UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
GRADUAÇÃO EM NUTRIÇÃO

Rafaela Diogo Silveira

EFEITO DO USO DE *Bacillus* NO DESENVOLVIMENTO DE *Aspergillus carbonarius* E SÍNTESE DE OCRATOXINAS EM UVAS

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Trabalho de conclusão do curso de graduação apresentado ao Curso de Nutrição da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do grau de Bacharel em Nutrição

Orientadora: Profª. Drª. Juliane Elisa Welke

Coorientador: Dr. Flávio Fonseca Veras

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RESUMO

Uvas podem ser contaminadas por fungos toxigênicos, como *Aspergillus carbonarius*, um dos principais responsáveis pela ocorrência de Ocratoxina A (OTA) em uvas. Tal micotoxina está associada a efeitos genotóxicos, teratogênicos e imunossupressores. A inibição do crescimento fúngico é considerada a melhor forma para evitar a contaminação por OTA. Neste caso, o controle de fungos é feito principalmente pela aplicação de fungicidas sintéticos, que podem trazer diversas consequências negativas, tais como: risco ocupacional, geração de resíduos no solo, na água e na própria uva, e alterar sua composição fenólica, perfil volátil, aparência e textura. Por esses motivos, alternativas de biocontrole vêm ganhando destaque na agricultura, principalmente por serem práticas mais sustentáveis para o controle de pragas e doenças. O objetivo deste estudo foi avaliar a capacidade de quatro cepas de *Bacillus* (P1, P7, P11 e P45) em inibir o desenvolvimento de *A. carbonarius* e a síntese de OTA em uvas Chardonnay, bem como avaliar o efeito da cepa mais promissora no perfil volátil. As bagas utilizadas foram inoculadas com suspensão de células de cada *Bacillus* (10^9 UFC mL^{-1}) e com *A. carbonarius* (10^3 esporos mL^{-1}). Todas as cepas apresentaram atividade antifúngica, com destaque para a cepa P1, que demonstrou 100% de inibição fúngica e síntese de OTA. Além disso, as uvas tratadas com essa cepa de *Bacillus* apresentaram maior concentração de compostos voláteis com odores agradáveis. As cepas de *Bacillus* investigadas são potenciais agentes de controle biológico para prevenir ou reduzir a ocorrência de *A. carbonarius* em uvas, e ainda inibir a síntese de OTA, proporcionando um alimento seguro e de qualidade.

PALAVRAS-CHAVE: biocontrole, micotoxinas, uva, *Aspergillus carbonarius*, *Bacillus*, Ocratoxina A

ABSTRACT

Grapes can be contaminated by toxigenic fungi, such as *Aspergillus carbonarius*, one of the main responsible for the occurrence of Ochratoxin A (OTA) in grapes. Such mycotoxin is associated with genotoxic, teratogenic and immunosuppressive effects. The inhibition of fungal growth is considered the best way to avoid contamination by OTA. In this case, the control of fungi is done mainly by the application of synthetic fungicides, which can bring several negative consequences, such as: occupational risk, generation of residues in the soil, in the water and in the grape itself, and change its phenolic composition, volatile profile, appearance and texture. For these reasons, biocontrol alternatives have been gaining prominence in agriculture, mainly because they are more sustainable practices for the control of pests and diseases. The aim of this study was to evaluate the ability of four strains of *Bacillus* (P1, P7, P11 and P45) to inhibit the development of *A. carbonarius* and the synthesis of OTA in Chardonnay grapes, as well as to evaluate the effect of the most promising strain on the profile volatile of grapes. The berries used were inoculated with cell suspension from each *Bacillus* (10^9 CFU mL^{-1}) and with *A. carbonarius* (10^3 spores mL^{-1}). All strains showed antifungal activity, especially the strain P1, which showed 100% fungal inhibition and OTA synthesis. In addition, the grapes treated with this strain of *Bacillus* showed higher concentration of volatile compounds with pleasant odors. The *Bacillus* strains investigated are potential biological control agents to prevent or reduce the occurrence of *A. carbonarius* in grapes, and also inhibit the synthesis of OTA, providing a safe and quality food.

KEY WORDS: biocontrol, mycotoxins, grape, *Aspergillus carbonarius*, *Bacillus*, Ochratoxin A.

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1. INTRODUÇÃO

A uva é um fruto oriundo da videira pertencente ao gênero *Vitis* e à família *Vitaceae*. Mais de sessenta espécies compõe o gênero *Vitis*, sendo que a espécie mais comum é a *Vitis vinifera*, que apresenta diversas cultivares com fins distintos, como: uvas destinadas para produção de vinho, uvas de mesa e uvas para produção de passas (Embrapa, 2012).

A viticultura é uma atividade econômica recente no Brasil e o Rio Grande do Sul é o principal estado produtor de uvas para processamento (Embrapa, 2003), contribuindo com aproximadamente 90% da produção nacional de uvas. Na safra de 2018 foram colhidos, no Rio Grande do Sul, cerca de 663,2 milhões de quilogramas de uvas destinadas ao processamento, sendo essa colheita acima da média histórica dos últimos dez anos que foi de 607,5 milhões de quilogramas (Conab, 2018). Em 2019, devido às adversidades climáticas, houve uma diminuição de 10 a 15% em relação à quantidade colhida na safra de 2018 (Conab, 2019). Já em 2020 houve uma queda de 17,8% na produção geral de uva, comparada à safra de 2019. Foram colhidos 504 milhões de quilogramas de uvas, e as possíveis causas para essa redução da safra são: o excesso de chuva na floração e a seca no enchimento da baga e na maturação (Secretaria da Agricultura, Pecuária e Desenvolvimento Rural, 2020).

A ocorrência de fungos nas uvas durante o seu cultivo é bastante comum, destacando a presença de *Aspergillus carbonarius* e *Aspergillus niger*, os quais são reconhecidos como os principais responsáveis pela ocorrência da micotoxina denominada Ocratoxina A (OTA) nesta fruta (DACHERY et al., 2015). Esse metabólito fúngico secundário está relacionado à genotoxicidade, nefrotoxicidade, teratogenicidade e carcinogenicidade (ROCHA et al., 2014).

Na produção convencional de uvas, o controle dos fungos é feito através da aplicação de fungicidas sintéticos. O uso destes pesticidas para o controle de pragas pode levar a geração de cepas resistentes aos fungicidas (DEISING; REIMANN; PASCHOLATI, 2008), resíduos indesejáveis nas uvas após a colheita (ROMANAZZI et al., 2016), alteração do perfil volátil (ALEM et al., 2019) e da composição fenólica (OLIVA et al., 2008). O uso intensivo de fungicidas em vinhedos pode levar à contaminação do solo e da água (KOMÁREK et al., 2010), além de apresentarem risco

toxicológico em organismos-testes (principalmente em *Danio rerio*) (LI et al., 2018), promoverem hepatotoxicidade em ratos Wistar (KNEBEL et al., 2019). Além disso, a exposição crônica à fungicidas está epidemiologicamente associada ao aumento da incidência de hipertireoidismo (SHRESTHA et al., 2019), leucemia mieloide (PATEL et al., 2019) e Parkinson (NARAYAN et al., 2017).

Os agrotóxicos são utilizados para proteger as plantas contra pestes, e contribuem, significativamente, para o aumento da produtividade agrícola (NAGY et al., 2019), para a redução de perdas causadas pela ação de ervas daninhas, patógenos, pragas e insetos (AKTAR; SENGUPTA; CHOWDHURY, 2009), e para a consequente redução no preço desses produtos que serão ofertados (VEIGA, 2007). Entretanto, a exposição (dérmica, oral e inalatória) a esses compostos está relacionada à ocorrência de doenças em humanos, tais como: leucemia, mieloma e cânceres de próstata, pâncreas, pulmão e ovário (HOPPIN et al., 2017). Além disso, em 2017, 2.548 casos de intoxicação por agrotóxicos foram registrados no Brasil, envolvendo 1.195 pessoas da região sul do país (SINITOX, 2017). Devido aos eventos citados, novas estratégias de controle fúngico vêm sendo estudadas, como é o caso do controle biológico.

O biocontrole caracteriza-se pelo uso de microrganismos, parasitoides ou insetos para inibir ou diminuir o desenvolvimento de fitopatógenos e controlar a presença de insetos transmissores de doenças, além de evitar os efeitos negativos das práticas agrícolas convencionais no ambiente, promovendo segurança ocupacional, sustentabilidade e um alimento seguro (TORRES et al., 2016). No caso dos biofungicidas, sua aplicação em qualquer etapa do desenvolvimento do alimento, até mesmo no dia da colheita, confere maior vantagem em relação ao uso de fungicidas sintéticos, uma vez que estes possuem um intervalo de segurança. O mecanismo de ação desses agentes pode ser justificado pela sua capacidade de afetar o patógeno através de parasitismo, competição por nutrientes e síntese de metabólitos, como os lipopeptídeos fengicina, iturina e surfactina (CALVO et al., 2019; CAULIER et al., 2019). Hanif et al. (2019) atribuem a atividade antifúngica de *Bacillus amyloliquefaciens* FZB42 à síntese de Fengicina. Já Dang et al. (2019) relatam que a produção de Iturina por *Bacillus amyloliquefaciens* C2LP inibiu o crescimento de diversas espécies fúngicas, como: *Alternaria alternata*, *Botrytis cinerea* e *Colletotrichum gloeosporioide.* A Surfactina, outro lipopeptídeo sintetizado por *Bacillus*, apresentou atividade antagonista contra *Plasmopara viticola*, em estudo conduzido por Li et al. (2019).

Diferentes cepas de *Bacillus* isoladas de intestino de peixes da região amazônica (*Potamorhina latior*, *Piaractus mesopotamicus* e *Leporinos* sp.) demonstraram o potencial como agentes antifúngicos. Experimentos *in vitro* confirmaram sua atividade antagonista, redução da germinação de esporos e da síntese de micotoxinas, além de serem capazes de produzir os lipopeptídeos citados anteriormente (VERAS et al., 2016).

As práticas agrícolas também podem afetar algumas características físico-químicas das uvas, como a composição fenólica, atividade antioxidante, perfil volátil, aparência e textura das bagas (MULERO et al., 2015). Em relação à composição das uvas, o seu perfil volátil está relacionado com a localização geográfica das videiras (KOUNDOURAS et al., 2006), tipo de solo, características climáticas (SABON et al., 2002) e variedade de uva (ARMANINO et al., 2008). O aroma é um dos fatores mais importantes relacionado à qualidade de vinhos e as práticas agrícolas podem afetar o perfil volátil das uvas (NICOLLI et al., 2018).

OLIVA et al. (2008) elucidaram que a aplicação de pesticidas sintéticos pode afetar negativamente a composição aromática de vinhos. Os vinhos que foram elaborados com uvas Monastrell na Espanha que tinham sido tratadas com os fungicidas sintéticos fluquinconazol e fenehexamida apresentaram menor concentração de ésteres etílicos e acetato de etila (aroma frutal e doce, respectivamente) do que os vinhos elaborados a partir de uvas que não receberam esses fungicidas.

Já em estudo conduzido por Escribano-viana et al. (2018), o agente de biocontrole comercial "Serenade Max", que contém 5,3 x 10^{10} UFC/mL de *Bacillus subtilis* QST 713, foi pulverizado em uvas da cultivar Tempranill. O biofungicida não afetou negativamente o perfil volátil das uvas e do vinho que foi produzido a partir das bagas tratadas, e por isso o agente natural não influenciou na qualidade de ambos. Em outro estudo, *Bacillus vanillea* XY18 foi utilizado no processo de cura de favas de baunilha. Houve um aumento na quantidade de vanilina, que é um aldeído formado a partir da hidrólise enzimática da glucovanilina (GU et al. 2015). E, por fim, Jeong et al. (2017) inocularam o *Bacillus licheniformis* em soja e verificaram um aumento nos níveis de álcoois e carbonetos, principalmente. Os voláteis obtidos em ambos os estudos são importantes para a qualidade desses alimentos, já que são compostos cuja presença é desejada tanto na soja, quanto na baunilha. Jeong et al. (2017) afirmam que bactérias "starters", como *B. licheniformis*, podem ser utilizadas para manipular e customizar o sabor da soja fermentada, comida tipicamente japonesa. Ademais, GU et al. (2015)

relatam que o uso de *Bacillus* aumenta a síntese de vanilina sem gerar atributos sensoriais desagradáveis.

Além de apresentarem compostos aromáticos, as uvas também possuem um elevado teor de compostos fenólicos (SOMKUWAR et al., 2018), que são os responsáveis pela atividade antioxidante capaz de ter efeito protetor contra o câncer e doenças cardiovasculares (AUNE et al., 2018). Tal ação pode ocorrer através da interferência no processo de oxidação das células, quelando metais e sequestrando radicais livres (SHAHIDI; AMBIGAIPALAN, 2015).

Tendo em vista que as práticas agrícolas interferem na qualidade das uvas, Mulero et al. (2015) avaliaram como o uso de fungicidas afeta a composição fenólica e a atividade antioxidante de vinhos produzidos a partir de uvas da cultivar Monastrell. Seis soluções diferentes compostas por fungicidas comerciais foram pulverizadas nas videiras. As uvas foram colhidas e passaram por um processo de microvinificação. Após a realização dos métodos utilizando o radical DPPH (2,2-difenil-1-picrilhidrazilo) e a cromatografia líquida de alta eficiência (do inglês: *High Performance Liquid Chromatography*; HPLC), foi observado que as quantidades de alguns compostos fenólicos, como os flavonoides, diminuíram e a atividade antioxidante também foi menor, quando comparada com a da amostra controle.

Além de induzirem o aumento da concentração de compostos voláteis, os *Bacillus* também são capazes de elevar a atividade antioxidante. Em estudo conduzido por Yang et al. (2019) *Bacillus amyloliquefaciens* SWJS22 aumentou a quantidade de fenólicos totais e de flavonoides em farelo de soja através da ação da β-glucosidase e da protease presente nessa cepa.

Considerando os riscos que tanto o uso de pesticidas, quanto a exposição à micotoxinas pode trazer para a saúde humana, animal, e gerar danos no ambiente, faz-se necessário o estudo e a introdução de alternativas de biocontrole, como o uso de novas cepas do gênero *Bacillus*.

2. JUSTIFICATIVA

O uso de agrotóxicos está relacionado ao risco de intoxicação, principalmente ocupacional (NARAYAN et al., 2017), e ao acúmulo de resíduos químicos no ambiente (DE MICCOLIS ANGELINI et al., 2014). Portanto, cada vez mais as alternativas ao seu uso vêm sendo estudadas e utilizadas, principalmente através do emprego de agentes de biocontrole. Testes *in vitro* demonstraram que as cepas P1, P7 e P11 de *Bacillus* sp. e P45 de *Bacillus subtilis*, isoladas do intestino de peixes da Bacia Amazônica, foram promissoras na redução do crescimento micelial de *Aspergillus carbonarius* e da síntese de OTA em meio de cultura (VERAS et al., 2016).

Levando isso em consideração, uma avaliação da aplicação prática dessas bactérias se faz necessário. Desta forma, a verificação do seu efeito sobre o crescimento de fungos toxigênicos na cultivar de uva Chardonnay, sendo considerada a cultivar mais suscetível à colonização do *Aspergillus carbonarius* e acúmulo de Ocratoxina A (VERAS et al., 2020), poderia favorecer seu uso como uma alternativa de biocontrole, mais especificamente, como um biofungicida.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Verificar o potencial de *Bacillus* sp. P1, P7, P11 e *Bacillus subtilis* P45 em inibir o desenvolvimento de *Aspergillus carbonarius* e a síntese de Ocratoxina A na cultivar de uva Chardonnay.

3.2. OBJETIVOS ESPECÍFICOS

- Avaliar a capacidade de cepas do gênero *Bacillus* em atuar como uma alternativa de biocontrole do desenvolvimento de *A. carbonarius* e produção de OTA em uva;
- Verificar o efeito da inoculação do *Bacillus* no perfil volátil das uvas.

4. REVISÃO BIBLIOGRÁFICA

4.1 *Aspergillus carbonarius*

O fungo filamentoso *A. carbonarius* é o principal produtor de OTA em uvas. O desenvolvimento desse fungo ocorre, frequentemente, em bagas que foram danificadas durante o seu período de amadurecimento. Entretanto, *A. carbonarius* é uma espécie tão invasiva que é capaz de penetrar nas bagas mesmo quando não há danos na superfície das uvas, e em qualquer estágio de maturação. Dessa forma, cachos sem aparente desenvolvimento fúngico também podem conter OTA (GONÇALVES et al., 2020).

Em meio de cultura *Synthetic Grape Juice* (SGM, composição: glicose, frutose, ácido tartárico, ácido málico, fosfato de amônio, fosfato monopotássico, sulfato de magnésio, cloreto de sódio, cloreto de cálcio, cloreto de cobre, sulfato de ferro, sulfato de zinco, hidrato de catequina e ágar), as condições ideais para desenvolvimento de *A. carbonarius* ocorreram entre 30 a 35 ºC com atividade de água entre 0,93 e 0,98 (MITCHELL et al., 2004). Entretanto, as condições ideais para a produção de OTA, analisadas em *Synthetic Nutrient Medium* (SNM) de composição semelhante ao SGM, foram 15 e 20 ºC e a atividade de água de 0,95 a 0,98 (BELLÍ et al., 2004). ESTEBAN et al., (2004) verificaram que *A. carbonarius* podem se desenvolver entre 10-40 ºC em meio de cultura *Yeast Extract Sucrose* (YES) composto por extrato de levedura, sacarose, sulfato de magnésio e ágar.

A ocorrência de *A. carbonarius* já foi detectada em uvas cultivadas em diversos países, conforme mostrado na Tabela 1, e a presença de OTA foi verificada em todos os estudos.

Tabela 1. Ocorrência de *Aspergillus carbonarius* e presença de OTA em cultivares de uva de diferentes países.

Cultivar	Níveis de OTA detectados (µg /g)	País	Referência
Alvarinho, Loureiro, Vinhão, Tinta Barroca, Touriga Franca e Cabernet Sauvignon	0,012 – 5,24	Portugal	(SERRA; MENDONÇA; VENÂNCIO, 2006)

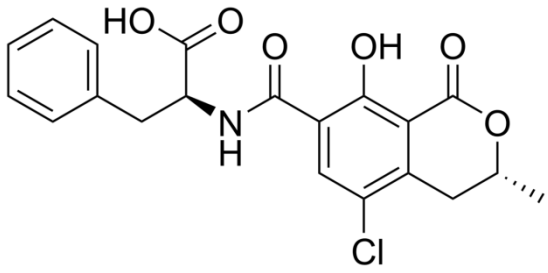
Não mencionada	0,5 - 87,5	França	(SAGE et al., 2002)
Não mencionada	8,38	Líbano	(EL KHOURY et al., 2008)
Negro Amaro, Malvasia Nero, Sangiovese, Primitivo, Verdeca e Trebbiano	< 0,001 - > 0,1	Itália	(BATTILANI et al., 2003)
Não mencionada	0,002 - > 0,04	Itália	(OLIVERI; TORTA; CATARA, 2008)
Negro Amaro	2,0 – 9,0	Itália	(COZZI et al., 2013)
Não mencionada	222,00	China	(HUANG et al., 2020)
Garnacha, Tempranillo, Bobal, Graciano, Cabernet Sauvignon, Merlot, Macabeo, Moscatel, Chardonnay e Sauvignon blanc	0,05 – 477,3	Espanha	(BELLÍ et al., 2006)
Cabernet Sauvignon, Garnatxa blanca, Macabeu, Moscatel e Palomino Fino	1,92 – 195,46	Espanha	(BAU et al., 2005)
Pinot, Cabernet Sauvignon, Tempranillo, Syrah, Merlot, Chardonnay, Xare-lo, Zalema, Chelva, Palomino, Ximénez e Montepila	263 - 2568	Espanha	(GARCÍA-CELA et al., 2015)
Xinomauro, Athiri, Cabernet Sauvignon, Sauvignon Blanc, Agiorgitiko, Athiri, Aidani, Asyrtiko, Mandilaria, Grenache Rouge, Limnio e Muscat	0 – > 0,025	Grécia	(TJAMOS; ANTONIOU; TJAMOS, 2006)
Malbec, Chardonnay, Merlot, Cabernet e Bonarda	18 - 234	Brasil	(ROSA et al., 2004)
Sultana, Zante Currant, Carina, Waltham Cross e Muscat Gordo Blanco	Não mencionado	Austrália	(LEONG et al., 2004)
Não mencionado	1,03	Eslováquia	(MIKUŠOVÁ et al., 2010)

4.2 Ocratoxina A

A OTA é um metabólito secundário produzido por fungos filamentosos toxigênicos quando há condições climáticas favoráveis, tais como temperatura e umidade, e em áreas geográficas específicas (WELKE; HOELTZ; NOLL, 2009). A exposição à OTA pode ocorrer através do consumo de alimentos contaminados por essa micotoxina, como milho (MAGNOLI et al., 2003; SEKIYAMA et al., 2005), trigo (RIBA et al., 2008), café (LEONG et al., 2007), e uva (LASRAM et al., 2007)

A OTA apresenta uma molécula de cloro em sua estrutura (Figura 1), fluorescência azul quando exposta à luz ultravioleta, além de ser solúvel em solventes orgânicos e ligeiramente solúvel em água (FRENETTE et al., 2008) A molécula de cloro presente na estrutura da OTA potencializa seu caráter tóxico (SCHMIDT-HEYDT et al., 2012), pois a torna uma substância lipossolúvel capaz de penetrar membranas celulares e se acumular nos tecidos conjuntivo, epitelial, muscular e adiposo (PÜSSA, 2008). Essa toxina também é conhecida por promover danos oxidativos através da produção de espécies reativas de oxigênio (ROS) e formar adutos covalentes com o DNA (Ácido desoxirribonucleico) (HADJEBA-MEDJDOUB et al., 2012).

Figura 1. Estrutura química da ocratoxina A (EL KHOURY; ATOUI, 2010)



Os efeitos tóxicos dessa micotoxina estão relacionados à genotoxicidade (EDITE BEZERRA DA ROCHA et al., 2014), caracterizada pela carcinogenicidade através da indução de mutações (NOHMI, 2018), nefrotoxicidade (JECFA, 2001), relacionada à indução de morte celular no epitélio do túbulo proximal renal (RACHED et al., 2008), teratogenicidade (EL KHOURY; ATOUI, 2010), sendo capaz de atravessar a barreira placentária e se acumular nos tecidos fetais (EL KHOURY; ATOUI, 2010),

neurotoxicidade (SAVA et al., 2006), estresse oxidativo, lipoperoxidação e danos no DNA de células neurais foram observados em estudo com camundongos (SAVA et al., 2006) e imunossupressão (ROSSIELLO et al., 2008), causada pela depleção dos linfócitos e redução da atividade dos anticorpos (KUMAR et al., 2008). Além disso, em nível celular, os efeitos da OTA estão relacionados com a inibição da síntese proteica através da competição com a fenilalanina-tRNA ligase (BAYMAN; BAKER, 2006), uma vez que, a OTA é uma isocumarina ligada à uma molécula de fenilalanina (PÜSSA, 2008).

A Agência Internacional de Pesquisa sobre o Câncer (IARC) classifica a OTA no grupo 2B, como possivelmente carcinogênica para humanos (IARC, 1993). O Comitê de Peritos em Aditivos Alimentares da Organização das Nações Unidas para Agricultura e Alimentação (FAO)/Organização Mundial da Saúde (OMS) determinou como parâmetro de exposição segura a ingestão semanal tolerável provisória (PTWI) de 112 ng/kg de peso corporal, o que corresponde a um exposição tolerável provisória de 16 ng/kg de peso corporal por dia (JECFA, 2007).

4.3 Uso de pesticidas sintéticos no cultivo das uvas

A ocorrência de fungos nas uvas pode ser observada durante o cultivo, colheita, transporte e armazenamento (DACHERY et al., 2016). Com o intuito de prevenir e combater a contaminação fúngica, fungicidas como Procimidona (ANVISA, 2015), Mancozebe (ANVISA, 2018), Pirimetanil (ANVISA, 2016), Famoxadona (ANVISA, 2016) e Fenamidona (ANVISA, 2015) são aplicados em diferentes estágios de maturação das uvas, e são permitidos, segundo a Agência Nacional de Vigilância Sanitária (ANVISA), para aplicação foliar, respeitando o limite máximo recomendado (LMR) e o intervalo de segurança, que é de 21 dias para o Pirimetanil e 7 dias para os demais.

Em 2018 foram registrados 450 agrotóxicos (Mapa, 2018), entre os quais encontra-se o Sulfoxaflor, classificado como medianamente tóxico (classe III), que passou a ser permitido para aplicação em uvas (ANVISA, 2018). Já em 2019, até o mês de março, 57 agrotóxicos tinham sido registrados pelo Ministério da Agricultura, Pecuária e Abastecimento (Mapa), sendo alguns deles, como o Piriproxifeno e o Glifosato, permitidos para aplicação em culturas de uva e classificados como

extremamente tóxicos (classe I) (Diário Oficial da União, 2019). O Glifosato apresenta risco para os trabalhadores de lavouras e para as pessoas que vivem próximo a essas áreas. Entretanto, ainda não foi classificado como carcinogênico para que o seu uso seja proibido (ANVISA, 2019). Já o Piriproxifeno está relacionado ao aumento da resistência de vários insetos, como é o caso da mosca branca (GRAFTON-CARDWELL et al., 2005), que causa danos às culturas e é vetor de várias espécies do gênero *Begomovirus* (WANG et al., 2010). Além disso, o uso de pesticidas pode levar a geração de cepas resistentes na população de patógenos (DEISING; REIMANN; PASCHOLATI, 2008), contaminação do solo e da água (KOMÁREK et al., 2010), alteração da composição fenólica (OLIVA et al., 2008) e do perfil volátil de uvas e vinhos (GARCÍA et al., 2004)

O efeito tóxico dos fungicidas é especialmente preocupante para os agricultores, bem como para a população que vive em torno dos vinhedos devido a dissipação destes produtos no ambiente após a pulverização. Estudos epidemiológicos têm demonstrado a relação entre a exposição ocupacional aos fungicidas e o aumento da incidência de hipertireoidismo em agricultores da Carolina do Norte e de Iowa, Estados Unidos (SHRESTHA et al., 2019). Leucemia mieloide aguda em crianças da *International Childhood Cancer Cohort Consortium* (Austrália, Dinamarca, Israel, Noruega e Reino Unido) cujos pais e mães se expuseram à fungicidas em Portugal (PATEL et al., 2019), doença de Parkinson nos Estados Unidos (NARAYAN et al., 2017), asma em agricultores dos Estados Unidos (HOPPIN et al., 2017), e melanoma na Itália e no Brasil (FORTES et al., 2016) são outros exemplos dos efeitos desses compostos. Entre os fungicidas usados nos vinhedos estão incluídos o captan, a piraclostrobina, hexaclorobenzeno (clorotalonil) e o tebuconazol (IBRAVIN, 2018). Estudos têm mostrado que o captan provoca estresse oxidativo, necrose e apoptose em linfócitos (INOUE et al., 2018), a piraclostrobina induz teratogenicidade e imunotoxicidade em *Danio rerio* (LI et al., 2018), o hexaclorobenzeno induz a progressão do quadro de endometriose em ratas (CHIAPPINI et al., 2019) e o tebuconazol promove hepatotoxicidade em ratos Wistar e em células hepáticas HepG2 e HepaRG (KNEBEL et al., 2019).

4.4 Estratégias de biocontrole

O biocontrole caracteriza-se pelo uso de microrganismos, parasitoides ou insetos para inibir ou diminuir o desenvolvimento de fitopatógenos e controlar a presença de insetos transmissores de doenças que servem como alternativa à utilização de pesticidas sintéticos, promovendo sustentabilidade, segurança ocupacional e do alimento. Esses microrganismos podem atuar competindo por espaço e nutrientes (TORRES et al., 2016), produzindo moléculas bioativas e induzindo respostas defensivas (CALVO et al., 2019; CAULIER et al., 2019). Um exemplo de biocontrole que vem sendo estudado é a utilização de cepas do gênero *Bacillus*.

Bacillus sp. é uma bactéria gram-positiva que pode formar esporos e atuar nas folhas e raízes de plantas, competindo com patógenos (FAN et al., 2018). Diversos estudos testaram espécies do gênero *Bacillus* como alternativa de biocontrole principalmente para fungos (Tabela 2). A maioria dos estudos mostrados na Tabela 2 foi realizada *in vitro* a partir de diferentes meios de cultura: *Potato dextrose agar* (VERAS et al., 2016; MA et al., 2018;MASSAWE et al., 2018; SAECHOW et al., 2018; LI et al., 2018; PAZ et al., 2018; MANNAA; KIM, 2018; GUARDADO-VALDIVIA et al., 2018; CAO et al., 2011; JIANG et al., 2014; WANG et al., 2018; CHEN et al., 2018; CALVO et al., 2017), *Yeast extract sucrose* (VERAS et al., 2016), *Luria agar* (PINTO et al., 2018), *Luria-Bertani* (LUO et al., 2018; (MASUM et al., 2018), *Plate count agar* (NIGRIS et al., 2018; ÖZTOPUZ et al., 2018), *Malt extract broth* (ÖZTOPUZ et al., 2018), *Minimal salt* (MASSAWE et al., 2018), *Nutrient broth* (LI et al., 2019a; SHUKLA et al., 2018), *Yeast malt extract agar* (PANDIN et al., 2018), *Brain-heart infusion* (WAMBACQ et al., 2018), *Corn silage infusion* (WAMBCQ et al., 2018) e *Tryptone yeast extract broth with glucose*

Tabela 2. Estudos focados na utilização de *Bacillus* spp. como biocontrole de fungos, vírus, bactérias e nematoides.

Cepa	Origem do *Bacillus*	Tipo de avaliação	Método	Objetivo	Principais resultados	País	Referência
Estudo *in vitro*							
Bacillus subtilis ATCC 19659 e P45B, *Bacillus* sp. P1, P7, P11, P11, P34, P39A, P51 e B312, *Bacillus licheniformis* P40	Intestino de peixes típicos da Amazônia brasileira (*Potamorhina latior*, *Piaractus mesopotamicus*, *Leporinus* sp., *Semaprochilodus* sp. e *Myletus edulis*)	*In vitro* (PDA[a] e YES[b])	Inoculação em meio de cultura contendo 10^7 UFC/mL do *Bacillus* e 10^6 esporos/mL de espécies de fungos toxigênicos *Aspergillus*, *Fusarium*, *Penicillium* e *Monascus*	Avaliar a capacidade dos *Bacillus* em afetar os parâmetros de crescimento dos fungos toxigênicos e da produção de micotoxinas, e a habilidade de produzirem lipopetídeos	Efeito antagonista, diminuição da quantidade de esporos e das micotoxinas Aflatoxina B1, OTA e citrinina e síntese dos lipopeptídeos Iturina, Fengicina e Surfactina	Brasil	(VERAS et al., 2016)
Bacillus amyloliquefaciens subsp. *Plantarum* F321	*Vitis vinifera* cv. Merlot	*In vitro* (LA[c])	Caracterização genômica do *Bacillus* cultivado em meio LA a 28 ºC por 24 horas. O DNA da cepa foi extraído utilizando o *Wizard Genomic DNA Purification kit*	Realizar e analisar o sequenciamento genômico do *B. amyloliquefaciens* e comparar com o genoma de outras espécies	Identificação dos genes responsáveis pela produção de lipopeptídeos, compostos bioativos, atividade antimicrobiana e crescimento da planta, tais como: baciloene, dificidina, macrolactina, surfactina, fengicina e sideróforo	Portugal	(PINTO et al., 2018)
Bacillus altitudinis Lc5	Arroz preto	*In vitro* (Illumina HiSeq 2500)	Sequenciamento genômico utilizando a plataforma Illumina HiSeq 2500. Os genes responsáveis pela síntese de antibióticos e metabólitos secundários foram analisados usando antiSMASH 4.4.0	Identificar os genes da cepa responsáveis pela promoção de crescimento da planta e biocontrole	Elucidação dos genes responsáveis pela síntese de enzimas antioxidantes (catalase, peroxidase e superoxido dismutase) e metabólitos secundários (bacilisina, fengicina, surfactina, fengicina, entre outros)	Índia	(POTSHANGBAM et al., 2018)
Bacillus thuringiensis, *Bacillus cereus e Bacillus mojavensis*	Solo de pomares de figo	*In vitro* (PCA[e] e MEB[f])	Meio de cultura contendo 10^5 esporos/mL^{-1} (para testar efeito antifúngico na germinação de esporos) ou 10^6 esporos/mL^{-1} (para testar o efeito antifúngico no crescimento micelial) do fungo testado (*Aspergillus niger*, *foetidus*, *ochraceus* e *Fusarium solani*) e 0,5-5 mL da cultura de *Bacillus* incubado a 28-30 ºC por 24 horas	Avaliar a produção de enzimas líticas e os efeitos antifúngicos das cepas	Inibição do crescimento micelial e da germinação de esporos, produção de enzimas líticas (quitosanase, quitinase, protease e N-acetil-β-hexosaminidase)	Turquia	(ÖZTOPUZ et al., 2018)
Bacillus velezensis PEBA20	-	*In vitro*	Sequenciamento genômico	Sequenciar e analisar os genes da	Caracterização dos genes	China	(KONG et al., 2018)

			utilizando a plataforma Ilumina HiSeq 2500	cepa responsáveis pela atividade de biocontrole	relacionados à promoção de crescimento da planta e produção de substâncias antimicrobianas (mersacidina, amilociclicina, bacilomicina D, fengicina, surfactina, dificidina, macrolactina e bacillaene)		
Bacillus velezensis B6	Solo	*In vitro*	Sequenciamento genômico utilizando a plataforma Ilumina HiSeq 2500	Realizar a sequência genômica da cepa e avaliar quais são os genes responsáveis pela atividade de biocontrole	Determinação dos genes responsáveis pela produção de lipopeptídeos (mycosubtilina, surfactina e fengicina) e antibióticos (bacilene e bacilisina) com atividade antibacteriana, antfúngica e hemolítica	China	(GAO et al., 2018)
Bacillus atrophaeus GQJK17	Solo contendo rizosfera de Goji Berry	*In vitro* (PDA[a])	*Fusarium solani* com 6mm de diâmetro foi inoculado no centro da placa e o *Bacillus* foi inoculado a 2 cm de distância do *Fusarium*, por 3 a 5 dias a 28 ºC; Sequenciamento genômico através da *Polymerase chain reaction* (PCR)	Analisar o mecanismo molecular da capacidade de biocontrole da cepa	Atividade antagonista contra *F. solani* elucidada através da observação da área de inibição; Identificação dos genes responsáveis pela produção de surfactina, fengicina, peligipeptina, xenocoumacina, bacilomicina e rizocticina	China	(MA et al., 2018)
Bacillus amyloliquefaciens e *Bacillus velezensis*	Sementes de milho	*In vitro* (PDA[a] e MS[g])	Técnica de cultura dupla realizada com 5 microlitros da cultura de *Bacillus* em meio MS, e micélio de 6 mm do fungo *Sclerotinia sclerotirum* em meio PDA incubados a 25 ºC por 5 dias	Destacar os efeitos dos compostos voláteis produzidos pelos *Bacillus* contra *S. sclerotiorum*	Inibição do crescimento micelial, atividade antibiótica e mudanças morfológicas nas células fúngicas	China	(MASSAWE et al., 2018)
Bacillus amyloliquefaciens BAS23	Solo de cultura de arroz	*In vitro* (PDA[a]) e *in vivo* (sementes de arroz)	*In vitro*: culturas duplas contendo micélios de 5 mm dos fungos *Curvularia lunata*, *Fusarium semitectum* e *Helminthosporium oryzae* a 4,5cm de distância do *Bacillus* (1 alçada); *In vivo*: Sementes de arroz imersas em suspensão bacteriana por 1 hora	Avaliar a atividade antagonista do *Bacillus* contra fungos patogênicos presentes no arroz	Inibição do crescimento micelial dos fungos, produção dos lipopeptídeos iturina, fengicina e surfactina, e promoção do crescimento da planta	Tailândia	(SAECHOW et al., 2018)
Bacillus subtilis KU-153	Comida fermentada	*In vitro*	*In vitro*: *Bacillus subtilis*	Avaliar a habilidade do *Bacillus*	Redução de 58,1% dos níveis de	Coreia do Sul	(SHUKLA et al., 2018)

	tradicional coreana (Kimchi)	(NB[h]) e em vinho tinto	foram inoculados em 25 mL de NB contendo 40µg/L de OTA e incubados por 48h a 35 ºC; Em vinho: 2 mL de vinho tinto inoculados com 40µg/L de OTA e 0,4g de *Bacillus*, por 24 horas as 35 ºC	em reduzir a quantidade de OTA	OTA em meio de cultura e de 90% no vinho tinto		
Bacillus subtilis SEM-9	Bicho da seda	*In vitro* (PDA[a] e NB[h]) e in planta (Repolho chinês)	*In vitro:* Meio NB contendo 5-6 µL da cultura de SEM-9 colocado em discos de papel filtro, que foram postos em contato com o meio PDA contendo espécies de *Fusarium* (*sambucinum*, *catenulatum*, *oxysporum*, *proliferatum*, *equiseti* e *graminearum*), a 30 ºC por 2-3 dias; In planta: Plantas de repolho chinês foram pulverizadas três vezes com suspensão de SEM-9 contendo 5 x 10^8 UFC/mL	Caracterizar a sequência genômica da cepa SEM-9 e a sua função na promoção de crescimento de plantas e na atividade antagonista contra patógenos; Avaliar a atividade antagonista do *Bacillus in vitro* e da promoção de crescimento na planta	Elucidação dos genes relacionados com a regulação da produção de biofilme e de lipopeptídeos (surfactina, fengicina, bacilisina e subtilosina-A); atividade antagonista contra *Fusarim* spp. (inibição do crescimento micelial e da germinação de esporos) e promoção de crescimento da planta	China	(LI et al., 2018)
B. amyloliquefaciens EUCB10	Eucalipto	*In vitro* (PDA[a]) e in planta (*Eucalyptus urophylla*, *grandis* e *urograndis*)	*In vitro*: culturas duplas contendo micélio de 1 cm de diâmetro das espécies fúngicas *B. cinerea* ou *Calonectria gracilis*, e o *Bacillus* incubadas por 7 dias a 28 ºC; *In planta:* Estacas de *E. urograndis* foram submersas em suspensão de *Bacillus* 10^3 UFC/mL por uma hora e depois suas folhas foram pulverizados com 10^6 esporos/mL de *C. gracilis* e o *B. cinerea* foi inoculado nos danos realizados nas folhas	Avaliar a cepa de *Bacillus* como agente de biocontrole contra *Botrytis cinerea* e *Calonectria gracilis*	Inibição do crescimento micelial do *B. cinerea* e do *C. gracilis*, e aumento do crescimento das raízes do Eucalipto	Brasil	(PAZ et al., 2018)
Bacillus velezensis QST713	Biofungicida comercial (Serenade Max)	*In vitro* (YMEA[i]) e *in vivo* (cogumelo)	*In vitro*: fungo inoculado no centro do meio e o *Bacillus*	Determinar a sequência genômica da cepa e realizar	Diminuição da incidência de *T. aggressivum*, inibição do	França	(PANDIN et al., 2018)

			em três pontos a 3 cm de distância, a 25 ºC por 5 dias; *In vivo*: meio de cultura contendo o cogumelo (*Agaricus bisporus*) foi contaminado com *Trichoderma aggressivum* e tratado com $5x10^6$ UFC/gr^{-1} da cepa QST713	experimentos para enfatizar sua atividade de biocontrole	crescimento micelial e da esporulação fúngica e identificação dos genes responsáveis pela formação de biofilme e síntese de metabólitos secundários (surfactina, bacilomisina D, macrolactina, fengicina, entre outros)		
Bacillus velezensis NRRL B-23189	-	*In vitro* (BHI[j] e CSI[k]) e *in vivo* (azevém e trevo branco)	*In vitro*: meios de cultura contendo diferentes concentrações de suspensão bacteriana e 1 x 10^4 conidiosporos/mL^{-1} de *P. roqueforti* e *paneum*; *In planta*: mini-silos contendo mistura de azevém e trevo branco, *Penicillium* e suspensão de *Bacillus*	Avaliar o efeito antagonista do *Bacillus* contra *Penicillium roqueforti* e *Penicillium paneum* em condições de silagem	Inibição do crescimento de *P.roqueforti* e *P. paneum* e redução na quantidade de conidiosporos	Bélgica	(WAMBACQ et al., 2018)
Bacillus sp. PPM3	Sedimento marinho do Mar Vermelho	*In vitro* (ISP1G[l]) e *in planta* (milho)	*In vitro*: 2 x 10^8 UFC/mL^{-1} de suspensão bacteriana e 1 x 10^6 esporos/mL^{-1} de *Aspergillus flavus*, *Fusarium graminearum*, *Mucor* sp. e *Alternaria* sp. inoculados por 5 dias a 25 ºC; *Polymerase chain reaction* (PCR) para identificação dos genes; *In vivo:* Sementes de milho imersas em 10^6 UFC/mL de suspensão bacteriana por uma hora e plantadas em solo previamente contaminado com 10^6 esporos/mL de *F. graminearum*	Identificar as características genéticas e fenotípicas da cepa e avaliar a atividade inibitória	Redução do crescimento micelial e inibição da germinação de esporos de todos os fungos analisados, identificação dos genes responsáveis pela síntese de surfactina, iturina, bacilomicina e mycosubtilina e promoção de crescimento da planta	Sérvia	(RADOVANOVIĆ et al., 2018)
Bacillus megaterium KU143	Grãos de arroz armazenados na Coreia	*In vitro* (PDA[a]) e *in vivo* (grãos de arroz)	*In vitro:* 10^5 conídios/mL de suspensão fúngica (*Aspergillus candidus* e *fumigatus*, *Penicillium fellutanum* e *islandicum*)	Avaliar a atividade antifúngica das cepas e dos compostos voláteis de *Bacillus* contra *Aspergillus* e *Penicillium* sp. e identificar esses voláteis	Inibição de crescimento micelial e atividade antagonista contra todas as espécies fúngicas estudadas, síntese de hexanona e dimetil-hexano	Coreia do Sul	(MANNAA; KIM, 2018)

			inoculada com 1 mL do filtrado de cultura bacteriana contendo 10^8 células/mL, a 28 ºC por 10 horas; *In vivo:* Grãos de arroz tratados com suspensão bacteriana contendo 10^8 células/mL e inoculados com 10^7 conídios/mL por 7 dias				
Bacillus atrophaeus B5	Coleção de cepas do Laboratório de Biotecnologia do Instituto Tecnológico de Tepic, México	*In vitro* (PDA[a]) e *in vivo* (graviola e abacate)	*In vitro*: 10^7 UFC/mL de suspensão bacteriana inoculada com 10^6 esporos/mL^{-1} de *Colletotrichum gloeosporioides* a 28ºC por 7 dias; *In vivo*: graviola e abacate foram imersos em 10^7 UFC/mL de suspensão bacteriana e inoculados com 15 µL de suspensão de esporos fúngicos contendo 10^6 esporos/mL, e armazenadas a 25 ºC por 5 a 10 dias	Avaliar a eficácia da cepa em controlar a ocorrência de antracnose[1] em graviola e abacate	Redução do desenvolvimento de *C. gloeosporioides*, diminuição dos sintomas de antracnose[1] e identificação dos genes envolvidos na síntese de surfactina, bacilomicina e iturina	México	(GUARDADO-VALDIVIA et al., 2018)
Bacillus subtilis SQR 9	Solo contendo rizosfera de pepino	In vitro (PDA[a]) e in planta (Pepino)	*In vitro*: *Fusarium oxysporum* inoculado no centro da placa e o *Bacillus* entre a borda da placa e o fungo, por 7 dias a 28ºC; *In planta*: suspensão bacteriana ($4x10^{10}$ UFC/mL) aplicada no solo das mudas de pepino contaminado com $4x10^6$ conídios/mL de *F. oxysporum*	Avaliar o potencial antagonista da cepa SQR 9 contra *F. oxysporum* e estudar o mecanismo de ação do *Bacillus*	Inibição do crescimento micelial, redução de até 64% da incidência de *F. oxysporum* e aumento do crescimento da planta	China	(CAO et al., 2011)

Bacillus subtilis CCTCC M 207209	-	*In vitro* (PDA[a)] e *in vivo* (uvas de mesa: Thompson, Kyoho e Red earth)	<u>*In vitro*</u>: a suspensão de $1x10^5$ esporos/mL de *Aspergillus carbonarius* foi espalhada no PDA e a cultura líquida contendo $1x10^8$ UFC/mL do *Bacillus* foi inoculada no centro do PDA, mantido a 25-30ºC por 5 dias; <u>*In vivo:*</u> suspensão de *Bacillus* ($1x10^8$ UFC/mL) e de *Aspergillus* ($1x10^5$ esporos/mL) foi injetada nas uvas a 2 mm sob a casca. As bagas foram mantidas a 25-30 ºC por 30 dias ou a -1 ºC por 80 dias	Investigar o efeito inibitório do *B.subtilis* sobre o fungo *A. carbonarius* que contamina as uvas de mesa durante o armazenamento refrigerado	A inibição do *A. carbonarius* foi observada em todas as amostras tratadas com o *B. subtilis*		(JIANG et al., 2014)
Bacillus subtilis WXCDD105	Solo de rizosfera de plantas de tomates saudáveis	*In vitro* (PDA[a]) e *in planta* (Tomate)	<u>*In vitro*</u>: meios contendo 10^8 UFC/mL^{-1} da cepa de *Bacillus* e 10^5 UFC/mL^{-1} de *Botrytis cinerea* ou de *Cladosporium fulvum* inoculados a 28 ºC por 5 dias; <u>*In planta*</u>: Mudas de tomate foram pulverizadas com uma solução contendo 10^8 UFC/ml^{-1} de *Bacillus* uma vez por semana, por três semanas, e após, inoculadas com 10^5 UFC/mL^{-1} de *Botrytis cinerea* e *Cladosporium fulvum*	Triar novas bactérias ativas e identificar seus efeitos antagonistas	Inibição de mais de 80% do crescimento fúngico, aumento do crescimento das mudas de tomate e da germinação das sementes	China	(WANG et al., 2018)
Bacillus velezensis LDO2	Raiz de amendoim	*In vitro* (PDA[a]) e *in vivo* (Amendoim)	<u>In vitro</u>: micélios de 6 mm de diâmetro dos fungos dos gêneros *Aspergillus*, e *Penicillium*, e 10^6 UFC/mL bactéria do gênero *Ralstonia* foram inoculados no centro da cultura e 10^8 UFC/mL do *Bacillus* foi inoculado a 25 mm de distância, a 28 ºC e até que os fungos da cultura	Avaliar a atividade de biocontrole da cepa LDO2 em amendoim e entender os mecanismos genéticos da atividade antimicrobiana, e de promoção de crescimento da planta	Aumento da altura da planta e comprimento das raízes, inibição do crescimento de *A. flavus* e *R. solanacearum* e identificação dos genes responsáveis pela síntese dos lipopeptídeos fengicina e surfactina	China	(CHEN et al., 2019)

			controle tivessem crescido por toda a placa, ou, no caso da bactéria patogênica, a 30 ºC por 24 horas; *In planta*: sementes pré-germinadas de amendoim foram cultivadas com 10^9 UFC/mL do *Bacillus* por 2 semanas, para avaliar a promoção de crescimento				
Bacillus licheniformis GL174	*Vitis vinifera* cultivar Glera (cultivada *in vitro*)	*In vitro* (PCA[e]) e *in vivo* (Uva)	*In vitro*: a cepa GL174 foi inoculada com os fungos *Phaeoacremonium aleophilum*, *Phaeomoniella* spp., *Botryosphaeria* spp., *Botrytis cinerea*, *Sclerotinia sclerotiorum* e *Phytophthora infestans*, por 1 semana a 28 ºC *In planta*: estacas da uva e suas folhas foram inoculadas com o *Bacillus* e com micélios de 10 mm de diâmetro de *Botrytis cinerea* e plantas com 60 dias tiveram suas folhas infiltradas com 10^3 células/mL de suspensão bacteriana	Investigar a atividade de biocontrole da cepa e os seus mecanismos de ação	Inibição do crescimento micelial, degradação da parede celular de fungos (quitinase), identificação dos genes envolvidos na atividade de biocontrole e produção de enzimas líticas (acetolactato sintase e acetoína redutase)	Itália	(NIGRIS et al., 2018)
Bacillus amyloliquefaciens BUZ-14	Superfície de frutos de pêssego	*In vitro* (PDA[a]) e *in vivo* (laranja, maçã, uva, cereja e pêssego).	*In vitro*: 10^5 UFC/mL de *Botrytis cinerea*, *Monilinia fructicola* e *laxa*, *Penicillium digitatum*, *P. expansum* e *P. italicum* foram inoculados no centro de cada cultura, e, após, 10^9 UFC/mL da cepa BUZ-14 foi inoculada distante do centro, por 7 dias a 25 ºC; *In vivo*: danos de 3 mm de profundidade e de diâmetro foram realizados nas frutas, que foram inoculadas com 10 µL de 10^5 conídios/mL de *B. cinerea*, *P. digitatum*, *P. expansum* ou *P. italicum*.	Avaliar o potencial da cepa BUZ-14 em controlar doenças que ocorrem após a colheita de laranja, maçã, uva e pêssego	Inibição do crescimento micelial de todos os fungos testados *in vitro* e redução da incidência das espécies de *Penicillium*, de *Monilinia* e de *Botrytis cinerea* nas frutas testadas	Espanha	(CALVO et al., 2017)

			e 10 µL de 10^8 UFC/mL da cepa BUZ-14, por 7 dias a 20 ºC				
Bacillus amyloliquefaciens	Rizosfera de soja, pepino, pimentão, berinjela e tomate	*In vitro* (LB[d]) e *in vivo* (arroz)	<u>*In vitro*</u>: meio LB inoculado com 1 x 10^8 UFC/mL^{-1} de *Acidovorax* oryzae, e, depois, com 1 x 10^8 UFC/mL^{-1} do *Bacillus*, a 30ºC por 48 horas; <u>*In vivo*</u>: sementes de arroz germinadas foram imersas em mistura contendo a bactéria patogênica e a bactéria antagonista (1:1; 1 x 10^8 UFC/mL^{-1}), e mantidas a 28 ºC por 7 dias	Isolar, caracterizar e determinar a atividade antibacteriana de *Bacillus amyloliquefaciens*	Os *Bacillus* reduziram em até 80% o número de células e em 65% a formação de biofilme da bactéria *A. oryzae*, além de promoverem o crescimento da planta	China e Bangladesh	(MASUM et al., 2018)
Estudos *in vivo*							
Bacillus pumilus SS-10.7 e *Bacillus amyloliquefaciens* SS-12.6 e SS-38.4	Coleção previamente estabelecida e caracterizada	Beterraba	As folhas de plantas de beterraba foram injetadas com 10^7 UFC/mL de *Pseudomonas syringae* pv. *Aptata* P53 e com 1 mg/mL^{-1} dos lipopeptídeos (Iturina A, Fengicina A e Surfactina) isolados ou com 10^7 UFC/mL de *B. amyloliqueaciens* SS-12.6	Avaliar o controle biológico de *Pseudomonas syringae* utilizando extratos lipopeptídicos de *B. amyloliquefaciens* e de *B. pumilus*	Diminuição de necrose nas folhas e supressão notável do patógeno.	Sérvia	(NIKOLIĆ et al., 2019)
Bacillus spp. KFP-5, KFP-7 e KFP-17	Coleção de cepas do Laboratório de Interação Planta-microrganismo, Paquistão	Arroz	Raízes de mudas de arroz foram imersas em suspensão bacteriana contendo 8x10^9 UFC/mL^{-1}, e o fungo *Pyricularia oryzae* foi inoculado após 40-60 dias do transplante da planta	Avaliar a eficácia das cepas de *Bacillus* em suprimir a incidência de brusone[2] em variedades de arroz	Diminuição da incidência da doença brusone[2] e aumento da atividade de enzimas antioxidantes (Superóxido dismutase, Peroxidase, Polifenol oxidase e Fenilalanina amônia-liase)	Paquistão	(RAIS et al., 2018)
Bacillus amyloliquefaciens GJ1	Folhas saudáveis de *Citrus sinensis*	Laranja	*Citrus sinensis* (laranjeira) irrigadas com uma solução de *Bacillus* (OD[3] 600 nm) uma vez a cada 7 dias, e o genoma da cepa GJ1 foi extraído utilizando o "*Pure Plasmid Mini Kit*"	Avaliar a atividade de biocontrole da cepa GJ1, e realizar transcrição gênica para elucidar as diferenças nos mecanismos de detoxificação	Redução de 50% da incidência de *Candidatus* Liberibacter e identificação de genes da cepa responsáveis pelas repostas defensivas	China	(TANG et al., 2018)
Bacillus cereus Jdm1	Isolado pelo Laboratório de	Tomate	Mudas de tomate irrigadas	Avaliar a eficiência da cepa Jdm1	Aumento do crescimento da	China	(XIAO et al., 2018)

	Nematologia do Instituto de Proteção Vegetal		com 40 mL de suspensão do *Bacillus* (10^7 UFC/mL) por 7 semanas, e 600 nematoides recém-eclodidos foram pipetados na região ao redor das raízes das mudas; No teste de campo, as mudas foram irrigadas com 1,5 x 10^6 UFC/mL do *Bacillus*	contra *Meloidogyne incognita* e o seu impacto nas comunidades bacterianas presentes na rizosfera do tomate	planta, alteração da comunidade bacteriana da rizosfera do tomate, aumento da mortalidade do *M. incognita* e diminuição da eclosão dos seus ovos		
Bacillus amyloliquefaciens MBI600	Biofungicida Serifel BASF SE	Tomate	Plantas irrigadas com solução contendo 5,5 x 10^7 UFC/mL do *Bacillus*, e, um dia após, as folhas do tomate foram esfregadas com inóculos de *Tomato spotted wilt* e *Potato vírus Y*	Testar a atividade antiviral da cepa MBI600 em plantas de tomate	Redução de 80% da incidência de *Tomato spotted wilt vírus* e diminuição do acúmulo sistêmico de *Potato vírus Y*	Estados Unidos	(BERIS et al., 2018)
Bacillus velezensis LM2303	-	Trigo	800 mL de caldo de cultura bacteriana ($2x10^8$ UFC/mL) foram pulverizados no trigo, e 300 mL de suspensão contendo *Fusarium graminearum* ($2x10^5$ esporos/mL) foram pulverizados dois dias depois	Avaliar a eficácia do *Bacillus* em inibir o crescimento fúngico no trigo, sequenciar e analisar o genoma da cepa LM2303	Redução da ocorrência do *Fusarium*, identificação dos genes envolvidos na formação de biofilme, atividade antibacteriana e promoção de crescimento da planta	China	(CHEN et al., 2018)

[a] PDA – Potato dextrose agar. [b] YES – Yeast extract sucrose. [c] LA – Luria-agar. [d] LB – Luria-Bertani [e] PCA – Plate count agar [f] MEB – Malt extract broth [g] MS – Minimal salt [h] NB – Nutrient broth [i] YMEA – Yeast malt extract agar [j] BHI – Brain-heart infusion [k] CSI – Corn silage infusion [l] ISP1G – Tryptone yeast extract broth com adição de glicose [m] MRS – De Man, Rogosa and Sharpe [1] Antracnose – doença fúngica causada por *Colletotrichum gloeosporioides* (EMBRAPA, 2015). [2] Brusone – doença causada pelo fungo *Pyricularia oryzae*, que causa necrose na planta e pode levar a sua morte (EMBRAPA, 2015). [3] OD – Optical density

Entre os estudos realizados *in vitro* pode-se destacar o que foi conduzido por ÖZTOPUZ et al. (2018), um dos únicos estudos com fungos ocratoxigênicos. Neste caso, os autores avaliaram a produção de enzimas líticas e os efeitos das cepas de *Bacillus thuringiensis*, *Bacillus cereus* e *Bacillus mojavensis*, isoladas do solo de pomares de figo, contra as espécies *Aspergillus niger*, *Aspergillus foetidus*, *Aspergillus ochraceus* e *Fusarium solani*. Os fungos *A. niger* e *A. ochraceus* são comumente encontrados em café, e responsáveis pelo apodrecimento dos grãos (Ministério da Agricultura, Pecuária e Abastecimento, 2003), já *A. foetidus* pode ser encontrado em milho e amendoim e sintetizar a micotoxina fumonisina (PALENCIA et al., 2014). E o *F. solani* é encontrado com frequência em batatas, e favorece a entrada de bactérias e de outros fungos no tubérculo (Ministério da Agricultura, Pecuária e Abastecimento, 2003). Para testar o efeito antifúngico dos *Bacillus* no crescimento micelial de *A. niger*, o meio de cultura *Malt extract broth* foi inoculado com 10^6 esporos mL^{-1} e 5 mL da cultura de *Bacillus*, e incubado a 30 ºC por 24 horas. Uma inibição de até 33% do crescimento micelial e da germinação de esporos do fungo foram verificadas, além da identificação das enzimas líticas quitosanase, quitinase, protease e N-acetil-β-hexosaminidase. A quitina é um polissacarídeo que está presente na parede celular dos fungos (YANG; ZHANG, 2019), já a quitosana é um derivado da desacetilação parcial da quitina (LOPES et al., 2017). Tanto a quitina quanto a quitosana são compostas por monômeros de N-acetil-D-glicosamina e D-glicosamina unidas por ligações β (1-4) (COSTA SILVA; DOS SANTOS; FERREIRA, 2006; FRÁGUAS et al., 2015). A enzima N-acetil-β-hexosaminidase é uma hidrolase que cliva as ligações entre esses monômeros (XI; PAN; ZHANG, 2015). Logo, todas essas enzimas identificadas, incluindo as proteases, causam danos na estrutura celular do fungo.

Outra abordagem de biocontrole é a trazida por MASSAWE et al. (2018) que visaram determinar os efeitos dos compostos voláteis produzidos pelos *Bacillus amyloliquefaciens* e *B. velezensis* contra *Sclerotinia sclerotirum*, um fungo comumente encontrado em alcachofra, alface, amendoim, batata e berinjela, e responsável pela ocorrência do chamado mofo branco, que causa podridão na região do caule da planta e amarelamento das folhas (Ministério da Agricultura, Pecuária e Abastecimento, 2003). As cepas de *Bacillus* foram isoladas de sementes de milho e a avaliação da atividade antibiótica dos seus compostos voláteis foi realizada da seguinte forma: 5 microlitros da

cultura contendo *Bacillus* foi inoculada em meio *Minimal salt* e um micélio de *S. sclerotirum* de 6 mm de diâmetro foi inoculado na extremidade central da partição que continha o meio *Potato dextrose agar*; o meio foi incubado por 5 dias a 25 ºC. Os efeitos observados foram uma inibição do crescimento micelial de 78,1% e 75,24% causada por *B. velezensis* VM10 e *B. amyloliquefaciens* VM42, respectivamente. Esses resultados sugerem que *Bacillus* produzem compostos aromáticos, como 2-undecanona, benzotiazol, 1,3-butadieno e N,N-dimetildodecilamina, capazes de prevenir o crescimento fúngico.

Em relação à avaliação da inibição da síntese de micotoxinas através da utilização de cepas de *Bacillus*, poucos estudos foram realizados com esse intuito. VERAS et al. (2016), por exemplo, utilizaram cepas de *Bacillus subtilis* ATCC 19659, *Bacillus* sp. P1, *Bacillus* sp. P7, *Bacillus* sp. P11, *Bacillus* sp. P34, *Bacillus* sp. P39A, *Bacillus licheniformis* P40, *Bacillus subtilis* P45B, *Bacillus* sp. P51 e *Bacillus* sp. B312 isoladas do intestino das seguintes espécies de peixes típicos da região amazônica brasileira: *Potamorhina latior*, *Piaractus mesopotamicus*, *Leporinus* sp., *Semaprochilodus* sp. e *Myletus edulis*. A determinação do efeito do *Bacillus* na produção da micotoxina OTA foi realizada por meio da inoculação de 10^6 esporos/mL de *Aspergillus carbonarius* ITAL293, e 10^7 UFC/mL de suspensão bacteriana em meio *Potato Dextrose agar*, que foi incubado por 10 dias a 25 ºC. A porcentagem de redução de OTA pelas cepas P1 e P11 foi de 97,5% e 97,3%, respectivamente. Sugere-se que essas cepas bacterianas sejam candidatas promissoras para a atividade de biocontrole de fungos toxigênicos dos gêneros *Aspergillus, Penicillium, Monascus* e *Fusarium*.

Em outros estudos, como os conduzidos por POTSHANGBAM et al. (2018) e PINTO et al. (2018), foi efetivada a caracterização genômica de *Bacillus altitudinis* Lc5 e de *Bacillus amyloliquefaciens* F321, respectivamente, para elucidar os seus mecanismos de ação na atividade de biocontrole. POTSHANGBAM et al. (2018) reportaram que *B. altitudinis* Lc5 foi a primeira cepa dessa espécie isolada das folhas de arroz negro. Entretanto, a espécie *B. altitudinis* já é conhecida pelo seu efeito antagonista contra o fungo *Monilinia fructicola* (LIU et al., 2018), por exemplo, causador da podridão-parda em ameixa, pêssego, uva e maçã (Ministério da Agricultura, Pecuária e Abastecimento, 2003). O sequenciamento genômico foi realizado através da plataforma Illumina HiSeq 2500. Foram identificados os genes responsáveis pela produção de enzimas antioxidantes, como a catalase e a peroxidase, e de metabólitos secundários, como a fengicina, surfactina e liquenisina. Já PINTO et al. (2018) isolaram

o *B. amyloliquefaciens* F321 de uvas *Vitis vinifera*, cultivar Merlot, e extraíram o seu DNA utilizando o *Wizard Genomic DNA Purification kit*. A espécie *B. amyloliquefaciens* foi capaz de inibir o crescimento de *Fusarium graminearum*, produtor das micotoxinas desoxinivalenol (DON) e zearalenona, em estudo concretizado por GU et al. (2017). Foram elucidados por PINTO et al. (2018) os genes bcr-BVY13_11500 e *fos*B-BVY13_12675, responsáveis pela síntese de bacitracina e fosfomicina (compostos que conferem resistência à ação de outros microrganismos), e grupos de genes envolvidos na produção de dificidina, macrolatina e fengicina (atividade de biocontrole).

Há ainda trabalhos em que foi avaliado o potencial de *Bacillus* no biocontrole de fungos em alimentos, como: tomate (WANG et al., 2018), laranja, maçã e uva (CALVO et al., 2017), arroz (RAIS et al., 2018) e trigo (CHEN et al., 2018). *Bacillus subtilis* WXCDD105 foi utilizado por WANG et al. (2018) para controlar a ocorrência de *Botrytis cinerea* e *Cladosporium fulvum* em tomate. *B. cinerea* é o causador do chamado mofo cinzento, encontrado com frequência em alface, abacate, berinjela, batata e cebola. Os sintomas típicos são a queima das pontas das folhas, manchas marrons nas folhas e apodrecimento das flores e dos frutos (Ministério da Agricultura, Pecuária e Abastecimento, 2003). *Cladosporium fulvum* é conhecido por causar manchas amarelas na superfície das folhas, bem como manchas brancas a verde-oliva, que se tornam marrons durante a esporulação do fungo (THOMMA et al., 2005) o que impede a realização de fotossíntese, diminuindo a produtividade e a qualidade da fruta (MESARICH et al., 2018). WANG et al. (2018) pulverizaram as mudas de tomate com uma solução contendo 10^8 UFC/mL^{-1} da cepa WXCDD105 uma vez por semana, durante três semanas. Após esse período, uma suspensão contendo 10^5 UFC/mL^{-1} de *B. cinerea* e *C. fulvum*, diluída em *Potato dextrose broth*, foi aplicada nas mudas. O resultado obtido foi uma redução de 74,7% e 72,07% da ocorrência de *B. cinerea* e *C. fulvum*, respectivamente. Os autores concluíram que além de ter atividade antifúngica, a cepa de *Bacillus* também foi capaz de promover o crescimento das mudas e manter a qualidade dos tomates.

Enquanto isso, CALVO et al. (2016) realizaram danos de 3 mm de profundidade e de diâmetro em laranjas (cultivar Valencia), maçãs (cultivar Golden delicious) e uvas (cultivar Sultanina), que foram inoculadas, respectivamente, com 10 μL de 10^5 conídios/mL de *Penicillium digitatum*, *P. italicum* e *P. expansum*, e *Botrytis cinerea*. Depois, as frutas foram inoculadas com 10 μL de 10^8 UFC/mL do *Bacillus*

amyloliquefaciens BUZ-14, durante 7 dias a 20 ºC. Os fungos pertencentes ao gênero *Penicillium* causam bolores verdes em grãos de milho e lesões aquosas sobre a casca de frutas, principalmente no período pós-colheita (Ministério da Agricultura, Pecuária e Abastecimento, 2003). CALVO et al. (2016) observaram que a incidência de *P. expansum* nas maçãs foi de apenas 20% e a redução do *B. cinerea* foi de 20% nas uvas, concluindo que a cepa de *Bacillus* é promissora para tratamentos preventivos contra espécies de *Penicillium*.

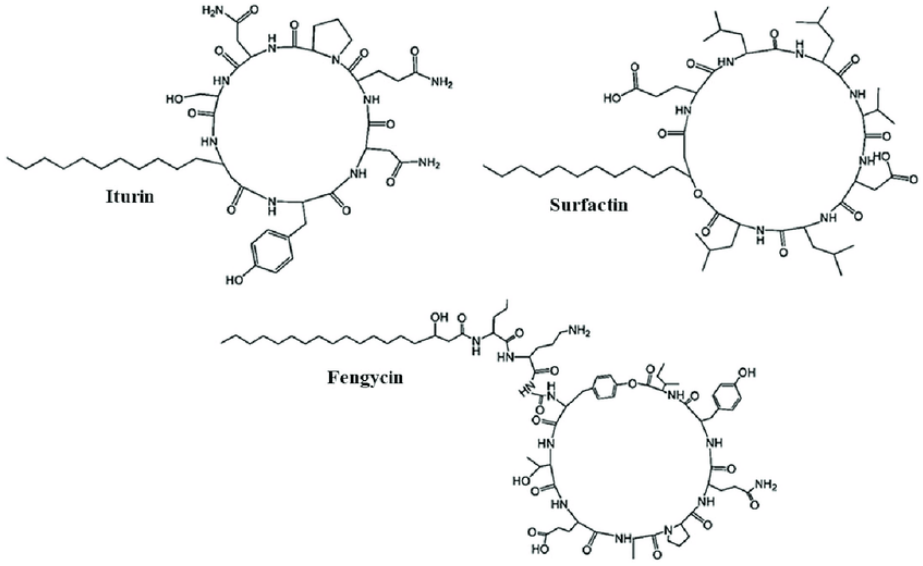
Bacillus spp. KFP-5, KFP-7 e KFP-17 foram utilizados por RAIS et al. (2018) para o controle do fungo *Pyricularia oryzae*, causador da doença brusone em arroz que leva à formação de lesões necróticas de coloração marrom na planta e a sua consequente morte (Embrapa, 2015). No estudo conduzido por RAIS et al. (2018), as raízes de mudas de arroz foram imersas em suspensão bacteriana contendo 8 x 10^9 UFC/mL^{-1}, e, após transcorridos 60 dias do cultivo do arroz, as mudas foram inoculadas com o fungo *P. oryzae*. As cepas de *Bacillus* reduziram em cerca de 44% a severidade da doença brusone nas mudas de arroz, além disso, os *Bacillus* induziram o aumento da ação das enzimas antioxidantes Superóxido dismutase, Peroxidase, Polifenol oxidase e Fenilalanina amônia-liase. Finalizando, *Bacillus* spp. KFP-5, KFP-7 e KFP-17 foram considerados os candidatos ideais para o manejo de brusone no arroz.

Produtos à base de *Bacillus* spp. estão disponíveis comercialmente como agentes de biocontrole contra fungos fitopatogênicos. O Serenade produzido pela Agro Bayer é composto por *Bacillus subtilis* (QST 713, 10^9 UFC g^{-1}) e recomendado para o controle de doenças como: mancha-de-alternaria (*Alternaria dauci*), mofo-cinzento (*Botrytis cinerea*), antracnose (*Colletotrichum gloeosporioides*) e mofo-branco (*Sclerotinia sclerotiorum*) em cenoura, coentro, alface, batata, uva, abacate, alho, maçã, amendoim, berinjela, tomate, entre outros alimentos (Agro Bayer, 2018). O Duravel produzido pela BASF é a base de *Bacillus amyloliquefaciens* (MBI 600, 10^{10} esporos viáveis g^{-1}), e é recomendado para controle de *Cryptosporiopsis perennans*, *Botrytis squamosa*, *Phyllosticta citricarpa*, *Xanthomonas campestris*, entre outros patógenos, em tomate, berinjela, pimentão, frutas cítricas e maçã (BASF, 2018). Os biofungicidas apresentam como vantagem em relação aos fungicidas sintéticos a possibilidade de serem aplicados em qualquer etapa do desenvolvimento do alimento e até o dia da colheita (IHARA, 2017), logo não possuem intervalo de segurança (Agro Bayer, 2018).

Em relação aos metabólitos secundários produzidos pelas espécies de *Bacillus*, os lipopeptídeos capazes de inibir o crescimento fúngico, incluindo as iturinas,

fengicinas e surfactinas, cujas estruturas são mostradas na Figura 2, têm sido identificados como um dos responsáveis pelo efeito antifúngico de *Bacillus* (DANG et al., 2019; HANIF et al., 2019; LI et al., 2019b).

Figura 2. Estrutura química dos lipopetídeos Iturina, Surfactina e Fengicina. (HAMLEY, 2015; KHAN; MAYMON; HIRSCH, 2017)



Hanif et al. (2019) atribuem a atividade antifúngica do *Bacillus amyloliquefaciens* FZB42, contra *Fusarium graminearum* em Potato dextrose agar (PDA), à síntese de fengicina. Por outro lado, Dang et al. (2019) relatam que a síntese de iturina por *Bacillus amyloliquefaciens* C2LP inibiu o crescimento de cinco espécies fúngicas: *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* e *Rhizoctonia solani* em meio PDA. A Surfactina apresentou atividade contra *Plasmopara viticola* em uvas *Vitis vinifera* cv. Marselan, em estudo conduzido por Li et al. (2019), e diminuiu o crescimento de *Magnaporthe grisea* em meio de cultura PDA (WU et al., 2019). As surfactinas não possuem atividade antifúngica, todavia, atuam de forma sinérgica amplificando a atividade das iturinas e das fengicinas (COUTTE et al., 2010); as iturinas alteram a estrutura e a permeabilidade da membrana celular dos fungos criando canais transmembrana que permitem a liberação de íons vitais, como o K^+, das células fúngicas (HSIEH et al., 2008), já as fengicinas atuam interagindo com os componentes lipídicos da membrana citoplasmática do fungo, como o ergosterol, inibindo o seu crescimento (DELEU et al., 2005).

Enzimas líticas como a quitinase (PAN et al., 2019), a glucanase (CAULIER et al., 2018), as celulases e as proteases (KARUPPIAH et al., 2019) também são sintetizadas por espécies de *Bacillus* e apresentaram atividade antifúngica contra *Alternaria solani*, *Fusarium solani* e *Rhizoctonia solani* em meio Luria- Bertani (LB) (CAULIER et al., 2018).

As espécies do gênero *Bacillus* apresentadas mostraram ser alternativas promissoras ao uso de pesticidas sintéticos, além disso, são capazes de promover o crescimento das plantas e manter a qualidade das frutas. Estudos com outros alimentos, cepas de *Bacillus*, e microrganismos patogênicos são necessários para dar continuidade a esse avanço biotecnológico e aumentar o espectro de ação do gênero.

4.6 Importância do perfil volátil para a qualidade das uvas

Os compostos voláteis possuem baixo ponto de ebulição e são capazes de estimular uma resposta sensorial no sistema olfativo humano (GĄSIOR; WOJTYCZA, 2016). Esses compostos pertencem às classes dos álcoois, aldeídos, cetonas, ácidos, tióis e ésteres (CANUTI et al., 2009). Metabólitos secundários das uvas também podem estar relacionados ao aroma, incluindo compostos das classes dos terpenos, C_{13}-norisoprenoides e fenóis, que podem ser encontrados nas formas livre ou como moléculas glicoconjugadas (ALEM et al., 2019)

Os terpenos presentes nas uvas ocorrem em maior quantidade na casca do que na polpa (ALEM et al., 2018) e são comumente encontrados conjugados à glicose, arabinose, ramnose e apiose. Os terpenos glicosilados são compostos não voláteis que durante o processo de fermentação sofrem hidrólise através da ação enzimática das glicosidases, e são transformados na sua forma ativa (livre) (MAICAS; MATEO, 2005) conferindo um aroma floral aos vinhos (WELKE et al., 2014).

Os norisoprenoides são derivados da biodegradação de carotenoides (ROBINSON et al., 2014) sendo α-ionona, β-ionona e a β-damascenona os principais compostos C_{13}-norisprenoides presentes em vinhos e que contribuem para a formação de aromas florais e frutados (WINTERHALTER; ROUSEFF, 2001). Algumas das formas glicoconjugadas mais comumente encontradas são: 3,4-di-hidro-3-oxo-actinidiol, 7,8-dihidrovomifoliol, 3-oxo-α-ionol e 3-oxo-retro-α-ionol (SÁNCHEZ-GÓMEZ et al., 2018). E, assim como os terpenos, também são hidrolisados durante a fermentação (BAHENA-GARRIDO et al., 2019).

Os fenóis também podem ser encontrados tanto na forma glicoconjugada quanto na forma livre. Alguns exemplos de fenóis ativos são o maltol, guaiacol, eugenol, 4-vinilguaiacol, siringol e *trans*-isoeugenol. Esses compostos podem contribuir para um aroma doce, caramelado, floral ou defumado (LAN et al., 2019)

Álcoois e aldeídos são oriundos da oxidação de moléculas de ácidos graxos e responsáveis pelo "green aroma" (adstringência) das uvas (ROBINSON et al., 2014). Além de alguns álcoois, como o 2-feniletanol, estarem relacionados ao aroma floral e aldeídos, como ao aroma amanteigado em vinhos (WELKE et al., 2014).

Em relação à classe dos ésteres, os compostos que se destacam são o éster de acetado e os ésteres de ácidos graxos, que são sintetizados pelas leveduras durante a fermentação alcóolica do vinho (SUMBY; GRBIN; JIRANEK, 2010). Os ésteres trazem atributos frutados e florais ao vinho (POIVET et al., 2018)Já os compostos ácidos, como o ácido succínico, ácido acético, ácido propanoico e ácido isobutanoico possuem aroma de melão (LASIK-KURDYS; MAJCHER; NOWAK, 2018), de vinagre, de especiarias e um aroma rançoso, respectivamente (TANG et al., 2019).

Os tióis são compostos sulfurados (PEÑA-GALLEGO et al., 2012), cuja origem se dá a partir de ácidos graxos e são encontrados ligados à cisteína ou à glutationa, e são compostos sem odor até que ocorra a lise enzimática durante a fermentação (BELDA et al., 2017). Alguns componentes majoritários são o 4-mercapto-metilpentan-2-ona, 3-mercaptohexan-1-ol e o acetato 3-mercaptohexil, que trazem para o vinho aromas de groselha e maracujá (KING et al., 2008). Já os ácidos podem possuir aroma pungente, amanteigado e gorduroso, enquanto as cetonas atribuem odores herbal e frutal (LIU et al., 2020).

As características aromáticas das uvas e dos vinhos são resultantes de diversos fatores, tais como: localização geográfica das videiras (KOUNDOURAS et al., 2006), tipo de solo, características climáticas (SABON et al., 2002), variedade de uva (ARMANINO et al., 2008), tipo de levedura utilizada na etapa de fermentação (TORRENS et al., 2004) e das técnicas utilizadas na produção de vinho (ESTI; TAMBORRA, 2006).

Outro fator que afeta o perfil volátil das uvas é o uso de fungicidas sintéticos. O efeito dos fungicidas ciprodinil, fludioxonil e pirimetanil na composição aromática do vinho branco de *Vitis vinifera*, cultivar Airen, foi estudado por GARCÍA et al. (2004). Os fungicidas foram dissolvidos em uma solução contendo água e etanol (9:1 v/v) e adicionados separadamente no mosto, que foi fermentado com *Saccharomyces*

cerevisiae, em duas doses (1 mg L^{-1} e 5mg L^{-1}). Através de análise cromatográfica, foi observado que a quantidade de álcoois isoamílicos aumentou, o que reduz a qualidade do vinho, pois em altas quantidades são considerados compostos indesejáveis; a quantidade aumentada de acetato isoamilo afetou negativamente o aroma do vinho, já que é um composto muito frutado.

Por outro lado, OLIVA et al. (2008) analisaram como a pulverização dos fungicidas comerciais Equatin Pro GR (famoxadona), Teldor WG (fenehexamida), Castellan GD (fluquinconazol), Stroby WG (metil-kresoxim), Arius SC (quinoxifeno) e Flint WG (trifloxistrobina) em uvas *Vitis vinifera*, cultivar Monastrell, diluídos e aplicados entre 14 a 35 dias antes da colheita das uvas, afeta o perfil volátil do vinho tinto. Ao final do tratamento, as uvas passaram pelo processo de microvinificação logo após as uvas terem sido colhidas. As quantidades de ácidos, acetatos de etila, ésteres etílicos e de terpenos encontradas nessas amostras foram inferiores às quantidades da amostra controle. Essas diminuições podem ter ocorrido por, principalmente, dois motivos: efeito sobre a atividade fermentativa da levedura, grande parte dos compostos voláteis estudados são oriundos desse processo, e sobre a atividade da glicosidase, enzima responsável pela transformação dos terpenos na sua forma livre/aromática. Portanto, os vinhos obtidos a partir das uvas Monastrell tratadas com esses fungicidas tinham um fraco potencial vínico.

Há ainda estudos que averiguaram como algumas cepas de *Bacillus* influenciam no perfil volátil de determinados alimentos. GU et al. (2015) avaliaram como a utilização de *Bacillus* pode estar envolvida na formação de vanilina, um aldeído, durante o processo de cura de favas de baunilha. Células de *Bacillus vanillea* XY18 e *Bacillus subtilis* XY20 foram utilizadas no processo de cura. As favas de baunilha passaram oito semanas sendo curadas através do processamento com ar quente e com os *Bacillus*. Cinco compostos voláteis (ácido hexanoico, ácido benzoico, 2-acetil-1H-pirrol, acetato de 2-feniletila e apocinina) foram identificados como resultantes do metabolismo de *Bacillus* e nenhum atributo sensorial desagradável foi gerado. Esse resultado sugere que a enzima β-D-glucosidase produzida por essas cepas participe da formação de vanilina através da hidrólise de glucovanilina. A cepa XY20 aumentou a produção de ácido butanoico na baunilha, que é utilizado na indústria alimentícia como um aditivo de sabor.

JEONG et al. (2017) por sua vez, utilizaram *Bacillus licheniformis*, isolados do "doenjang", uma pasta de soja fermentada, e do "meju", soja fermentada seca, para

avaliar a sua efetividade na produção de compostos aromáticos nesses alimentos. A soja esterilizada foi inoculada com $5x10^5$ UFC/g do *Bacillus* e incubada por vinte e oito dias em uma temperatura de 25 ºC. A análise dos voláteis foi realizada através do método cromatográfico GC-MS. Os principais voláteis formados foram o álcool oct-1-en-3-ol e o carboneto 3-hidroxibutan-2-ona. Espécies de *Bacillus* são essenciais para produzir o sabor tradicional do "doenjang" (HONG; JUNG; KIM, 2012) tradicionalmente consumido na Coreia.

E, em estudo realizado por AZOKPOTA et al. (2010) sementes de alfarroba africanas foram fermentadas com *Bacillus subtilis* B51, B52 e B53 e foi realizada uma avaliação qualitativa e quantitativa dos compostos voláteis. As cepas de *B. subtilis* foram isoladas dos condimentos fermentados "afitin", "iru" e "sonru", comumente consumidos em Benim. Cerca de 0,5 mL da diluição de cada cepa de *Bacillus* foi adicionado a 50g de alfarroba esterilizada, que foi mantida incubada a 37 ºC por 24 horas ou 48 horas. A extração e a identificação dos compostos voláteis foram realizadas através do método de destilação-extração "Likens-Nickerson". Os compostos identificados são das classes dos álcoois, aldeídos, cetonas, pirazinas, ésteres, ácidos e sulfurados. *B. subtilis* foi considerado um potencial starter para ser utilizado na fermentação de alfarroba africana para produzir condimentos Benineses.

Até o momento, apenas um estudo avaliou o perfil volátil de uvas e vinho após inoculação com espécie de *Bacillus*. ESCRIBANO-VIANA et al. (2018) pulverizaram em videiras de uvas *Vitis vinifera* cultivar Tempranillo, localizadas na Espanha, o biofungicida comercial "Serenade Max" contendo 5,3 x 10^{10} UFC/mL de *Bacillus subtilis* QST 713. O biofungicida foi aplicado em dois momentos: 21 dias e 3 dias antes da colheita. O processo de vinificação foi realizado através de fermentação alcoólica espontânea a 25 ºC, e fermentação malolática a 20 ºC utilizando as bactérias ácido láticas comerciais "Uvaferm alpha". Os testes de pH, acidez total e concentrações de ácido málico, ácido glucônico e potássio não mostraram diferenças significativas quando comparados com as amostras controle. A aplicação do biofungicida reduziu 7% da quantidade de álcoois, como o 1-hexanol, nas amostras de vinho. Concluiu-se que o tratamento com o biofungicida não influenciou negativamente a qualidade das uvas ou do vinho, e que foi positivo do ponto de vista microbiológico, já que ocasionou um aumento na produção das uvas e melhorou a implantação da bactéria utilizada na fermentação malolática.

5 ARTIGO ORIGINAL 1

Bacillus velezensis P1 inhibits the *Aspergillus carbonarius* growth and the synthesis of ochratoxins in grapes

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Abstract

The use of *Bacillus* sp. as a strategy biocontrol to eliminate/reduce the use of toxic fungicides in viticulture has emerged, while fungi resistant to commonly used products have become frequent. Therefore, the search for new strains with potential fungicide must be recurrent. *Aspergillus carbonarius* is the main fungus responsible for the production of ochratoxins, including ochratoxin A (OTA) that presents maximum permissible limit established by legislation for grape products. The aim of this study was to evaluate the potential of four strains of *Bacillus* (P1, P7, P11 and P45) as a biocontrol agent for *A. carbonarius* and to inhibit the synthesis of ochratoxins in grapes. P1 was the most promising strain, since it inhibited *A. carbonarius* in all berries at 10^9 CFU mL^{-1}, whereas the grapes treated with P7, P11 and P45 resulted in no fungal growth in 58, 74 and 25% of the berries. Six forms of ochratoxin were produced by *A. carbonarius* in grapes, including ochratoxin α, ochratoxin β, ochratoxin α methyl-ester, ochratoxin α amide, N-formyl-Ochratoxin α amide, and OTA, which were identified using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. All *Bacillus* strains inhibited the synthesis of OTA, which is the most toxic form of ochratoxin. No ochratoxins were found when *Bacillus* P1 and P7 were used on grapes. Despite some forms of ochratoxin were found in grapes treated with *Bacillus* P11 and P45, the levels were lower than those found in control grapes. *Bacillus* P1 successfully identified as *B. velezensis* proved to be the most promising strain, since it inhibited fungal growth and all ochratoxins.

Keywords: Biocontrol, Biofungicide, *Bacillus*, *A. carbonarius*, ochratoxin A, Chardonnay

1. Introduction

The control of fungi is one of the main challenges for viticulture since the climate of the vine regions worldwide favors the occurrence of these microorganisms, impairing grape quality. Among the toxigenic fungi reported in grapes, *Aspergillus carbonarius* is the main fungus responsible to produce ochratoxin A (OTA) (Welke 2019). This mycotoxin is related to genotoxicity, nephrotoxicity, teratogenicity, neurotoxicity and immunosuppression (Cimbalo, Font, & Manyes, 2020). OTA is classified as a possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1993). Due to toxicological concern, the European Union (EFSA, 2006) and Brazil (ANVISA, 2011) set 2 μg kg^{-1} as the maximum limit for OTA in wines and grape juices.

There is controversy regarding how OTA biosynthesis occurs and some compounds has been identified as precursors of OTA including ochratoxin α (OTα), ochratoxin β (OTβ), ochratoxin B (OTB) and ochratoxin C (OTC) (Gil-Serna et al., 2020). As OTA is reported as the most toxic form among ochratoxins, followed by OTC, OTB, OTα, and OTβ (Heussner & Bingle 2015). Modified forms of these mycotoxins have been detected in wines, including OTα methyl ester, OTB methyl ester, OTA methyl ester, ethylamide OTA and OTA glucose (Freire et al. 2020). In grapes, a product of OTA degradation named 14-decarboxy-OTA and an OTA conjugation compound (ethylamide OTA) were identified by Freire et al. (2018; J. Agric. Food Chem. 2018, 66, 8824−8831). These modified forms of ochratoxins can be produced by fungi, or by conjugation of ochratoxins with other molecules present in grape products or by reactions of hydrolysis, reduction and oxidation during grape maturation or

winemaking. Modified forms of OTA can be converted into the parent mycotoxin during digestion, causing adverse health impacts (Freire et al. 2018).

The use of synthetic fungicides is the main practice to control fungal infection and ochratoxins in order to achieve adequate quality and yield of grape production. However, the prolonged use these products can increase pathogens' resistance to chemicals, leading to the use of greater quantities of these products and/or other more toxic fungicides. In addition, they can also harm the agricultural ecosystem and the human health (Zhang et al. 2020).

Bordeaux mixture (copper sulphate, lime and water) is also used as an antifungal even in organic viticulture. However, the use of this product repeatedly and in large quantities results in accumulation of copper in the soils of vineyards worldwide. Excessive copper can cause damage to the roots making nutrient absorption difficult, reducing photosynthetic rate and delaying the development of the vine (Bortoluzzi, Korchagin, Moterle, dos Santos, & Caner, 2019).

Due to the negative impact caused by synthetic fungicides and Bordeaux mixture, the study of alternative methods as the use of biological control agents has emerged. *Bacillus* species have be explored for their potential to inhibit fungal growth and mycotoxin biosynthesis (Ren et al., 2020). Products containing *Bacillus* spp. are commercially available as biocontrol agents against phytopathogenic fungi. However, the emergence of fungi resistant to commonly used products is frequent, which also endorses the need to research new strains for biocontrol.

The antifungal activity of *Bacillus* spp. may be related to their ability to synthesize lipopeptides (mainly iturins and fengycins), polyketides,

siderophores, volatile compounds and enzymes, in addition to the competition for space and nutrients with the pathogens (Calvo, Mendiara, Arias, Blanco, & Venturini, 2019). Jiang et al. (2020) reported iturin A, produced by *Bacillus subtillis,* as the major compound that inhibits *A. carbonarius* in vitro. This lipopeptide disturbs the transport, energy metabolism, and osmotic pressure of fungal as revealed by transcriptomics analysis. Regarding grape berries, some studies have tested species of the genus *Bacillus* as a biocontrol proposal to inhibit *Botrytis cinerea*, Chen et al., 2019, Rotolo et al., 2017), *Colletotrichum gloeosporioides* (Aoki, Aoki, Ishiai, Otoguro, & Suzuki, 2017), *Aspergillus niger, A. parasiticus, A. tubingensis* (El Shanshoury et al., 2018), *A. ochraceus, A. flavus* (Kasfi et al. 2018) and *A. carbonarius* (Jiang, Shi, Liu, & Zhu, 2014). However, this is the first study to assess the ability of *Bacillus* strains to inhibit the synthesis of different forms of ochratoxins by *A. carbonarius*.

In a previous study, ten *Bacillus* strains isolated from aquatic environment of the Amazon region, Brazil, were assessed against toxigenic fungi in culture media (Veras, Correa, Welke, & Brandelli, 2016). Four strains were promising to reduce the *A. carbonarius* growth and OTA synthesis in vitro. Based on these preliminary results, the objective of this study was to evaluate, for the first time, these four strains of *Bacillus* as a biocontrol agent for *A. carbonarius* in grapes and to inhibit the synthesis of different forms of ochratoxin.

2. Materials and methods

2.1. Reagents and chemicals

Water was purified in a Millipore Milli-Q Plus system (Molsheim, France). Acetonitrile (LC grade) was supplied by J. T. Baker (Phillipsburg, New Jersey, USA). Acetic acid, magnesium sulfate heptahydrate and sodium chloride were purchased from Panreac Quimica S.A. (Barcelona, Spain). C18 sorbent was acquired from Macherey-Nagel (Duren, Germany) and ammonium acetate (LC-MS grade) was from Sigma-Aldrich (Darmstadt, Germany).

Analytical standard of OTA (purity higher than 98%) was from Sigma-Aldrich (Saint Louis, MO, USA). OTA stock solution (100 mg L^{-1}) was prepared in acetonitrile. An intermediate OTA standard solution (3 mg L^{-1}) was obtained by dilution of the stock solution in acetonitrile and it was used to prepare the analytical curves containing eight points with OTA ranging from 0.125 to 20 µg L^{-1} using the matrix-matched calibration approach.

OTα, OTα methyl-ester and OTα amide were prepared using OTA standard solution (3 mg L^{-1} in acetonitrile). Solutions of OTα and OTα amide were obtained by OTA thermal degradation according to Bittner et al. (2015). OTα methyl-ester solution was prepared by an esterification reaction using a solution of 6 mol L^{-1} HCl according to Li, Marquardt, & Frohlich (1998).

2.2 Microrganisms and inocula preparation

Aspergillus carbonarius ITAL293 was cultivated in Potato dextrose agar (PDA) medium for 7 days at 25°C. This strain was confirmed to produce OTA as described in a previous study (Dachery et al., 2019). For the inoculation of the grape berries, a spore suspension was prepared by adding 5 mL of sterile

distilled water containing 0.1% (v/v) Tween 80 over the *A. carbonarius* colony. The spore count was performed in the Neubauer chamber and the concentration of the suspension was adjusted to 10^3 spores mL^{-1}.

Bacillus strains used in this study (P1, P7, P11 and P45) were isolated from aquatic environment of the Amazon region, Brazil, (3°06′S, 60°01′W) as described in a previous study by Veras et al. (2016), who reported these four strains were the most promising against toxigenic fungi evaluated in culture medium.

Bacillus were cultivated in Brain-Heart infusion (BHI) broth at 37 °C. Cell suspension were made as follows: BHI broth was placed in tubes and centrifuged for 15 minutes at 10,000 rpm and 4 °C to obtain a pellet. After suspending the pellet in saline water (NaCl 0.85% w/v), the cell concentration was determined by measuring the optical density at 600 nm in a spectrophotometer (Shimadzu, UVmini-1240, Japan) and adjusted to 10^6, 10^7, 10^8 and 10^9 CFU mL^{-1}.

2.3. Identification of the Bacillus strains

In previous studies, the analysis of 16S rDNA sequence indicates that both strains P7 and P11 were clustered with *B. subtilis, B. velezensis* and *B. amyloliquefaciens*. The genetic variation of 16S rRNA gene fragment (1,060 bp) was not enough to distinguish between these three species (Giongo, Lucas, Casarin, Heeb, & Brandelli, 2007). The isolate *Bacillus* sp. P45 was clustered with the *Bacillus subtilis* group (Sirtori, Cladera-Olivera, Lorenzini, Tsai, & Brandelli, 2006). GenBank accession number of these isolates is done as follows: P7 (DQ387865), P11 (DQ387864) and P45 (AY962474).

For identification of the isolate *Bacillus* sp. P1, the genomic DNA was extracted as described previously (Ambrosini et al., 2012) and the 16S rRNA gene sequence was amplified using the primers 27F and 1525R (Pereira et al., 2014). The obtained sequence was analyzed with the Basic Local Alignment Search Tool (BLAST) at the NCBI and EZBiocloud databases.

Sequences of type strains of the most closely related species were obtained from the RDP database and aligned using MAFFT (Katoh et al., 2019). Type stains are descendants of the original isolates used in species and subspecies descriptions, as defined by the Bacteriological Code, that exhibit all the relevant phenotypic and genotypic properties cited in the original published taxonomic circumscription (Lapage et al., 1990). The phylogenetic tree was reconstructed using the Maximum Likelihood approach through the default settings of the RaxML v.8.0.0 software provided at Cipres Gateway (Miller et al., 2010).

2.4. *Grapes*

Chardonnay grapes (*Vitis vinifera*) harvested in 2019 in Canela (29°21'56.4"S 50°46'06.6"W), Rio Grande do Sul, Brazil were superficially disinfected by immersing the bunches in a 1% sodium hypochlorite solution for three minutes, followed by rinsing with sterile distilled water. Excess water was removed by placing the berries in a laminar flow cabinet for one hour before the microorganisms inoculation, as described by Lappa et al. (2018). Chardonnay was chosen for this study because this grape is grown in vineyards in more than 40 countries, which is important for viticulture worldwide (OIV, 2020). In addition, Chardonnay has been identified as the cultivar most susceptible

among nine other grape varieties to the colonization of *A. carbonarius* and accumulation of OTA in a previous study (Veras et al. 2020).

2.5. Effect of Bacillus strains on A. carbonarius growth and fungal colony count and in grapes

The effect of *Bacillus sp.* P1, P7, P11 and P45 *on A. carbonarius growth* in grapes was evaluated by immersing the berries for two minutes in the *Bacillus* cell suspension containing 10^6, 10^7, 10^8 or 10^9 CFU mL^{-1}. The berries were kept for one hour to air dry in a laminar flow cabinet, and immersed in the spore suspension of *A. carbonarius* (10^3 spores mL^{-1}) for two minutes. The inoculated grape berries were placed in Petri dishes and remained for one hour in a laminar flow cabinet. The incubation occurred at 30°C for 7 days. Humidity was maintained using cotton moistened with distilled water, both sterile. The berries inoculated only with *A. carbonarius* were used as positive control. For the negative control, uninoculated berries were incubated in the same conditions.

The percentage of grapes with no A. *carbonarius* infection was determined using the formula:

$$\text{Grapes with no A. } carbonarius \text{ infection (\%)} = \frac{\text{number of berries with no fungal growth}}{\text{number of total berries}} \times 100$$

The inhibitory effect of the *Bacillus* strains was also evaluated by the counting fungal colonies, according to Jiang et al. (2014) with some modifications. Grape berries were macerated in a 60 mL sterile solution of 0.1% (w/v) peptone. The mixture was shaken in a vortex mixer (Kasvi, Korea) for 2 minutes to dislodge the fungi from the surface of the grape berries. The solution

was serially diluted 6-fold in 0.1% peptone solution. Aliquots of each diluted solution (0.1 mL) were plated onto Dichloran Rose Bengal Chloramphenicol agar (DRBC) medium (Merck, Germany). Plates were incubated at 25°C for 5 days and the number of colonies was counted after this period. The results were expressed as colony forming units per mililiter (CFU mL^{-1}).

2.6. Evaluation of ochratoxins produced by A. carbonarius in grapes using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOFMS)

The ochratoxins were extracted according to a previous study (Nievierowski et al., 2020). Grapes (2 g) were mixed with 5 mL of acetonitrile:formic acid 1% (v/v) using a vortex for 2 minutes, followed by shaking for 60 minutes at 200 rpm in an orbital shaker (Marconi, Brazil). The organic extract was purified dispersive solid-phase extraction (d-SPE) with octadecyl ($C_{18,}$ Macherey-Nagel, Germany) and $MgSO_4$ (Vetec, Brazil) and centrifugation at 3000 rpm for 5 minutes (Thermo Fisher, USA). Samples were completely evaporated under a nitrogen stream and reconstituted in mobile phase B of liquid chromatographic analysis (acetonitrile with 0.1% acetic acid and 4 mM ammonium acetate).

Analyses were carried out in a liquid chromatography (LC, Shimadzu, Japan) coupled to electrospray ionization quadrupole flight time mass spectrometry (ESI-QTOFMS, Bruker Daltonics, micrOTOF-Q III model, Germany) and a C_{18} column (Kinetex Core-Shell Technology 2.6 µm F5 100 A, USA) at a flow rate of 0.4 mL min^{-1} and a column temperature of 35 °C. Ultrapure water (phase A) and acetonitrile (phase B) both containing 0.1%

acetic acid and 4 mM ammonium acetate were used as mobile phase in the following gradient of phase B: 0 – 4 min 40%; 4 – 5 min 44.6%; 5 – 9 min 63%; 9 – 14 min 63%; 14 – 18 80%; 18 – 19 min 80%; 19 – 23 min 40%.

The MS was operated in the multiple reaction monitoring (MRM) mode to identify the ochratoxins, using capillary voltage of 3.5 kV, source and desolvation at 120 and 400 °C, respectively. Standard solutions of the OTA, OTα, OTα methyl-ester and OTα amid were analyzed in both positive and negative ESI modes. The ochratoxins evaluated were listed in Table S1 of Supplementary Material. For each compound, the suspected adducts were: $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M-H]^-$ and $[M+Cl]^-$ (Klitgaard et al., 2014).

The identification of OTA, OTα, OTα methyl-ester and OTα amide was based on retention time, accurate mass and fragmentation pattern (MS/MS) in comparison to the analytical standards analyzed under the same condition. Forms of ochratoxins were identified using these same parameters and elution order in the C18 column compared to the literature data (Bittner, Cramer, Harrer, & Humpf, 2015; González-Arias, Marín, Rojas-García, Sanchis, & Ramos, 2017; Zhang et al., 2016) .

The analytical performance of LC-QTOFMS method was evaluated through linearity, accuracy, intermediate precision (four analyses performed in four different days, *n*=16), repeatability (six analyses performed in the same day, *n*=6), limit of detection (LOD) and quantification (LOQ) according to the International Conference on Harmonization (ICH, 2005).

2.7. Data analysis

Statistical analyses were performed using the statistical software XLSTAT2017 (Addinsoft, New York, USA) for Microsoft Excel. Student's t-test was used to compare the fungal colony count of the control grapes (inoculated only with *A. carbonarius*) with that verified in the grapes treated with each strain of *Bacillus*. ANOVA followed by Tukey Test was used to compare the fungal infection of grapes treated with different concentrations of the four strains of *Bacillus*.

3. Results & Discussion

3.1. Bacillus sp. P1 identification through partial genomic sequencing

The 1,447 bp 16S rRNA gene sequence indicated this isolate belongs to the *Bacillus velezensis* species and was deposited in GenBank under the accession number MT299679. The phylogenetic reconstruction (Figure 1) corroborated this result and positioned strain P1 in the same cluster of a well-known biological control and plant growth promotion features strain, *Bacillus velezensis* FZB42 (Fan et al., 2018). *Bacillus velezensis* FZB42 was isolated from beet rhizosphere in 1998 and has been genome sequenced in 2007. The strain FZB42 is successfully used as biofertilizer and biocontrol bacteria in agriculture being especially efficient against fungal and bacterial pathogens as reported for potato, cotton, strawberry, wheat, lettuce and tomato. This strain exhibited biocontrol activity *in vitro* against *Fusarium graminearum* in corn and wheat (Fan et al., 2018). However, no study has been performed on the potential of this strain to inhibit *A. carbonarius* and mycotoxin production.

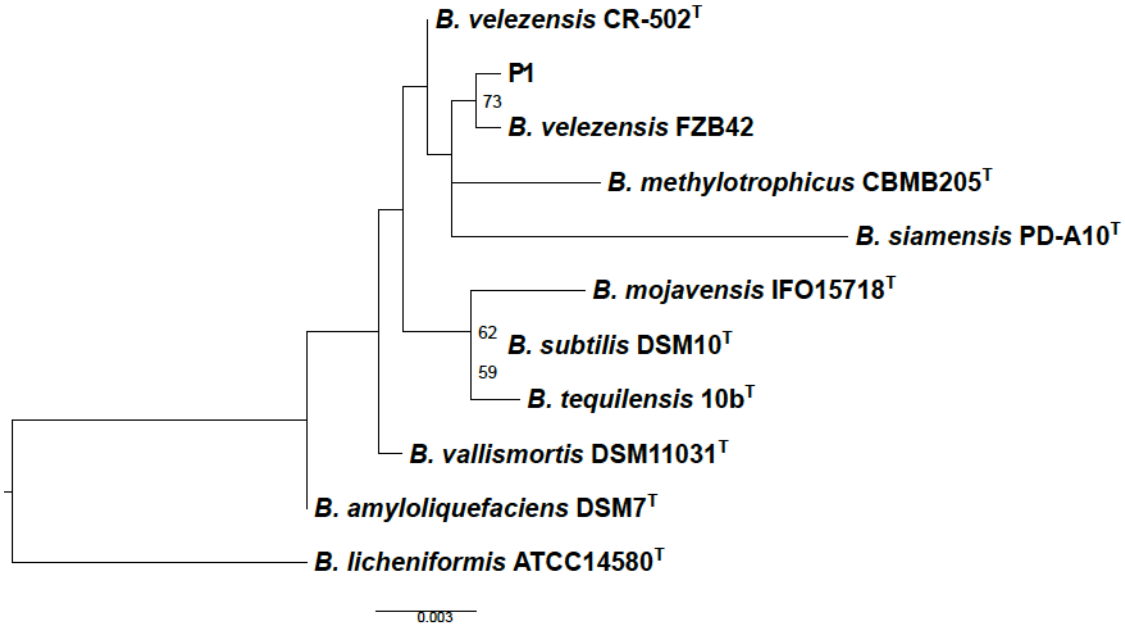

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequence (1,447 bp) of *Bacillus* sp. P1 and the type strains of the most closely related species. The tree was constructed using the Maximum Likelihood method with a bootstrap of 1,000 replicates. Bootstraps below 50 are not shown. *Bacillus licheniformis* ATCC14580^{T} was set as the outgroup. The superscript "T" denotes the sequence is from the type strain for the species. The scale bar represents the expected mean number of nucleotide substitutions per site.

3.2. Effect of Bacillus strains on the A. carbonarius growth in grapes

Figure 2A shows the percentage of grape berries that showed no *A. carbonarius* growth when treated with different concentrations (10^{6} – 10^{9} CFU mL^{-1}) of each Bacillus strain. *A. carbonarius* was verified in all grapes treated with the lowest concentration (10^{6} CFU mL^{-1}) of the four strains of *Bacillus*. Biocontrol activity started from 10^{7} CFU mL^{-1} for P1, P7 and P11 strains, reaching its best performance at 10^{9} CFU mL^{-1} with the P1 strain. For P45 strain, the biocontrol activity was verified only at the highest concentration (10^{9} CFU mL^{-1}). The P1 strain inhibited *A. carbonarius* in all berries at 10^{9} CFU mL^{-1}, whereas the grapes treated with P7, P11 and P45 presented no fungal growth in 58, 74 and 25% of the berries.

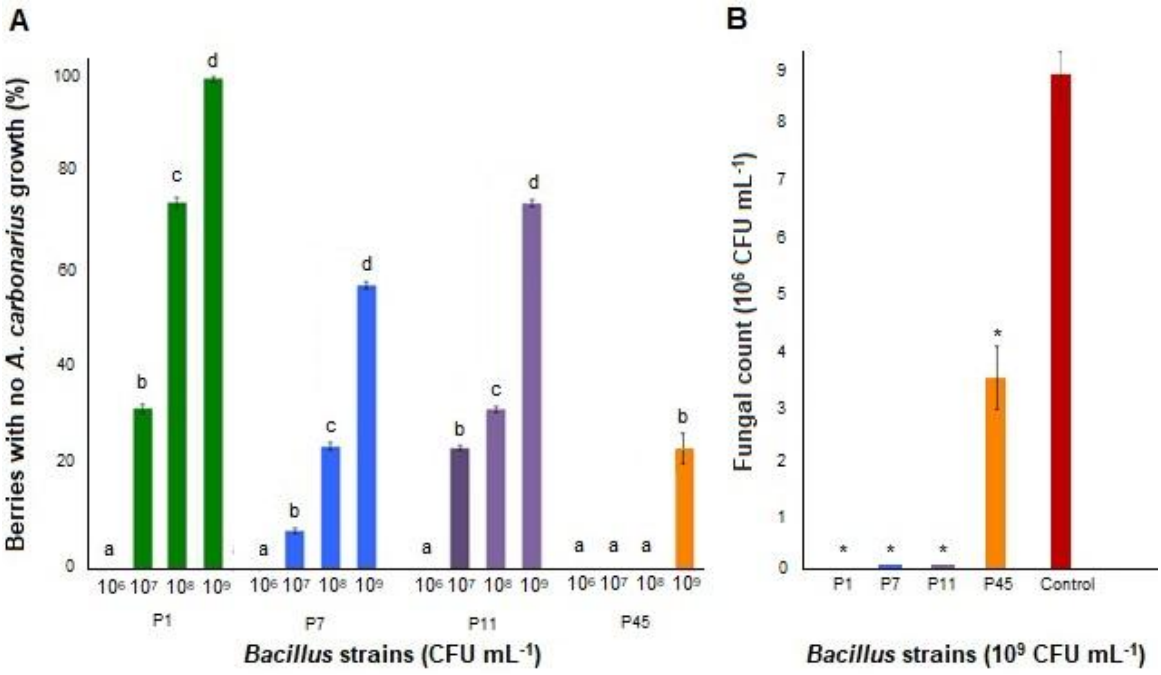

Figure 2. Effect of the *Bacillus* strains (P1, P7, P11 and P45) on the *Aspergillus carbonarius* growth in Chardonnay grapes. (A) Percentage of grape berries with no *A. carbonarius* growth when treated with different concentrations (10^6 – 10^9 CFU mL^{-1}) of each *Bacillus* strain. For each strain, different letters indicate a significant difference ($p < 0.05$) according to ANOVA followed by Tukey Test. (B) Counting of fungal colonies verified in berries treated with the *Bacillus* strains (10^9 CFU mL^{-1}). The control refers to the grapes inoculated only with *A. carbonarius* (10^3 spores mL^{-1}). Asterisk indicates significant difference between the grapes treated with *Bacillus* and the control grapes ($p < 0.05$ according to the Student's t-test).

Figure 2B presents the count of fungal colonies verified in berries treated with the *Bacillus* strains (10^9 CFU mL^{-1}). All *Bacillus* strains caused a significant reduction ($p < 0.05$ according to Student's t-test) in the counting of fungal colonies compared to the control grapes (grapes inoculated with *A. carbonarius*). Strains P1, P7 and P11 were the most efficient to reduce the count of fungal colonies. The berries treated with the P1 strain presented no fungal colonies (100% reduction), while both P7 and P11 strains caused a

reduction of around 95% on the fungal count. The lowest percentage of reduction in fungal colony count was found for the P45 strain (61% reduction).

The concentration of *Bacillus* cells (10^9 CFU mL^{-1}) proposed in the present study to inhibit the growth of *A. carbonarius* is similar to that used in commercial biopesticides. According to the manufacturers' information, these products contain at least 10^9 CFU g^{-1} of *Bacillus subtilis* QST-713 (Serenade®) or *Bacillus pumilus* QST 2808 (Sonata®), which are produced by Bayer. Other commercial biopesticides contain more than 5 x 10^{10} CFU g^{-1} of *Bacillus amyloliquefaciens* D-747 (Eco-shot®, Ihara), *Bacillus subtilis* GB03 (Companion®, Growth Products) or *Bacillus amyloliquefaciens* MBI 600 (5.5 x 10^{10} viable spores g^{-1}, Duravel®, BASF). It is important to mention that none of these commercially available products are proposed by the manufacturers to control *A. carbonarius*.

Jiang et al. (2014) reported that *Bacillus subtilis* CCTCCM207209 (10^8 CFU mL^{-1}), obtained from a Chinese Culture Collection Center, reduced the rot damage ratio caused by *A. carbonarius* in 94, 92, and 12% for Red Earth, Kyoho, and Thompson Seedless grapes, respectively. Therefore, the *Bacillus* inhibitory effect may differ according to grape cultivar. Differences in nutrients, pH, and other conditions between the grape cultivars might be the intrinsic factor affecting the diversity of the inhibitory effect of *B. subtilis* on the contamination of *A. carbonarius* in grapes (Jiang et al., 2014). In addition, the thickness and hardness of grape skins are the key factors for the colonization of *A. carbonarius*. Cultivars with lower skin thickness and hardness, as the Chardonnay grapes under study, are the most susceptible to fungal colonization

than other cultivars. Therefore, controlling the *A. carbonarius* growth becomes more challenging in cultivars with these skin characteristics (Veras et al., 2020).

3.3. Inhibitory effect of Bacillus on the synthesis of ochratoxins by A. carbonarius in grapes

Table 3 presents the molecular formula, retention time, monoisotopic mass, mass-to-charge ratio (*m/z*) of precursor and product ions of the mycotoxins detected using LC-QTOFMS. All compounds were detected in the positive ESI mode and **Figure S1** presents the fragmentation pattern of the mycotoxins. Six forms of ochratoxin were produced by *A. carbonarius* in Chardonnay grapes after 7 days of incubation at 30°C, including ochratoxin α (OTα), ochratoxin β (OTβ), ochratoxin α methyl-ester (OTα methyl-ester), ochratoxin α amide (OTα amide), N-formyl-Ochratoxin α amide (N-formyl-OTα amide), and OTA. To our knowledge, this is the first time that OTα, OTβ, OTα methyl-ester, OTα amide, N-Formyl-OTα amide are reported in grapes.

Table 3. Molecular formula, retention time, monoisotopic mass, mass-to-charge ratio (*m/z*) of the precursor and product ions of the ochratoxins produced by *Aspergillus carbonarius* in Chardonnay grapes after 7 days of incubation at 30°C and evaluated by liquid chromatography coupled to quadrupole time of flight mass spectrometry (LC-QTOFMS).

Mycotoxin	Molecular formula	T_R (min)[a]	Monoisotopic mass	*m/z* precursor ion and mode	*m/z* product ions
Ochratoxin α (OTα)[b]	$C_{11}H_9ClO_5$	1.0	256.0139	294.9776 $[M+K]^+$	276 $[M+K-H_2O]^+$ 258 $[M+K-2H_2O]^+$ 230 $[M+K-2H_2O-CO]^+$
Ochratoxin β (OTβ)	$C_{11}H_{10}O_5$	1.1	222.0528	245.0426 $[M+Na]^+$	213 $[M+Na-2O]^+$ 195 $[M+Na-2O-H_2O]^+$ 177 $[M+Na-2O-2H_2O]^+$ 149 $[M+Na-2O-2H_2O-CO]^+$
Ochratoxin α methyl-ester (OTα methyl-ester)[b]	$C_{12}H_{11}ClO_5$	1.2	270.0295	253.0268 $[M+H-H_2O]^+$	235 $[M+H-2H_2O]^+$ 210 $[M+H-2H_2O-CH_3]^+$ 133 $[M+H-2H_2O-CH_3-C_6H5]^+$
Ochratoxin α amide (OTα amide)[b]	$C_{11}H_{10}O_4NCl$	1.4	255.0298	273.0642 $[M+NH_4]^+$	241 $[M+NH_4-O_2]^+$ 213 $[M+NH_4-C_2H_4]^+$ 195 $[M+NH_4-C_2H_4-H_2O]^+$ 177 $[M+NH_4-C_2H_4-2H_2O]^+$
N-Formyl-Ochratoxin α amide (N-Formyl-OTα amide)	$C_{12}H_{10}O_5NCl$	1.5	283.0247	284.0326 $[M+H]^+$	266 $[M+H-H_2O]^+$; 238 $[M+H-H_2O-CO]^+$; 162 $[M+H-2H_2O-2CO-CH_2NH_2]^+$
Ochratoxin A (OTA)[b]	$C_{20}H_{18}ClNO_6$	2.5	403.0823	404.0901 $[M+H]^+$	386 $[M+H-H_2O]^+$; 358 $[M+H-H_2O-CO]^+$; 257 $[M+H-H_2O-CO-CO_2-H_2O-C_3H_3]^+$; 239 $[M+H-2H_2O-CO-CO_2-H_2O-C_3H_3]^+$

[a] Retention time in minutes on a C18 column and solvent: gradient of water and acetonitrile with 0.1% acetic acid and 4 mM ammonium acetate; [b] identification using analytical standard.

The effect of different *Bacillus* strains on ochratoxins synthesis by *A. carbonarius* in grapes is presented in **Table 4**. Ochratoxins were quantified as OTA equivalent using an analytical curve (y=317673-6255.1) obtained for OTA from 0.125 to 20 µg L^{-1}. The analytical curve displayed adequate linearity with coefficient of determination (R^2) of 0.9998. The LOD (0.01 µg kg^{-1}) and LOQ (0.05 µg kg^{-1}) showed that the method is sufficiently sensitive to quantify ochratoxins in grapes, since these values are, respectively, 200 and 40 times lower than the maximum limit allowed for OTA in grape products (2 µg kg^{-1}) by European Commission (2006) and Brazil (2011). Furthermore, the relative standard deviation obtained in repeatability and intermediate precision assays were lower than 3 and 8%, respectively; while a suitable recovery was obtained (98%), indicating the quantitative efficiency of the LC-QTOFMS method according to the guidelines of International Conference on Harmonization (ICH, 2005).

Table 4. Effect of the *Bacillus* strains (10^9 CFU mL^{-1}) on the synthesis of forms of ochratoxins in grapes inoculated with *A. carbonarius* (10^3 spores mL^{-1}).

Grape treatment	**Mycotoxin levels ± standard deviation (μg kg^{-1})[a]**						
	Ochratoxin α (OTα)	Ochratoxin β (OTβ)	Ochratoxin α methyl-ester (OTα methyl-ester)	Ochratoxin α amide (OTα amide)	N-Formyl-Ochratoxin α amide (N-Formyl-OTα amide)	Ochratoxin A (OTA)	**Total (μg kg^{-1})[b]**
Control grapes - *A. carbonarius*[c]	43.20 ± 3.20^{A}	16.58 ± 1.60^{A}	18.40 ± 1.50^{A}	5.17 ± 0.20^{A}	27.49 ± 2.20^{A}	15.21 ± 1.27^{A}	126.05
Bacillus strains[d]							
P1	ND^{C}	ND^{C}	ND^{C}	ND^{B}	ND^{C}	ND^{B}	ND
P7	ND^{C}	ND^{C}	ND^{C}	ND^{B}	ND^{C}	ND^{B}	ND
P11	0.47 ± 0.03^{B}	0.42 ± 0.02^{B}	ND^{C}	ND^{B}	ND^{C}	ND^{B}	0.89
P45	0.35 ± 0.04^{B}	0.28 ± 0.03^{B}	11.03 ± 1.00^{B}	ND^{B}	19.34 ± 1.92^{B}	ND^{B}	31.00

[a] Quantified as OTA equivalent; [b] Total of ochratoxins; [c] Control grapes inoculated with *A. carbonarius*; [d] Grapes inoculated with *A. carbonarius* and treated with *Bacillus* strains; ND: Not detected (values lower than limit of detection of the LC-QTOF method, 0.01 μg kg^{-1});
Different capital letters in the columns indicate statistically significant differences between groups by ANOVA followed by Tukey test ($P < 0.05$).

All *Bacillus* strains inhibited the synthesis of OTA, which is the most toxic form of ochratoxin. As previously mentioned and shown in **Figure 2**, the grapes treated with *Bacillus* P1 showed no fungal growth. Accordingly, no ochratoxins were found when this strain was used on grapes as shown in **Table 4**. *Bacillus* P7 also inhibited the synthesis of all ochratoxins, which were found in control grapes. However, some other forms of ochratoxins were found when the grapes were treated with these strains. In grapes treated with *Bacillus* P11, OTα and OTβ were detected. Similar levels of these ochratoxins were found in grapes treated with Bacillus P45, in addition other forms of ochratoxins were detected as OTα methyl-ester and N-formyl-OTα amide. Although the strains of *Bacillus* P11 and P45 did not inhibit the synthesis of all ochratoxins, the levels found in the grapes treated with these strains were lower than those found in the control grapes.

The α and β ochratoxins produced by *A. carbonarius* in Chadonnay grapes (**Table 4**) are included in the biosynthetic pathways of OTA, as summarized in **Figure S2**. *Bacillus* P11 and P45 seem to interfere in the OTA biosynthetic rote, since some intermediate compounds of this pathway were detected in grapes treated with these strains, while OTA was not found. Inhibition of OTA synthesis was also verified when epiphytic bacteria (*B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29) were used as a biocontrol strategy for *A. niger* Taify grape berries. However, other ochratoxins have not been evaluated (El-Shanshoury et al. 2018).

4. Conclusions

Bacillus strains P1, P7 and P11, P45 showed potential to be used as a biological control agent to prevent or reduce the *A. carbonarius* growth and the occurrence of ochratoxins. The synthesis of ochratoxin A, the most toxic form of ochratoxin, was inhibited by the four strains of *Bacillus*.

P1 was successfully identified as *Bacillus velezensis* and it was the most promising strain, since grapes treated with this strain showed no fungal growth and consequently no ochratoxin was found. Regarding *Bacillus* P7, although some fungal growth was observed in the grapes, the ochratoxins were not detected. Despite some forms of ochratoxin (ochratoxin α, ochratoxin β, ochratoxin α methyl-ester, ochratoxin α amide and N-formyl ochratoxin α amide) were found in grapes treated with *Bacillus* P11 and P45, the levels were significantly lower than those found in control grapes, also indicating the efficiency of these strains in reducing the synthesis of ochratoxins.

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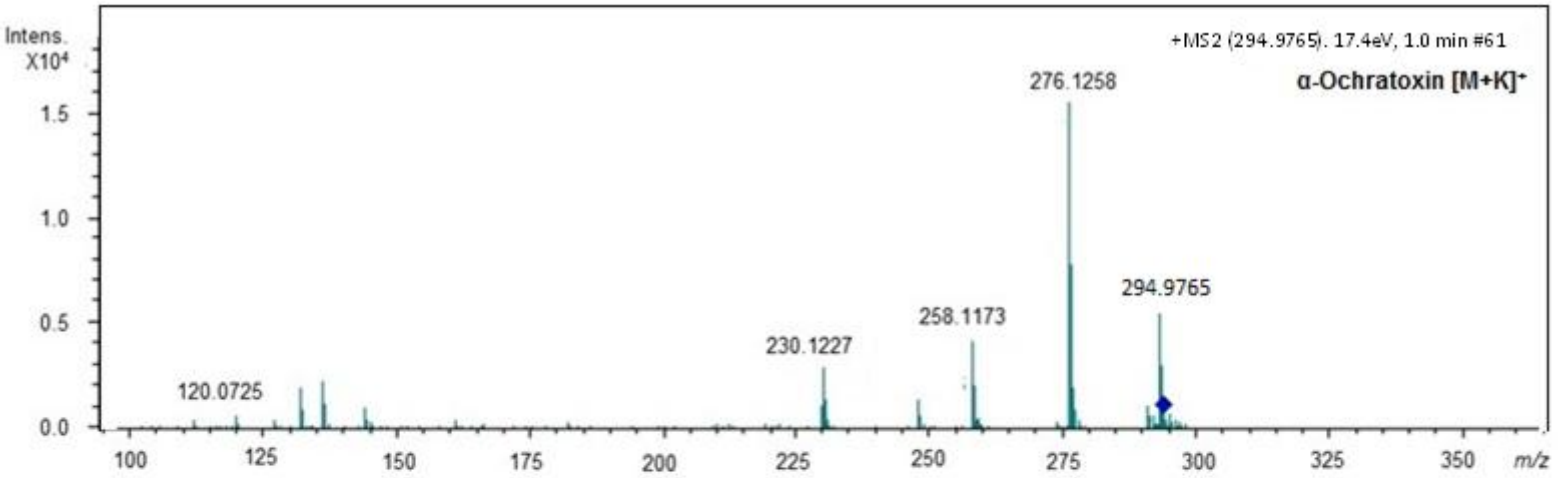
Supplementary Material

Table S1. Monoisotopic mass of the forms of ochratoxins evaluated using LC-ESI-QTOFMS.

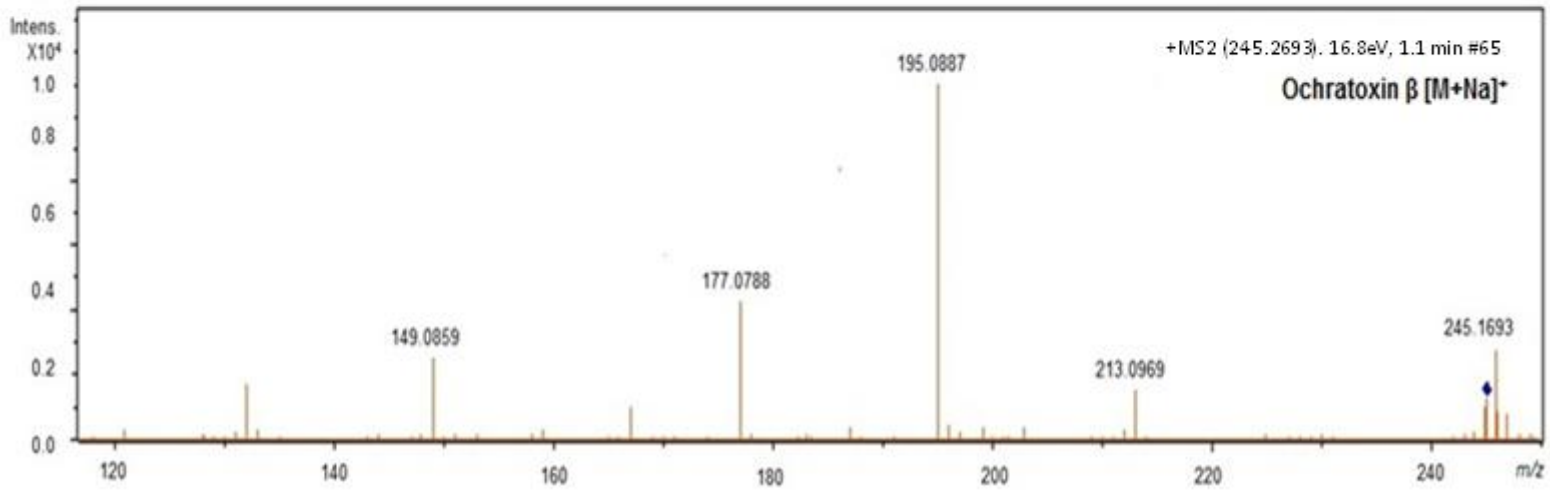
Compound	Molecular Formula	Monoisotopic mass (g mol^{-1})	Reference
Ochratoxin A	$C_{20}H_{18}ClNO_6$	403.0823	[1]
Ochratoxin β	$C_{11}H_{10}O_5$	222.0528	[2–4]
Ochratoxin α amide	$C_{11}H_{10}O_4NCl$	255.0298	[5]
α-Ochratoxin	$C_{11}H_9ClO_5$	256.0139	[2–4]
α-Ochratoxin methyl ester	$C_{12}H_{11}ClO_5$	270.0295	[6]
N-formyl-ochratoxin α amide	$C_{12}H_{10}O_5NCl$	283.0247	[7]
Ochratoxin α ethyl-ester	$C_{13}H_{13}ClO_5$	284.0451	[8]
14-Decarboxy-ochratoxin A	$C_{19}H_{18}O_4NCl$	359.0924	[4,5]
Ochratoxin B	$C_{20}H_{19}NO_6$	369.1212	[2–4]
Ochratoxin quinone	$C_{20}H_{17}NO_7$	383.1005	[3]
Ochratoxin B methyl-ester	$C_{21}H_{21}NO_6$	383.1369	[2–4,6]
Ochratoxin A hydroquinone	$C_{20}H_{19}NO_7$	385.1162	[3,4]
Ochratoxin B etil-ester	$C_{22}H_{23}NO_6$	397.1525	[2,3]
Ochratoxin A methyl-ester	$C_{21}H_{20}ClNO_6$	417.0979	[8]
4-Hidroxyochratoxin A	$C_{20}H_{18}ClNO_7$	419.0772	[2–4,6]
Ethylamide ochratoxin A	$C_{22}H_{23}ClN_2O_5$	430.1296	[3]
Ochratoxin A ethyl-ester	$C_{22}H_{22}ClNO_6$	431.1135	[8]
Ochratoxin C	$C_{22}H_{22}ClNO_6$	431.1136	[2–4]
Hydroxy-ochratoxin A methyl ester	$C_{21}H_{20}ClNO_7$	433.0928	[6]
Ochratoxin A glucose ester	$C_{26}H_{28}ClNO_{11}$	565.1351	[3,5]
Ochratoxin A-methyl-α-glucopyranoside ester	$C_{27}H_{30}ClNO_{11}$	579.1507	[9]
Ochratoxin A cellobiose ester	$C_{32}H_{38}ClNO_{16}$	727.1879	[6]
Ochratoxin A diglucoside	$C_{32}H_{38}ClNO_{16}$	727.1879	[6]

(Ochratoxin A diglucoside ester)			

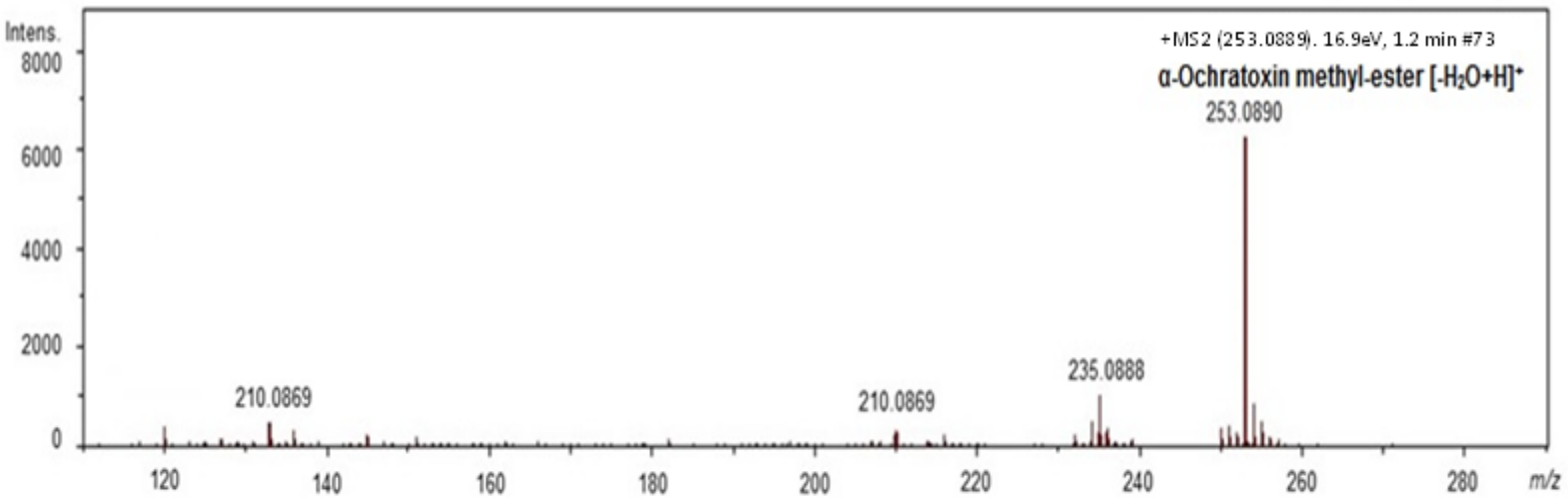
(a)


Intens.
X10⁴
1.5
1.0
0.5
0.0
+MS2 (294.9765). 17.4eV, 1.0 min #61
α-Ochratoxin [M+K]⁺
276.1258
294.9765
258.1173
230.1227
120.0725
100
125
150
175
200
225
250
275
300
325
350
m/z

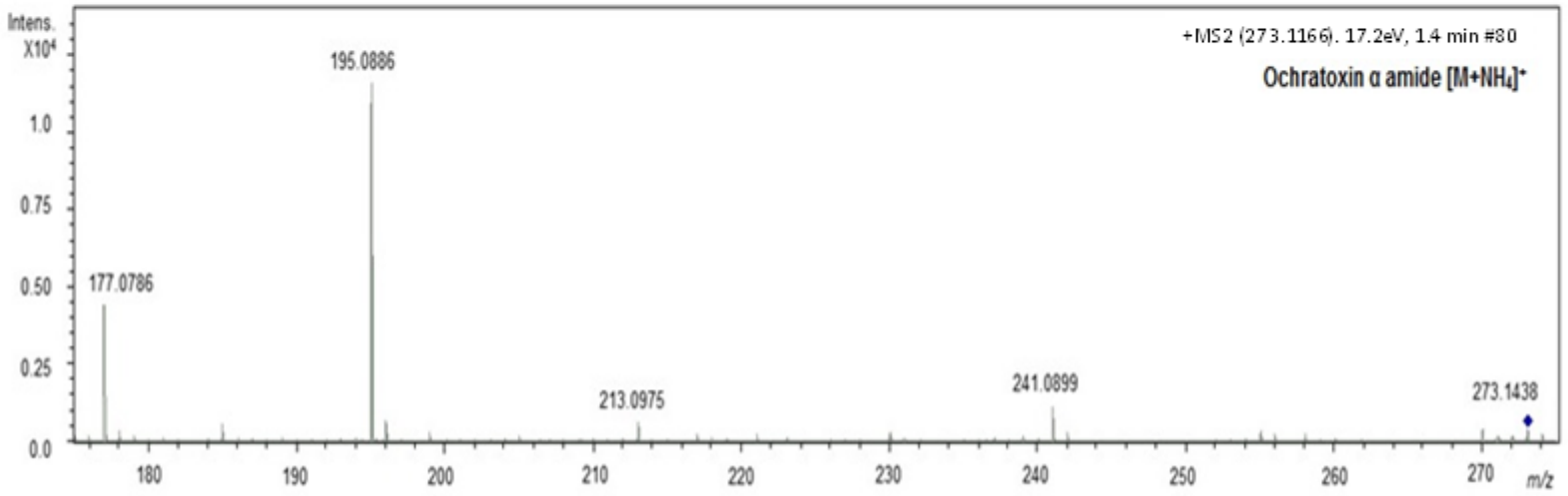
(b)


Intens.
X10⁴
1.0
0.8
0.6
0.4
0.2
0.0
+MS2 (245.2693). 16.8eV, 1.1 min #65
Ochratoxin β [M+Na]⁺
195.0887
177.0788
149.0859
245.1693
213.0969
120
140
160
180
200
220
240
m/z

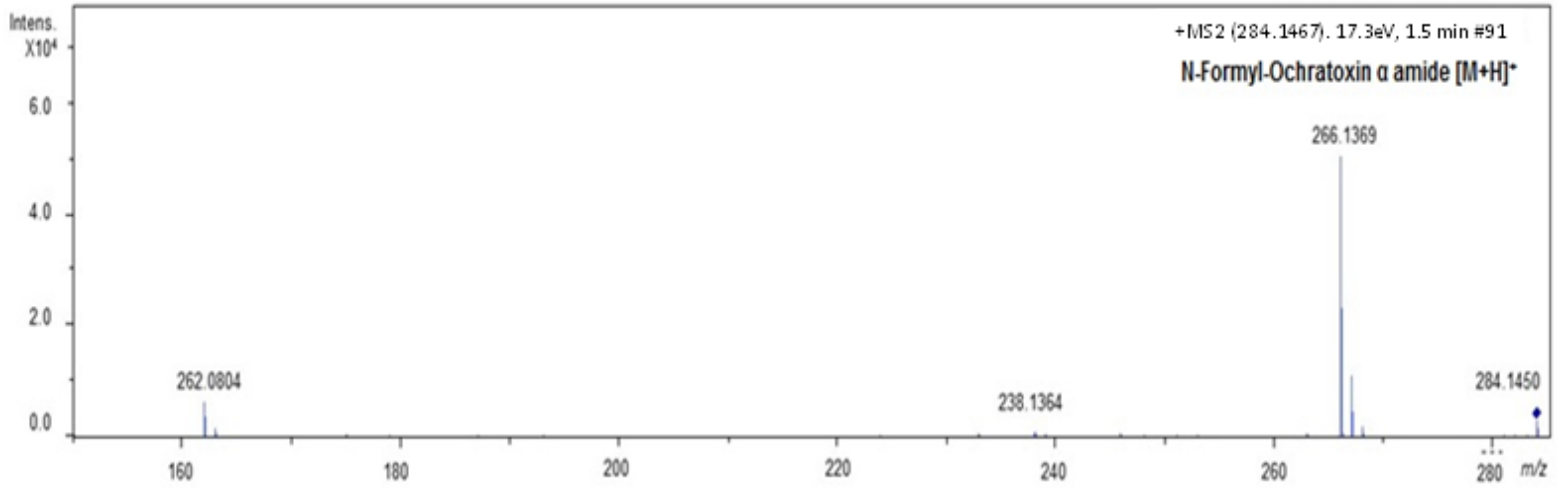
(c)


Intens.
8000
6000
4000
2000
0
+MS2 (253.0889), 16.9eV, 1.2 min #73
α-Ochratoxin methyl-ester [-H2O+H]+
253.0890
235.0888
210.0869
210.0869
120
140
160
180
200
220
240
260
280
m/z

(d)


Intens.
X10^4
1.0
0.75
0.50
0.25
0.0
+MS2 (273.1166), 17.2eV, 1.4 min #80
Ochratoxin α amide [M+NH4]+
195.0886
177.0786
213.0975
241.0899
273.1438
180
190
200
210
220
230
240
250
260
270
m/z

(e)


Intens.
X10⁴
6.0
4.0
2.0
0.0
+MS2 (284.1467), 17.3eV, 1.5 min #91
N-Formyl-Ochratoxin α amide [M+H]⁺
266.1369
262.0804
238.1364
284.1450
160
180
200
220
240
260
280
m/z

(f)

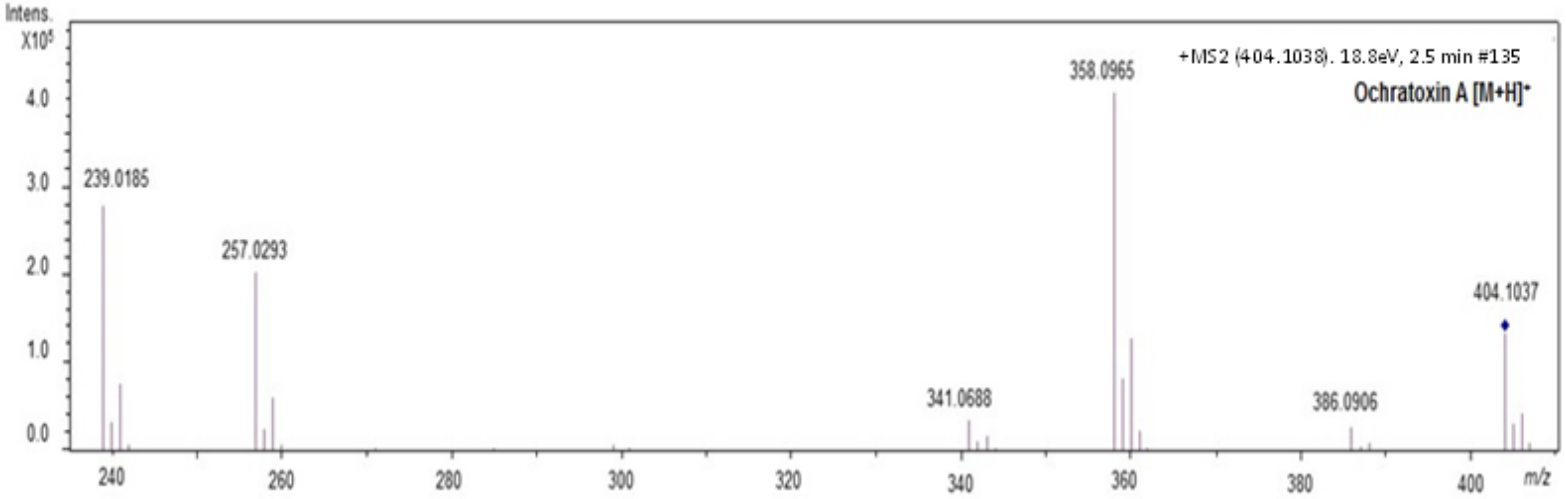

Intens.
X10⁵
4.0
3.0
2.0
1.0
0.0
+MS2 (404.1038), 18.8eV, 2.5 min #135
Ochratoxin A [M+H]⁺
358.0965
239.0185
257.0293
404.1037
341.0688
386.0906
240
260
280
300
320
340
360
380
400
m/z

Figure S1. Mass spectra showing the fragmentation pattern of the ochratoxins produced by *Aspegillus carbonarius* (10^3 spores mL^{-1}) in Chardonnay grapes incubated for 7 days at 25°C and analyzed by LC-ESI-QTOFMS: **(a)** α-Ochratoxin $[M+K]^+$, **(b)** Ochratoxin β $[M+Na]^+$, **(c)** α-Ochratoxin methyl-ester $[-H_2O+H]^+$, **(d)** Ochratoxin α amide $[M+NH_4]^+$, **(e)** N-Formyl-Ochratoxin α amide $[M+H]^+$ and **(f)** Ochratoxin A $[M+H]^+$. The blue diamond (♦) represent the precursor ion (*m/z*)

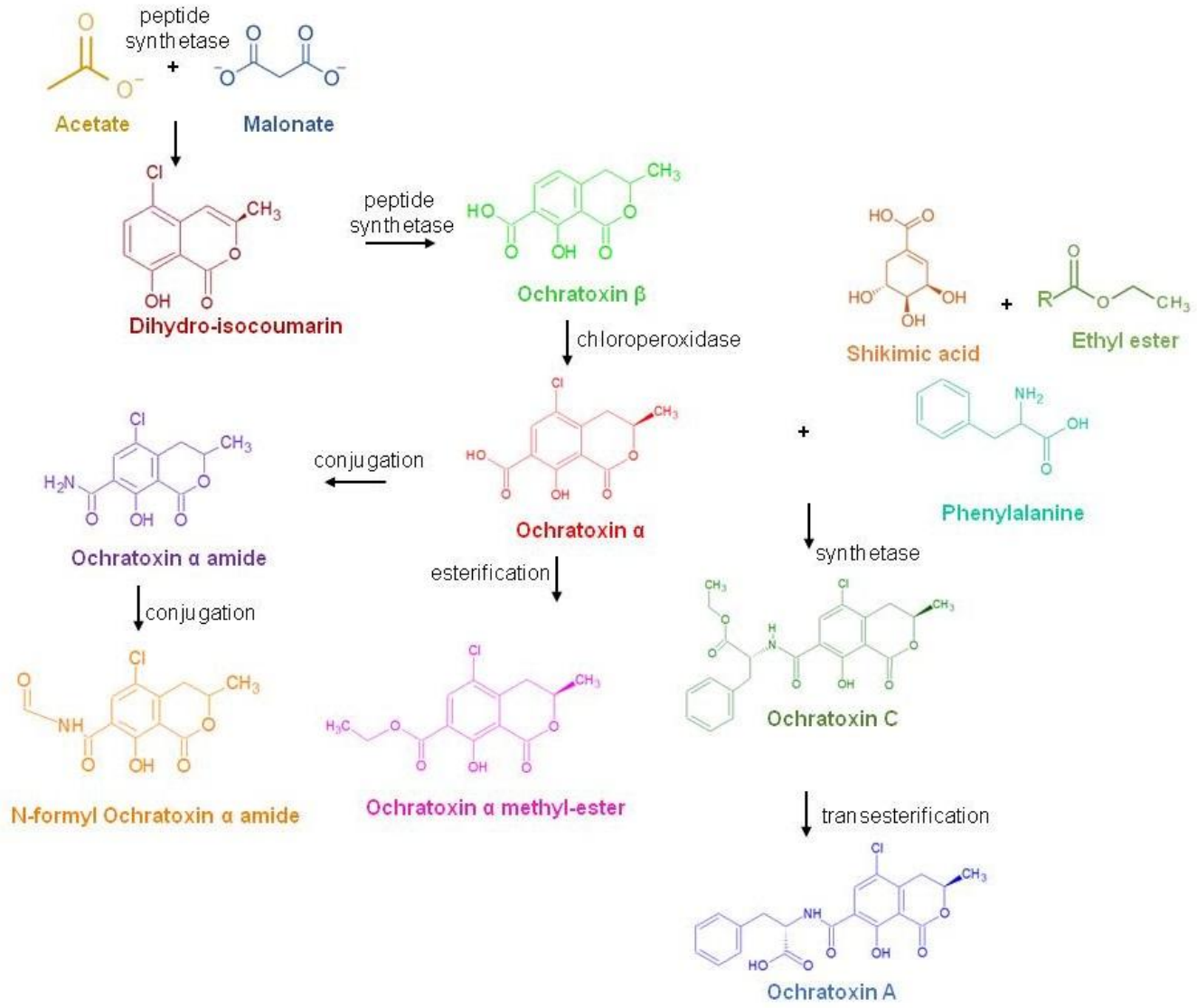


Figure S2. Metabolic pathways of ochratoxins synthetized by *Aspergillus carbonarius* according to Gallo et al. (2012); Harris & Mantle (2001); Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman (2016) and Ringot, Chango, Schneider, & Larondelle (2006).

References:

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3. Malir F, Ostry V, Pfohl-Leszkowicz A, Malir J, Toman J: **Ochratoxin A: 50 years of research**. *Toxins (Basel)* 2016, **8**:12–15.
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6 ARTIGO ORIGINAL 2

Volatile profile of the must of Chardonnay grapes treated with *Bacillus velezensis* as a biocontrol strategy for *Aspergillus carbonarius* and Ochratoxin

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ABSTRACT

Conventional agronomic practices can negatively affect physico-chemical characteristics of grapes, including its volatile profile, besides not being sustainable for the environment. That is one of the reasons why the interest in biological control of plant diseases has significantly increased. An example is the use of *Bacillus* strains that are producers of a wide array of antagonistic compounds like lipopeptides and volatiles. In a previous study, *Bacillus velezensis* P1 was the most promising strain presenting 100% inhibition of *Aspergillus carbonarius* development and Ochratoxin A synthesis in Chardonnay grapes. The aim of this study was to evaluate for the first time the effect of *B.velezensis* P1 on the volatile profile of Chardonnay grape must. Grape samples inoculated with *B.velezensis* P1 showed the highest level of compounds with pleasant odors, like: 3-hexenoic acid (sweet odor), ethyl-3-methylbutanoate (fruity odor) and nonanal (floral odor). Furthermore, a high level of terpene group compounds were identified in samples inoculated with *A.carbonarius* and *Bacillus* strain, which might be justified by glycosidases synthesis by *Bacillus* P1. Grapes inoculated only with *A.carbonarius* were identified compounds that indicates its presence (hexanoic acid and α-terpineol). Those compounds occur in response to pathogens and may be involved in

defense strategies. *B.velezensis* P1 significantly improved the odor and taste of Chardonnay grapes without bringing unpleasant characteristics.

Key words: grape, volatile, *Bacillus*, *Aspergillus carbonarius*, biocontrol

1. INTRODUCTION

The interest in biological control of plant diseases has significantly increased, due to the need for introduction of more environmentally friendly alternatives to the exacerbated use of chemical pesticides. An example are bacteria from the *Bacillus* genus that are Gram-positive microorganisms and well known as producers of a wide array of antagonistic compounds of different structures, like: lipopeptides, polyketide, bacteriocins, volatiles and siderophores (Fira, Dimkić, Berić, Lozo, & Stanković, 2018). There are commercial biopesticides with *Bacillus* strains in its composition (*B. subtilis, B.amyloliquefaciens, B.pumilus*, and others). They can be applied to different crops, such as: carrot, apple, grape, strawberry and tomato to control the pathogens *Sclerotinia sclerotiorum* (white mold), *Podosphaera fuliginea* (powdery mildew), *Alternaria dauci* (carrot leaf blight), *Botrytis cinerea* (gray mold), *Fusarium oxysporum* (*Fusarium* wilt), and others. It is important to research the potential of new *Bacillus* strains because their constant use, as well as chemical pesticides, can lead to the generation of resistant pathogens. Furthermore, it is recommended by the companies to apply biofungicides in rotation with other biocontrol agents. Also, new strains are important to have a greater spectrum of action against pathogens.

In a previous study (SILVEIRA et al., 2020) four strains of *Bacillus* (identified as P1, P7, P11 and P45) were tested to inhibit the development of *Aspergillus carbonarius* and the synthesis of Ochratoxin A (OTA), including their

modified forms in Chardonnay grapes. All strains showed antifungal activity ranging from 25% to 100% of fungal development inhibition. The suggested mechanisms are lipopeptide synthesis, competition of nutrients and space, endospore and volatile compounds synthesis. *Bacillus* sp. P1 was the most promising strain, inhibiting 100% of fungal development and 100% of OTA synthesis in Chardonnay grape berries, also strain P1 was successfully identified as *Bacillus velezensis*.

Aspergillus carbonarius is the main fungus responsible to produce OTA in grapes (Dachery, Manfroi, Berleze, & Welke, 2015). OTA is a mycotoxin related to genotoxicity, nephrotoxicity, teratogenicity, neurotoxicity and immunosuppression (Cimbalo, Font, & Manyes, 2020). OTA is classified in group 2B of the International Agency for Research on Cancer (IARC, 1993), as a possibly carcinogenic to humans. Brazil (ANVISA, 2011) and the European Union (EFSA, 2006) set a maximum limit (2 μg kg^{-1}) of OTA for wines and grape juices. Furthemore, it is known that the manifestation of *A.carbonarius* affects negatively the volatile profile of grapes and its respective wines, due to the synthesis of acids that are precursor of esters that might impart negative aroma for wine (Dachery et al., 2019).

Volatile profile is one of the most important factors in determining wine character and quality. Part of those molecules is biosynthesized in berries, and the other part results from winemaking and aging process. Aroma compounds in plants are found as free or bound (glycosylated) to a sugar moiety. Glycosylated volatile compounds have little or no active odor. Another factor that affects grapes volatile profile is agronomic practices, in two ways: the biosynthesis of the molecules change as a result of the agronomic practice (directly), and variation in the

concentration of the molecules due to changes in fruit volume and weight (Alem, Rigou, Schneider, Ojeda, & Torregrosa, 2019)

Bacteria in general, including *Bacillus* strains, release a high diversity of volatile secondary metabolites including hydrocarbons, ketones, alcohols, sulfur and nitrogen containing compounds, terpenes, and others. These metabolites are primarily considered as infochemicals but they also act as antimicrobial or antifungal agents (Kai, 2020). Few studies have evaluated the effect of *Bacillus* strains with biocontrol activity on the volatile profile of food matrices. Gao, Li, Xu, Zeng, & Guan, (2018) showed that benzothiazole synthesized by *B. subtilis* CF-3 was the volatile compound with the strongest inhibitory effect on the mycelial growth of *Monilinia fruticola* and *Colletotrichum gloeosporioides* in peaches and lichi fruit. Gu, Chen, Fang, Wu, & Tan, (2015) reported that *B.subtilis* and *B.vanillea* are able to produce the enzyme β-D-glucosidase that participates in the formation of vanillin, an aldehyde, through the hydrolysis of glucovaniline in vanilla beans. In a study conducted by Jeong et al.(2017). *B.licheniformis* was used in soybean to produce a fermented paste (doenjang). The main volatiles produced were the alcohol oct-1-en-3-ol and the carbide 3-hydroxybutan-2-one. Escribano-Viana et al. (2018) sprayed the commercial biofungicide Serenade Max (*Bacillus subtilis* QST 713) in Tempranillo grapes in two moments: 21 days and 3 days before the harvest. After, the vinification process was carried out through spontaneous alcoholic an malolctic fermentation. The application of the fungicide reduced 7% of the amount of the alcohols such as 1-hexanol. It was concluded that the treatment with the biofungicide did not negatively influence the quality of the grapes or wine, and that it was positive to malolactic fermentation improving the implantation of the bacteria used in this process.

The aim of this study was to evaluate for the first time the effect of a *Bacillus* strain with biocontrol activity in the volatile profile of Chardonnay grape must. Also, this is the first time that the effect of *Bacillus* and *A.carbonarius* mutual inoculation and the effect of the *Bacillus velezensis* P1 itself in the volatile profile of a food matrix was verified.

2. MATERIAL AND METHODS

2.1. Grapes

Vitis vinifera grapes (cultivar Chardonnay) harvested in 2019 in Canela (29°21'56.4"S 50°46'06.6"W), Rio Grande do Sul, Brazil were sanitized with 1% solution of sodium hypochlorite for three minutes. Then, were rinsed with sterile distilled water and left to dry in the laminar flow for one hour before the microorganisms inoculation, as described by Lappa et al. (2018).

2.2. Sample preparation

Bacillus Velezensis (sp. P1), strain reported as most promising for biocontrol of *Aspergillus carbonarius* in grapes in a previous study (SILVEIRA et al., 2020) was cultivated in Brain-Heart infusion (BHI) at 37^{o}C. Cell suspension was made as follows: BHI broth was placed in Falcon tubes and centrifuged for 15 minutes at 10,000 rpm and 4 ^{o}C. The final concentration was adjusted to 10^{9} CFU mL^{-1} in saline water.

Aspergillus carbonarius ITAL293 was cultivated in Potato dextrose agar (PDA) medium (infusion from potato, dextrose and agar) for 7 days at 25^{o}C. For the inoculation of grape berries, a spore suspension was made from this culture and a 0.1% Tween 80 solution was used to remove spores from the Petri dish. The spore count was performed in the Neubauer chamber and the concentration of the suspension was adjusted to 10^{3} spores mL^{-1}.

Grapes samples were immersed for 2 minutes in the *Bacillus* cell suspension and left to dry for 1 hour. After, the berries were immersed in spore suspension for the same time and dried for another hour (Figure 1). As positive control (grapes inoculated only with *A. carbonarius*), the *Bacillus* suspensions were replaced by distilled water.

Negative control (no inoculated microorganism) was prepared replacing both bacterial and fungal suspensions by distilled water. To evaluate the effect of the *Bacillus* strain grapes were inoculated only with it. Grapes were placed in Petri dishes and kept in an incubator at 30°C for 7 days. Humidity was maintained using cotton moistened with distilled water.

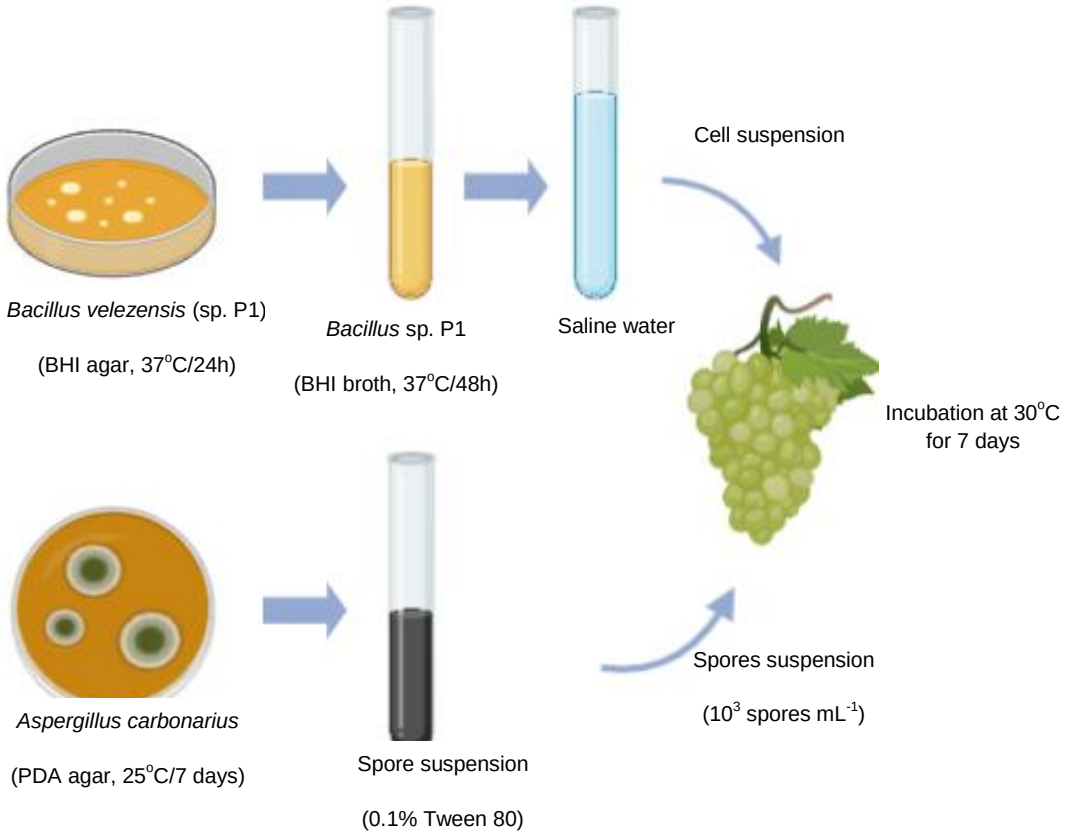

Figure 1. Representation of Chardonnay grapes inoculation with *Bacillus velezensis* sp. P1 (10^9 CFU mL^{-1}) and *Aspergillus carbonarius* (10^3 spores mL^{-1}). After 7 days of incubation at 30°C the effect of the inoculations on the volatile profile of grapes was evaluated by GC/qMS.

2.3. *Determination of profile volatile of grapes*

Volatile compounds were extracted by headspace solid phase microextraction (HS-SPME) with a 2 cm divinylbenzene/carboxen/polydimethylsiloxane fiber (50/30 μm DVB/CAR/PDMS, Supelco, Bellefonte, USA) according to a previous optimized protocol: 1 mL of sample, 30% of NaCl (w/v, Nuclear, São Paulo, Brazil), for 45 min at

55 °C (Welke, Zanus, Lazarotto, Schmitt, & Zini, 2012). Analyses were performed in a Shimadzu gas chromatograph with quadrupole mass spectrometric detection (GC/qMS, QP2010, Kyoto, Japan) coupled to an autosampler (CTC CombiPAL, Zwingen, Switzerland). Compounds were separated with the following columns (30 m × 0.25 mm × 0.25 μm; J&W Scientific Inc., Folsom, USA): a 5% diphenyl-95% dimethyl polysiloxane (nopolar; DB-5) and a polyethyleneglycol (polar; DB-Wax). Other chromatographic conditions of analysis were the same described by Soares et al. (2015).

Identification of the compounds was carried through comparisons of retention data and mass spectra of standard compounds and those found in samples. Standard compounds (Aldrich, Steinheim, Germany; purity higher than 98%) used in the identification were indicated in Table S1. For unavailable analytical standards, tentative identification was achieved when retention indices (RI) reported in literature (RIlit) had not differed more than 18 units from those experimentally obtained (RIexp) using a series of n-alkanes (C9–C24, Supelco) analyzed under the same chromatographic conditions as the samples. In addition, similarity between mass spectrometric information of each chromatographic peak and NIST (National Institute of Standards and Technology, Gaithersburg, USA) mass spectra library was at least 80%.

Internal standards (IS) were used to normalize the area of identified volatile compounds, having in mind that their chemical structure should be similar to the analytes: 1-propanol (alcohols), 2-methoxyphenol (phenols), trans-p-menth-6-ene-2,8-diol (terpenes), 2-methylvaleric acid (acids), octanal (aldehydes), 2-heptanone (ketone), phenyl acetate. Former tests were performed to verify their absence in wine samples. These IS were purchased from Sigma. A solution of each IS (1000 mg L^{-1}) was prepared in double distilled ethanol. A solution (10 mg L^{-1}) containing the three IS was prepared in double distilled ethanol and 10 μL of this mix was added to each sample before HS-SPME.

2.4. Statistical analysis

The statistical analysis was performed using XLSTAT2017 (Addinsoft, New York, USA) for Microsoft Excel. Data of volatile compounds were evaluated using Fisher ratio to determine the features that best discriminate the samples and to reduce

the dimension of the original variables before performing hierarchical clustering analysis (HCA) and heat map. Fisher ratios were calculated according to the approach previously applied by Welke et al. (2014), considering the ratio between the variance of the normalized area of a compound verified in the different samples (grape without inoculation, grape inoculated with A. carbonarius, grape inoculated with B. velezensis P1, grape inoculated with A. carbonarius and B. Velezensis P1) and within each sample. Compounds with the highest Fisher ratios are the ones that contributed the most to differentiate the samples and were used in HCA and heat map.

3. Results and discussion

A total of 56 volatile compounds were found in grapes, including 11 esters, 10 alcohols, 6 terpenes, 15 acids, 4 ketones, 6 aldehyde, 2 furans and 2 phenols compounds Table S1 presented the volatile compounds, their RI (experimentally acquired and from literature), odors and Fisher ratios.

Twenty-eight compounds that presented Fisher ratio corresponding to at least 15% of the Fisher ratio value of the most discriminant compound were used to perform the heat map (Figure 2). This approach has been successfully applied in previous studies to discriminate sparkling wines produced with different yeasts (Costa, Nicolli, Welke, Manfroi, & Zini, 2018), wines produced following different agronomic practices (Nicolli et al., 2018), grapes and wines inoculated with *A. carbonarius* (Dachery et al., 2019).

Heat map and hierarchical cluster (Figure 2) analyses were used to visualize the similarities/differences between the volatile profile of the grapes with and without fungal and *B. velezensis* P1 inoculation. These tools help visualizing the contributions of the volatile compounds that differentiate samples. The color scale of the heat map ranging from red, orange and yellow represents higher, medium and lower normalized chromatographic area, respectively. Clusters related to the grouping of samples and volatiles were designated on the upper horizontal axis. Furthermore, on the vertical axis the four major clusters grouped the compounds that presented higher chromatographic areas.

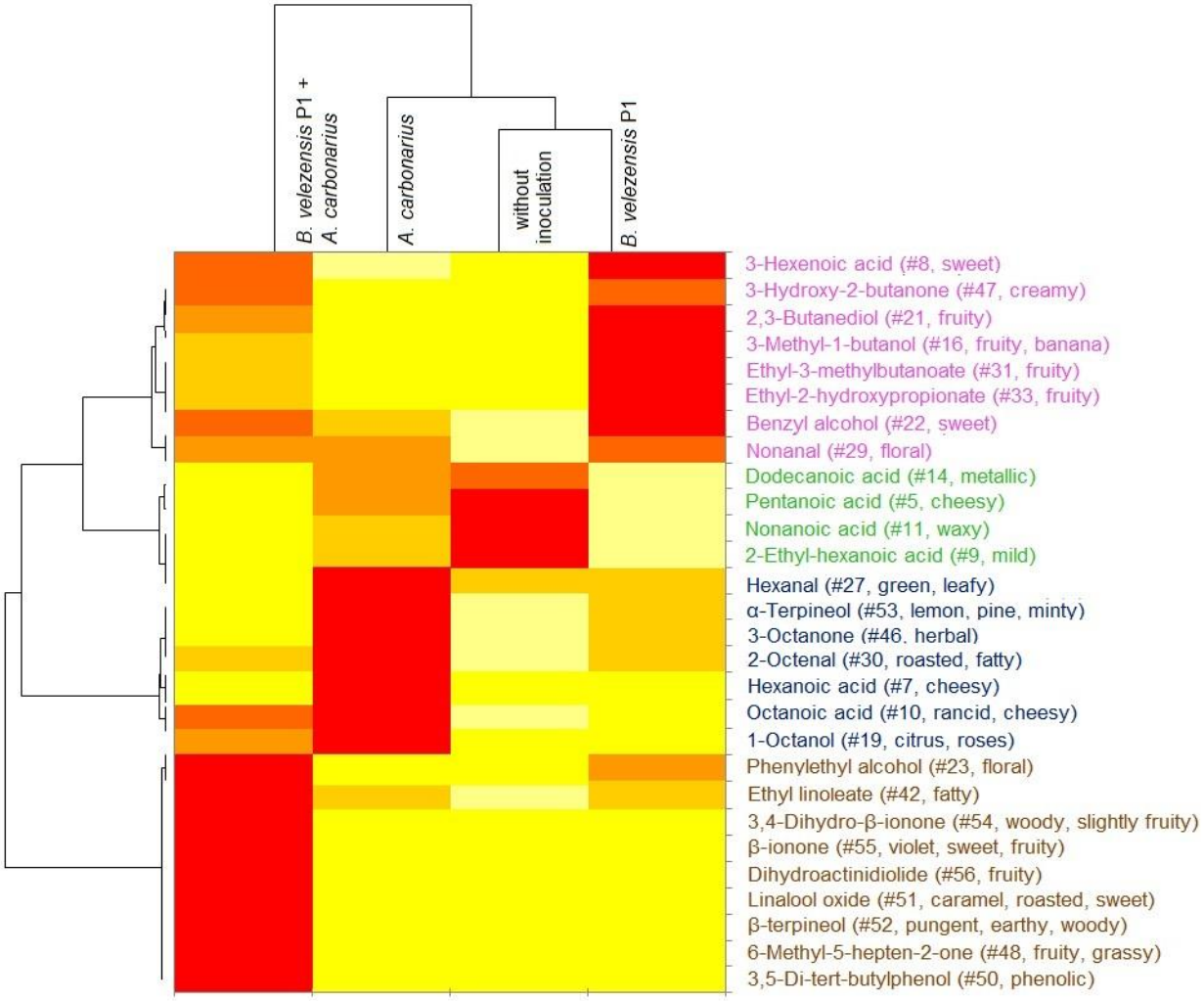

Figure 2. Heat map and hierarchical cluster analysis obtained using normalized chromatographic areas of volatile compound (Table S1) found in Chardonnay grapes with and without *A. carbonarius* and/or *B. velezensis* P1. Red, orange and yellow colors represent high, medium and low chromatographic areas, respectively.

Compounds of each cluster were indicated with different colors. Cluster 1 (pink) consists of the compounds that showed the highest levels in the grapes in which *B. velezensis* P1 was inoculated. Among these compounds, 3-hydroxy-2-butanone has also been identified as the volatile compound produced by *Bacillus velezensis* G341 isolated from roots of Korean ginseng and showed antifungal activity against *Botrytis cinerea*, *Alternaria panax*, *Magnaporthe oryzae* and other fungi evaluated in culture medium (Lim et al., 2017). This ketone is precursor of 2,3 butanediol (Ferreira et al., 2018), which was also found at higher level in grapes treated with *B. velezensis* P1. The

formation of 2,3-butanediol (fruity odor) from acetoin (cream odor) may have a positive contribution to the quality of grape products. The other compounds in this cluster also have pleasant odor descriptions, including sweet (3-hexenoic acid and benzyl alcohol), fruity (3-methyl-1-butanol, ethyl 3-methylbutanoate and ethyl hydroxypropionate) and floral (nonanal). 3-methyl-1-butanol was identified as the compound produced in greater quantity by *Bacillus licheniformis* against *Aspergillus flavus* in maize ears, and indicated as the possible responsible for the antifungal activity of the strain (Ul Hassan, Al Thani, Alnaimi, Migheli, & Jaoua, 2019).

Cluster 2 (green) contains the four acids (pentanoic, ethyl-hexanoic, nonanoic and dodecanoic) found at higher levels in grapes without microorganisms' inoculation. An important point to be detached that the lowest levels of the acids as pentanoic, nonanoic and dodecanoic were found when *B. velezensis* P1 was inoculated in grapes, which may be interesting for grape quality since these acids has odor described as cheesy and metallic.

Cluster 3 (blue) is formed by volatiles found at higher levels in grapes with *A. carbonarius* such as compounds with 8 carbons (3-octanone, 2-octenal, octanoic acid and 1-octanol; odor description of herbal, fatty, cheesy and citrus, respectively). Fungal enzymes may favor the oxidation of fatty acids (e.g. linoleic acid) of grapes giving rise to C-8 compounds as previously reported by Dachery et al. (2019). Cheng et al. (2018) proved that the formation of these compounds follows the same metabolic route of ochratoxin A production by *A. carbonarius* in agar, requering acetyl- CoA, malonate and polyketide synthases. In cluster 3 are also included hexanoic acid (odor: cheesy) and α-terpineol (odor: lemon, pine, minty), which were found in previous work as an indication of the presence of *A. carbonarius* in Moscato Italico and Cabernet Sauvignon grapes, as well as in the respective wines (Dachery et al., 2019). The formation of terpenes, as α-terpineol, is well-konwn in the literature and occurs in response to pathogens by two ways: (1) the mevalonate (MVA) pathway and (2) the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (DOXP/MEP) pathway (Hammerbacher, Coutinho, & Gershenzon, 2019). Another compound of cluster 3 that may be involved in defense strategies against *A. carbonarius* is hexanal. This aldehyde is produced from free fatty acids by lipoxygenases action as response to fungal diseases as reported by Lin et al. (2019). Lipoxygenase products are often described as having an

herbaceous, leafy “green” aroma and can be a negative contributor to grape aroma (Lin et al., 2019).

Cluster 4 included terpenic compounds **(**3,4-dihydro-β-ionone, β-ionone, dihydroactinidiolide, linalool oxide, and β-terpineol), esters (phenylethyl alcohol and ethyl linoleate), a ketone (6-methyl-5-hepten-2-one) and a phenol (3,5-di-tert-butylphenol). These compounds were found at higher levels in the grapes in which *A. carbonarius* was inoculated and that received the biocontrol treatment with *B. velezensis* P1.

Expression of key enzymes in the MVA, DOXP/MEP and/o methylerythritol phosphate (MEP) pathways may be related to the of terpenes synthesis of cluster 4 by *Bacillus* spp. Volatile terpenes are generally recognized for their ability to inhibit bacteria, fungi and nematodes. The mechanism of action might be explained by their lipophilic nature that destabilize the cell membrane integrity. (Caulier et al., 2019). Another mechanism to explain the highest levels of terpenes is related to glycosidases produced by *Bacillus spp*. Glycosidases are involved in the release of free aroma compounds, which are linked to sugars in the grape. This is being reported on grapes for the first time. Terpenes exist in two forms: the free form is volatile and directly contributes to aroma, while the glycoside form is non-volatile but can be transformed into the volatile form via hydrolysis.

6-Methyl-5-hepten-2-one is (fruity and grassy odors) an important intermediate in the synthesis of terpenoids as myrcene and geraniol (Parshikov & Sutherland, 2014). These terpenes were not found in grapes (Table S1). Chardonnay grapes are extensively used to elaborate wine and reactions during winemaking may result in the free forms of these terpenes. This subject will be addressed in a future work.

Ethyl linoleate (fatty odor) is an ester derived from linoleic acid, which is present in berry skin and seeds (Pérez-Navarro et al., 2019). In this way, the biocontrol strategy may have an important role on the esterification reaction of this fatty acid.

Phenylethyl alcohol (floral odor) is a compound with known antifungal activity that was previously reported as product of the metabolism of *B. velezensis* C2 isolated from Tunisian soil. This alcohol was identified among the compounds responsible by the protection of tomato against *Verticillium* wilt disease (Dhouib et al., 2019). In a

study conducted in India Phenylethyl alcohol presented antifungal activity against *Aspergillus flavus* and *Aspergillus parasiticus* (two aflatoxin-producing fungi) in soybean seeds and *in vitro* tests (Boukaew & Prasertsan, 2018). Also, that compound showed inhibitory activity on mycelial growth, sporulation and sporangian germination of *Peronophythora litchi* (litchi downy blight) in lichi fruit and *in vitro* assays (Xing et al., 2018).

The cluster 4 also contains 3,5-di-tert-butylphenol (DTBP), which was verified as the only compound with unpleasant odor (phenolic odor) found in grapes treated with *B. velezensis* P1. Zhao, Wang, Lucardi, Su and Li, (2020) verified that this lipophilic phenol is produced by around 16 species of bacteria including *Bacillus spp* and the different analogs of DTBF exhibit antioxidant, anti-Inflammatory, insecticidal, nematicidal and antiviral activitiy. DTBP was found to be effective against *Fusarium oxysporum in vitro* (potato dextrose agar) by inhibiting spore germination and hyphal growth (Dharni et al., 2014), also reduced mycellium growth of *Cladosporium fulvum* in tomato (Bao et al., 2013) and in *Aspergillus flavus in vitro* tests (Gong et al., 2015).

CONCLUSION

The volatiles produced in grapes treated with *B.velezensis* P1 have a pleasant odor description, which can contribute positively to the aroma and flavor characteristics of Chardonnay grapes. The higher levels of terpenes may be due to glycosidases produced by *Bacillus* spp. In the presence of *A.carbonarius,* the biocontrol strategy resulted in higher concentrations of other volatile compounds that present mostly fruity odor. In grapes inoculated only with *A.carbonarius* were identified compounds that indicates its presence (hexanoic acid and α-terpineol). Also, hexanal and α-terpineol occurs in response to pathogens and may be involved in defense strategies.

In addition to being successful as a biocontrol agent in a previous study, *B. velezensis* P1 significantly improved the odor and taste of Chardonnay grapes without bringing unpleasant characteristics. Which is an important discovery that is being reported for the first time in grapes.

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SUPPLEMENTARY MATERIAL

Table S1. Volatile profile of Chardonnay grape berries using the DVB/Car/PDMS fiber and gas chromatography with mass spectrometric detection. Compounds are listed in increasing order of RI for the distinct classes.

#	Compound	CAS[a]	RI exp (WAX)[b]	RI lit (WAX)[c]	RI exp (DB5)[d]	RI lit (DB5)[e]	Odor
	Acid						
1	Propanoic acid	79-09-4	1533	1535 [1]	-	-	Pungent, acidic, cheesy [2]
2	2-Methyl-propanoic acid	79-31-2	1560	1568 [1]	1370	1373 [11]	Cheesy, buttery, rancid [4]
3	Butanoic acid	107-92-6	1623	1630 [1]	**-**	**-**	Sweaty [5]
4	3-Methyl-butanoic acid	503-74-2	1662	1667 [1]	**-**	**-**	Sweaty [6]
5	Pentanoic acid	109-52-4	1732	1733 [22]	**-**	**-**	Cheesy [8]
6	2-Methyl-pentanoic acid	97-61-0	1760	1764 [7]	**-**	**-**	Cheesy [10]]
7	Hexanoic acid	142-62-	1845	1855 [1]	**-**	**-**	Cheesy [8]

		1					
8	3-Hexenoic acid	4219-24-3	1964	1954 [34]	-	-	Sweet [5]
9	2-Ethyl-hexanoic acid	149-57-5	1951	1950 [35]	-	-	Mild [13]
10	Octanoic acid	124-07-2	2055	2055 [14]	-	-	Rancid, cheesy [6]
11	Nonanoic acid	112-05-0	2159	2168 [1]	-	-	Waxy [16]
12	Decanoic acid	334-48-5	2264	2266 [1]]	-	-	Fatty [17]
13	Benzoic Acid	65-85-0	2426	2423 [28]	-	-	Fresh, faint gingery, grass and milky [19]
14	Dodecanoic acid	143-07-7	2472	2470 [37]	-	-	Metallic [7]
15	Benzeneacetic acid	103-82-2	2550	2550 [41]	-	-	Sweet, honey, floral [23]
	Alcohol						
16	3-Methyl-1-butanol	123-51-3	1208	1213 [31]	-	-	Fruity, banana [15]
17	Pentanol	71-41-0	1245	1249 [1]	770	766 [26]	Mellow, balsamic [27]
18	2-Hexen-1-ol	2305-21-7	1390	1394 [9]	865	868 [21]	Fruity, green, leafy [30]
19	1-Octanol	111-87-5	1557	1557 [1]	1075	1078 [30]	Intense citrus, roses [4]
20	1-Octen-3-ol	3391-86-4	1451	1451 [18]	979	979 [1]	Green, mushroom-like [19]
21	2,3-Butanediol	513-85-9	1575	1580 [12]	-	-	Fruity [2]
22	Benzyl Alcohol	100-51-6	1871	1869 [1]	-	-	Sweet [36]
23	Phenylethyl alcohol	60-12-8	1898	1898 [1]	-	-	Floral [37]
24	1-Dodecanol	112-53-8	1969	1977 [1]	1482	1478 [33]	Waxy, fatty [39]
25	2-Phenoxy-ethanol	122-99-6	2136	2142 [4]	-	-	Floral, rose [41]
	Aldehyde						
26	Pentanal	110-62-3	978	979 [16]	701	699 [26]	Almond, malt, pungent [27]
27	Hexanal	66-25-1	1077	1073 [7]	848	848 [15]	Green, leafy [44]

28	2-Heptenal	2463-63-0	1313	1319 [16]	952	953 [11]	Green [45]
29	Nonanal	124-19-6		1388 [1]	-	1112 [2]	Floral
30	2-Octenal	2363-89-5	1440	1442 [16]	1058	1060 [20]	Roast, fatty [12]
31	Benzaldehyde	100-52-7	1508	1506 [1]	-	-	Fruity, green, gum, almond [19]
	Ester						
32	Ethyl 3-methylbutanoate	108-64-5	1068	1071 [12]	-	-	Fruity [14]
33	Isoamyl acetate (1-Butanol, 3-methyl-, acetate)	123-92-2	1115	1117 [8]	876	876 [3]	Sweet, fruity, banana [52]
34	Ethyl 2-hydroxypropanoate (Propanoic acid, 2-hydroxy-, ethyl ester)	97-64-3	1330	1334 [1]	820	815 [41]	Fruity [1]
35	2-Phenylethyl acetate	103-45-7	1430	1448 [13]	-	-	Floral, rose [54]
36	Ethyl phenylacetate	101-97-3	1775	1779 [12]	-	-	Sweet, floral [55]
37	Methyl tetradecanoate	124-10-7	2005	2008 [35]	-	-	Fatty, waxy [56]
38	Methyl hexadecanoate	112-39-0	2216	2213 [8]	1926	1926 [32]	Oily, waxy [58]
39	7-Hexadecanoic acid, methyl ester, (Z)	56875-67-3	2216	2213 [8]	-	-	-
40	Ethyl hexadecanoate	628-97-7	2255	2246 [1]	-	-	Fatty, fruity [14]
41	Methyl dihydrojasmonate	24851-98-7	2287	2276 [1]	-	-	Floral, jasmine [59]]
42	Linoleic acid ethyl ester	544-35-4	2530	2532 [42]	-	-	Fatty [61]
	Furan						
43	5-Ethyldihydro-2(3H)-furanone	695-06-7	1690	1694 [29]	1066	1062 [10]	Herbal, coconut [64]
44	Dihydro-5-pentyl-2(3H)-furanone	104-61-0	2018	2007 [39]	-	-	Milky, creamy, floral [19]
	Ketone						
45	3-Methyl-2-butanone	563-80-4	932	929 [13]	-	-	Camphor [65]
46	3-Octanone	106-68-3	1266	1261 [24]	977	970 [40]	Herbal [14]
47	3-Hydroxy-2-butanone	513-86-0	1275	1277 [19]	-	-	Cream [69]
48	6-Methyl-5-hepten-2-	110-93-	1335	1332 [1]	992	983	Fruity, grassy

	one	0					[70]
	Phenol						
49	Phenol	108-95-2	1995	2004 [17]	**-**	**-**	Phenolic, rubber [72]
50	3,5-Di-tert-butylphenol	1138-52-9	2324	2328 [23]	-	-	Phenolic [7]
	Terpene						
51	Linalool oxide	60047-17-8	1425	1423 [25]	1075	1074 [6]	Caramel, roasted, sweet [19]
52	β-terpineol	138-87-4		1616	-	1144	Pungent, earthy, woody [9]
53	α-Terpineol	98-55-5	1661	1659 [1]	1190	1189 [2]	Lemon, pine, minty [9]
54	3,4-Dihydro-β-ionone	17283-81-7	1856	1854 [5]	1435	1433 [11]	Woody, slightly fruity [78]
55	β-Ionone	14901-07-6		1947 [79]	1480	1486 [38]	Violet, sweet fruity [11]
56	Dihydroactinidiolide	17092-92-1	2330	2337 [24]	1498	1493 [27]	Fruity [82]

[a] CAS: chemical abstract service registry number.

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7 CONCLUSÃO

As cepas de *Bacillus* estudadas têm potencial para serem utilizadas como agente de controle biológico em uvas a fim de prevenir ou reduzir a ocorrência de *A. carbonarius*, bem como na síntese de OTA e suas formas modificadas, sendo uma alternativa sustentável e promovendo um alimento seguro. Uma vantagem dos biofungicidas sobre os pesticidas sintéticos é que podem ser aplicados em qualquer etapa do desenvolvimento do alimento e até o dia da colheita.

Bacillus sp. P1 se destacou entre as demais por apresentar 100% de inibição fúngica e síntese de OTA, incluindo suas formas modificadas. Os voláteis produzidos em uvas tratadas com *Bacillus* sp. P1 possuem uma descrição de odor agradável, o que pode contribuir positivamente para as características de aroma e sabor das uvas Chardonnay. Através desses achados, pode-se concluir que a cepa de *Bacillus* sp. é uma alternativa sustentável para o controle fúngico em uvas, proporcionando um alimento seguro e com melhor descrição de odor.

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