were determined by probit analysis using SPSS software (IBM, Armonk, NY, USA).

MFC was evaluated with plates obtained in MIC assay. After MIC assay evaluation 5 µL of each well was spotted in YEPD agar. Plates were incubated at 28°C and after 48 h colony formation was visualized, MFC was determined as the minimal concentration without yeast growth.

Viability assay

Exponentially growth yeasts were washed in saline solution and resuspend in SD medium with a cell concentration of 1 x 10^7 cells/mL, different concentrations of monoterpenes with lowest MIC and MFC were evaluated. Cell viability was evaluated at different times by

drop plating in YEPD and incubation at 28°C, after 48 h colonies formation units (c.f.u.) were counted and the viability curves were constructed.

Flow cytometry analysis

Cell vitality and viability (membrane integrity) were evaluated by flow cytometry in a FACSCalibur flow cytometer (Becton-Dickinson) equipped with an argon-ion laser emitting at 488 nm. Flow cytometer data of 10,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc).

Cell membrane integrity and viability were determined using the Membrane Integrity Yeast Viability kit (ThermoFisher) following the manufacturing instructions. The kit contains the cell-permeant DNA binding dye SYTO 9, and the cell-impermeant nucleic acid stain propidium iodide (PI), that binds to DNA of cell membrane damaged cells.

Exponential growing cells were collected, washed, suspended in saline solution, and treated for 180 min with 1.25 mM of selected monoterpenes (citral, geraniol, citronellal, or citronellol). Fluorescence was evaluated using an excitation of 488 nm, and emission on FL1 (530 ± 15 nm) and FL3 (650 ± 20 nm).

Statistical analysis

All the experiments were conducted in triplicate and the results were statistically analyzed by two-way ANOVA, and multiple comparisons of means (Tukey test) using the SPSS software (IBM, Armonk, NY, USA).

Results and Discussion

In the present study, twenty monoterpenes, including four monoterpenes hydrocarbons and 16 oxygenated monoterpenes (monoterpenoids) were evaluated against the model yeast *S.*

cerevisiae. As can be observed in Figure 2, 75% of the monoterpenes evaluated were able to inhibit yeast growth in concentrations lower than 25 mM. The terpenes that did not exhibit significant antifungal activity were menthone, myrcene, α-terpinene, rose oxide, and 1,8-cineole. These compounds are hydrocarbons or oxygenated terpenes of the pyran class or cyclic ether in which the oxygen is not exposed, except for menthone that is classified as a cyclic aldehyde derived from menthol (Figure 1). DL-limonene and β-pinene, two cyclic hydrocarbons, exhibited antifungal activity with MIC values of 20 and 16 mM, respectively. An almost 100x higher MIC value for DL-limonene on *S. cerevisiae* (2000 mg/L) was reported[26,27], and associated to programmed cell death.

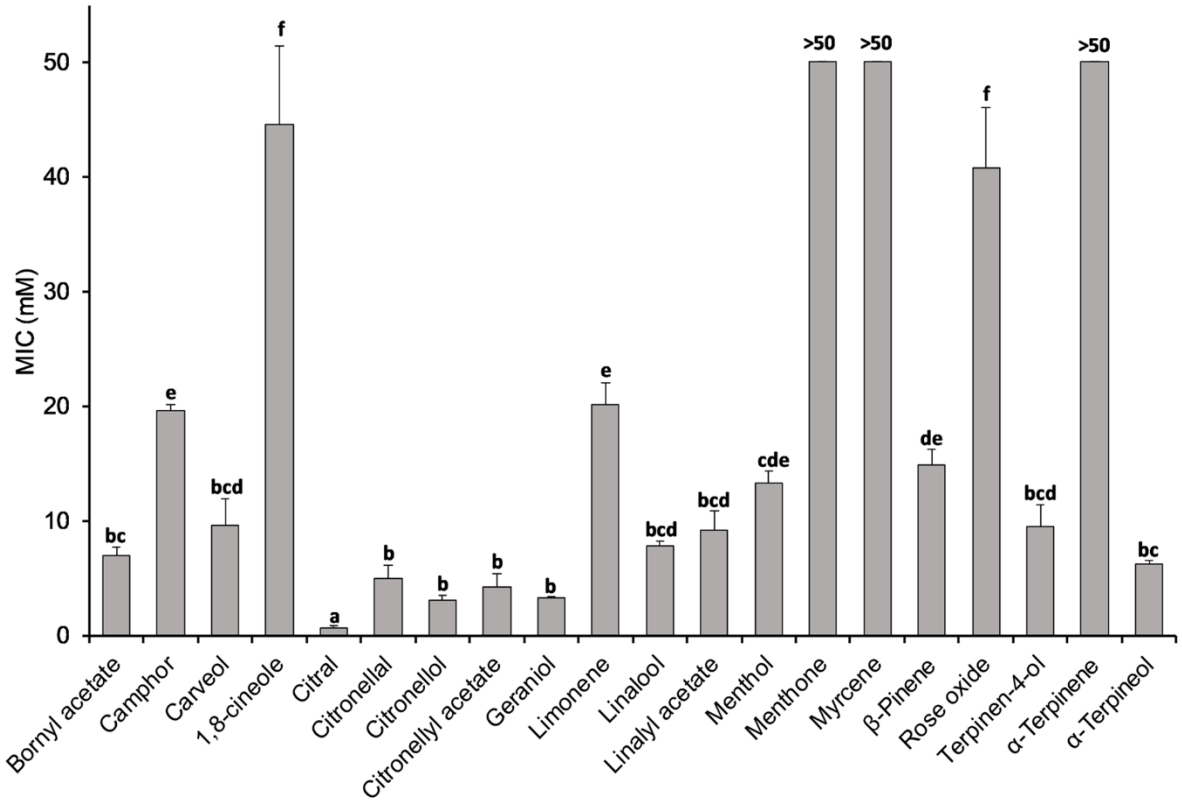


Figure 2. Minimal inhibitory concentration (MIC) of 20 monoterpenes on *Saccharomyces cerevisiae* BY4741. The different letters indicate statistically significant differences between the treatments ($p \leq 0.05$).

The most efficient monoterpenes were oxygenated compounds with alcohol and aldehyde radicals (Fig. 2). Among these, citral (a mixture of geranial and neral) exhibited the lowest MIC value (0.636 mM or 96.7 mg/L), followed by geraniol, citronellol, citronellal,

citronellyl acetate, α-terpineol, bornyl acetate, terpinen-4-ol, and carveol with MIC values that ranged between 2 and 9 mM. MIC reported for citral and geraniol exhibited very discrepant values, some similar to those obtained in the present work, with MIC values of 0.23 and 0.66 mM on pathogenic *Candida* species[24], 0.42 and 1.68 mM on *C. albicans*[28], and a higher MIC of approximately 3.29 mM on *S. cerevisiae*[25]. Ünal et al.[26] showed an antimicrobial effect of D-limonene, the most important constituent of citrus essential oils, against several grape and wine yeasts with MIC values ranging between 3.68 and 7.36 mM on *S. cerevisiae*. Moreover, the anticandidal activity of citronellal against *C. albicans*, and other yeast species[29], with a MIC of 6.5 mM, similar to the value obtained in the present work on *S. cerevisiae* (6.25 mM).

Although many of the monoterpenes have not been previously evaluated on yeasts, antibacterial and antifungal activity of essential oils with a high concentration of the monoterpenes included in the present work have been reported. For example, *Mentha spicata* essential oil with high content of carvone (51.7%) and carveol (24.35%), and the essential oil of *Aeolanthus suaveolens* with a high concentration of linalool (34.2 to 34.9%) exhibited antimicrobial activity against bacteria and fungi[30,31], and commercial essential oils of cinnamon, oregano, marigold, clove, and laurel oil inhibited the growth of several food yeasts, including *S. cerevisiae*, with MIC values ranging from 7.8 mg/L to 500 mg/L[32]. Eventual discrepancies of antifungal activities of essential oils or terpene on bacterial, yeasts or fungi have been attributed to differences in experimental conditions and extraction procedures, as well as microbial species and strains used in each work[2,18].

The MFC results are presented in Figure 3 and showed that all the monoterpenes evaluated have fungicidal activity. As expected a high correlation was observed between MIC and MFC (R=0.88), and in general, MFC were higher than the MIC values. Citral showed the lowest (1,56 mM) MFC between evaluated monoterpenes, followed by citronellol (3,13 mM), citronellal and geraniol (6,25 mM). Conversely, camphor, 1,8-cineole, menthone, myrcene and α-terpinene exhibited MFC of ≥50 mM and were the monoterpenes with lower efficiency

against *S. cerevisiae*. However, menthone that showed an MIC value of >50 mM, exhibited an MFC value of 25 mM, indicating that this monoterpene allows several divisions of yeast, but leads to cell death after 48 hours exposure.

The antifungal mechanisms of essential oils and their constituents indicate that they can be responsible for cell wall and membrane damage, mitochondrial dysfunction, reactive oxygen species accumulation, and inhibition of efflux pumps, which in turn can trigger apoptotic or necrotic cell death[21,22]. For instance, experimental data showed that citral interacts with ergosterols causing yeast membrane destabilization[24], inducing osmotic stress and Na-K leakage[25]. However, while citral exhibits necrotic death, geraniol induces DNA damage and possible apoptotic cell death[25], with no effect on the cell wall and ergosterol[33]. Thakre et al.[25] suggest that limonene inhibits *C. albicans* by cell wall/membrane damage induced by oxidative stress that triggers DNA damage, cell cycle arrest, and metacaspase-dependent apoptotic cell death. Conversely, Saibabu et al.[34] showed that citronellal induces reactive oxygen species (ROS) accumulation, mitochondrial membrane depolarization, and DNA damage on *C. albicans*, and Ferreira et al.[21] reported apoptotic *Saccharomyces* cell death associated with ROS-mediated damage induced by *Mentha piperita* essential oil. Despite these works, the mode of action of terpenes on yeasts and other microorganisms is still controversial as discrepant results have been obtained by different authors with several essential oils and monoterpenes[2,22].

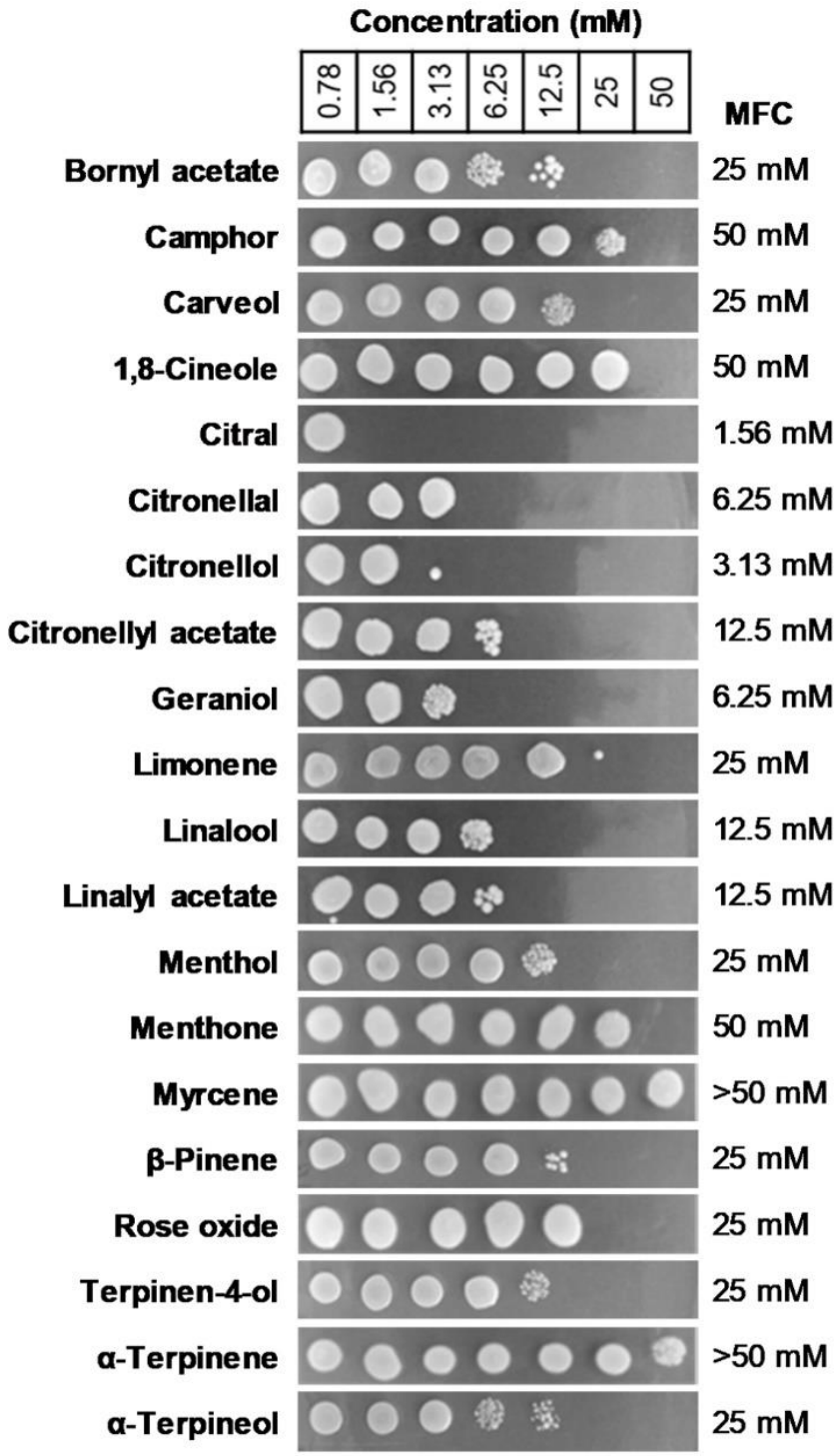


Figure 3. Minimal fungicidal concentration (MFC) of 20 monoterpenes on *Saccharomyces cerevisiae* BY4741.

Based in the effect observed in the previous assays were select four monoterpenes (citral, citronellol, citronellal, and geraniol) for time/cell death curve evaluation in acute treatments with 0 to 1.25 mM concentrations. As can be observed in Figure 4.A, 1.25 mM citral treatment reduce 100% of yeast viability in just 60 min, while 1.0 mM citral treatment leads to

a 98% reduction on yeast viability after 180 min, and 0.75 mM killed 78% of yeast cells in 360 min. In the lowest concentrations, the killing effect was time-dependent and almost linear in the lowest concentration (0.75 mM), which corresponds to 1/2 MFC. On the other hand, geraniol (Fig. 4.B) induced 98% mortality after 360 min at 1.25 mM concentration, and 68% after 360 min using a 1 mM. In both concentrations, the reduction of yeast viability was time-dependent. At the lowest concentration (0.75 mM) yeast viability was reduced to 80% in the first two hours but increase again in the last hour, indicating that the remaining viable yeast rapidly adapted to the stress imposed by geraniol, and resumed growth. It is important to point out that geraniol is the alcoholic counterpart of one of the two isomers (geranial) present in citral. Despite their structural similarity, geraniol is involved in apoptotic yeast cell death[25], while citral affects membrane stability by interaction with ergosterol, and induce necrotic death[24].

Citronellal (1.25 mM), approximately 1/4 MFC, drastically reduced yeast cell viability in just 1 hour, while 1.0 mM caused a 50% reduction of UFC in the same exposure time (Fig. 4.C), reaching approximately 95% in 360 min. Similar results were obtained with citronellol (Fig. 4. D) using 1.25 mM (1/3 MFC) and 1 mM (1/5 MFC). However, in the lowest concentration (0.75 mM) citronellol was more efficient than citronellal. Data obtained by Saibabu et al.[34] in *C. albicans* indicates that citronellal induces ROS accumulation with apoptotic cell death signals, but there are no data on the effect of citronellol on yeasts.

Cell viability and membrane integrity analyzed by flow cytometry (Figure 5 A-E) showed that the exposure of yeast cells to 1.25 mM of the four monoterpenes for 180 min determined the loss of cell membrane integrity (propidium iodide positive cells).

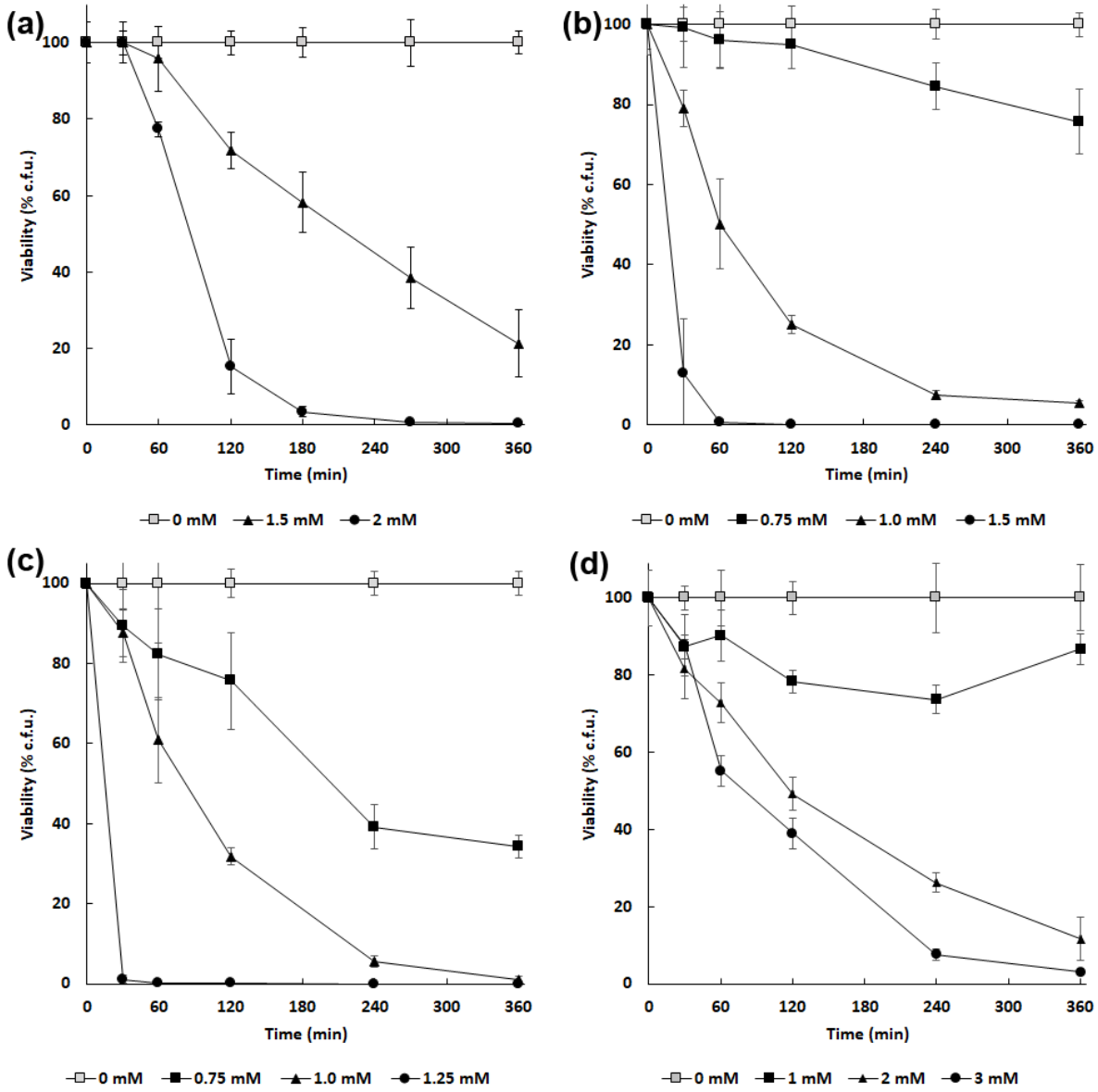


Figure 4. Effect of exposure time and monoterpenes concentration on *Saccharomyces cerevisiae* BY4741 of A (citral), B (geraniol), C (citronellal) e d (citronellol). Mean and standard deviations of three replications.

Cell membrane destabilization by citral, geraniol, and citronellal, as well as, several essential oils have been previously reported[13,24,25]. The loss of cell membrane integrity has been associated with both necrotic or apoptotic cell death[21,22,24,25], and result in changes in the redox potential, DNA damage[21,27,34], Na-K leakage[25], and modification of H(+)-efflux by the inhibition of H(+)-ATPase[35].

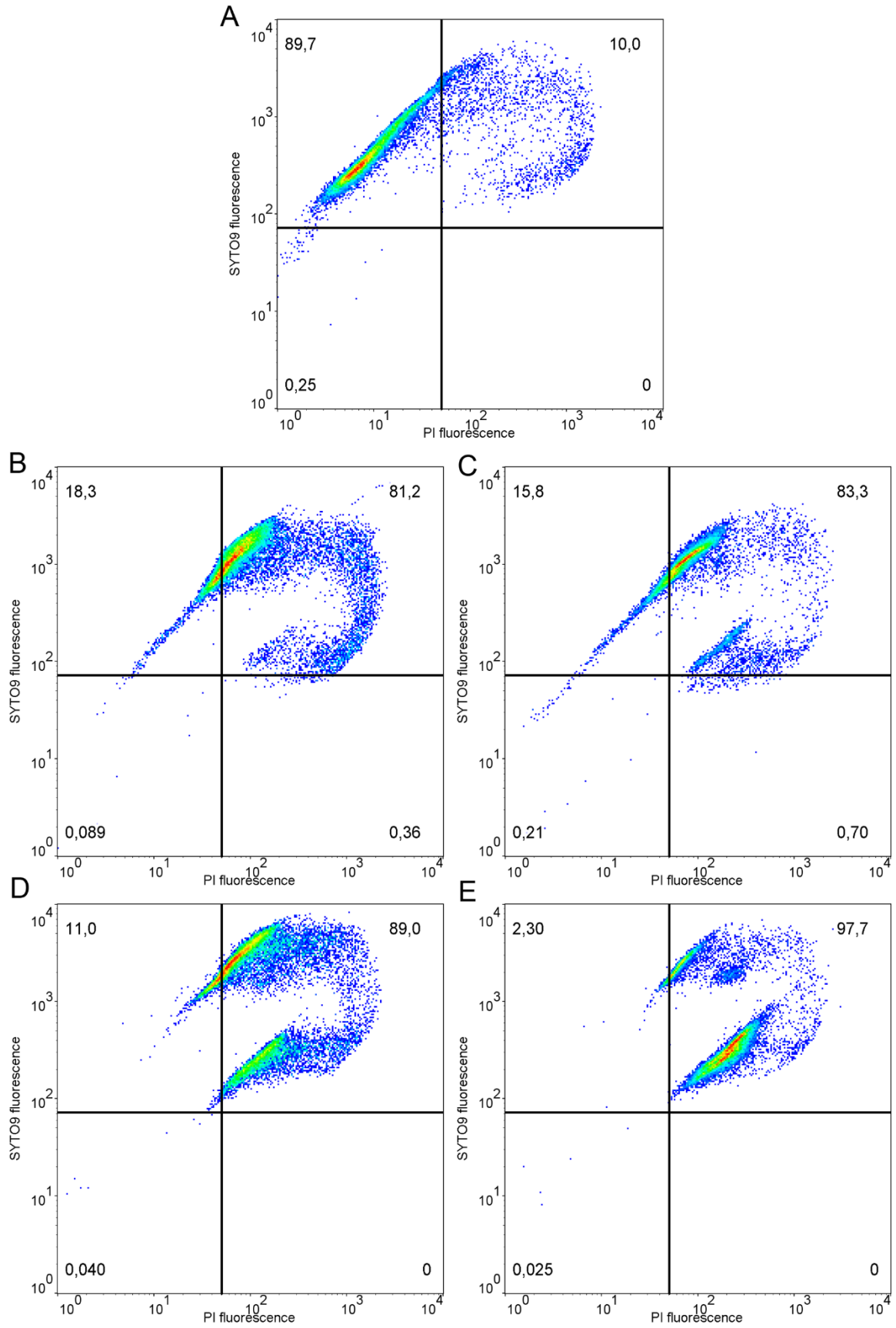


Figure 5. Cell membrane integrity of control and monoterpenes treated (1.25 mM -180 min) yeast cells. A. control, B- citral, C- geraniol, D- citronellal, E- citronellol.

Based on the present data, it can be concluded that oxygenated monoterpenes had greater antifungal activity than hydrocarbons ones. For instance, myrcene and α-terpinene did not show any antifungal activity at the highest concentration evaluated (50 mM). Among oxygenated monoterpenes, alcohol and aldehyde derivatives have greater activity than ketones. The lowest MIC and MFC values were obtained with the aliphatic monoterpenes citral, geraniol, citronellal, and citronellol, indicating that essential oils with a high concentration of these compounds may be more effective on the control of yeasts, and eventually, other fungi. Moreover, acute treatment with the selected monoterpenes leads to a rapid reduction of viability, and the loss of yeast cell membrane integrity.

Acknowledgements

This work was financially supported by the The National Council for Scientific and Technological Development-CNPq (Universal- 431538/2016-6); and the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES, finance code 001).

Disclosure statement

There is no conflict of interest of any authors in relation to the submission.

References

1. Turek C, Stintzing FC. Stability of essential oils: a review. *Compr Rev Food Sci Food Saf.* 2013;12:40-53.
2. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils–a review. *Food Chem Toxicol.* 2008;46:446-475.
3. Andrade BFMT, Barbosa LN, Probst IS, Júnior AF. Antimicrobial activity of essential oils. *J Essent Oil Res*. 2014;26:34-40.

4. Perricone M, Arace E, Corbo MR, Sinigaglia M, Bevilacqua A. Bioactivity of essential oils: a review on their interaction with food components. *Front Microbiol*. 2015;6:76.

5. Sharifi-Rad J, Sureda A, Tenore GC, et al. Biological activities of essential oils: from plant chemoecology to traditional healing systems. *Molecules.* 2017;22:70.

6. Marei GIK, Rasoul MAA, Abdelgaleil SA. Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. *Pestic Biochem Physiol*. 2012;103:56-61.

7. Trombetta D, Castelli F, Sarpietro MG, et al. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemothe*. 2005;49:2474-2478.

8. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytother Res*. 2010;24:673-679.

9. Echeverrigaray S, Zacaria J, Beltrão R. Nematicidal activity of monoterpenoids against the root-knot nematode *Meloidogyne incognita*. *Phytopath.* 2010;100:199-203.

10. Vardar-Ünlü G, Yağmuroğlu A, Ünlü M. Evaluation of in vitro activity of carvacrol against *Candida albicans* strains. *Nat Prod Resear*. 2010;24:1189-1193

11. Tserennadmid R, Takó M, Galgóczy L, et al. Anti-yeast activities of some essential oils in growth medium; fruit juices and milk. *Int J Food Microbiol*. 2011;144:480-486.

12. Nicola AM, Albuquerque P, Martinez LR, et al. Macrophage autophagy in immunity to *Cryptococcus neoformans* and *Candida albicans*. *Infect Immun*. 2012;80:3065-3076.

13. Konuk HB, Ergüden B. Antifungal activity of various essential oils against *Saccharomyces cerevisiae* depends on disruption of cell membrane integrity. *Biocell*. 2017;41:13-18.

14. Bachirraho G, Bevilacqua A, Corbo MR, Sinigaglia M, Pignatiello S. Antifungal properties of some essential oils against *Saccharomyces cerevisiae*. *Curr Tr Biotech & Microbio*. 2018;1:8-11.

15. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. *Ind Crops Prod.* 2014;62:250-264.

16. Tripathi P, Dubey NK, Shukla AK. Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. *World J Microbiol Biotechnol*. 2008;24:39-46.

17. Pedrotti C, Marcon AR., Delamare APL, et al. Alternative control of grape rots by essential oils of two *Eucalyptus* species. *J Sci Food Agr*. 2019;99:6552-6561.

18. Cowan MM. 1999. Plants products as antimicrobial agents. *Clin Microbiol Rev*. 1999;12:564-582

19. Krisch J, Tserennadmid R, Vagvolgyi C. Essential oils against yeasts and moulds causing food spoilage. In: Méndez-Vilas A, editor. *Science against microbial pathogens: communicating current research and technological advances*. Badajoz: Formatex; 2011. p.1135-1142.

20. Kunicka-Styczynska A. Activity of essential oils against food-spoiling yeast. A review. *Flavour Frag J*. 2011;26:326-328.

21. Ferreira P, Cardoso T, Ferreira F, et al. *Mentha piperita* essential oil induces apoptosis in yeast associated with both cytosolic and mitochondrial ROS-mediated damage. *FEMS Yeast Res.* 2014;14:1006-1014.

22. Nazzaro F, Fratianni F, Coppola R, De Feo V. Essential oils and antifungal activity. *Pharmaceuticals*. 2017;10:86.

23. Chami F, Chami N, Bennis S, Bouchikhi T, Remmal A. Oregano and clove essential oils induce surface alterations of *Saccharomyces cerevisiae*. *Phytother Res*. 2005;19:405-408.

24. Miron D, Battisti F, Silva FK, et al. Antifungal activity and mechanism of action of monoterpenes against dermatophytes and yeasts. *Rev Bras Farmacogn*. 2014;24:660-667.

25. Gaonkar R, Avti PK, Hedge G. 2018. Differential antifungal efficiency of geraniol and citral. *Nat Prod Commun*. 2018;13:1609-1614.

26. Ünal MU, Uçan F, Sener A, Dinçer S. Research on antifungal and inhibitory effects of DL-limonene on some yeasts. *Turk J Agric For*. 2012;36:576-582.

27. Thakre A, Zore G, Kodgire S, et al. Limonene inhibits *Candida albicans* growth by inducing apoptosis. *Med Mycol.* 2018;56:565-578.

28. Leite MCA, Bezerra APB, de Souza JP, Guerra FQS, Lima EO. Evaluation of antifungal activity and mechanism of action of citral against *Candida albicans*. *Evid Based Complement Alternat Med*. 2014;Article ID: 378280.

29. Singh S, Fatima Z, Hameed S. Citronellal-induced disruption of membrane homeostasis in *Candida albicans* and attenuation of its virulence attributes. *Rev Soc Bras Med Trop*. 2016;49:465-472.

30. Simionatto E, Porto C, Stüker CZ, Dalcol II, da Silva UF. Chemical composition and antimicrobial activity of the essential oil of *Aeolanthus suaveolens*. *Mart Ex Spreng Quim Nova*. 2007;30:1923-1925.

31. Hussain AI, Anwar F, Shahid M, Ashraf M, Przbylski R. Chemical composition; and antioxidant and antimicrobial activities of essential oil of spearmint (*Mentha spicata* L.) from Pakistan. *J Essent Oil Res*. 2010;22:78-84.

32. Çoskun F, Tirpanci G, Demirci AS. Anti-yeast effects of some commercial essential oils against food-related yeasts. *IOSR J Environ Sci Tox Food Techn*. 2016;10:83-88.

33. Lima ALA, Pérez ALAL, Sousa JP, et al. Antifungal activity of geraniol on *Candida albicans* isolates of pediatric clinical importance. *IJPPR*. 2017;9:581-586.

34. Saibabu V, Singh S, Ansari MA, Fatima Z, Hameed S. Insights into the intracellular mechanisms of citronellal in *Candida albicans*: implications for reactive oxygen species-mediated necrosis; mitochondrial dysfunction; and DNA damage. *Rev Soc Bras Med Trop*. 2017;50:524-529.

35. Bhatia R, Shreaz S, Khan N, et al. Proton pumping ATPase mediated fungicidal activity of two essential oil components. *J Basic Microbiol*. 2012;52:504-512.

4.4 CAPÍTULO 4

Citral and geraniol induce necrotic and apoptotic cell death on *Saccharomyces cerevisiae*

Fernando Joel Scariot; Mariliza Salete Pansera; Ana Paula Longaray Delamare; Sergio Echeverrigaray

Artigo publicado na revista *World Journal of Microbiology and Biotechnology*.

World Journal of Microbiology and Biotechnology (2021) 37:42
https://doi.org/10.1007/s11274-021-03011-8

ORIGINAL PAPER

Citral and geraniol induce necrotic and apoptotic cell death on *Saccharomyces cerevisiae*

Fernando J. Scariot[1] · Mariliza S. Pansera[1] · Ana Paula L. Delamare[1] · Sergio Echeverrigaray[1]

Received: 24 November 2020 / Accepted: 19 January 2021

Abstract
Essential oils and their main components, monoterpenes, have been proven to be important alternatives for the control of pathogenic and spoiling microorganisms, but the mode of action of these compounds is poorly understood. This work aimed to determine the mode of action of citral and geraniol on the model yeast *Saccharomyces cerevisiae* using a flow cytometry approach. Exponentially growing yeast cells were treated with different concentrations of citral and geraniol for 3 h, and evaluated for cell wall susceptibility to glucanase, membrane integrity, reactive oxygen species (ROS) accumulation, mitochondrial membrane potential, and metacaspase activity. Results provide strong evidence that citral and geraniol acute fungicidal activity against *Saccharomyces* cells involves the loss of membrane and cell wall integrity resulting in a dose-dependent apoptotic/necrotic cell death. However, yeast cells that escape this first cell membrane disruption, particularly evident on sub-lethal concentration, die by metacaspase-mediated apoptosis induced by the accumulation of intracellular ROS. The deleted mutant on the yca1 gene showed high tolerance to citral and geraniol.

Citral and geraniol induce necrotic and apoptotic cell death on *Saccharomyces cerevisiae*

Fernando J. Scariot*; Mariliza S. Pansera; Ana Paula L. Delamare; Sergio Echeverrigaray

Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil.

* Corresponding author. Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil.

E-mail address: ferjscariot@gmail.com (F.J. Scariot).

Abstract

Essential oils and their main components, monoterpenes, have been proven to be important alternatives for the control of pathogenic and spoiling microorganisms, but the mode of action of these compounds is poorly understood. This work aimed to determine the mode of action of citral and geraniol on the model yeast *Saccharomyces cerevisiae* using a flow cytometry approach. Exponentially growing yeast cells were treated with different concentrations of citral and geraniol for 3 h, and evaluated for cell wall susceptibility to glucanase, membrane integrity, reactive oxygen species (ROS) accumulation, mitochondrial membrane potential, and metacaspase activity. Results provide strong evidence that citral and geraniol acute fungicidal activity against *Saccharomyces* cells involves the loss of membrane and cell wall integrity resulting in a dose-dependent apoptotic/necrotic cell death. However, yeast cells that escape this first cell membrane disruption, particularly evident on sub-lethal concentration, die by metacaspase-mediated apoptosis induced by the accumulation of intracellular ROS. The deleted mutant on the yca1 gene showed high tolerance to citral and geraniol.

Keywords: membrane integrity, monoterpenoids, reactive oxygen species, YCA1

Introduction

The increase in the resistance to antifungal molecules and the restricted number of available compounds to control spoilage and pathogenic microorganisms stimulate the search for new alternatives. In this context, plant extracts could provide a large number of molecules with antimicrobial activity considered generally as safe for use. Among antimicrobial plant extracts, essential oils obtained by distillation of different organs of aromatic plants, and their main compounds, are considered as promising alternatives (Pandey et al. 2017; Winska et al. 2019).

The main constituents of essential oils are monoterpenes like citral, geraniol, carveol, linalool, among others. The presence and concentration of these compounds in essential oils varied among plant species, and are affected by genetic and environmental conditions. Monoterpenes are biosynthesized by the condensation of isoprene units through the mevalonate or the chloroplastic methyl D-erythritol 4-phosphate (MEP) pathways and accumulated in glandular trichomes of plants (Bakkali et al. 2008; Bergman et al. 2019). Citral is the main constituent of the essential oils of several aromatic plants including lemon myrtle, lemongrass, lemon, orange, and several other plant species. This terpenoid is the mixture of two aldehyde stereoisomers geranial (trans-citral) and neral (cis-citral). Geraniol is an acyclic alcohol monoterpenoid found in the essential oils of roses, palmarosa, citronella, and in small, but significant concentrations in geranium, lemon, aromatic grapes, and many other oils. Citral and geraniol are biosynthetically related molecules, as geraniol (geranyl pyrophosphate) is the precursor of geranial, as well as other monoterpenoids (Ramak et al. 2014).

Citral and geraniol, like other monoterpenes, are highly volatile compounds, exhibit a lipophilic behavior, and show low solubility in water. In vitro studies showed that citral and geraniol possess biological activity including antibacterial (Shi et al. 2016; Wang et al. 2019), anti-inflammatory (Ye et al. 2019), and antifungal effects (Leite et al. 2014; Li et al. 2014; Lima et al. 2012; Tang et al. 2018).

The antimicrobial mode of action of citral and geraniol, as well as other terpenoids, is still not completely understood. Studies on bacteria showed that citral alters the lipid content of *Salmonella enteritidis* cell membranes, increasing the proportion of saturated fatty acids (Dubois-Brissonnet and Naitali, 2011). Moreover, cells of *Cronobacter sakazakii* treated with citral exhibited a reduction in ATP production, intracellular pH modification, and cell membrane hyperpolarization (Shi et al. 2016). Chueca et al. (2014) showed that citral increases the oxidative stress of *Escherichia coli*, and its effect was protected by the scavenging molecules like thiourea, indicating an oxidation-dependent mechanism. However, these authors did not verify intracellular ROS accumulation.

Studies in eukaryotic cells showed that citral interacts with membrane fatty acids of the filamentous fungi *Geotrichum citri-aurantii* decreasing the total content of lipids and modifying membrane permeability (Zhou et al. 2014). In addition, this monoterpenoid also affects mitochondrial morphology, decreases intracellular ATP content, and inhibits several enzymes of the tricarboxylic acid cycle pathway of *Penicillium digitatum* (Zheng et al. 2015).

In yeasts, *Candida*, and *Saccharomyces*, geraniol enhances the rate of potassium leakage by an increase in membrane fluidity, affecting the central portion of membrane bilayer (Bard et al. 1988). Moreover, *Candida* species treated with geraniol evidenced cell wall rupture and a reduction in pseudohyphae and clamidoconidea differentiation. Sharma et al. (2016) and Singh et al. (2018) evidenced a significant reduction of ergosterol levels, and ATP-dependent efflux, associated with a loss of the mitochondrial membrane potential of *C. albicans* cells treated with geraniol. These results are supported by the bioinformatics data that showed a potential interaction of geraniol with membrane and apoptotic related genes (Zhang et al. 2019). Conversely, Leite et al. (2014) concluded that, although showing high antifungal activity, citral and geraniol did not affect cell wall and ergosterol on *Candida albicans.*

In *S. cerevisiae* the apoptotic process could be metacaspase dependent when mediated by the activation of the metacaspase (YCA1). Alternatively, there is a pathway that is

independent of the metacaspase activation and is dependent on the apoptosis-inducing factor (AIF1) activation together with the proapoptotic endonuclease (NUC1) activity (Carmona-Gutierrez et al. 2010).

Citral and geraniol have activity over mammalian diseases, Chaouki et al. (2009) showed that the effect of citral on the human breast cancer cell line MCF-7 included apoptotic induction and a cell cycle arrest in the G2/M phase. The apoptotic effect of citral was showed also in the leukemia cell line NB4, and in that cell, caspase-3 activation was observed (Xia et al. 2013). Geraniol shows activity on PC-3 prostate cancer cells, the mechanism of action included cell cycle arrest and apoptosis induction (Kim et al. 2011).

Considering the discrepancies among data on the mode of action of citral and geraniol on eukaryotic cells, we used the industrial, spoilage, and model organism *Saccharomyces cerevisiae* (Karathia et al. 2011) to better define the cellular mechanisms involved in their antifungal activity. *S. cerevisiae* has been used to elucidate the effect of several compounds on fungi, like mancozeb (Scariot et al. 2016), captan (Scariot et al. 2017), and other fungicides (Karathia et al. 2011). In this context, the present study aimed to better understand the cell death mechanisms of citral and geraniol on aerobically exponential cells of wild type *Saccharomyces cerevisiae* and its isogenic mutants, evaluating individual cell response using several flow cytometry analyses.

.

Material and Methods

Yeast and media

S. cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) strain and its isogenic mutants Y06233 (*yca1*::kanMX4), Y0233 (*aif1*::kanMX4), and Y01217 (*nuc1*::kanMX4) were obtained from Euroscarf (Frankfurt, Germany). Yeasts were grown in YEPD broth (2% yeast extract, 2% glucose, 1% peptone, pH 6.5) for inoculum. SD medium (0.67% Yeast Nitrogen

Base without amino acids, 2% glucose, with 20 mg/L histidine, methionine, and uracil, and 60 mg/L leucine, pH 6.5) was used for assays.

Citral and geraniol were purchased from Acros-Organics (cod. 110441000, and 163333, respectively), and stock solutions were diluted in Tween (final concentration lower than 0.5% v/v).

Yeast viability assay

Yeasts were grown until exponential phase in YEPD medium, washed in saline solution (NaCl 0.9% w/v), and cellular concentration was adjusted to 1 x 10^7 cells/mL in SD medium. Yeasts treated with citral or geraniol (0 to 3.0 mM) were incubated for 180 min in an orbital shaker (150 rpm; 28°C). Yeast cells were harvested, diluted at tenfold series, and 10 µL aliquots were plated in YEPD plates. Plates were incubated for 48 h at 28°C. Colonies forming units (CFU) were counted, and results were expressed as a percentage compared with the control. All assays were made in triplicate.

Cell wall integrity assay

The interference of citral or geraniol on cell wall was determinate by cell wall sensitivity to enzymatic degradation by β-1,3 glucanase from *Arthrobacter luteus* (Lyticase, Sigma-Aldrich) as described for Brennan et al. (2013). Briefly, control and terpenes treated cells were washed with NaCl (0.9% w/v), diluted in TE buffer (50 mM Tris-HCl; 5 mM EDTA, pH 7,5), and treated with 5.0 µg/L of Lyticase at 28°C with gentle agitation. Samples were collected every 30 min and the absorbance (600 nm) was evaluated. The results were expressed as the percentage of absorbance compared with time 0 (initial values).

Flow cytometry analysis

Cell membrane integrity, intracellular esterase activity, phosphatidylserine

externalization, intracellular ROS accumulation, mitochondrial membrane potential, and metacaspase activity were measured by flow cytometry. A FACSCalibur (Becton-Dickison) flow cytometer equipped with an argon-ion laser emitting at 488 nm was utilized. Flow cytometer data of 10,000 cells were acquired by Cell Quest Pro software (Becton-Dickison) and analyzed by FlowJo v.10 software (TreeStar, Inc).

For flow analysis, exponentially growth yeasts (1 x 10^7 cells/mL) were treated with 0 (control), 1.5, 2.0 or 3.0 mM of citral or geraniol. After 180 min of incubation in an orbital shaker (28°C; 150 rpm) cells were harvested, washed, and suspended in phosphate-buffered saline - PBS (pH 7.4). The assays were conducted in triplicate.

Cell membrane integrity and intracellular esterase activity were evaluated using the FungaLight™ Yeast CFDA, AM/Propidium Iodide Vitality Kit (Invitrogen), that include two dyes: propidium iodide (PI), a nucleic acid dye which penetrates only membrane-damaged cells, and 5-carboxyfluorescein diacetate acetoxymethyl (CFDA) a cell-permeable esterase substrate, that is cleaved by nonspecific esterases, resulting in a green fluorescent product. The staining and flow cytometer analyses were conducted following manufacturing instructions.

Intracellular reactive oxygen species (ROS) were quantified using the oxidant-sensitive fluorescent dye dihydroethidium (DHE, Sigma). Yeast cells were stained using a DHE final concentration of 5 µg/mL (DHE stock solution at 5 mg/mL in DMSO). Samples were incubated at 30°C for 30 min in the dark and evaluated by flow cytometry using the FL3 filter (488/670).

To evaluate mitochondrial membrane potential, cells were stained with 175 nM of 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$, Sigma) and incubate for 30 min at 30°C protected from light. Cell fluorescence was analyzed by flow cytometry using the FL1 filter (488/533).

Apoptotic cells were detected using FITC Annexin V/PI Dead Cell Apoptosis Kit (Invitrogen). Yeast cells spheroplasts preparation and dyeing were performed according to Scariot et al. (2016). Flow cytometry analysis was performed as described in manufacturer instructions.

Metacaspase activation was detected using a fluorescent dye, CaspACE FITC-VAD-FMK (Promega), that irreversibly binds to active caspases. Citral and geraniol treated (1.5 mM) and control cells were incubated with the dye for 30 min in the dark. Their fluorescence was measured by flow cytometry using the FL1 filter.

Results

In the first group of experiments, we evaluate the effect of different concentrations of citral and geraniol on the model *S. cerevisiae* strain BY4741 to define the concentrations that determine 50 to 10% reduction in yeast cell viability on acute systems. Thus, exponentially aerobic grown yeast cells were exposed to 0 to 6 mM of terpenoids for 180 min, and yeast viability was evaluated by plating and counting of colony-forming units (CFU/ml). Results showed that yeast cells treated with 2 mM citral reached a 95% reduction on CFU/ml, while in the same concentration, geraniol treated cells exhibited just a 71% reduction. However, 1.5 mM concentration of both terpenoids reduced 40 to 50% of CFU/ml. These results allowed to select a sub-lethal concentration of 1.5 mM, and lethal concentrations (MIC) of 2 and 3 mM, for further experiments.

The cell membrane integrity and metabolic activity of *S. cerevisiae* determined by PI and CFDA fluorescence by flow cytometry analysis of individual cells showed that both terpenoids have a dose-dependent increase of PI-positive cells (Fig. 1). Yeasts treated with sub-lethal concentrations of citral and geraniol (1.5 mM) exhibited an increase in the populations of cells with high PI fluorescence without loss of CFDA fluorescence, indicating that at this concentration both terpenoids induce cell membrane damage and a 3x increase in esterase activity. Moreover, at higher concentrations (2 and 3 mM), geraniol showed a higher percentage of PI-positive cells with low esterase activity than yeasts treated with citral (Fig. 1).

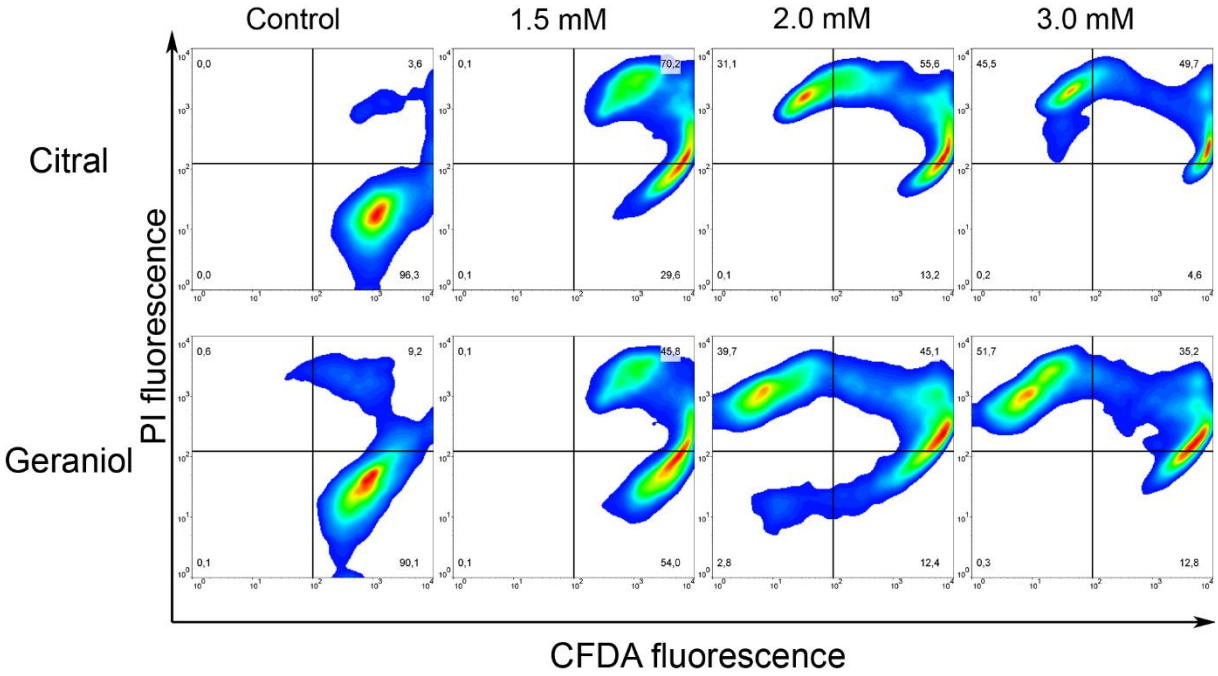


Fig. 1 Cell membrane integrity (PI fluorescence) and vitality (CFDA fluorescence) of wild-type BY4741 yeast treated for 3 h with 0 (control), 1.5 mM, 2.0 mM, or 3.0 mM of citral or geraniol. A total of 10,000 cells were analyzed, and numbers within figures indicate the percentage of cells within each quadrant.

The more external structure of yeasts is cell walls, whose main constituents are glucans, chitin, and mannoproteins. The impact of citral and geraniol on yeast cell wall was evaluated by measuring the susceptibility of treated cells to digestion with glucanase. Citral and geraniol-treated cells show more susceptibility to glucanase digestion than the untreated control. This effect is observed just after 30 min of assay and is more evident in higher monoterpenes concentrations (Fig. 2). When the two monoterpenes are compared, the citral-treated yeasts were more susceptible to glucanase treatment than those treated with geraniol, indicating that citral induces more damage in yeast cell wall than geraniol.

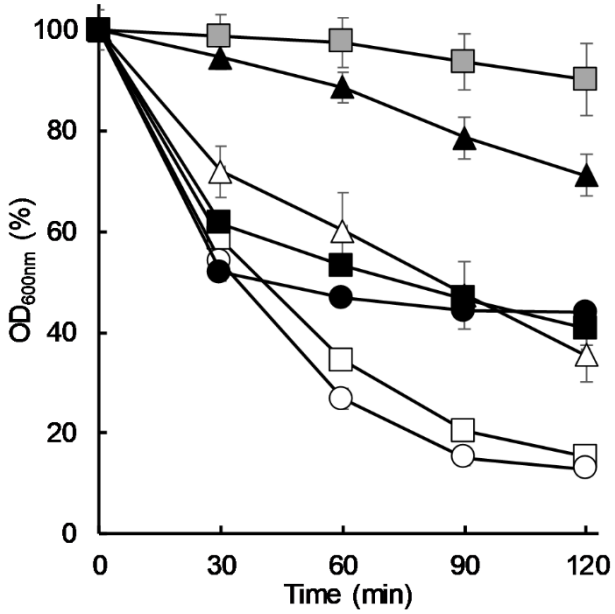


Fig. 2 Cell wall integrity based on Lyticase sensitivity. Control (■), citral 1.5mM (△), 2.0 mM (□), 3.0 mM (○), and geraniol 1.5 mM (▲), 2.0 mM (■), 3.0 mM (●). Error bar represent the standard error of mean of three replicates.

Citral and geraniol increased the intracellular reactive oxygen species (ROS) with a dose-dependent effect (Fig. 3). In the sub-lethal concentration (1.5 mM), two populations with different ROS accumulation were evident. In this concentration, the average increase of DHE fluorescence was 6x for citral and 2.3x for geraniol.

An increase in the intracellular concentration of ROS can be associated with modifications on mitochondrial activity, so we evaluated the mitochondrial membrane potential using the DioC6 dye. As can be observed in Fig. 3, citral treated cells showed an increase in mitochondrial potential, while those treated with geraniol maintained the same behavior as the control cells.

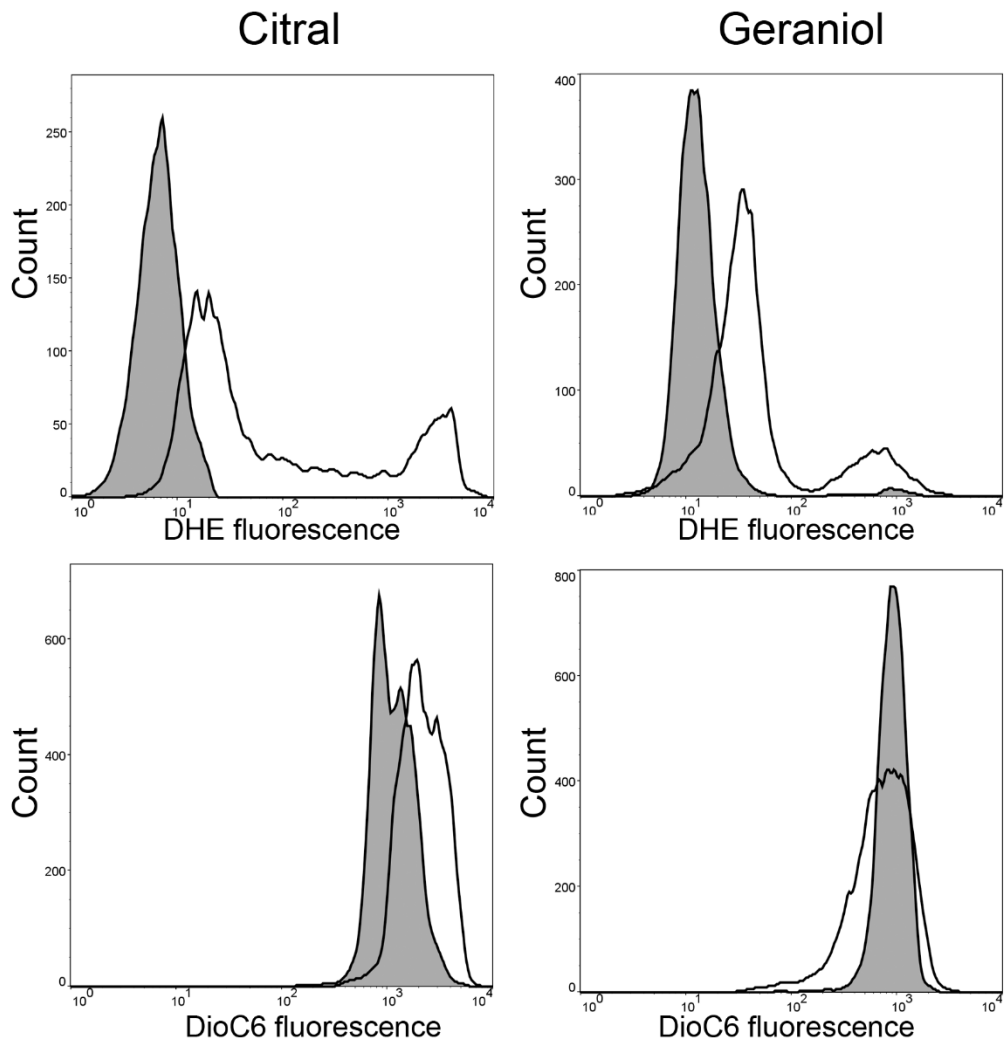


Fig. 3 ROS Intracellular ROS accumulation (DHE fluorescence intensity) and mitochondrial membrane potential ($\Delta\psi_m$) (DioC6 fluorescence) in control yeast (grey area) versus cells treated with 2.0 mM citral or geraniol for 3 h.

Considering that intracellular ROS accumulation can trigger apoptotic cell death in microbial and animal, we evaluated apoptosis on control and monoterpene-treated *Saccharomyces* cells using the AnnexinV-FITC dye, a marker of apoptotic cells that detect phosphatidylserine externalization (Wloch-Salamon and Bem, 2012). As can be observed in Fig. 4, citral and geraniol treated cells showed a substantial increase in AnnexinV-FITC fluorescence, indicating apoptotic cell death of yeast cells treated with both monoterpenoids.

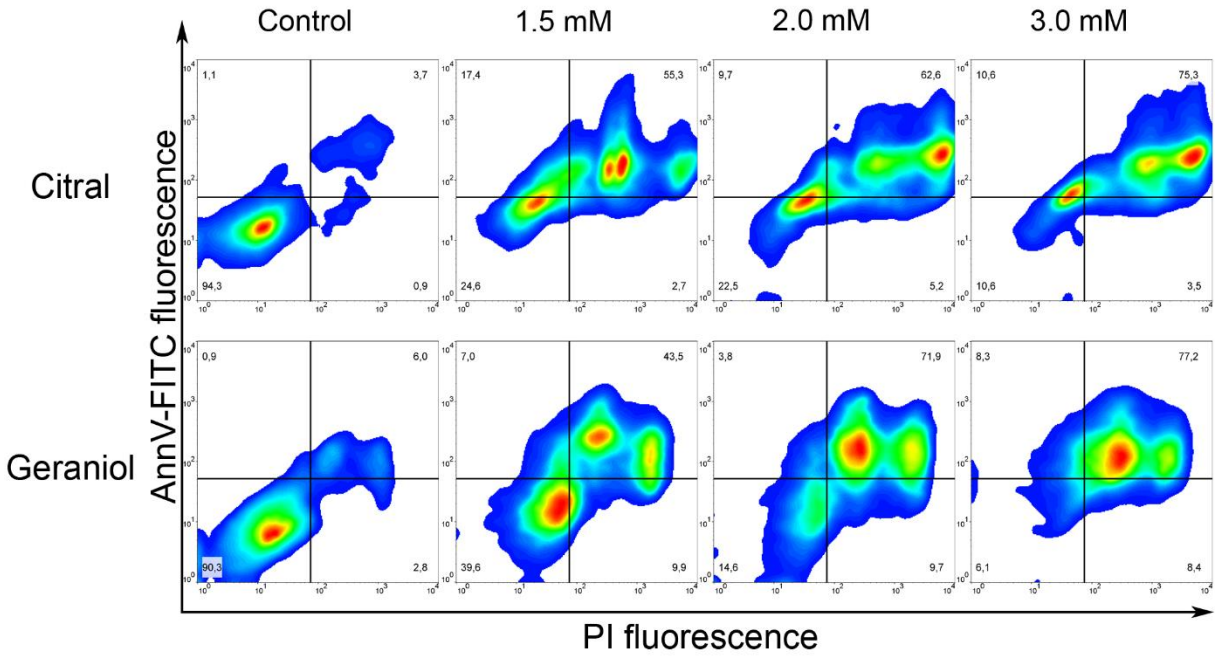


Fig. 4 Apoptosis determined by Annexin V/ PI staining on *S. cerevisiae* in the absence (Control) and the presence of 1.5 mM, 2.0 mM, and 3.0 mM of citral and geraniol for 3 h. A total of 10,000 cells were analyzed, and numbers within figures indicate the percentage of cells within each quadrant.

To better understand the apoptotic processes caused by citral and geraniol in *S. cerevisiae*, we evaluated the metacaspase activity using CaspACE FITC-VAD-FMK, which irreversibly binds to active metacaspase. Results showed that the exposition to both monoterpenes leads to an activation of yeast metacaspase (Fig. 5a and Fig. 5b). These data indicate that citral and geraniol induce an apoptosis cell death by the metacaspase dependent pathway.

To confirm the main role of metacaspase activation and the potential effect of other apoptotic pathways on yeast cell death induced by citral and geraniol, we evaluated the behavior of *Δyca1* (metacaspase), *Δaif1* (apoptotic inducing factor), and *Δnuc1* (mitochondrial nuclease) deleted mutants. The *Δaif1* and *Δnuc1* mutant showed the same cell death behavior of wild type yeast cells (data not shown), while, as can be evidenced in Fig. 5, *Δyca1* deleted mutants treated with citral (Fig. 5c) and geraniol (Fig. 5d) exhibited higher viability than the wild strain ($P < 0.05$), confirming that the apoptotic metacaspase dependent pathway is the main responsible

for *Saccharomyces cerevisiae* cell death induced by citral and geraniol.

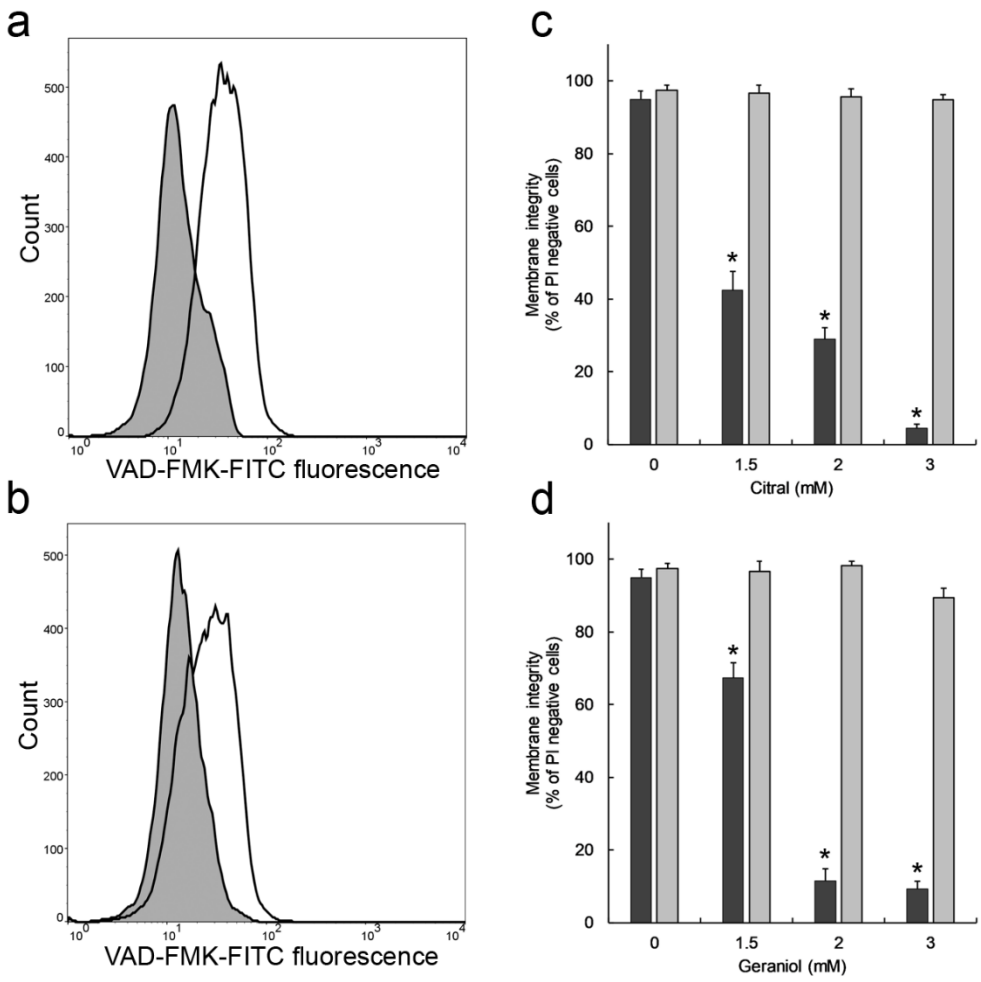


Fig. 5 Caspase activity (VAD-FMK-FITC fluorescence) in control yeasts (grey area) and cells treated with 2.0 mM citral (a) or geraniol (b), and cell membrane integrity in wild-type BY4741(black bars) and a *yca1* deleted mutant (grey bars) treated with 2.0 mM citral (c) and geraniol (d). Error bar represent the standard error of mean of three replicates. The asterisk indicates significant differences according to Student's *t*-test (*P < 0.05).

Discussion

The present data indicate that citral is more effective than geraniol, with a drastic viability reduction upon exposure to 2.0 and 3.0 mM of both terpenes for a short period (180 min). These concentrations are similar to those previously reported in *Candida albicans* and *Saccharomyces cerevisiae* (Bard et al. 1988; Lima et al. 2012; Gaonkar et al. 2018) on both chronic and acute systems.

Our data, obtained by flow cytometry analysis, showed that citral and geraniol induce a disruption of cell membrane integrity evidenced by high PI fluorescence. Also, cell membrane integrity was inversely proportional to monoterpenoids concentration, indicating a direct relationship between these compounds and cell membrane target molecules. These results confirm previous experimental data which showed that the main mode of action of essential oils and monoterpenes on yeasts and other microorganisms is related to their interaction with the cell membrane, causing damages that can lead to cell death (Bard et al. 1988; Lima et al. 2012; Chueca et al. 2014; Leite et al. 2014; Sharma et al. 2016; Shi et al. 2016, Konuk and Ergüden., 2017). Furthermore, we observed a drastic reduction in the CFDA fluorescent cells in PI-negative cells, indicating a loss of cellular homeostasis that reduces cell metabolism (esterase activity) in a cell population with integral membrane structures.

Cell membrane dysfunction induced by citral and geraniol were reported in *Candida albicans* (Leite et al. 2014) and *Saccharomyces cerevisiae* (Bard et al. 1988) and has been associated with the interaction of terpenoids with the lipids of membrane bilayer (Bard et al. 1988), causing leakage of potassium and cell constituents (Bard et al. 1988; Gaonkar et al. 2018).

The effect of monoterpenoids on the yeast cell wall is somewhat controversial. While some authors did not evidence cell wall modifications (Leite et al. 2014), other experiments showed important modifications of yeast cell walls (Bennis et al. 2004) or the expression of cell wall stress genes (Chatrath et al. 2019) in response to monoterpenoids. Our results showed that both citral and geraniol modify yeast cell walls, increasing their susceptibility to glucanase activity. This effect is observed just after 30 min of assay and is more evident in higher monoterpenes concentrations. Moreover, comparing the two monoterpenes, the citral-treated yeasts are more susceptible to glucanase than those treated with geraniol, indicating that citral induces more damage in yeast cell wall than geraniol. The action of monoterpenoids on cell walls can be explained by monoterpenes/mannoprotein interaction which can disarrange cell

wall compact structure increasing glucanase access and consequent activity.

The intracellular concentration of ROS can be drastically increased under several stress conditions affecting cell metabolism and viability (Farrugia and Balzan, 2012). In our experiments, *S. cerevisiae* cells treated with citral and geraniol exhibited a dose-dependent accumulation of intracellular ROS. In the lowest concentration (1.5 mM), two populations were evidenced. These populations correspond to viable (PI-) and death (PI+) cells, indicating that high ROS accumulation is related to yeast cell death by citral and geraniol. These data corroborate those previously obtained on filamentous fungi that pointed out intracellular ROS accumulation in citral and geraniol treated conidia and hyphae (Tang et al. 2018; Scariot et al. 2020).

One of the main sources of ROS is cell respiration, which in turn is related to mitochondrial activity. In this sense, *S. cerevisiae* cells treated with citral (2.0 mM) exhibited a mitochondrial hyperpolarization that can be responsible for the increase of intracellular ROS (Fedoseeva et al. 2017). However, this effect was not detected in geraniol treated cells, indicating a different mode of action or reactivity between these compounds, as reported by Gaonkar et al. (2018).

In normal cells, phosphatidylserine is located in the inner part of the cytoplasmic membrane. However, at the early stages of apoptosis, when membrane integrity is still retained, phosphatidylserine is exposed at the outer surface. To check apoptosis, control and monoterpenoid treated cells were stained with FITC-Annexin V and PI, and we observed a dose-dependent increase on early apoptotic (Annexin +/ PI -) and late apoptotic/necrotic cells (Annexin V +/ PI +) cells. ROS and apoptosis. In this sense, Chaouki et al. (2009) reported apoptotic induction and cell cycle arrest in the G2/M phase on human breast cancer cell line MCF-7 treated with citral, and Xia et al. (2013) observed apoptotic effect and caspase-3 activation upon exposure of the leukemia cell line NB4 to citral. Moreover, in *Candida albicans*, Zore et al. (2011) reported citral induction of cell cycle arrest at S phase, an indicator

of apoptosis.

Yeast programmed cell death or apoptosis has been detected in several stress conditions, like aging, ethanol, acetic acid and salt stress, drugs, hydrogen peroxide, fungicides, among others (Carmona-Gutierrez, 2010; Scariot et al. 2016). ROS and redox homeostasis are implicated in apoptosis processes in yeasts (Strich 2015), and can switch a metacaspase (*YCA1*) and/or *AIF1/NUC1* pathways of cellular catabolism (Carmona-Gutierrez et al. 2010). Our results showed an important increase of metacaspase (*YCA1*) in citral and geraniol treated cells, and a clear resistance of *YCA1* deleted mutants to both monoterpenoids, indicating that citral and geraniol induce a metacaspase dependent apoptotic death in *S. cerevisiae*. Additionally, as metacaspase apoptosis is related to ROS accumulation, the fungicidal of these monoterpenoids can be associated with their direct or indirect ROS increase.

Taken together, our experimental results provide strong evidence that citral and geraniol acute fungicidal activity in *Saccharomyces* cells involves a loss of membrane and cell wall integrity resulting in a typical apoptotic/necrotic cell death. However, yeast cells that escape this first cell membrane disruption, particularly evident on sub-lethal concentration, die by metacaspase-mediated apoptosis induced by the accumulation of intracellular ROS.

Acknowledgements

This work was financially supported by the National Council for Scientific and Technological Development-CNPq (431538/2016-6, and 304906/2017-4) and the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES, finance code 001). The authors acknowledge Cytogene Diagnósticos Moleculares Ltda. for access to flow cytometry equipment.

Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

References

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils–a review. Food Chem Toxicol 46:446-475

Bard M, Albrecht MR, Gupta N, Guynn CJ, Stillwell W (1988) Geraniol interferes with membrane functions in strains of *Candida* and *Saccharomyces*. Lipids 23:534-538

Bennis S, Cami F, Chami N, Bouchikhi T, Remmal A (2004) Surface alteration of *Saccharomyces cerevisiae* induced by thymol and eugenol. Let Appl Microbiol 38:454-458

Bergman ME, Davis B, Phillips MA (2019) Medically useful plant terpenoids: biosynthesis, occurrence, and mechanism of action. Molecules 24:3961

Brennan TC, Krömer JO, Nielsen LK. (2013) Physiological and transcriptional responses of *Saccharomyces cerevisiae* to d-limonene show changes to the cell wall but not to the plasma membrane. Appl Environ Microb 79:3590-3600

Carmona-Gutierrez D, Eisenberg T, Büttner S, Meisinger C, Kroemer G, Madeo F (2010) Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ 17:763-773

Chaouki W, Leger DY, Liagre B, Beneytout JL, Hmamouchi M (2009) Citral inhibits cell proliferation and induces apoptosis and cell cycle arrest in MCF-7 cells. Fundam Clin Pharm 23:549-556

Chatrath A, Gangwar R, Kumari P, Prasad R (2019) *In Vitro* anti-biofilm activities of citral and thymol against *Candida tropicalis*. J Fungi 5:13

Chueca B, Pagan R, Garcia-Gonzalo D (2014) Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic

acid cycle and Fenton reaction. Int J Food Microbiol 189:126–131

Dubois-Brissonnet F, Naitali M, Mafu AA, Briandet R (2011) Induction of fatty acid composition modifications and tolerance to biocides in *Salmonella enterica* serovar *typhimurium* by plant-derived terpenes. Appl Environ Microbiol 77:906–910

Farrugia G, Balzan R (2012) Oxidative stress and programmed cell death in yeast. Front Oncol 2:64

Fedoseeva IV, Pyatrikas DV, Stepanov AV, Fedyaeva AV, Varakina NN, Rusaleva TM, Rikhvanov EG (2017) The role of flavin-containing enzymes in mitochondrial membrane hyperpolarization and ROS production in respiring *Saccharomyces cerevisiae* cells under heat-shock conditions. Sci Rep 7:1-14

Gaonkar R, Aytic P, Hegdeb G (2018) Differential antifungal efficiency of geraniol and citral. Nat Prod Com 13:1609-1614

Karathia H, Vilaprinyo E, Sorribas A, Alve R (2011) *Saccharomyces cerevisiae* as a model organism: a comparative study. PLoS One 6:e16015

Kim SH, Bae HC, Park EJ, Lee CR, Kim BJ, Lee S, Park HH, Kim S, So I, Kim TW, Jeon JH (2011) Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways. Biochem Biophys Res Commun 407:129-134

Konuk HB, Ergüden B (2017) Antifungal activity of various essential oils against *Saccharomyces cerevisiae* depends on disruption of cell membrane integrity. BioCell 41:13-18

Leite MCA, Bezerra APDB, Sousa JPD, Guerra FQS, Lima EDO (2014) Evaluation of antifungal activity and mechanism of action of citral against *Candida albicans*. Evid-based Compl Alt Med. http://dx.doi.org/10.1155/2014/378280

Li RY, Wu XM, Yin XH, Liang JN, Li M (2014) The natural product citral can cause significant damage to the hyphal cell walls of *Magnaporthe grisea*. Molecules 19:10279-10290

Lima IO, de Medeiros Nóbrega F, de Oliveira WA, de Oliveira Lima E, Albuquerque Menezes

E, Afrânio Cunha F, de Fátima Formiga Melo Diniz M (2012) Anti-*Candida albicans* effectiveness of citral and investigation of mode of action. Pharm Biol 50:1536-1541

Pandey AK, Pradeep K, Pooja S, Tripathi NN, Bajpai VK (2017) Essential oils: sources of antimicrobials and food preservatives. Front Microbiol 7:2161

Ramak P, Osaloo KS, Sharifi M, Ebrahimzadeh H, Behmanesh M (2014) Biosynthesis, regulation and properties of plant monoterpenoids. J Med Plants Res 8:983-991

Scariot FJ, Jahn LM, Maianti JP, Delamare APL, Echeverrigaray S (2016) The fungicide Mancozeb induces metacaspase-dependent apoptotic cell death in *Saccharomyces cerevisiae* BY4741. Apoptosis 21:866–872

Scariot FJ, Jahn L, Delamare APL, Echeverrigaray S (2017) Necrotic and apoptotic cell death induced by Captan on *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 33:159

Scariot FJ, Foresti L, Delamare APL, Echeverrigaray S (2020) Activity of monoterpenoids on the in vitro growth of two *Colletotrichum* species and the mode of action on *C. acutatum*. Pestic Biochem Phys 170:104698

Sharma Y, Khan LA, Manzoor N (2016) Anti-*Candida* activity of geraniol involves disruption of cell membrane integrity and function. J Mycol Med 26:244-254

Shi C, Song K, Zhang X, Sun Y, Sui Y, Chen Y, Jia Z, Sun H, Sun Z, Xia X (2016) Antimicrobial activity and possible mechanism of action of citral against *Cronobacter sakazakii*. PLoS One 11:e0159006

Singh S, Fatima Z, Ahmad K, Hameed S (2018) Fungicidal action of geraniol against *Candida albicans* is potentiated by abrogated CaCdr1p drug efflux and fluconazole synergism. PLoS One 13:e0203079

Strich R, (2015) Programmed cell death initiation and execution in budding yeast. Genetics 200:1003-1014

Tang X, Shao YL, Tang YJ, Zhou WW (2018) Antifungal activity of essential oil compounds (geraniol and citral) and inhibitory mechanisms on grain pathogens (*Aspergillus flavus*

and *Aspergillus ochraceus*). Molecules 23:2108

Wang CY, Chen YW, Hou CY (2019) Antioxidant and antibacterial activity of seven predominant terpenoids. Int J Food Prop 22:230-238

Winska K, Maczka W, Tyczko J, Grabarczyk M, Czubaszek A, Szummy A (2019) Essential Oils as Antimicrobial Agents—Myth or Real Alternative? Molecules 24: 2130

Wloch-Salamon DM, Bem AE (2012) Type of cell death and methods of their detection in yeast *Saccharomyces cerevisiae*. J Appl Microbiol 114:287-298

Xia H, Liang W, Song Q, Chen X, Chen X, Hong J (2013) The in vitro study of apoptosis in NB4 cell induced by citral. Cytotechnology 65:49-57

Ye CJ, Li SA, Zhang Y, Lee WH (2019) Geraniol targets KV1. 3 ion channel and exhibits anti-inflammatory activity *in vitro* and *in vivo*. Fitoterapia 139:104394

Zhang YF, Huang Y, Ni YH, Xu ZM (2019) Systematic elucidation of the mechanism of geraniol via network pharmacology. Drug Des Devel Ther 13:1069-1075

Zheng S, Jing G, Wang X, Ouyang Q, Jia L, Tao N (2015) Citral exerts its antifungal activity against *Penicillium digitatum* by affecting the mitochondrial morphology and function. Food Chem 178:76–81

Zhou HE, Tao NG, Jia L (2014) Antifungal activity of citral, octanal and alpha-terpineol against *Geotrichum citri-aurantii*. Food Control 37:277–283

Zore GB, Thakre AD, Jadhav S, Karuppayil SM (2011) Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine 18:1181-1190

5 DISCUSSÃO GERAL

A utilização de compostos botânicos para o controle de fungos é uma importante alternativa em substituição às moléculas fungicidas sintéticas. Neste sentido, os óleos essenciais apresentam importante atividade e de forma especial seus compostos majoritários, os monoterpenos. Neste trabalho, o potencial fungicida de monoterpenos foi mostrado sobre o fungo *Colletotrichum* e sobre a levedura modelo *S. cerevisiae*.

Inicialmente foi avaliada a diversidade de espécies de *Colletotrichum* presentes em uvas com sintomas de podridão da uva madura. Foram identificadas as espécies *C. viniferum, C. fructicola, C. kahawae, C. nymphaeae, C. limitticola* e *C. karstii*. As espécies *C. limitticola* e *C. karstii* foram associadas a podridão da uva madura pela primeira vez, enquanto as outras espécies já foram relatadas previamente causando a doença em outras regiões vitivinícolas (Lei *et al*., 2016; Oo & Oh, 2017; Guginski-Piva *et al*., 2018). Observou-se ainda que os isolados pertencentes as espécies *C. viniferum* e *C. fructicola*, que foram mais abundantes, apresentaram maior virulência em comparação as outras espécies.

Visando avaliar o controle de *Colletotrichum* com a utilização de monoterpenos, foram selecionados os isolados CA002 (*C. fructicola* – grupo gloeosporioides) e A44/17 (*C. limitticola* – grupo acutatum). O isolado CA002 foi selecionado devido a relevância da espécie *C. fructicola*, tanto em videiras, quanto em outras plantas (Sharma & Shenoy, 2014; Alaniz *et al*., 2015; Li *et al*., 2016), enquanto o isolado A44/17 foi selecionado pela sua capacidade anormal de esporulação. Fungos de gênero *Colletotrichum* em geral apresentam dificuldades para realizar o processo de esporulação, com muitos isolados falhando em esporular em sistemas artificiais *in vitro* (Suzaki, 2011; Wang *et al*., 2017). Portanto, o isolado A44/17 apresenta enorme potencial de utilização em ensaios que necessitem de conídios, como os ensaios de inibição e citometria de fluxo.

A avaliação da atividade fungicida de diferentes monoterpenos sobre os isolados de *Colletotrichum* mostrou que monoterpenos oxigenados (álcoois e aldeídos) apresentaram maior

atividade fungicida, quando comparados com os outros monoterpenos (hidrocarbonetos, cetonas e ésteres). Quando a atividade fungicida dos monoterpenos foi avaliada sobre *S. cerevisiae*, resultado similar foi observado. Estes resultados indicam que a atividade fungicida desses monoterpenos está provavelmente associada com a presença de grupos hidroxila ou carbonila em sua estrutura. Conclusão similar foi obtida por Iraji *et al*. (2020), quando a atividade fungicida de monoterpenos foi avaliada em diferentes isolados de leveduras do gênero *Candida*.

Para a definição do modo de ação desses monoterpenos sobre *Colletotrichum*, apenas os conídios do isolado A44/17 foram utilizados, uma vez que os marcadores de integridade da membrana celular, acúmulo intracelular de espécies reativas de oxigênio e potencial da membrana mitocondrial foram avaliados por citometria de fluxo. Apesar de não ser tão convencional, a aplicabilidade da citometria de fluxo em ensaios com fungos já foi revisada por Bleichrodt & Read (2019). Observou-se que o tratamento dos conídios com os monoterpenos citral, citronelol, geraniol, carvacrol e timol levou a perda da integridade da membrana celular e acúmulo intracelular de ERO, não foram observadas alterações no potencial de membrana mitocondrial nos conídios tratados com os monoterpenos. A perda de integridade da membrana celular indica a presença morte celular por necrose (Eisenberg *et al*., 2010), enquanto a presença de ERO é um indicio de morte celular apoptótica (Gonçalves *et al*., 2017). Portanto, visando melhorar a elucidação da forma de morte causado pelos monoterpenos em *Colleotrichum*, a levedura modelo *S. cerevisiae* foi utilizada. *S. cerevisiae* foi utilizada principalmente por ser um fungo unicelular, o que permite seu emprego em ensaios que necessitem de células livres, como a citometria de fluxo (Gonçalves *et al*., 2017), além disso, a linhagem utilizada, BY4741, possui uma ampla coleção de mutantes isogênicos (Brachmann *et al*., 1998), facilitando estudos com ela.

Após uma avaliação inicial do conjunto de monoterpenos sobre *S. cerevisiae*, foram selecionados os monoterpenos citral e geraniol para um estudo mais aprofundado e definição

do seu modo de ação e forma de morte. Estes dois monoterpenos também possuem importante atividade fungicida sobre *Colletotrichum*. Similar ao observado em *Colletotrichum*, citral e geraniol causam perda na integridade de membrana celular e acúmulo intracelular de ERO em *S. cerevisiae*. Entretanto, diferente do observado em *Colletotrichum*, o potencial de membrana mitocondrial foi aumentado nas leveduras tratadas com citral, enquanto não houveram diferenças após o tratamento com geraniol. O aumento no potencial de membrana mitocondrial ocasionado por citral, apesar de não ter sido observado em *Colletotrichum*, foi observado anteriormente no fungo *Penicillium roqueforti* (Ju *et al*., 2020).

Além disso, foram observadas alterações na parede celular das leveduras tratadas com os monoterpenos. A composição estrutural da parede celular de fungos é heterogênea entre as diferentes espécies (Gow *et al*., 2017), portanto, apesar do efeito sobre a parede celular observado em *S. cerevisiae*, é difícil prever se o mesmo efeito vá ocorrer em *Colletotrichum*. Independente disso, a parede celular como alvo atrativo para o desenvolvimento de moléculas com atividade fungicida (Garcia-Rubio *et al*., 2020).

Por fim, as leveduras tratadas com citral e geraniol apresentaram exposição de fosfatidilserina e ativação da metacaspases, que complementarmente com a sobrevida observada na linhagem com deleção em *YCA1*, indicam um processo de morte celular apoptótica metacaspase dependente. Em *S. cerevisiae* a ativação do processo apoptótico pela metacaspase já é um fato conhecido (Madeo *et al*., 2002), entretanto, em *Colletotrichum* a definição detalhada do papel das metacaspases ainda precisa de estudos. Como referência, diferentemente de *S. cerevisiae*, que possui apenas uma metacaspase, no fungo filamentoso *Aspergillus fumigatus* estão presentes duas metacaspase (Richie *et al*., 2007).

A aplicabilidade da utilização de monoterpenos em ensaios *in vivo* para o controle de doenças associadas a *Colletotrichum*, como a podridão da uva madura, ainda precisa ser estuda de forma mais aprofundada. Por exemplo, estratégias envolvendo a utilização de sistemas para a estabilização das moléculas de monoterpenos parecem ser necessárias, uma vez que os

monoterpenos apresentam problemas de volatilidade e fotodegradação (Wadhwa *et al*., 2019). A utilização de estruturas combinando óleo essencial de *Ruta graveolens* e quitosana para a formação de revestimentos, que posteriormente foram utilizados para o controle de *Colletotrichum gloeosporioides* em sistemas de pós colheita, já foi mostrado em goiabas (Grande Tovar *et al*., 2019). O efeito de revestimentos de quitosana contendo óleo essencial de *Mentha piperita* mostrou potencial de controle de *Colletotrichum* durante o armazenamento de frutos de manga (de Oliveira *et al*., 2017). Outra opção, que contribui para a estabilidade dos monoterpenos é a sua incorporação em nanocápsulas. Por exemplo, Antonioli *et al*. (2020) mostraram que nanocápsulas com óleo essencial de capim-limão apresentaram efeito fungicida sobre isolados de *Colletotrichum* quando aplicadas sobre frutos de maçã.

O isolamento e identificação de espécies de *Colletotrichum* associadas com a podridão da uva madura mostrou a predominância das espécies *C. viniferum* e *C. fructicola*. A utilização de monoterpenos é uma alternativa para o controle de *Colletotrichum*, além disso o modo de ação dos monoterpenos está envolvido com uma perda da integridade da membrana celular e acúmulo intracelular de ERO, levando as células a uma morte celular apoptótica metacaspase dependente.

6 CONCLUSÕES

A partir dos resultados obtidos neste trabalho podemos concluir que:

- As espécies de *Colletotrichum* associadas com a podridão da uva madura em videiras da Serra Gaúcha foram *C. fructicola*, *C. kahawae, C. karstii*, *C. limitticola*, *C. nymphaeae* e *C. viniferum.*

- Os isolados de *C. fructicola* e *C. viniferum* apresentaram maior virulência em comparação com os isolados das outras espécies.

- Não houve diferenças na distribuição das espécies de *Colletotrichum* entre as variedades de *Vitis labrusca* e *V. vinifera*.

- As atividades antifúngicas dos monoterpenos sobre *Colletotrichum* spp. e *S. cerevisiae* indicam que monoterpenos oxigenados apresentam maior potencial fungicida em comparação com os demais monoterpenos, com destaque para citral, geraniol, citronelal e citronelol.

- Esses monoterpenos apresentaram capacidade de inibir o crescimento micelial e a germinação de conídios de *Colletotrichum* spp.

- A atividade desses monoterpenos sobre a germinação de conídios está envolvida com a perda de integridade de membrana celular e acúmulo intracelular de ROS.

- Citral e geraniol apresentam atividade fungicida sobre *S. cerevisiae*, em que a morte celular ocorre devido a perda da integridade da membrana e da parede celular.

- Em concentrações subletais esses monoterpenos levam a uma morte celular apoptótica, com ativação de metacaspase, induzida pelo acúmulo intracelular de ROS.

BIBLIOGRAFIA COMPLEMENTAR

Adaskaveg, J.E.; Förster, H. (2000). **Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California**. In: Prusky, D.; Freeman, S.; Dickman, M.B. (eds.), **Colletotrichum: Host Specificity, Pathology, and Host-Pathogen Interaction**. The American Phytopathological Society. St. Paul. pp. 317-336.

Ahmad, A.; Khan, A.; Akhtar, F.; Yousuf, S.; Xess, I.; Khan, L.A.; Manzoor, N. (2011). Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. **Eur. J. Clin. Microbiol. Infect. Dis.**, 30(1): 41-50.

Alaniz, S.; Hernández, L.; Mondino, P. (2015). *Colletotrichum fructicola* is the dominant and one of the most aggressive species causing bitter rot of apple in Uruguay. **Trop. Plant Pathol.** 40(4): 265-274.

Antonioli, G.; Fontanella, G.; Echeverrigaray, S.; Delamare, A.P.L.; Pauletti, G.F.; Barcellos, T. (2020). Poly (lactic acid) nanocapsules containing lemongrass essential oil for postharvest decay control: *In vitro* and *in vivo* evaluation against phytopathogenic fungi. **Food Chem.** 326: 126997.

Avenot, H.F.; Michailides, T.J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. **Crop Protect.** 29(7): 643-651.

Bailey, J.A.; O'connell, R.J.; Pring, R.J.; Nash, C. (1992). **Infection strategies of *Colletotrichum* species**. In: Bailey, J.A.; Jeger, M.J. (Ed.) ***Colletotrichum:* biology, pathology and control**. Wallingford: CAB International. pp 88-120.

Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. (2008). Biological effects of essential oils–a review. **Food Chem. Toxicol.** 46(2): 446-475.

Baroncelli, R.; Sreenivasaprasad, S.; Lane, C.R.; Thon, M.R.; Sukno, S.A. (2014). First report of *Colletotrichum acutatum* sensu lato (*Colletotrichum* godetiae) causing anthracnose on grapevine (*Vitis vinifera*) in the United Kingdom. **New Dis. Rep.** 29: 26-26.

Bartlett, D.W.; Clough, J.M.; Godwin, J.R.; Hall, A.A.; Hamer, M.; Parr-Dobrzanski, B. (2002). The strobilurin fungicides. **Pest Manag. Sci.**, 58(7): 649-662.

Bhunjun, C.S.; Phukhamsakda, C.; Jayawardena, R.S.; Jeewon, R,; Promputtha, I.; Hyde, K.D. (2021). Investigating species boundaries in *Colletotrichum*. **Fungal Divers**. 107(1): 107-127.

Bleichrodt, R.J.: Read, N.D. (2019). Flow cytometry and FACS applied to filamentous fungi. **Fungal Biol. Rev.** 33(1): 1-15.

Bonomelli, B.; Martegani, E.; Colombo, S. (2020). Lack of SNF1 induces localization of active Ras in mitochondria and triggers apoptosis in the yeast *Saccharomyces cerevisiae*. **Biochem. Biophys. Res. Commun.** 523(1): 130-134.

Brachmann, C.B.; Davies, A.; Cost, G.J.; Caputo, E.; Li, J.; Hieter, P.; Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. **Yeast**. 14(2): 115-132.

Bragança, C.A.; Damm, U.; Baroncelli, R.; Júnior, N.S.M.; Crous, P.W. (2016). Species of the *Colletotrichum acutatum* complex associated with anthracnose diseases of fruit in Brazil. **Fungal Biol**. 120(4): 547-561.

Brandhorst, T.T.; Klein, B.S. (2019). Uncertainty surrounding the mechanism and safety of the post-harvest fungicide fludioxonil. **Food Chem. Toxicol**. 123: 561-565.

Campos-Martínez, A.; Velázquez-del Valle, M.G.; Flores-Moctezuma, H.E.; Suárez-Rodríguez, R.; Ramírez-Trujillo, J.A.; Hernández-Lauzardo, A.N. (2016). Antagonistic yeasts with potential to control *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Colletotrichum acutatum* JH Simmonds on avocado fruits. **Crop Prot.** 89: 101-104.

Cannon, P.F.; Damm, U.; Johnston, P.R.; Weir, B.S. (2012). *Colletotrichum*–current status and future directions. **Stud. Mycol.** 73: 181-213.

Chatrath, A.; Gangwar, R.; Kumari, P.; Prasad, R. (2019). *In Vitro* Anti-Biofilm Activities of Citral and Thymol Against *Candida tropicalis*. **J. Fungi**. 5(1): 13.

Corda, A.C.I. (1837). **Sturm's Deutscblands Flora**. Nurnberg, Germany. p. 144.

Dai, Z.; Huang, M.; Chen, Y.; Siewers, V.; Nielsen, J. (2018). Global rewiring of cellular metabolism renders *Saccharomyces cerevisiae* Crabtree negative. **Nat. Comm.** 9(1): 1-8.

Damm, U.; Cannon, P.F.; Woudenberg, J.H.C.; Crous, P.W. (2012). The *Colletotrichum acutatum* species complex. **Stud. Mycol**. 73: 37-113.

De Castro, R.D.; de Souza, T.M.P.A.; Bezerra, L.M.D.; Ferreira, G.L.S.; de Brito Costa, E.M.M.; Cavalcanti, A.L. (2015). Antifungal activity and mode of action of thymol and its synergism with nystatin against *Candida* species involved with infections in the oral cavity: an in vitro study. **BMC Complement. Altern. Med.** 15(1): 1-7.

de Oliveira, K.Á.R.; Berger, L.R.R.; de Araújo, S.A.; Câmara, M.P.S.; de Souza, E.L. (2017). Synergistic mixtures of chitosan and *Mentha piperita* L. essential oil to inhibit *Colletotrichum* species and anthracnose development in mango cultivar Tommy Atkins. **Food Microb**. 66: 96-103.

De Silva, D.D.; Crous, P.W.; Ades, P.K.; Hyde, K.D.; Taylor, P.W. (2017). Life styles of *Colletotrichum* species and implications for plant biosecurity. **Fungal Biol. Rev.** 31(3): 155-168.

Dean, R.; Van Kan, J.A.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A. Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, R.; Ellis, J.; Foster, G.D. (2012). The Top 10 fungal pathogens in molecular plant pathology. **Mol. Plant Pathol.** 13(4): 414-430.

Devkatte, A.; Zore, G.B.; Karuppayil, S.M. (2005). Potential of plant oils as inhibitors of *Candida albicans* growth. **FEMS Yeast Res**. 5: 867–873.

Echeverrigaray S, Delamare APL, Fontanella G, Favaron F, Stella L, Scariot FJ. (2019). *Colletotrichum* species associated to ripe rot disease of grapes in the "Serra Gaucha" region of Southern Brazil. **BIO Web of Conferences**. 12: 01008.

Echeverrigaray, S.; Zacaria, J.; Beltrão, R. (2010). Nematicidal activity of monoterpenoids against the root-knot nematode *Meloidogyne incognita*. **Phytopathology**. 100(2): 199-203.

Eisenberg, T.; Carmona-Gutierrez, D.; Büttner, S.; Tavernarakis, N.; Madeo, F. (2010). Necrosis in yeast. **Apoptosis**. 15(3): 257-268.

Gan, P.; Ikeda, K.; Irieda, H.; Narusaka, M.; O'Connell, R.J.; Narusaka, Y.; Takano, Y.; Kubo, Y.; Shirasu, K. (2013). Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. **New Phytol**. 197(4): 1236-1249.

Gao, S.; Singh, J. (1998). *In vitro* percutaneous absorption enhancement of lipophilic drug tamoxifen by terpenes. **J. Control Release**. 51: 193–199.

Gao, T.; Zhou, H.; Zhou, W.; Hu, L.; Chen, J.; Shi, Z. (2016). The fungicidal activity of thymol against *Fusarium graminearum* via inducing lipid peroxidation and disrupting ergosterol biosynthesis. **Molecules**. 21(6): 770.

Garcia-Rubio, R.; de Oliveira, H.C.; Rivera, J.; Trevijano-Contador, N. (2020). The fungal cell wall: *Candida*, *Cryptococcus*, and *Aspergillus* species. **Front. Microbiol.** 10: 2993.

Gonçalves, A.P.; Heller, J.; Daskalov, A.; Videira, A.; Glass, N.L. (2017). Regulated forms of cell death in fungi. **Front. Microbiol.** 8: 1837.

Gopinath, K.; Radhakrishnan, N.V.; Jayaraj, J. (2006). Effect of propiconazole and difenoconazole on the control of anthracnose of chilli fruits caused by *Colletotrichum capsici.* **Crop Prot.** 25(9): 1024-1031.

Gow, N.A.; Latge, J.P.; Munro, C.A. (2017). The fungal cell wall: structure, biosynthesis, and function. **The fungal kingdom**. 267-292.

Grande Tovar, C.D.; Delgado-Ospina, J.; Navia Porras, D.P.; Peralta-Ruiz, Y.; Cordero, A.P.;

Castro, J.I.; Valencia, M.N.C.; Mina, J.H.; Chaves López, C. (2019). *Colletotrichum gloesporioides* inhibition in situ by chitosan-*Ruta graveolens* essential oil coatings: Effect on microbiological, physicochemical, and organoleptic properties of guava (*Psidium guajava* L.) during room temperature storage. **Biomolecules**, 9(9): 399.

Guginski-Piva, C.A.; Bogo, A.; Gomes, B.R.; Menon, J.K.; Nodari, R.O.; Welter, L.J. (2018). Morphological and molecular characterization of *Colletotrichum nymphaeae* and *C. fructicola* associated with anthracnose symptoms of grape in Santa Catarina State, southern Brazil. **J. Plant Dis. Prot.** 125(4): 405-413.

Gupta, K.; Bishop, J.; Peck, A.; Brown, J.; Wilson, L.; Panda, D. (2004). Antimitotic antifungal compound benomyl inhibits brain microtubule polymerization and dynamics and cancer cell proliferation at mitosis, by binding to a novel site in tubulin. **Biochemistry.** 43(21): 6645-6655.

Han, Y.C.; Zeng, X.G.; Guo, C.; Zhang, Q.H.; Chen, F.Y.; Ren, L.; Chen, W.; Qin, L. (2021). Reproduction response of *Colletotrichum* fungi under the fungicide stress reveals new aspects of chemical control of fungal diseases. **Microb. Biotechnol.** doi: 10.1111/1751-7915.13754.

Iraji, A.; Yazdanpanah, S.; Alizadeh, F.; Mirzamohammadi, S.; Ghasemi, Y.; Pakshir, K.; Yang, Y.; Zomorodian, K. (2020). Screening the antifungal activities of monoterpenes and their isomers against *Candida* species. **J. Appl. Microbiol.** 129(6): 1541-1551.

Irkin, R.; Korukluoglu, M. (2009). Effectiveness of *Cymbopogon citratus* L. essential oil to inhibit the growth of some filamentous fungi and yeasts. **J. Med. Food** 12: 193–197.

Ishikawa, T.; Lisiecki, K. (2020). Anti-prion drug screening system in *Saccharomyces cerevisiae* based on an artificial [LEU2+] prion. **Fungal Genet. Biol.** 134: 103280.

Jing, G.X.; Tao, N.G.; Jia, L.; Zhou, H.E. (2015). Influence of α-terpineol on the growth and morphogenesis of *Penicillium digitatum*. **Bot. Stud.**, 56(1): 35.

Ju, J.; Xie, Y.; Yu, H.; Guo, Y.; Cheng, Y.; Zhang, R.; Yao, W. (2020). Major components in

Lilac and Litsea cubeba essential oils kill *Penicillium roqueforti* through mitochondrial apoptosis pathway. **Ind. Crops Prod.** 149: 112349.

Kaczmarek Michaels, K.; Mohd Mostafa, S.; Ruiz Capella, J.; Moore, C.L. (2020). Regulation of alternative polyadenylation in the yeast *Saccharomyces cerevisiae* by histone H3K4 and H3K36 methyltransferases. **Nucleic Acids Res.** 48(10): 5407-5425.

Karathia, H.; Vilaprinyo, E.; Sorribas, A.; Alves, R. (2011). *Saccharomyces cerevisiae* as a model organism: a comparative study. **PLoS One**. 6(2): e16015.

Kawai, M.; Yamagishi, J.I. (2009). Mechanisms of action of acriflavine: electron microscopic study of cell wall changes induced in *Staphylococcus aureus* by acriflavine. **Microbiol. Immunol**. 53(9): 481-486.

Krysan, D.J.; Didone, L. (2008). A high-throughput screening assay for small molecules that disrupt yeast cell integrity. **J. Biomol. Screen**. 13(7): 657-664.

Kumar, A.; Changwal, C.; Hada, A.; Singh, P.K. (2020). **Molecular Insight of Plant–Pathogen Interaction**. In: Roychowdhury, R.; Choudhury, S.; Hasanuzzaman, M.; Srivastava, S. (Eds) **Sustainable Agriculture in the Era of Climate Change.** Springer, Cham. pp 481-511.

Kumari, P.; Arora, N.; Chatrath, A.; Gangwar, R.; Pruthi, V.; Poluri, K. M.; Prasad, R. (2019). Delineating the Biofilm Inhibition Mechanisms of Phenolic and Aldehydic Terpenes against *Cryptococcus neoformans*. **ACS Omega**, 4(18): 17634-17648.

Lang, G.; Buchbauer, G. (2012). A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals: a review. **Flav. Frag. J.** 27: 13–39.

Latunde-Dada, A.O.; O'Connell, R.J.; Nash, C.; Pring, R.J.; Lucas, J.A.; Bailey, J.A. (1996). Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum*) from cowpea (*Vigna unguiculata*). **Mycol. Res.** 100(9): 1133-1141.

Lei, Y.; Fu, P.; Jun, X.; Cheng, P. (2019). Pharmacological Properties of Geraniol–A

Review. **Planta Med.**, 85(01): 48-55.

Lei, Y.; Tang, X.B.; Jayawardena, R.S.; Yan, J.Y.; Wang, X.D.; Liu, M.; Chen, T.; Liu, X.M.; Wang, J.C.; Chen, Q.X. (2016). Identification and characterization of *Colletotrichum* species causing grape ripe rot in southern China. **Mycosphere**, 7(8): 1177-1191.

Leite, M.C.A.; de Brito Bezerra, A.P.; de Sousa, J.P.; de Oliveira Lima, E. (2014). Investigating the antifungal activity and mechanism (s) of geraniol against *Candida albicans* strains. **Med. Mycol.** 53(3): 275-284.

Li, H.; Zhou, G.Y.; Liu, J.A.; Xu, J. (2016). Population genetic analyses of the fungal pathogen *Colletotrichum fructicola* on tea-oil trees in China. **PLoS One.** 11(6): e0156841.

Li, R.Y.; Wu, X.M.; Yin, X.H.; Liang, J.N.; Li, M. (2014). The natural product citral can cause significant damage to the hyphal cell walls of *Magnaporthe grisea*. **Molecules.** 19(7): 10279-10290.

Li, R.Y.; Wu, X.M.; Yin, X.H.; Long, Y.H.; Li, M. (2015). Naturally produced citral can significantly inhibit normal physiology and induce cytotoxicity on *Magnaporthe grisea.* **Pestic. Biochem. Physiol.** 118: 19-25.

Lopez-Mirabal, H.R.; Winther, J.R. (2008). Redox characteristics of the eukaryotic cytosol. Bio**chim. Biophys. Acta**. 1783: 629–640.

Ma, B.; Ban, X.; Huang, B.; He, J.; Tian, J.; Zeng, H.; Chen, Y.; Wang, Y. (2015). Interference and mechanism of dill seed essential oil and contribution of carvone and limonene in preventing *Sclerotinia* rot of rapeseed. **PLoS One**. 10(7): e0131733.

Madeo, F.; Herker, E.; Maldener, C.; Wissing, S.; Lachelt, S.; Herlan, M.; Fehr, M.; Lauber, K.; Sigrist, S. J.; Wesselborg, S.; Frohlich, K. U (2002). A caspase-related protease regulates apoptosis in yeast. **Mol. Cell**. 9(4): 911-917.

Marchese, A.; Orhan, I.E.; Daglia, M.; Barbieri, R.; Di Lorenzo, A.; Nabavi, S.F.; Gortzi, O.; Izadi, M.; Nabavi, S.M. (2016). Antibacterial and antifungal activities of thymol: A brief review of the literature. **Food Chem.** 210: 402-414.

Marrink, S.J.; Corradi, V.; Souza, P.C.; Ingólfsson, H.I.; Tieleman, D.P.; Sansom, M.S. (2019). Computational modeling of realistic cell membranes. **Chem. Rev**. 119(9): 6184-6226.

Matellán, L.; Monje-Casas, F. (2020). Regulation of mitotic exit by cell cycle checkpoints: lessons from *Saccharomyces cerevisiae*. **Genes**. 11(2): 195.

Mills, P.R.; Hodson, A.; Brown, A.E. (1992). **Molecular differentiation of *Colletotrichum gloeosporioides* isolates infecting tropical fruits**. In: Bailey, J.A.; Jeger, M.J. ***Colletotrichum*: biology, pathology and control**. Wallingford: CAB International. pp. 269–288.

Moral, J.; Agustí-Brisach, C.; Agalliu, G.; de Oliveira, R.; Pérez-Rodríguez, M.; Roca, L.F.; Romero, J.; Trapero, A. (2018). Preliminary selection and evaluation of fungicides and natural compounds to control olive anthracnose caused by *Colletotrichum* species. **Crop Prot.** 114, 167-176.

Moreira, R.R.; Hamada, N.A.; Peres, N.A.; De Mio, L.L.M. (2019). Sensitivity of the *Colletotrichum acutatum* Species Complex From Apple Trees in Brazil to Dithiocarbamates, Methyl Benzimidazole Carbamates, and Quinone Outside Inhibitor Fungicides. **Plant Dis.** 103(10): 2569-2576.

Nóbrega, R.D.O.; Teixeira, A.P.D.C.; Oliveira, W.A.D.; Lima, E.D.O.; Lima, I.O. (2016). Investigation of the antifungal activity of carvacrol against strains of *Cryptococcus neoformans*. **Pharm. Biol.** 54(11): 2591-2596.

O'Connell, R.J.; Thon, M.R.; Hacquard, S.; Amyotte, S.G.; Kleemann, J.; Torres, M.F.; et al. (2012). Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. **Nat. Genet.** 44(9): 1060-1065.

Oo, M. M.; Oh, S.K. (2017). Identification and characterization of new record of grape ripe rot disease caused by *Colletotrichum viniferum* in Korea. **Mycobiology.** 45(4): 421-425.

OuYang, Q.; Tao, N.; Jing, G. (2016). Transcriptional profiling analysis of *Penicillium digitatum*, the causal agent of citrus green mold, unravels an inhibited ergosterol

biosynthesis pathway in response to citral. **BMC Genomics.** 17(1): 599.

OuYang, Q.; Tao, N.; Zhang, M. (2018). A damaged oxidative phosphorylation mechanism is involved in the antifungal activity of citral against *Penicillium digitatum*. **Front. Microbiol.** 9: 239.

Peng, L.J.; Sun, T.; Yang, Y.L.; Cai, L.; Hyde, K.D.; Bahkali, A.H.; Liu, Z.Y. (2013). *Colletotrichum* species on grape in Guizhou and Yunnan provinces, China. **Mycoscience**, 54(1): 29-41.

Pereira, F.D.O.; Mendes, J.M.; Lima, I.O.; Mota, K.S.D.L.; Oliveira, W.A.D.; Lima, E.D.O. (2015). Antifungal activity of geraniol and citronellol, two monoterpenes alcohols, against *Trichophyton rubrum* involves inhibition of ergosterol biosynthesis. **Pharm. Biol.** 53(2): 228-234.

Peres, N.A.; Timmer, L.W.; Adaskaveg, J.E.; Correll, J.C. (2005). Lifestyles of *Colletotrichum acutatum.* **Plant Dis.** 89(8): 784-796.

Perina, F.J.; Amaral, D.C.; Fernandes, R.S.; Labory, C.R.; Teixeira, G.A.; Alves, E. (2015). Thymus vulgaris essential oil and thymol against *Alternaria alternata* (Fr.) Keissler: effects on growth, viability, early infection and cellular mode of action. **Pest Manag. Sci.** 71(10): 1371-1378.

Phoulivong, S. (2011). *Colletotrichum*, naming, control, resistance, biocontrol of weeds and current challenges. **Curr. Res. Environ. Appl. Mycol.** 1(1): 53-73.

Prada-Luengo, I.; Møller, H.D.; Henriksen, R.A.; Gao, Q.; Larsen, C.E.; Alizadeh, S.; Maretty, L.; Houseley, J.; Regenberg, B. (2020). Replicative aging is associated with loss of genetic heterogeneity from extrachromosomal circular DNA in *Saccharomyces cerevisiae*. **Nucleic Acids Res.** 48(14): 7883-7898.

Ramage, G.; Milligan, S.; Lappin, D.F.; Sherry, L.; Sweeney, P.; Williams, C.; Bagg, J.; Culshaw, S. (2012). Antifungal, cytotoxic, and immunomodulatory properties of tea tree oil and its derivative components: potential role in management of oral candidosis in

cancer patients. **Front.Microb.** 3: 220.

Ramos-Guerrero, A.; González-Estrada, R.R.; Hanako-Rosas, G.; Bautista-Banos, S.; Acevedo-Hernández, G.; Tiznado-Hernández, M.E.; Gutiérrez-Martínez, P. (2018). Use of inductors in the control of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* isolated from soursop fruits: *in vitro* tests. **Food Sci. Biotechnol.** 27(3): 755-763.

Rathinasamy, K.; Panda, D. (2006). Suppression of microtubule dynamics by benomyl decreases tension across kinetochore pairs and induces apoptosis in cancer cells. **FEBS J**. 273(17): 4114-4128.

Richie, D.L.: Miley, M.D.; Bhabhra, R.; Robson, G.D.; Rhodes, J.C.; Askew, D. S. (2007). The *Aspergillus fumigatus* metacaspases CasA and CasB facilitate growth under conditions of endoplasmic reticulum stress. **Mol. Microbiol**. 63(2): 591-604.

Saeidnia, S.; Gohari, A.R. (2012). Trypanocidal monoterpenes: lead compounds to design future trypanocidal drugs. **Stud. Nat. Prod. Chem.** 37: 173–189.

Saibabu, V.; Singh, S.; Ansari, M.A.; Fatima, Z.; Hameed, S. (2017). Insights into the intracellular mechanisms of citronellal in *Candida albicans*: implications for reactive oxygen species-mediated necrosis, mitochondrial dysfunction, and DNA damage. **Rev. Soc. Bras. Med. Trop.** 50(4): 524-529.

Santos, P.L.; Matos, J.P.S.; Picot, L.; Almeida, J.R.; Quintans, J.S.; Quintans-Júnior, L.J. (2019). Citronellol, a monoterpene alcohol with promising pharmacological activities-A systematic review. **Food Chem. Toxicol.** 123: 459-469.

Schnitzler, P.; Koch, C.; Reichling, J. (2007). Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. **Antimicrob. Agents Chemother.** 51: 1859–1862.

Serrano, M.; Coluccia, F.; Torres, M.; L'Haridon, F.; Métraux, J.P. (2014). The cuticle and plant defense to pathogens. **Front. Plant Sci.** 5: 274.

Sharma, G.; Shenoy, B.D. (2014). *Colletotrichum fructicola* and *C. siamense* are involved in

chilli anthracnose in India. Arch. Phytopathol. Pflanzenschutz. 47(10): 1179-1194.

Sharma, Y.; Khan, L.A.; Manzoor, N. (2016). Anti-*Candida* activity of geraniol involves disruption of cell membrane integrity and function. **J. Mycol. Med.** 26(3): 244-254.

Sharma, Y.; Rastogi, S.K.; Perwez, A.; Rizvi, M.A.; Manzoor, N. (2020). β-citronellol alters cell surface properties of *Candida albicans* to influence pathogenicity related traits. **Med. Mycol.** 58(1): 93-106.

Shen, Q.; Zhou, W.; Li, H.; Hu, L.; Mo, H. (2016). ROS involves the fungicidal actions of thymol against spores of *Aspergillus flavus* via the induction of nitric oxide. **PLoS One**. 11(5): e0155647.

Sherriff, C.; Whelan, M.J.; Arnold, G.M.; Lafay, J.F.; Brygoo, Y.; Bailey, J.A. (1994). Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. **Exp. Mycol**. 18(2): 121-138.

Singh, S.; Fatima, Z.; Ahmad, K.; Hameed, S. (2018). Fungicidal action of geraniol against *Candida albicans* is potentiated by abrogated CaCdr1p drug efflux and fluconazole synergism. **PLoS One**, 13(8): e0203079.

Singh, S.; Fatima, Z.; Hameed, S. (2016). Citronellal-induced disruption of membrane homeostasis in *Candida albicans* and attenuation of its virulence attributes. **Rev. Soc. Bras. Med. Trop.** 49(4): 465-472.

Sreenivasaprasad, S.; Brown, A.E.; Mills, P.R. (1992). DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. **Physiol. Mol. Plant Pathol.** 41(4): 265-281.

Sukul, P.; Spiteller, M. (2000). Metalaxyl: persistence, degradation, metabolism, and analytical methods. **Rev. Environ. Contam**. T. 164: 1-26.

Sun, Y.; Guo, D.; Hua, Z.; Sun, H.; Zheng, Z.; Xia, X.; Shi, C. (2019). Attenuation of multiple *Vibrio parahaemolyticus* virulence factors by citral. **Front. Microbiol.** 10: 894.

Sussman, A.; Huss, K.; Chio, L.C.; Heidler, S.; Shaw, M.; Ma, D.; et al. (2004). Discovery of

cercosporamide, a known antifungal natural product, as a selective Pkc1 kinase inhibitor through high-throughput screening. **Eukaryot. Cell.** 3(4): 932-943.

Sutton, B.C. (1992). **The genus *Glomerella* and its anamorph *Colletotrichum***. In: Bailey, J.A.; Jeger, M.J. ***Colletotrichum*****: biology, pathology and control**. Wallingford: CAB International. pp. 1-26.

Suzaki K. (2011) Improved method to induce sporulation of *Colletotrichum gloeosporioides*, causal fungus of grape ripe rot. **J. Gen. Plant Pathol.** 77: 81–84.

Takayama, T.; Yamamoto, K.; Saito, H.; Tatebayashi, K. (2019). Interaction between the transmembrane domains of Sho1 and Opy2 enhances the signaling efficiency of the Hog1 MAP kinase cascade in *Saccharomyces cerevisiae*. **PLoS One**. 14(1): e0211380.

Talhinhas, P.; Sreenivasaprasad, S.; Neves-Martins, J.; Oliveira, H. (2002). Genetic and morphological characterization of *Colletotrichum acutatum* causing anthracnose of lupins. **Phytopathology**. 92(9): 986-996.

Tani, N.; Rahnasto-Rilla, M.; Wittekindt, C.; Salminen, K.A.; Ritvanen, A.; Ollakka, R.; Koskiranta, J.; Raunio, H.; Juvonen, R.O. (2012). Antifungal activities of novel non-azole molecules against *S. cerevisiae* and *C. albicans*. **Eur. J. Med. Chem.** 47: 270-277.

Tebbets, B.; Stewart, D.; Lawry, S.; Nett, J.; Nantel, A.; Andes, D.; Klein, B.S. (2012). Identification and characterization of antifungal compounds using a *Saccharomyces cerevisiae* reporter bioassay. **PLoS One**. 7(5): e36021.

Thakre, A.; Zore, G.; Kodgire, S.; Kazi, R.; Mulange, S.; Patil, R.; Shelar, A.; Santhakumari, B.; Kulkarni, M.; Kharat, K.; Karuppayil, S.M. (2018). Limonene inhibits *Candida albicans* growth by inducing apoptosis. **Med. Mycol.** 56(5): 565-578.

Tode, H.J. (1790). **Fungi Mecklenbergensis Seclecti.** Lungeburg. p. 64.

Trindade, L.A.; de Araújo Oliveira, J.; de Castro, R.D.; de Oliveira Lima, E. (2015). Inhibition of adherence of *C. albicans* to dental implants and cover screws by *Cymbopogon nardus*

essential oil and citronellal. **Clin Oral Investig**. 19(9): 2223-2231.

Vermaas, J.V.; Bentley, G.J.; Beckham, G.T.; Crowley, M.F. (2018). Membrane Permeability of Terpenoids Explored with Molecular Simulation. **J. Phys. Chem. B.** 122(45): 10349-10361.

Vilaplana, R.; Pazmiño, L.; Valencia-Chamorro, S. (2018). Control of anthracnose, caused by *Colletotrichum musae*, on postharvest organic banana by thyme oil. **Postharvest Biol. Technol.** 138: 56-63.

Vinnere, O.; Fatehi, J; Wright, S.A.; Gerhardson, B. (2002). The causal agent of anthracnose of *Rhododendron* in Sweden and Latvia. **Mycol. Res.** 106(1): 60-69.

von Arx, J.A. (1957). Die arten der gattung *Colletotrichum* Cda. **Phytopathol Z**. 29: 413-468.

Wadhwa, G.; Kumar, S.; Mittal, V.; Rao, R. (2019). Encapsulation of babchi essential oil into microsponges: Physicochemical properties, cytotoxic evaluation and anti-microbial activity. **J. Food Drug Anal.** 27(1): 60-70.

Wang, L.; Jiang, N.; Wang, D.; Wang, M. (2019). Effects of Essential Oil Citral on the Growth, Mycotoxin Biosynthesis and Transcriptomic Profile of *Alternaria alternata*. **Toxins**. 11(10): 553.

Wang, W.; Liang, X.; Zhang, R.; Gleason, M. L.; Sun, G. (2017). Liquid shake culture overcomes solid plate culture in inducing conidial production of *Colletotrichum* isolates. **Australas. Plant Pathol**. 46(3): 285-287.

Wharton, P.S.; & Schilder, A.C. (2008). Novel infection strategies of *Colletotrichum acutatum* on ripe blueberry fruit. **Plant Pathol.** 57(1): 122-134.

Wharton, P.S.; Diéguez-Uribeondo, J. (2004). The biology of *Colletotrichum acutatum*. **An. Jard. Bot. Madr.** 61(1): 3-22.

Wu, Y.; OuYang, Q.; Tao, N. (2016). Plasma membrane damage contributes to antifungal activity of citronellal against *Penicillium digitatum*. **J. Food Sci. Technol.** 53(10): 3853-3858.

Yan, J.Y.; Jayawardena, M.M.R.S.; Goonasekara, I.D.; Wang, Y.; Zhang, W.; Liu, M.; Huang, J.; Wang, Z.; Shang, J.; Peng, Y.; Bahkali, A.; Hyde K.D.; Li, X.H. (2015). Diverse species of *Colletotrichum* associated with grapevine anthracnose in China. **Fungal Divers.** 71(1): 233-246.

Zhang, C.; Diao, Y.; Wang, W.; Hao, J.; Imran, M.; Duan, H.; Liu, X. (2017). Assessing the risk for resistance and elucidating the genetics of *Colletotrichum truncatum* that is only sensitive to some DMI fungicides. **Front. Microbiol**., 8: 1779.

Zhang, M.; Ge, J.; Yu, X. (2018). Transcriptome analysis reveals the mechanism of fungicidal of thymol against *Fusarium oxysporum* f. sp. *niveum*. **Curr. Microbiol.** 75(4): 410-419.

Zheng, S.; Jing, G.; Wang, X.; Ouyang, Q.; Jia, L.; Tao, N. (2015). Citral exerts its antifungal activity against *Penicillium digitatum* by affecting the mitochondrial morphology and function. **Food Chem.**, 178: 76-81.

Zhou, D.; Wang, Z.; Li, M.; Xing, M.; Xian, T.; Tu, K. (2018). Carvacrol and eugenol effectively inhibit *Rhizopus stolonifer* and control postharvest soft rot decay in peaches. **J. Appl. Microbiol.** 124(1): 166-178.

Zore, G.B.; Thakre, A.D.; Rathod, V.; Karuppayil, S.M. (2011). Evaluation of anti-*Candida* potential of geranium oil constituents against clinical isolates of *Candida albicans* differentially sensitive to fluconazole: inhibition of growth, dimorphism and sensitization. **Mycoses.** 54(4): 99-109.

ANEXOS

Durante o período do doutorado foram executados outros trabalhos em colaboração com outros pesquisadores, estes trabalhos estão apresentados aqui.

Food and Chemical Toxicology 112 (2018) 383–392

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Apoptosis induction by *Pleurotus sajor-caju* (Fr.) Singer extracts on colorectal cancer cell lines

Tiane C. Finimundy[a,c], Rui M.V. Abreu[c], Natalia Bonetto[a], Fernando J. Scariot[b], Aldo J.P. Dillon[d], Sergio Echeverrigaray[b,1], Lillian Barros[c], Isabel C.F.R. Ferreira[c,*], João A.P. Henriques[a], Mariana Roesch-Ely[a,**]

[a] Laboratory of Genomics, Proteomics and DNA Repair, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil
[b] Laboratory of Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil
[c] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[d] Laboratory of Enzyme and Biomass, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Brazil

ARTICLE INFO

Keywords:
Pleurotus sajor-caju
Sterols
Cytotoxicity
Apoptosis

ABSTRACT

Pleurotus sajor-caju (PSC) is an edible mushroom used in food supplements, presenting antitumor properties through induction of cell death pathways. The PSC potential against colorectal cancer was analyzed by exposing HCT116^{wt} cells to different PSC extracts. The PSC n-hexane extract (PSC-hex) showed the highest cytotoxicity effect (IC_{50} value 0.05 mg/mL). The observed cytotoxicity was then associated to apoptosis-promoting and cell cycle-arrest pathways. PSC-hex was able to induce apoptosis related to breakdown of mitochondrial membrane potential and ROS generation. The absence of cytotoxicity in HTC116^{-p53} and HTC116^{-Bax} cells, alongside with an increase in p53, Bax and Caspase-3 expression, and decrease in Bcl-2 expression, supports that the pro-apoptotic effect is probably induced through a p53 associated pathway. PSC-hex induced cell cycle arrest at G2/M in HCT116^{wt} without cytotoxicity in HTC116^{-p21} cells. These findings suggest that a p21/p53 cell cycle regulation pathway is probably disrupted by compounds present on PSC-hex. Identification of the major components was then performed with ergosta-5,7,22-trien-3β-ol representing 30.6% of total weight. In silico docking studies of ergosta-5,7,22-trien-3β against Bcl-2 were performed and results show a credible interaction with the Bcl-2 hydrophobic cleft. The results show that PSC-hex can be used as supplementary food for adjuvant therapy in colorectal carcinoma.

International Journal of Medicinal Mushrooms, 20(1):31–46 (2018)

Extrinsic and Intrinsic Apoptotic Responses Induced by Shiitake Culinary-Medicinal Mushroom *Lentinus edodes* (Agaricomycetes) Aqueous Extract against a Larynx Carcinoma Cell Line

Tiane C. Finimundy,[1] Gustavo Scola,[2] Fernando J. Scariot,[3] Aldo J.P. Dillon,[4] Sidnei Moura,[5] Sérgio Echeverrigaray,[3,6] João Pegas Henriques,[1] & Mariana Roesch-Ely[1,*]

[1]Laboratory of Genomics, Proteomics and DNA Repair, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Rio Grande do Sul, Brazil; [2]Departments of Psychiatry and Pharmacology, University of Toronto, and Centre for Addiction and Mental Health, Toronto, Canada; [3]Laboratory of Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Rio Grande do Sul, Brazil; [4]Laboratory of Enzyme and Biomass, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Rio Grande do Sul, Brazil; [5]Laboratory of Biotechnology of Natural and Synthetics Products, Technology Department, Institute of Biotechnology, University of Caxias do Sul, Caxias do Sul, Rio Grande do Sul, Brazil; [6]Cytogenes Diagnósticos Moleculares Ltda., Lajeado, Rio Grande do Sul, Brazil

*Address all correspondence to: Mariana Roesch-Ely, Laboratory of Genomics, Proteomics and DNA Repair, Institute of Biotechnology, University of Caxias do Sul, Rua Francisco Getúlio Vargas 1130, 95070-560 Caxias do Sul, Rio Grande do Sul, Brazil; mrely@ucs.br

ABSTRACT: Cumulative evidence from research studies has shown that the shiitake culinary-medicinal mushroom, *Lentinus edodes*, is an excellent source of natural antitumor agents and is capable of inhibiting cancer cell growth. However, the cell signaling pathway that leads tumor cells to apoptosis is not well understood because many chemical compounds may be acting. This study investigated the chemopreventive effects of an *L. edodes* aqueous extract on human HEp-2 epithelial larynx carcinoma cells and normal human MRC-5 lung fibroblasts by identifying proliferative and apoptotic pathways. The chemical characterization of the dry powder was assessed by high-performance liquid chromatography. Antiproliferative and proapoptotic effects induced by the extract were evaluated by assessing proliferative markers, cell sorting through flow cytometry, and expression levels of apoptotic proteins with Western blotting. The results suggest that inhibition of cell proliferation was more prominent in HEp-2 than in MRC-5 cells. Cell death analysis showed the appearance of cell populations in the sub-G_1 phase, with late apoptotic signal increased in a dose-dependent manner. In addition, the aqueous extract induced depolarization of mitochondria, activating the generation of intracellular reactive oxygen species in HEp-2 cells. These observations suggest that *L. edodes* extract may exert a chemopreventive effect, regulating mitotic induction of apoptogenic signals. These findings highlight the mushroom's pharmacological potential in cancer treatment.

KEY WORDS: cytotoxicity, flow cytometry, HEp-2, *Lentinus edodes*, medicinal mushrooms, multitarget

ANTICANCER RESEARCH *38*: 6231-6236 (2018)
doi:10.21873/anticanres.12978

Piperlongumine Induces Apoptosis in Colorectal Cancer HCT 116 Cells Independent of Bax, p21 and p53 Status

FERNANDA DA SILVA MACHADO, FERNANDA MOSENA MUNARI, FERNANDO JOEL SCARIOT, SERGIO ECHEVERRIGARAY, CESAR AGUZZOLI, CLAUS TRÖGER PICH, MASSUO JORGE KATO, LYDIA YAMAGUCHI, SIDNEI MOURA, JOÃO ANTONIO PÊGAS HENRIQUES and MARIANA ROESCH-ELY

Laboratory of Genomics, Proteomics and DNA Repair, Biotechnology Institute, University of Caxias do Sul, Caxias do Sul, Brazil

Abstract. *Background/Aim: Colorectal cancer is a common type of cancer with reported resistance to treatment, in most cases due to loss of function of apoptotic and cell-cycle proteins. Piperlongumine (PPLGM) is a natural alkaloid isolated from Piper species, with promising anti-cancer properties. This study investigated whether PPLGM is able to induce cell death in colorectal carcinoma HCT 116 cells expressing wild-type or deficient in Bax, p21 or p53. Materials and Methods: PPLGM was extracted from roots of Piper tuberculatum. Cell viability was determined by reduction of 3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assay. Cell death was evaluated by acridine orange/ethidium bromide staining and flow cytometry. Plasmid cleavage activity and circular dichroism DNA interaction were also analyzed. Results: PPLGM induced selective cell death in all cell lines (IC_{50} range from 10.7 to 13.9 μM) with an increase in the number of late apoptotic cells and different profiles in cell-cycle distribution. Plasmid DNA analysis showed that PPLGM does not interact directly with DNA. Conclusion: This paper suggests that PPLGM may be a promising candidate in colorectal cancer therapy.*

International Journal of Food Microbiology 314 (2020) 108383

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Anthocyanin adsorption by *Saccharomyces cerevisiae* during wine fermentation is associated to the loss of yeast cell wall/membrane integrity

Sergio Echeverrigaray*, Fernando Joel Scariot, Morgana Menegotto, Ana Paula Longaray Delamare

Institute of Biotechnology, University of Caxias do Sul, Francisco Getúlio Vargas 1130, Brazil

ARTICLE INFO

Keywords:
Anthocyanins
Wine color
Yeast pigment adsorption
Cell viability

ABSTRACT

Yeasts contribute to anthocyanin extraction during red wine fermentation, but they also reduce wine color by adsorption of pigments on their cell walls. *Saccharomyces cerevisiae* strains vary on their pigment adsorptive properties, but the mechanisms involved in this phenomenon remain unclear. In this work, we evaluated high, medium and low pigment-adsorbing *S. cerevisiae* winemaking strains during red wine fermentation. Flow cytometry protocols were devised to measure different populations of yeast and their pigment adsorption in association with other variables such as fermentation rate, cell viability, and cell wall/membrane integrity. The results showed that the high pigment adsorbing strain accumulated anthocyanin towards the end of alcoholic fermentation observed as an increase in the population of fluorescent cells. By contrast, low adsorbing strain displayed a single low adsorbing cell population. Pigment adsorption was negatively correlated with cell viability and cell wall/membrane integrity. Independent of their adsorptive potential during wine fermentation, viable cells displayed a low capacity to adsorb anthocyanins, while permeabilized yeast cells exhibited high pigment adsorption capacity. Our findings suggest that yeast pigment adsorption requires a breach to the inner part of the cell wall, for pigments to access constitutively expressed pigment-binding factors, and that the phenotypic differences among strains are determined by their fermentative live-span and cell wall/membrane integrity. These findings will help to rationalize and optimize the match of winemaking strains and vines.

Systematic Botany (2020), 45(4): pp. 707–748

DOI 10.1600/036364420X16033962925150
Date of publication December 8, 2020

Revision of *Hymenophyllum* subg. *Sphaerocionium* (Hymenophyllaceae) in the Atlantic Forest Domain (Brazil), Based on Molecular and Morphological Evidence

Felipe Gonzatti,[1,2,4] Paulo G. Windisch,[2] Fernando J. Scariot,[3] Sergio Echeverrigaray,[3] and Mara R. Ritter[2]

[1]Herbário da Universidade de Caxias do Sul, Universidade de Caxias do Sul – UCS,
Rua Francisco Getúlio Vargas, 1130, CEP 95070-560, Caxias do Sul, RS, Brazil
[2]Programa de Pós-graduação em Botânica, Universidade Federal do Rio Grande do Sul-UFRGS,
Campus do Vale Prédio 43433, CEP 91501-970, Porto Alegre, RS, Brazil
[3]Laboratório de Microbiologia Aplicada da Universidade de Caxias do Sul, Universidade de Caxias do Sul- UCS,
Rua Francisco Getúlio Vargas, 1130, CEP 95070-560, Caxias do Sul, RS, Brazil
[4]Author for correspondence (fgonzatti@ucs.br)

Communicating Editor: Alejandra Vasco

Abstract*—*Sphaerocionium is the largest subgenus of *Hymenophyllum* and occurs mainly in tropical forests of the Neotropical Region. Species of *Hymenophyllum* occurring in Brazil are poorly known due to difficulties in species delimitation and the absence of detailed studies. The aim of the present study was to present a synopsis of *Hymenophyllum* subg. *Sphaerocionium* in the Atlantic Forest domain, based on molecular and morphological data. Field and herbarium specimens were morphologically analyzed by stereomicroscopy, optical microscopy, and scanning electron microscopy. Phylogenetic relationships were evaluated by sequence comparison of the plastidial regions *trnG-R* and *rps4-trnS*. Morphological and phylogenetic evidence led to the recognition of 14 taxa throughout the Atlantic Forest, five of which are endemic. The taxonomy and nomenclature of *Hymenophyllum* in Brazil are revised and 19 new lectotypifications are proposed. The conservation status and geographical distributions of all species are updated and a dichotomous key is provided.

Ciência Rural, Santa Maria, v.51:9, e20200728, 2021 http://doi.org/10.1590/0103-8478cr20200728

ISSNe 1678-4596
CROP PROTECTION

Morphological characterization and molecular identification of *Colletotrichum* species associated to sweet persimmon anthracnose in Southern Brazil

Joel Andrioli[1] Fernando Joel Scariot[1*] Ana Paula Longaray Delamare[1] Sergio Echeverrigaray[1]

[1]Laboratório de Enologia e Microbiologia Aplicada, Instituto de Biotecnologia, Universidade de Caxias do Sul (UCS), 95070-560, Caxias do Sul, RS, Brasil. E-mail: ferjscariot@gmail.com. *Corresponding author.

ABSTRACT: *The highlands of Southern Brazil contribute with 40% of Brazilian persimmon production. Although expanding, persimmon production faces major problems caused by anthracnose disease (black spot), including fruit rot and necrosis of leaves. Several Colletotrichum species (C. horii, C. gloeosporioides, among others) are implicated in persimmon anthracnose around the world. To identify Colletotrichum species associated with persimmon anthracnose in the highlands of Southern Brazil, 34 isolates were analyzed by ITS-rDNA partial region, GAPDH, and TUB2 partial gene sequences, morphological characteristics, and virulence on persimmon fruits and leaves. Data showed a high prevalence of C. horii (85.3%), that associated with its high virulence on fruits and leaves, confirm a considerable degree of host preference. Moreover, other species C. aenigma, C. asianum, C. fructicola, and C. nymphaeae, were identified, but the last three ones exhibited low virulence on fruits and were not able to produce symptoms on leaves. As far as we know this is the first reference on C. asianum in persimmon. The present data may contribute to a better understanding of the etiology of anthracnose in sweet persimmon in Southern Brazil, and it will be useful for epidemiological studies and the development of disease management measures.*

Key words: *black spot, molecular identification, virulence.*

International Journal of Food Microbiology 347 (2021) 109200

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Yeast biodiversity in honey produced by stingless bees raised in the highlands of southern Brazil

Sergio Echeverrigaray, Fernando Joel Scariot *, Luciano Foresti, Luisa Vivian Schwarz, Ronaldo Kauê Mattos Rocha, Gabriela Priscila da Silva, Jennifer Pereira Moreira, Ana Paula Longaray Delamare

Laboratory of Enology and Applied Microbiology, Institute of Biotechnology, University of Caxias do Sul, Brazil

ARTICLE INFO

Keywords:
Meliponini
Physicochemical properties
Starmerella
Yeast species

ABSTRACT

The physicochemical characteristics and yeasts diversity in honey samples from 17 species of stingless bees of the genera *Nannotrigona, Melipona, Plebeia, Scraptotrigona,* and *Tetragonisca* cultivated in Southern Brazil were determined. The sugar content, moisture, water activity, pH, reducing sugars/total sugar ratio, and total yeast population varied significantly among the honey from the different bee species. The highest yeast population was found in the *Plebeia*'s honey samples and correlated with their high water-activity. Sixteen yeast species were identified based on the nuclear large subunit (26S) ribosomal RNA partial sequences. The genera *Starmerella* and *Zygosaccharomyces* were found predominant, with a high prevalence of *Starmerella* sp., *S. etchellsii*, and *S. apicola*. Some yeast species were only identified in honey samples from specific bee species indicating a close relationship between the yeasts and the insects. For the first time, *Wickerhamomyces sydowiorum* in honey is being reported. In general, the yeast species isolated from stingless bee honey samples demonstrated high osmotolerance and low sugar assimilation.