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PROSPECÇÃO DE ÓLEOS ESSENCIAIS COM ATIVIDADE ANTIFÚNGICA PARA ALTERNATIVA NO CONTROLE DE *Botrytis cinerea* (PERS) e *Colletotrichum acutatum* (SIMMONDS) EM VIDEIRA

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CAXIAS DO SUL

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

Orientador(a): Prof. Dra. Joséli Schwambach

Co-orientador(a): Dra. Rute Terezinha da Silva Ribeiro

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DISSERTAÇÃO APROVADA EM 17 DE NOVEMBRO DE 2016.

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RESUMO

A viticultura brasileira apresenta-se como uma das atividades mais importantes social e economicamente dentro da fruticultura nacional. A Serra Gaúcha é a principal região produtora de uvas e vinhos do Brasil. Todavia, possui condições climáticas desfavoráveis ao cultivo, devido ao elevado índice pluviométrico e à alta umidade relativa do ar. Sendo assim, a vitivinicultura na região somente se torna possível com um rigoroso sistema de controle preventivo e curativo de doenças fúngicas. Dentre as muitas doenças que acometem a cultura da videira, destacam-se *Botrytis cinerea* e *Colletotrichum acutatum,* causadores da podridão cinzenta e podridão da uva madura, respectivamente. Estes fungos atacam principalmente as bagas, o que reduz a qualidade e produtividade, acarretando elevados prejuízos econômicos. As medidas de controle para estas doenças geralmente consistem na aplicação de fungicidas sintéticos, porém este método pode apresentar consequências indesejáveis, tanto para a saúde humana quanto para o meio ambiente. Assim, se faz necessária a busca por novos métodos de controle de baixo impacto ambiental, que sejam economicamente viáveis e que possam ser integrados em programas de controle de doenças de plantas. Muitos óleos essenciais possuem propriedades fungicidas. Assim, pesquisas nesta área que confirmem esta atividade, segundo protocolos científicos, tornam-se cada vez mais necessárias. Diante desta situação, o objetivo do presente estudo foi avaliar a atividade antifúngica de óleos essenciais de *Eucalyptus staigeriana, Eucalyptus globulus, Foeniculum vulgare, Baccharis trimera* e *Baccharis dracunculifolia* sobre o crescimento *in vitro e* no controle *in vivo* de *Botrytis cinerea* e *Colletotrichum acutatum* em videiras (*Vitis* spp. *e Vitis vinifera*). Foram realizados testes *in vitro* para avaliar a ação fungicida dos óleos essenciais sobre a germinação de conídios e o crescimento micelial dos fungos (fase de contato e volátil) e a partir dos resultados obtidos, foram definidos os óleos essenciais a serem utilizados nos testes *in vivo*. Os testes *in vivo* foram realizados com o intuito de avaliar a atividade antifúngica dos óleos essenciais no

controle das doenças causadas por *B. cinerea* e *C. acutatum* em uvas da cultivar "Isabel" (*Vitis* spp.*)* no pós-colheita e em videiras das cultivares "Isabel" (*Vitis* spp.*)* e "Tannat" (*V. vinifera)* no campo. As uvas provenientes dos tratamentos realizados nas videiras foram vinificadas e o vinho analisado quanto as suas propriedades químicas e seus compostos voláteis. Os resultados *in vitro* revelam que os óleos essenciais de *E. staigeriana, E. globulus, B. trimera* e *F. vulgare* possuem ação fungicida, inibindo ambos fungos em pequenas concentrações que variaram de 50 a 600 ppm de acordo com o óleo e o fungo testados. Nos testes pós-colheita foram utilizados os óleos essenciais de *E. staigeriana, F. vulgare* e *B. trimera,* todos reduziram a incidência e a severidade das doenças, tanto no tratamento preventivo quanto no curativo. O tratamento curativo foi mais eficiente, inibindo completamente os sintomas das doenças em concentrações que variaram de 100 a 600 ppm de acordo com o óleo e o fungo testados. Para o tratamento *in vivo* em videiras, foi utilizado o óleo essencial de *E. staigeriana,* nas concentrações de 100 e 500 ppm, que apresentaram resultados promissores, reduzindo a incidência e severidade das doenças em ambas as variedades de videiras. As análises químicas dos vinhos demonstram que o óleo essencial não interfere nas características do vinho. Nas análises dos compostos voláteis dos vinhos, foi identificado pequenas concentrações do composto 1,8-cineol em ambos os vinhos e o do composto citral no vinho produzido com as uvas de *Vitis* spp. var. "Isabel", mas que não interferem nas características sensoriais do vinho. Assim, conclui-se que os óleos essenciais apresentam potencial uso como biofungicidas no controle de *B. cinerea* e *C. acutatum* em videiras.

ABSTRACT

The Brazilian viticulture is presented as one of the most important activities socially and economically within the national fruit production. The Serra Gaúcha is the main producing region of grapes and wine in Brazil. However, it has unfavorable climatic conditions for cultivation due to high rainfall and high relative humidity. Thus, the wine industry in the area becomes possible only with a rigorous preventive and curative control system of fungal diseases. Among the many diseases that affect the growing of vines, there are *Botrytis cinerea* and *Colletotrichum acutatum*, causing botrytis rot and ripe rot grapes, respectively. These fungi attack mainly berries, which reduces the quality and productivity, resulting in high economic losses. Control measures for these diseases generally consist in applying synthetic fungicides, but this method can have undesirable consequences, both for the human health and for the environment. Thus, it is necessary to search for new methods of low environmental impact control, which are economically viable and can be integrated into plant disease control programs. Many essential oils have fungicidal properties and research in this area to confirm this activity, according to scientific protocols, become increasingly necessary. In this situation, the aim of this study was to evaluate the antifungal activity of essential oils of *Eucalyptus staigeriana, Eucalyptus globulus, Foeniculum vulgare, Baccharis trimera* and *Baccharis dracunculifolia* on *in vitro* and *in vivo* growth control of *Botrytis cinerea* and *Colletotrichum acutatum* on vines (*Vitis* spp. and *Vitis vinifera*). *In vitro* tests were performed to evaluate the fungicidal action of essential oils on the germination of conidia and mycelial growth of fungi (contact phase and volatile) and from the results it was defined the essential oils to be used *in vivo*. *In vivo* tests were performed in order to evaluate the antifungal activity of essential oils in the control of diseases caused by *B. cinerea* and *C. acutatum* on grape variety “Isabela” (*Vitis* spp.) in the postharvest and vines varieties “Isabela” (*Vitis* spp.) and “Tannat” (*V. vinifera*) in the field. The grapes from the treatments performed on the vines

were vinificated and the wine analyzed for their chemical properties and their volatile compounds. *In vitro* results show that *E. staigeriana, E. globulus, B. trimera* and *F. vulgare* essential oils have fungicidal action, inhibiting both fungi in small concentrations ranging from 50 to 600 ppm according to the essential oil and fungus tested. In postharvest tests it was used *E. staigeriana, F. vulgare* and *B. trimera* esssential oils. All of them reduced the incidence and severity of diseases, on both preventive and curative treatment. The curative treatment was more effective, inhibiting completely the symptoms of the diseases in concentrations ranging from 100 to 600 ppm according to the essential oil and fungus tested. For the *in vivo* treatment of vines, we used the essential oil of *E. staigeriana* at concentrations of 100 and 500 ppm, which has promising results, reducing incidence and severity of disease on both varieties of vines. The chemical analysis of wines demonstrates that the essential oil does not interfere in the characteristic of the wine. In the analysis of volatile compounds of wines, it was identified low concentrations of 1.8-cineole compound in both wines and the compound citral in wine made from the grapes *Vitis* spp. cv. 'Isabela", but that does not interfere with the sensory characteristics of the wine. Thus, we can conclude that the essential oils presents potencial use as biofungicides in control of *B. cinerea* and *C. acutatum* on vines.

1. INTRODUÇÃO

A videira (*Vitis* spp.) é considerada uma das plantas frutíferas de maior relevância econômica mundial. No Brasil, a videira vem sendo explorada comercialmente a mais de um século e se firmou como atividade socioeconômica de grande importância em diversas regiões do país. A região da Serra Gaúcha no Rio Grande do Sul destaca-se no âmbito nacional como a maior produtora de uvas e vinhos. Porém, a cultura da videira enfrenta graves problemas quanto à fitossanidade de suas cultivares, sendo alvo de organismos como fungos, insetos, nematóides, vírus e bactérias. Dentre as doenças de grande relevância para essa cultura destaca-se a podridão cinzenta e a podridão da uva madura, causadas por *Botrytis cinerea* e *Colletotrichum acutatum*, respectivamente.

Os sintomas destas doenças são observados principalmente durante a maturação das bagas e o controle com fungicidas sintéticos causam uma série de consequências indesejáveis a saúde humana e ao meio ambiente. Em razão disto, faz-se importante e necessário a procura por novos defensivos agrícolas alternativos, de baixo impacto ambiental, viáveis economicamente e de possível aplicação, que possam controlar doenças de plantas reduzindo, assim, a dependência a fungicidas sintéticos. Algumas técnicas de controle alternativo de doenças de plantas utilizam extratos ou óleos vegetais e microrganismos, que enquadram-se nestes parâmetros, sendo importante sua investigação para possível utilização. Os óleos essenciais são metabólitos secundários de plantas e possuem grande potencial no combate a doenças fitopatogênicas, pois possuem propriedades antifúngicas, antibacterianas e inseticidas, além de serem pouco tóxicos ao meio ambiente e ao ser humano.

Diante desta situação, o objetivo do presente estudo foi avaliar a ação fungicida dos óleos essenciais de *Eucalyptus staigeriana*, *Eucalyptus globulus*, *Foeniculum vulgare*, *Baccharis*

trimera e *Baccharis dracunculifolia* sobre o crescimento *in vitro e* no controle *in vivo* de *Botrytis cinerea* e *Colletotrichum acutatum* em videiras (*Vitis* spp. *e Vitis vinifera*).

2. REVISÃO BIBLIOGRÁFICA

2.1. Cultura da videira

A família Vitaceae está dividida em 12 gêneros e estes são constituídos por 700 espécies que se distribuem nas regiões tropicais e subtropicais do planeta e até em algumas regiões temperadas (Heywood, 1993). O gênero *Vitis* possui 46 espécies e, é o principal representante desta família devido à importância econômica da uva (Giovannini, 2008; 2014).

As plantas da família Vitaceae possuem folhas alternas, simples ou compostas, frequentemente palminérveas e com estípulas, inflorescência cimosa ou paniculada, terminal, axilar ou oposta às folhas. A videira é uma planta perene, lenhosa, caducifólia e sarmentosa, com órgão de sustentação chamado gavinha. Possui sistema radicial amplamente ramificado, caule ereto, folhas com a presença de pelos na parte abaxial, flores completas ou hermafroditas e frutos tipo baga (Kishino, 2007; Giovannini, 2008).

Apesar de ser considerada uma cultura de clima temperado, o gênero *Vitis* apresenta adaptabilidade a variadas condições climáticas (Guimarães, 2008). No Brasil, a cultura da videira está difundida desde o Rio Grande do Sul, a 31°S de latitude, até o Rio Grande do Norte e Ceará, a 05°S de latitude. A variação de altitude também é grande, havendo considerável diversidade ambiental entre as zonas de produção, incluindo regiões de clima temperado, subtropical e tropical (Camargo *et al.*, 2011).

Na região Sul do Brasil, a viticultura praticada é a tradicional, com a realização de apenas um ciclo anual, no qual a planta, após a poda, inicia a brotação, floresce, frutifica, amadurece e entra novamente em repouso vegetativo (Rodrigues, 2009). A Serra Gaúcha é considerada a principal região vitícola do país. Está localizada no Nordeste do Estado do Rio Grande do Sul, cujas coordenadas geográficas e indicadores climáticos médios são: latitude 29ºS, longitude 51ºW, altitude 600-800 m, precipitação 1.700 mm, temperatura 17,2º C e umidade relativa do

ar de 76 % (Protas & Camargo, 2011). A uva produzida na região serrana é utilizada, em sua maioria, na elaboração de vinhos, concentrando mais de 90% da produção nacional (MAPA, 2015) e uma fração das uvas cultivadas é destinada ao consumo *in natura.*

A produção de uvas na Serra Gaúcha está dividida em três grupos: um grupo formado pelas *Vitis vinifera* (uvas finas ou européias) e pelas uvas *Vitis labrusca*, *Vitis bourquina* e *Vitis* spp. (uvas comuns, rústicas, americanas ou híbridas) (Nachitigal & Schneider, 2007). Estes grupos apresentam características diferenciadas quanto à produção, resistência a pragas e doenças e produtos que originam.

2.1.1. *Vitis vinifera*

A espécie *V. vinifera* é originária da Europa ocidental e da Zona Trans-caucasiana, de onde foi difundida por toda a costa mediterrânea, onde ao longo de séculos de cultivo, foram selecionadas diversas variedades (Arnold *et al*., 1998; Zohary & Hopf, 2000; Arroyo-García *et al.,* 2006). Esta espécie apresenta a melhor qualidade dentre o gênero *Vitis*, a qual ocupa a maior parte da área cultivada com videiras no mundo. São plantas exigentes quanto a condições de clima, preferindo os secos, com baixa umidade relativa do ar e bastante insolação, normalmente apresentam baixa resistência às principais doenças da cultura (Kuhn *et al.*, 1996).

As uvas da espécie *V. vinifera* são utilizadas para elaboração de vinhos finos ou para consumo *in natura*. Os produtos elaborados a partir dessas cultivares são mais valorizados, porém apresentam um custo de produção mais elevado (Nachtigal & Schneider, 2007).

A cultivar "Tannat" (Fig. 1) é originária da região de Madiram, no sul da França. Foi introduzida no Rio Grande do Sul em 1947 e passou a ser cultivada comercialmente na Serra Gaúcha em 1987 (Rizzon & Miele, 2004; Camargo *et al*, 2008; Silveira *et al*., 2015). A uva "Tannat" é uma variedade de *V. vinifera* que possui médio vigor e alta produtividade, sendo

amplamente difundida na região vitícola do sul Brasil, onde em 2015 representou 9,22% da produção total de uvas viniferas tintas (Camargo, 1994; Rizzon & Miele, 2004; Camargo *et al.*, 2008; Mello & Machado, 2014). Esta variedade é utilizada principalmente para a produção de vinho, que é rico em cor e em extrato, servindo para corrigir as deficiências dessas características em outros vinhos de viníferas, além de também ser comercializado com sucesso, como vinho varietal (Camargo *et al.*, 2008).

Figura 1. *Vitis vinifera* var. "Tannat" (Arquivo pessoal, 2015).

2.1.2. *Vitis labrusca*, *Vitis bourquina* e *Vitis* spp.

V. labrusca, *V. bourquina* e *Vitis* spp. (híbridos) são espécies oriundas da América do Norte, que aclimatam-se bem em ambientes quentes e úmidos e de invernos rigorosos (Thomé *et al.*, 1999; Silveira *et al.*, 2015) adaptando-se bem às condições ambientais do Sul do Brasil. De modo geral, as uvas produzidas por estas espécies se caracterizam pela alta produtividade e resistência às principais doenças fúngicas (Kunh *et al.*, 1996; Sônego *et al.*, 2005; Nachitigal & Schneider, 2007).

As uvas das espécies *V. labrusca*, *V. bourquina* e *Vitis* spp. podem ser utilizadas para elaboração de sucos, vinho de mesa e para o consumo *in natura*. Apresentam um menor custo de produção, porém, normalmente, são comercializadas por um valor menor do que as uvas *V. vinifera* (Nachitigal & Schneider, 2007).

Embora haja alguma discordância sobre a origem da cultivar de uva "Isabel" (Fig. 2), a mesma é considerada por muitos autores como um hibrido natural entre as espécies de *V. labrusca* e *V.* vinifera, que foi inicialmente propagada no estado da Carolina do Sul, na costa leste americana (Silveira *et al.,* 2015). Foi introduzida no Rio Grande do Sul entre 1839 e 1842 (Camargo, 1994). A uva "Isabel" é uma cultivar muito rústica de fácil adaptação a variabilidade de condições edafoclimáticas, também possui uma elevada produtividade e longevidade (Giovannini, 2008). É a cultivar mais plantada no Rio Grande do Sul, onde em 2015 representou 47,7% da produção total de uvas americanas tintas (Camargo, 1994; Camargo *et al.,* 2008; Mello & Machado, 2015; Silveira *et al.,* 2015). Os principais destinos da uva "Isabel" são a produção de vinho tinto de mesa, suco de uva, vinagre, geléias e consumo *in natura* (Zanuz, 1991; Rizzon *et al*., 2000; Camargo *et al.,* 2008; Rombaldi *et al.,* 2004). O vinho produzido a partir desta variedade apresenta aroma e gosto foxados. Mesmo assim, pelo hábito de consumo, associado às informações que indicam os benefícios de pigmentos e taninos existentes nesse vinho, faz com que ainda seja o mais consumido no País e tenha grande potencial de expansão. Essa mesma lógica é válida para outros produtos derivados dessa cultivar, como vinagre, suco, geléia e a própria uva para consumo direto (Zanuz, 1991; Rizzon *et al.*, 2000).

Figura 2. *Vitis* spp. var. “Isabel” (Arquivo pessoal, 2015).

2.2. Importância econômica da cultura da videira

O cultivo de videira assume um importante papel no contexto social e econômico a nível mundial, sendo uma das principais frutíferas cultivadas. A vitivinicultura brasileira, com relação aos demais países produtores de uvas e vinhos, ocupou o 19° lugar em área cultivada com uvas (82.603 hectares), o 12° em produção de uvas (1.514.768 toneladas) e o 13° em produção de vinhos (350.000 toneladas) em 2012, segundo dados da FAO (Melllo, 2015 a).

Em 2014, no Brasil a produção de uvas foi de 1.436.074 toneladas, sendo que deste montante, 673,422 milhões de quilos de uvas foram destinadas ao processamento (vinho, suco e derivados), representando 46,89% da produção nacional, o restante da produção (53,11%) foi destinado ao consumo *in natura.* As exportações brasileiras do setor vitivinícola somaram 89,239 milhões de dólares. A quantidade de vinhos exportada foi de 2.32 milhões de litros e a exportação de uva de mesa foi de 28,348 toneladas (Mello, 2015 a). Neste mesmo ano, o Rio Grande do Sul destacou-se como a maior região produtora (812,537 toneladas), sendo o

estado que possui maior área plantada (51,152 hectares) e área colhida (49,900 hectares) do Brasil (Mello, 2015 b).

Além da produção de uvas, vinhos e derivados, a vitivinicultura brasileira tem oportunizado a agregação de valor em outras atividades da economia como o turismo e a gastronomia. Essas atividades são importantes para a sustentabilidade da pequena propriedade de agricultura familiar (Mello, 2015 b).

2.3. Doenças fúngicas da parte aérea da Videira

As doenças fúngicas constituem-se num dos principais entraves para a produção qualitativa e quantitativa de uva. Vários patógenos fúngicos podem infectar a videira, variando sua importância com a região geográfica e a resistência varietal. O ambiente tem um papel muito importante neste contexto, podendo contribuir para aumentar ou limitar o desenvolvimento das doenças (Sônego *et al.*, 2005). Na região da Serra Gaúcha, as condições climáticas podem apresentar-se desfavoráveis ao cultivo da videira em alguns aspectos. A frequência e distribuição de chuvas são elementos climáticos de grande importância neste processo produtivo, sendo que nesta região Sul do Brasil há uma série histórica pluviométrica com tendência ao excesso se comparada a regiões vitícolas tradicionais de outros países (Westphalen & Maluf, 2000), o que favorece o desenvolvimento destas doenças. E com relação à resistência varietal, de um modo geral, as variedades *V. labrusca*, *V. bourquina* e *Vitis* spp. (híbridas) são menos suscetíveis a doenças do que as variedades de *V. vinifera* (Sônego *et al*., 2005; Chavarria *et al*., 2008; Neroni, 2009).

Considerando as doenças causadas por fungos ou chromistas que acometem os vinhedos, podem-se destacar as doenças da parte aérea da videira, que causam elevadas perdas na produção, pois atacam ramos, gemas, folhas, inflorescências e as bagas (Sônego *et al.*, 2005). As doenças mais comuns são: o míldio (*Plasmopara viticola*), a antracnose (*Elsinoe*

ampelina), a escoriose (*Phomopsis viticola*), a podridão do cacho (*Greeneria uvicola),* a podridão cinzenta (*Botrytis cinerea*) e a podridão da uva madura *(Colletotrichum acutatum)* (Sônego *et al.*, 2005; Giovannini, 2014).

E dentre estas doenças destacamos a podridão cinzenta e a podridão da uva madura, onde os sintomas são observados principalmente durante a maturação das bagas. Com isto, além das perdas quantitativas, muitos produtores realizam colheitas antecipadas, em comparação ao ponto ideal de maturação com o intuito de evitar perdas ocasionadas por estas podridões e isto resulta no comprometimento da qualidade da uva e na qualidade enológica do mosto pela paralisação do processo de maturação (Tonietto & Falcade, 2003).

2.3.1. *Botrytis cinerea*

A podridão cinzenta da uva, mofo cinzento ou podridão de *Botrytis* causada pelo fungo *Botrytis cinerea* Pers. Fr. (*Botryotinia fuckeliana* (de Bary) Whetzel na sua forma sexuada) existe em praticamente todos os vinhedos do mundo, reduzindo qualitativa e quantitativamente a produção. A literatura menciona mais de 200 plantas hospedeiras deste fungo, o que vem a contribuir para sua disseminação (Garrido & Sônego, 2005; Giovannini, 2014).

No vinhedo, *B. cinerea* sobrevive no solo na forma de micélio em restos culturais e gemas e na forma de escleródios na casca dos ramos. Na primavera, conídios (Fig. 3 C) são produzidos infectando folhas e cachos novos antes da floração. O fungo, quando infecta as flores, pode permanecer inativo (latente), devido ao baixo conteúdo de açúcares e alto conteúdo de ácido, até a infecção dos frutos, no início da maturação. Infecções diretamente dos frutos, costumam iniciar quando as bagas apresentam 5 a 8% de açúcar e estendem-se até a colheita. Conídios abundantes são produzidos em frutos infectados, conduzindo a ciclos secundários. A dispersão dos conídios é auxiliada pela chuva, irrigação, orvalho e suco de bagas rompidas. O

vento também desempenha um papel importante na dispersão. Os danos mecânicos causados por pássaros ou pragas criam pontos de entrada para o fungo aumentando as perdas devido a esta doença. O fungo se desenvolve melhor na temperatura de 18 a 23º C e umidade relativa alta. Conídios germinados penetram nos tecidos através de ferimentos ou após crescimento sobre pétalas de flores velhas ou folhagem seca. Os escleródios de *B. cinerea* germinam produzindo hifas que podem infectar tecidos diretamente ou em alguns casos produzem apotécio e ascósporos. As infecções de uvas por *B. cinerea* ocorrem durante períodos de pelo menos 16 horas de temperatura entre 15 e 20º C e umidade relativa alta. Nas temperaturas 10; 15,5; 22,5; 26,5 e 39º C são necessárias 30, 18, 15, 22 e 35 horas de condições de molhamento, respectivamente, para sucesso da infecção (Garrido & Sônego, 2005).

O fungo pode atacar quase todos os órgãos da videira, porém, é nas bagas, durante a maturação que os cachos apresentam a máxima suscetibilidade, uma vez que, a concentração de açúcares nas bagas é a base nutritiva para o desenvolvimento do fungo (Perez, 1998; Garrido & Sônego, 2005). Os primeiros sintomas da doença nas bagas de uvas são a presença de pontuações ligeiramente claras, circulares de 2 a 3 mm, que podem ser observadas 72 horas após a penetração do fungo nos tecidos. Depois que a infecção se estabeleceu por cerca de 5 a 7 dias, o fungo cresce através das rachaduras na casca das bagas, emitindo os conídios que podem recobrir toda a baga, formando um mofo cinzento (Fig. 3 A e B) (Garrido & Sônego, 2005).

Na produção de uvas de mesa, *B. cinerea* é o maior causador de perdas pós-colheita, pois além de perdas significativas na qualidade dos frutos no campo, também causa perdas durante o armazenamento e transporte, já que o patógeno também é ativo em baixas temperaturas e causa consideráveis perdas em produtos armazenadas por longos períodos, mesmo com temperatura entre 0 e 10º C (Bulit & Dubos, 1990; Lichter, 2002; Garrido & Sônego, 2005). Enquanto que, na produção de uvas para processamento, os danos mais severos são

qualitativos, pela modificação da composição química das bagas afetadas, pois o fungo converte açúcares simples (glicose ou frutose) em glicerol e ácido glucônico, produz enzimas que catalisam a oxidação de compostos fenólicos e secreta polissacarídeos como β-glucano que impedem a clarificação do vinho, além de causar perdas no aroma, torna-os frágeis, sensíveis a oxidação e sujeitos a contaminação bacteriana (Dobourdieu, 1982; Pearson & Goheen, 1988; Meneguzzo, 1996; Garrido & Sônego, 2005).

Figura 3. Sintomas da podridão cinzenta. A: Sintomas da doença em *Vitis* spp. var. "Isabel" B: Sintomas da doença em *Vitis vinifera* var. "Tannat" C: Conídiósporo e conídios de *B. cinerea* (Arquivo pessoal, 2015).

2.3.2. *Colletotrichum acutatum*

A podridão da uva madura da videira é causada pelo fungo *Colletotrichum acutatum* (Simmonds) (*Glomerella acutata* (Guerber & Correll) na sua forma sexuada) (Guerber & Correll, 1997; 2001; Meunier e Steel 2009). O patógeno está associado principalmente a regiões vitícolas com clima quente e úmido durante a fase de maturação da uva (Meunier & Steel, 2009; Greer *et al*., 2011).

Steel *et al*. (2012), demonstraram que as inflorescências da videira são suscetíveis a infecção por *C. acutatum* e que o manejo da doença deve iniciar ainda nesta fase. Mas os sintomas da doença são observados nas bagas em fase de maturação, que inicialmente perdem o turgor e

murcham. Isto é seguido pelo aparecimento de uma massa de conídios (Fig. 4 C) alaranjados na superfície da baga (Fig. 4 A e B) (Meunier & Steel, 2009).

As perdas relacionadas a *C. acutatum* estão associadas com qualidade da uva. Na produção de uvas de mesa, as bagas afetadas possuem um sabor amargo. O mesmo observa-se na produção de uvas para processamento, onde o vinho feito a partir da fruta afetada pela podridão apresenta um pH, concentração de álcool, glicerol, ácido glucônico, açúcar residual e acidez volátil mais elevados e uma menor concentração de acidez titulável (Meunier & Steel 2009; Greer *et al*., 2011).

Apesar de uma quantidade considerável de literatura sobre a biologia e o manejo de *Colletotrichum acutatum* em plantas hospedeiras que não as videiras (Prusky *et al.,* 2000), pouco se sabe sobre sua epidemiologia em *Vitis.* O ciclo de vida de *Colletotrichum acutatum* em videiras e a disseminação entre videiras e vinhedos ainda não foram descritas (Greer *et al.,* 2011).

Na Serra Gaúcha, a podridão da uva madura está associada a *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (*Glomerella cingulata* (Stonemam) Spauld & Schrenk na sua fase sexuada) (Garrido & Sônego, 2004). Isolados de *Colletotrichum spp*. obtidos em videiras da Serra Gaúcha e inicialmente identificados com *C. gloeosporioides*, foram encaminhados para o sequenciamento genético e os resultados revelaram que trata-se de outra espécie, denominada *C. acutatum* (dados não apresentados). No passado, *C. gloeosporioides* foi considerado o único organismo causador da podridão da uva madura, no entanto, em diversos países do mundo, tanto *C. acutatum* quanto *C. gloeosporioides* estão associados à doença (Whitelaw-Weckert *et al*., 2007; Greer *et al.,* 2011). No Brasil, ainda não foi reportada a podridão da uva madura associada a *C. acutatum*, somente a *C. gloesporioides*. Isso pode ter

ocorrido devido a existência de um nome anterior para a espécie *C. acutatum*, provavelmente classificado como um sinônimo de *C. gloeosporioides* por Arx (1957) (Damm *et al*., 2012).

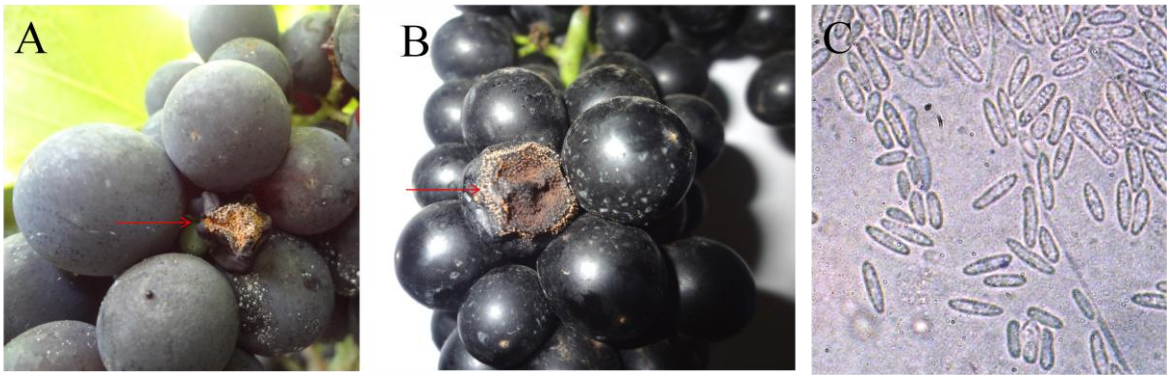

Figura 4. Sintomas da podridão da uva madura. A: Sintomas da doença em *Vitis* spp. var. "Isabel" B: Sintomas da doença em *Vitis vinifera* var. "Tannat" C: Conídios de *C.acutatum* (Arquivo pessoal, 2015).

2.4. Controle alternativo de doenças de plantas

O manejo das doenças da videira requer a aplicação de fungicidas diversas vezes durante os diferentes estágios fenológicos (Madden *et al.*, 2000). Apesar do controle químico estar contribuindo há vários anos para a viabilidade econômica e eficiência no controle das doenças na cultura, atualmente esse manejo convencional de doenças de plantas tem levado ao uso contínuo e abusivo de produtos químicos, o que acaba gerando a seleção de patógenos resistentes a esses produtos (Meinerz *et al.*, 2008), ou ainda causar sérios desequilíbrios no agroecossistema e sérios problemas para a saúde humana (Bettiol & Ghini, 2003). Além disso, pode ocasionar a redução da biodiversidade, alterações na ciclagem da matéria orgânica e dos nutrientes, no controle biológico natural de doenças e pragas, nas atividades microbianas no solo e alterações nas populações de organismos do solo e água, entre outros. Assim, é necessária a busca por métodos alternativos à proteção de plantas que reduzam ou eliminem o uso de pesticidas (Bettiol *et al*., 1997; Bettiol *et al*., 2005).

Devido a esses problemas, houve um aumento da atividade agrícola orgânica em todo o mundo (Silva *et al*., 2007). A agricultura orgânica é um sistema de produção agrícola que elimina o uso de fertilizantes, pesticidas, reguladores de crescimento e aditivos na alimentação de animais (Ronald & Fouche, 2006). No foco da agricultura alternativa para o controle de doenças de plantas, técnicas recentes favorecem o emprego de substâncias alternativas, como as derivadas de extratos naturais de plantas, aminoácidos e óleos essenciais (Nascimento *et al*., 2008; Fawzi *et al*., 2009). Além do uso de microorganismos vivos para o controle microbiológico e a indução de resistência em plantas (Bettiol, 1991; Huffaker & Messenger, 2012). As pesquisas desenvolvidas com óleos essenciais apresentaram controle de doenças, tanto por sua ação fungistática, que inibe o crescimento micelial e a germinação de esporos, quanto pela presença de compostos eliciadores (Medice *et al.*, 2007).

2.5. Óleos essenciais

Óleos essenciais, também conhecidos como óleos voláteis, óleos etéreos ou simplesmente essências, são definidos pela International Standard Organization (ISO) como produtos obtidos de partes de plantas, através de destilação por arraste com vapor d'água. São misturas complexas de substâncias orgânicas voláteis, lipofílicas, geralmente odoríferas e que apresentam-se sob a forma de líquidos oleosos. A volatilidade e a insolubilidade em água e a solubilidade em solventes orgânicos usuais permite caracterizá-los e promover seu isolamento (Costa, 1994; Simões & Spitzer, 2000).

Os óleos essenciais são encontrados em várias plantas, na forma de pequenas gotas entre as células, onde agem como hormônios, reguladores e catalisadores. Seu papel é ajudar a planta a se adaptar ao meio ambiente, por isso, sua produção aumenta em situações de estresse. Sua função natural está relacionada à proteção da planta contra doenças e parasitas, além de atrair certos insetos que realizam a polinização (Taiz & Zeiger, 2010).

Os métodos de extração dos óleos essenciais variam conforme a localização do óleo essencial na planta e com a proposta de utilização do mesmo. Para extração de óleos essenciais de plantas, os métodos convencionais são o arraste a vapor e a hidrodestilação (Dorman & Deans, 2000; Simões & Spitzer, 2000; Prabuseenivasan *et al.*, 2006; Bakkali *et al*., 2008; Bizzo *et al*., 2009).

Os constituintes dos óleos essenciais são hidrocarbonetos terpênicos, sendo mais encontrados os monoterpenos e sesquiterpenos, além de compostos oxigenados, como álcoois, aldeídos, cetonas, fenóis, ésteres, éteres, lactonas e compostos nitrogenados e sulfurados (Dorman & Deans, 2000; Simões & Spitzer, 2000; Prabuseenivasan *et al*., 2006; Knaak & Fiuza, 2010; Nerio *et al.*, 2010). A composição dos óleos essenciais pode variar consideravelmente entre espécies e variedades de plantas aromáticas e mesmo dentro da mesma variedade, localizada em diferentes áreas geográficas; depende ainda do estado de desenvolvimento do vegetal, além das condições ambientais de crescimento e da técnica de extração (Bakkali *et al*., 2005; Rakotonirainy & Lavédrine, 2005; George *et al.*, 2009; Knaak & Fiuza, 2010; Nerio *et al.*, 2010).

Os óleos essenciais são conhecidos pelas suas atividades antisséptica, antioxidante, bactericida, antiviral, fungicida, inseticida, além de suas propriedades medicinais, que são: analgésica, adstringente, anticarcinogênica, antidepressiva, antipirética, anestésica, bacteriostática, béquica, citofilática, desodorante, espasmolítica, estimulante, fungistática, imunoestimulante e sedativa (Cimanga *et al*., 2002; Sacchetti *et al*., 2005; Lima *et al.*, 2006; Prabuseenivasan *et al*., 2006; Bakkali *et al.*, 2008; Cansian *et al.*, 2010; Knaak & Fiuza, 2010). E por isso podem ser utilizados em sínteses químicas ou como novos materiais para uso científico, tecnológico e aplicações comerciais. Essas substâncias naturais são utilizadas por um grande número de indústrias: farmacêutica (fármacos: taxol, efedrina); alimentícia

(flavorizantes e corantes naturais); cosmética (produtos naturais: cânfora, linalol); química, agroquímica (fungicida, inseticida), dentre outras (Simões & Spitzer, 2000).

A mistura dos compostos de um óleo essencial é complexa, podendo ser de centenas de compostos químicos diferentes, que possuem ação sinérgica ou complementar entre eles, originando, assim, sua atividade (Siqueira *et al*., 2007; Bakkali *et al*., 2008; Nerio *et al*., 2010). Tais compostos apresentam-se em diferentes concentrações, sendo um deles, normalmente o composto majoritário, existindo outros em menores teores e alguns em baixíssimas quantidades, chamados traços. Porém sabe-se que os compostos em menor quantidade também contribuem para a bioatividade dos óleos essenciais (Simões & Spitzer, 2000; Bakkali *et al*., 2008; Knaak & Fiuza, 2010; Nerio *et al.*, 2010). É provável que os vários compostos dos óleos essenciais tenham papel importante em definir as características dos óleos, como fragrância, densidade, textura, cor, além das características de ação, como a penetração celular, a atração lipofílica ou hidrofílica, a distribuição celular e a fixação nas paredes celulares e membranas, quando atuam sobre microorganismos (Bakkali *et al.*, 2008). Dentre as atividades biológicas dos óleos essenciais, vários estudos têm comprovado a sua ação como fungicida natural, inibindo a atividade fúngica (Chao & Young, 2000).

2.5.1. *Eucalyptus staigeriana* e *Eucalyptus globulus*

A família Myrtaceae abrange 3.000 espécies distribuídas em 150 gêneros, que se desenvolvem em regiões tropicais e temperadas de todo o mundo, sendo predominantes na América e Austrália (Lee *et al*., 2008; Araújo *et al.*, 2010). *Eucalyptus* é um grande gênero da família Myrtaceae, nativo da Austrália, que possui 900 espécies e subespécies (Gilles *et al.*, 2010; Tyagi & Malik, 2011).

Os eucaliptos são plantas perenes e altas, apresentam copa rala e alongada, tronco quase sempre retilíneo e cilíndrico, raízes profundas com ectomicorrizas, fruto seco e capsular

lenhoso com sementes pequenas, folhas opostas na parte inferior e alternas na parte superior (Vitti & Brito, 2003; Macedo, 2008; Malinowski, 2010). O eucalipto armazena seu óleo essencial, principalmente, nas folhas, sendo produzido em pequenas cavidades globulares, chamadas glândulas, as quais estão distribuídas em todo parênquima foliar da espécie. O óleo essencial é utilizado pela planta para defesa contra insetos, herbivoria, resistência ao frio quando plântulas, redução de perda de água e provoca efeito alelopático (Vitti & Brito, 2003; Batish *et al*., 2008). As folhas das plantas desse gênero são aromáticas e ricas em óleos, sendo uma excelente fonte de óleo essencial, muito comercializado mundialmente (Batish *et al*., 2008). O uso do óleo essencial de eucalipto é dividido em três grupos principais, dependendo de sua função, são eles: óleos medicinais, óleos industriais e óleos para perfumaria (Vitti & Brito, 2003; Batish *et al*., 2008).

As plantas deste gênero podem ser utilizadas para produção de madeira e lenha, pasta para papel (Rocha & Santos, 2007; Malinowski, 2010), carvão vegetal (Bizzo *et al.*, 2009), além de serem usados para combater várias doenças provocadas por infecções microbianas (Ghalem & Mohamed, 2008).

Os óleos essenciais de *Eucalyptus* apresentam várias propriedades biológicas, como ação antibacteriana, antifúngica, repelente, anti-helmíntica, nematicida, antisséptica, adstringente, desinfetante, cicatrizante, anti-inflamatória, antitumoral, anti-hiperglicêmica e antioxidante (Siqueira *et al*., 2007; Ashour, 2008; Batish *et al*., 2008; Macedo *et al.*, 2010; Malinowski, 2010; Tyagi & Malik, 2011). Vários estudos têm demonstrado a atividade antifúngica dos óleos essenciais de *Eucalyptus* (Fiori *et al*., 2000; Ramezani *et al*., 2002; Dhaliwal *et al*., 2004; Lee *et al*., 2007; Somda *et al.*, 2007; Tripathi *et al*., 2008; Jhalegaret *et al*., 2015).

A espécie *Eucalyptus staigeriana* tem sua origem natural na península de Cape York, no Estado de Queensland, na Austrália (Vitti & Brito, 2003). É uma árvore de porte médio, com

copa reduzida e espalhada que pode chegar até 22 metros de altura (Figura 5 A). Suas folhas vão da forma oval à lanceolada, com aproximadamente 13 cm de comprimento, de coloração verde-azulada, levemente pontiagudas, com pedúnculos axilares ou laterais, apresentando de três a seis flores, geralmente na região terminal, com cheiro cítrico, devido ao armazenamento de grandes quantidades de óleo essencial (Figura 5 B), seus frutos vão da forma semi-ovóide a hemisférica, em pedicelos finos e com borda fina. São adaptadas a climas quentes e subúmidos, a solos pobres e bem drenados e matas ou florestas abertas (Baker & Smith, 1920; Vitti & Brito, 2003).

Esta espécie foi introduzida no Brasil em 1943, no estado de São Paulo. Sua produção ocorre para extração de óleo essencial, rico em citral e sua madeira é vendida como lenha (Vitti & Brito, 2003). O óleo essencial de *E. staigeriana* apresenta o aldeído citral (mistura de geranial e neral) como componente majoritário, caracterizando-o com aroma agradável, cítrico, e coloração verde clara, quando combinado com geraniol proporciona odor agradável e refrescante (Baker & Smith, 1920; Vitti & Brito, 2003; Bizzo *et al*., 2009). Já foram relatadas quantidades de até 30% de citral em óleos dessa espécie (Wilkinson & Cavanagh, 2005).

Gilles *et al*. (2010) identificaram 29 compostos no óleo essencial de *E. staigeriana*, representando 97,7% do óleo, são eles: 1,8-cineol, neral, geranial, α- felandreno e geranato de metila. Macedo *et al*. (2010) encontraram citral (24,93%) em maior quantidade no óleo essencial dessa planta, além de α-pineno, β-pineno, β-mirceno, α-felandreno, o-cimeno, l-limoneno, eucaliptol, γ-terpineno, α-terpinoleno, linalol, citronelal, 4-terpineol, geraniol, geranato de metila, acetato de nerila e acetato de geranila. Seu rendimento gira em torno de 1,2 a 2,53%, com teor de citral variando de 10 a 65% (Baker & Smith, 1920; Pinto *et al*., 1976; Vitti & Brito, 2003; Bizzo *et al*., 2009).

A aplicação do óleo essencial de *E. staigeriana* ocorre nas indústrias de perfumaria, onde faz parte da composição de perfumes para diversos fins, na produção de produtos de limpeza (sabões, sabonetes, fragrância de detergentes e desinfetantes, entre outros) e faz parte da composição de cosméticos (Pinto *et al*., 1976; Chagas *et al*., 2002; Vitti & Brito, 2003). O óleo essencial de *E. staigeriana* possui ação antimicrobiana contra os organismos *Staphylococcus aureus, Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* e *Candida albicans* (Wilkinson & Cavanagh, 2005; Gilles *et al*., 2010), além de apresentar ação inseticida sobre *Zabrotes subfasciatus*, *Callosobruchus maculatus*, *Boophilus microplus* e *Lutzomyia longipalpis* (Chagas *et al*., 2002; Brito *et al*., 2006; Batish *et al.*, 2008; Costa *et al.*, 2008; Maciel, 2009), bem como ação anti-helmíntica sobre *Haemonchus contortus* (Macedo *et al*., 2010).

Figura 5. A: *Eucalyptus staigeriana*; B: folhas e ramos (Eliza Zorzi Tomazoni, 2014).

Eucalyptus globulus é uma planta nativa da Austrália e está espalhada por quase todas as regiões tropicais e subtropicais do globo, sendo cultivada no Brasil, Espanha, Portugal, Zimbábue e China. Esta planta é facilmente adaptável a diversas altitudes, latitudes,

umidades, solos ricos em sais minerais, entre outras circunstâncias, sendo uma das árvores mais altas do mundo, chegando até 70 m de altura em florestas densas, porém em matas abertas são árvores pequenas e arbustivas (Figura 6 A), possuem casca lisa, acinzentada ou castanha, que se desprende em placas; com raízes profundas e galhos grandes, folhas alternas, pecioladas, falciforme-lanceoladas e longas, com até 35 cm de comprimento e 10 cm de largura, apresentando heterofilia, onde as folhas jovens são largas e curtas, e as folhas adultas são compridas e pontiagudas (Figura 6 B), suas flores são grandes, brancas e vistosas (Figura 6 C), seu fruto é em capsular e suas sementes são arredondadas (Vitti & Brito, 2003; Rocha & Santos, 2007).

No Brasil, a espécies *E. globulus* foi introduzida em 1855 e é muito utilizada para reflorestamento, drenagem de regiões pantanosas, fornecimento de madeira, na fabricação de papel e de carvão vegetal. O uso de seu óleo essencial está concentrado na indústria farmacêutica, devido ao alto teor de 1,8-cineol, sendo utilizado para produção de inalantes, estimulantes de secreção nasal, produtos de higiene bucal e para dar sabor e aroma aos medicamentos, é utilizado também em aromatizantes de ambiente (Vitti & Brito, 2003; Mantero *et al*., 2007; Rocha & Santos, 2007; Siqueira *et al*., 2007; Macedo, 2008).

Os óleos essenciais de *E. globulus* apresentam como componente principal o 1,8-cineol ou eucaliptol, que aparece em quantidades de até 80% (Vitti & Brito, 2003; Bosnic *et al.*, 2006; Silva *et al*., 2006; Mantero *et al*., 2007; Rocha & Santos, 2007; Siqueira *et al*., 2007; Batish *et al*., 2008; Maciel, 2009). Chagas *et al.* (2002) identificaram no óleo essencial de *E. globulus*, as substâncias α-pineno, β-pineno, β-mirceno, l-felandreno, γ-terpineno, α-terpinoleno e 1,8-cineol. Seu rendimento pode variar de 0,77 a 5,5% com teor de 1,8-cineol de 33 a 89% (Cimanga *et al*., 2002; Vitti & Brito, 2003; Silva *et al.*, 2006; Mantero *et al.*, 2007; Rocha & Santos, 2007; Bizzo *et al.*, 2009; Hendry *et al*., 2009).

O óleo essencial de *E. globulus* apresenta ação alelopática e antisséptica (Mantero *et al*., 2007; Rocha & Santos, 2007), além de atividade bacteriostática e bactericida contra *Staphylococcus aureus, Escherichia coli, Streptococcus agalactiae, Enterococcus faecalis, Klebsiella pneumoniae, Stenotrophonas maltophilia* e *Streptococcus pneumoniae* (Siqueira *et al.*, 2007; Ghalem & Mohamed, 2008; Costa *et al*., 2008), ação antifúngica sobre A*spergillus fumigatus, Aspergillus niger* (Bansod & Rai, 2008), *Candida tropicalis, Candida albicans, Trichophyton mentagrophytes, Microsporum canis, Pythium aphanidermatum* (Cimanga *et al*., 2002; Batish *et al.*, 2008; Castro & Lima, 2010) e *Monilia fructicola* (Knaak & Fiuza, 2010) e atividade inseticida sobre *Boophilus microplus, Musca domestica, Sitophilus zeamais, Acanthoscelides obtectus, Aedes aegypti* (Chagas *et al*., 2002; Batish *et al.*, 2008; Macedo, 2008), *Callosobruchus maculatus* (Brito *et al*., 2006), *Zabrotes subfasciatus* (Rocha & Santos, 2007) e *Lutzomuia longipalpis* (Maciel, 2009).

Neste contexto, a produção de óleo essencial de eucalipto é muito vantajosa, pois, além das diversas propriedades do óleo essencial, que pode ser utilizado para fins diversos, este pode ser extraído das folhas do eucalipto que são um bioproduto do corte das árvores, utilizadas para indústria madeireira e de papel (Silvestre *et al*., 1997; Vitti & Brito, 2003; Mantero *et al*., 2007; Batish *et al.*, 2008; Malinowski, 2010).

Figura 6. A: *Eucalyptus globulus*; B: folhas; C: flores (Adaptado de Rocha & Santos, 2007).

2.5.2. *Foeniculum vulgare*

Foeniculum vulgare Mill. é uma espécie pertencente à família Apiaceae (Umbeliferae), da ordem Apiales, que possui cerca de 300 gêneros, com mais de 3.000 espécies. Desde 1.500 a.C., as plantas umbelíferas, de origem mediterrânea, já eram cultivadas pelos egípcios como alimento, bebida e medicamento (Choi & Hwang, 2004; Simões *et al.*, 2004; Tognolini *et al.*, 2007).

Conhecida como erva-doce ou funcho, é uma antiga erva sazonal. É originária da região Sul do Mediterrâneo que cresce do forma selvagem ou cultivada em todo o Norte, Leste e hemisférios ocidentais, especialmente na Ásia, América do Norte e Europa. A erva era bem conhecido para os antigos egípcios, romanos, índios e chinenes. Os romanos cultivavam esta espécie para obter as sementes aromáticas e os brotos carnudos comestíveis, e ainda hoje é um vegetal muito comum no sul da Itália. O Imperador Carlos Magno era conhecido por ter incentivado seu cultivo na Europa Central. É um ingrediente indispensável na moderna cozinha francesa e italiana. Todas as partes da planta são aromáticas e podem ser usadas de muitas maneiras (Krishnamarthy, 2011; Badgujar *et al.*, 2014). Possivelmente, veio para o Brasil junto com os colonizadores portugueses (Silva, 2001).

F. vulgare é uma planta herbácea, perene, anual ou bianual, que mede de 80 cm a 2 m de altura (Fig. 7 A). Possui caule estriado, folhas pinadas com segmentos filiformes de até 4 cm de comprimento (Fig. 7 C), ramos verticais rígidos e muitas folhas divididas em segmentos lineares (Fig. 7 B). As flores são pequenas, amarelas e encontradas em grandes umbelas achatadas (Fig. 7 D). Os frutos são oblongos a ovóides com 3-5 mm de comprimento e 1.5-2.0 mm de largura (Fig. 7 E) (Badgujar *et al.*, 2014).

Os frutos maduros de *F. vulgare* e seu óleo essencial são usados como agentes aromatizantes nos produtos alimentícios e também como componente de produtos cosméticos e

farmacêuticos (Telci *et al*., 2009; Rather *et al.*, 2012). Trabalhos prévios mostraram que o óleo essencial de *Foeniculum vulgare* Mill. possui como compostos majoritários o trans-anetol, funchone, estragol e limoneno. O óleo essencial também apresenta os compostos α-tujeno, α-pineno, canfeno, sabineno, β-pineno, mirceno, α-felandrene, o-cimeno, γ-terpineno, canfora, terpineno-4-ol, estragol, isocariofileno, palustrol, *(E)*-nerolidol, cis-anetol, *p*-anisaldeido, δ-3-careno e D-germacreno. (Telci *et al*., 2009; Viuda-Martos *et al.*, 2011; Kazemi *et al*., 2012; Roby *et al.*, 2013; Diao *et al.*, 2014; Mota *et al*., 2015 a).

Diversas pesquisas tem demonstrado várias atividades biológicas do óleo essencial, como efeito hepatoprotetivo (Ozbek *et al*., 2003), atividade antidiabética (El-Soud *et al.*, 2011), atividade antitumoral (Pradhan *et al*., 2008), atividade antioxidante (Ruberto, *et al.*, 2000; Singh *et al.*, 2006), atividade antitrombótica (Tognolini *et al.*, 2007), atividade anti-inflamatória (Choi & Hwang, 2004) e atividade acaricida (Lee, 2004). Além disso, o óleo essencial de *F. vulgare* possui significante atividade antifúngica contra *Sclerotinia sclerotiorum*, *Aspergillum niger, Aspergillum flavus, Fusarium graminearum* e *Fusarium moniliforme* (Singh *et al.*, 2006; Soylu *et al*., 2007).

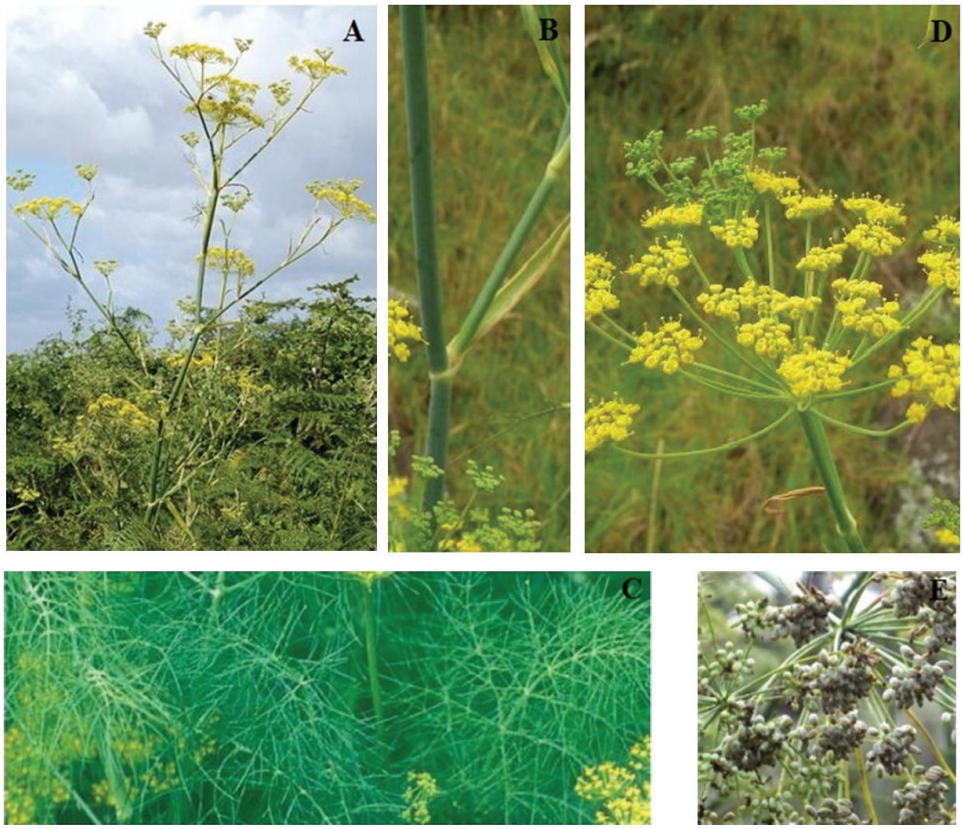

Figura 7. A: *Foeniculum vulgare*; B: caule; C: folhas; D: inflorescência e flores; E: frutos (Adaptado de Badgujar *et al*., 2014).

2.5.3. *Baccharis trimera* e *Baccharis dracunculifolia*

A família Asteraceae é o grupo sistemático mais numeroso dentro das Angiospermas, compreendendo cerca de 1.100 gêneros e 25.000 espécies. São plantas de aspecto extremamente variado, incluindo principalmente pequenas ervas ou arbustos e raramente árvores (Heywood, 1993). Cerca de 98% dos gêneros são constituídos por plantas de pequeno porte e são encontradas em todos os tipos de habitats, mas principalmente nas regiões tropicais montanhosas na América do Sul (Joly, 1967).

O gênero *Baccharis* (tribo Astereae) está representado por mais de 500 espécies distribuídas principalmente no Brasil, Argentina, Colômbia, Chile e México, ocupando as regiões mais elevadas (Dupont, 1966; Malagarriga, 1976). A alta concentração de espécies no Brasil e nos Andes indica que uma dessas áreas é o provável centro de origem desse gênero (Budel *et al.*,

2005). As espécies do gênero Baccharis são no geral arbustos e medem em média de 0,5 a 4,0 m de altura. Apresentam elevado valor socioeconômico, com ampla dispersão nos estados de Santa Catarina, Paraná, São Paulo e Rio Grande do Sul, entre outras regiões do país, onde grande número delas são utilizadas na medicina popular, para controle ou tratamento de várias doenças. São consumidas principalmente na forma de chás com indicações para males do estômago, fígado, anemias, inflamações, diabetes, doenças na próstata, sendo também descritas como remédio para o processo de desintoxicação do organismo (Corrêa, 1984; Korbes, 1995; Franco, 1995).

Muitas espécies deste gênero tiveram a atividade biológica estudada, destacando-se as atividades alelopática, analgésica, antidiabética, anti-inflamatória, antileucêmica, antimicrobiana, antimutagênica, antioxidante, citotóxica, espasmolítica, gastroprotetora, hepatoprotetora, vasorrelaxante, antiviral, inseticida, antibactericida, esquisatomicida e antifúngica (Kupchan *et al*., 1976; Soicke & Leng-Peschlow, 1987; He *et al.*, 1996; De las Heras *et al.*, 1998; Nakasugi e Komai, 1998; Torres *et al*., 2000; Avancini *et al.*, 2000; Weimann *et al*., 2002; Feresin *et al.*, 2003; Oliveira *et al*., 2005; Blanco & Benedetti, 2009; Gonçalves, 2010; Oliveira *et al*., 2012; Vieira *et al*., 2014, Caneschi *et al*., 2015).

Baccharis trimera (Less.) DC. conhecida popularmente como “carqueja, carqueja-amargosa, carqueja-do-mato, carquejinha e tiririca-de-balaio”, é uma das espécies melhor estudadas em termos botânicos, químicos e farmacológicos. É um subarbusto perene, ereto, ramificado na base, caules alados denominados cladódios com ramos verdes de expansões trialadas, com 50 a 80 cm de altura, é nativa do sul e sudeste do Brasil (Fig. 8 A e B) (Lorenzi, 2000). As alas dos cladódios apresentam mesófilo homogêneo, estômatos com poros espessados, fibras e tricomas pluricelulares que exsudam óleos voláteis (Chicourel *et al.*, 1998). Possui inflorescências em capítulo, dispostas ao longo dos ramos, de cor esbranquiçada e produz frutos do tipo aquênio (Fig. 8 C e D) (Lorenzi, 2000; Lorenzi & Matos, 2002). Apresenta

ampla dispersão nos estados de Santa Catarina, Paraná, São Paulo e Rio Grande do Sul (Verdi *et al.*, 2005).

B. trimera é amplamente estudada em relação à sua composição química e atividade biológica incluindo atividade antidiabética (Oliveira *et al.*, 2005), antioxidante (Abad *et al.*, 2007; Dias *et al.*, 2009; Pádua *et al*., 2010), anti-inflamatória (Abad *et al*., 2007), antiulcerogênica (Dias *et al.*, 2009) esquistosomicida (Oliveira *et al*., 2012), antibactericida (Avancini *et al.*, 2000; Blanco & Benedetti, 2009; Gonçalves, 2010) e atividade antifúngica (Vieira *et al*., 2014, Caneschi *et al*., 2015).

Chialva & Doglia (1990) e Simões-Pires *et al*. (2005) caracterizaram 35 e 23 compostos, respectivamente, no óleo essencial de plantas de *B. trimera* do sul do Brasil. Dentre estes compostos, diversos autores destacam o carquejol acetato como composto majoritário (Simões-Pires *et al*., 2005; Besten *et al*., 2013) além de α-pineno, saboneno, β-pineno, mirceno, limoneno, carquejol, β-elemeno, β-cariofileno, γ- gurjeneno, α- selineno, α-muuroleno, elemol, ledol, espatulenol, globulol, epiglobulol, δ- cadineno, 1 –epi-cubenol, β-eudesmol e α-cadinol (Simões-Pires *et al.*, 2005). O carquejol acetato presente na família Asteraceae está relacionada a atividade antifúngica (Tabassum & Vidyasagar, 2013). A ação fungicida deste óleo essencial foi confirmada por Caneschi *et al*. (2015) que testou o óleo essencial contra os fungos *Trichophyton rubrum* e *Microsporum canise*.

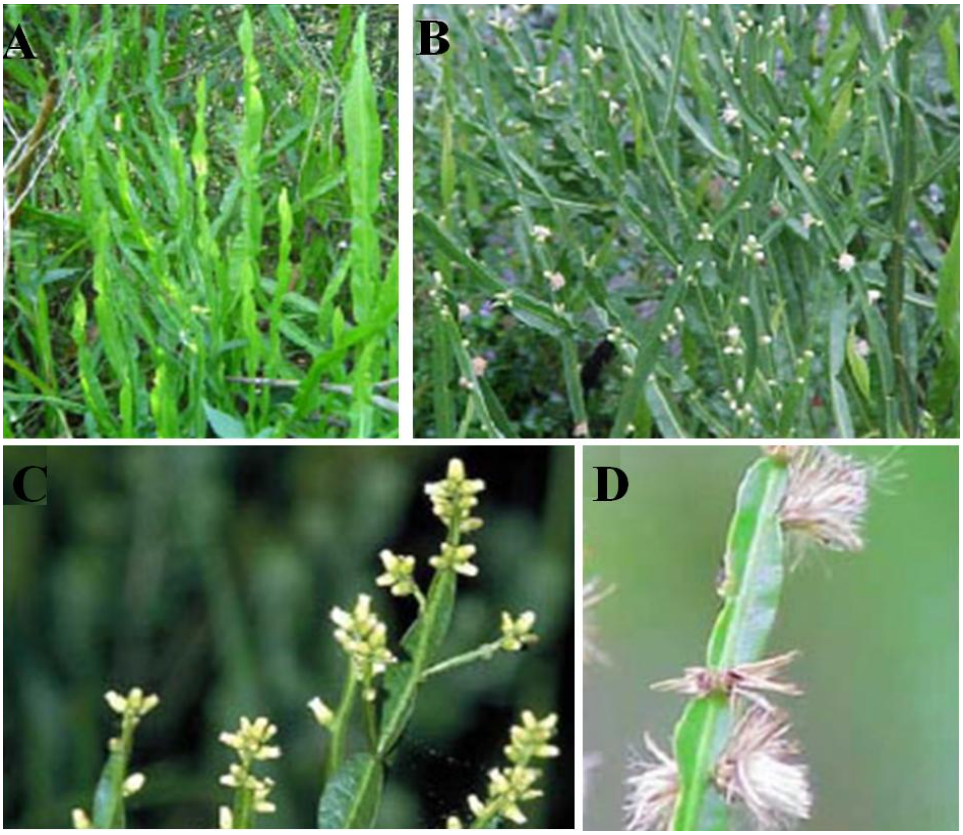

Figura 8. A: *Baccharis trimera* em estágio vegetativo; B: *Baccharis trimera* em estágio reprodutivo; C: ramo de inflorescência; D: detalhe dos frutos aquênios (Adaptado de Carreira, 2007).

A *Baccharis dracunculifolia* D.C. (De Candole), Asteraceae, é popularmente conhecida como "vassoura, alecrim-do-campo, alecrim vassoura, carqueja, chilca, cilca, erva-de-são-joão-maria, suncho, thola, vassoureira ou vassourinha" e é amplamente utilizada na medicina popular. A planta já foi muito utilizada na confecção de vassouras rústicas, de onde vem um de seus nomes populares (Takeda & Farago, 2001). A forma de infusão de suas folhas é empregada para problemas hepáticos, disfunções estomacais, cansaço físico, inapetência, afecções febris, debilidade orgânica e como anti-inflamatória (Budel *et al*., 2004).

B. dracunculifolia (Fig. 9 A) é um arbusto com ocorrência no Brasil, Uruguai, Argentina, Paraguai e encontrada também nos altos vales da Bolívia, chegando até 3.280 m de altitude (Cassel *et al*., 2000). É uma planta dióica com inflorescências (Fig. 9 B) masculinas e

femininas em indivíduos separados, possui folhas lanceoladas, membranáceas, uninérvias, com 1 a 2 cm de comprimento e 3 a 4 mm de largura, densamente pontuadas de glândulas, com margens inteiras, 1 a 3 denteadas, raramente apresentando entre 5 e 7 dentes. Possui flor feminina com corola de 2 a 3 mm de comprimento e flor masculina com corola de 2,5 a 3 mm de comprimento. Os frutos são denominados cipselas ou aquênios, sendo que, o mais adequado é o uso do termo cipsela, conforme Marzinek *et al*. (2008). Os pápus são bem desenvolvidos e o carpopódio distinto, onde se dá a abscisão do fruto no momento da dispersão (Bremer, 1994).

O óleo essencial de *B. dracunculifolia* tem como significativos os teores de β-Pineno, limoneno e espatulenol (Frizzo *et al.,* 2008; Massignani *et al*., 2009; Parreira *et al*., 2010). Cassel *et al.* (2000) identificaram 14 constituintes, contra 100 constituintes obtidos por Weyerstahl *et al.* (1996) no óleo de *B. dracunculifolia*, com ambas amostras coletadas no sul do Brasil. Ferracini *et al.* (1995) identificaram em plantas masculinas e femininas os compostos α-pineno, β-pineno, limoneno, α-terpineol, trans-cariofileno, aromadendreno, α-humuleno, δ-cadineno, espatulenol, nerolidol e globulol. Cassel *et al.* (2000), encontraram trans-nerolidol e espatulenol como componentes principais em óleos essenciais obtidos município de Campestre da Serra - RS. Fabiane *et al.* (2008) encontraram os compostos β-pineno, trans-nerolidol, limoneno e espatulenol com majoritários em plantas coletadas no Sudeste do Paraná.

Parreira *et al*. (2010) encontraram atividade leishmanicida e esquistossomicida para o óleo essencial. O óleo essencial também foi sugerido para o tratamento de úlceras gástricas por Massignani *et al*. (2009). Oliveira *et al*. (2015) avaliaram a atividade do óleo essencial de *B. dracunculifolia*, que em altas concentrações demonstrou que possui uma boa atividade antifúngica contra *Candida albicans.*

Figura 10. *Baccharis dracunculifolia* em fase vegetativa; B: inflorescência (Adaptado de Rigotti, 2011).

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a eficácia dos óleos essenciais de *Eucalyptus globulus*, *Eucalyptus staigeriana*, *Foeniculum vulgare*, *Baccharis trimera* e *Baccharis dracunculifolia* no controle de *Botrytis cinerea* e *Colletotrichum acutatum* em videiras (*Vitis* spp. e *Vitis vinifera*) e o efeito residual do óleo essencial no vinho.

3.2. Objetivos Específicos

- Avaliar *in vitro* a atividade antifúngica do óleo essencial de *Eucalyptus globulus, Eucalyptus staigeriana, Foeniculum vulgare, Baccharis trimera e Baccharis dracunculifolia* sobre o crescimento micelial de *Botrytis cinerea* e *Colletotrichum acutatum;*

- Avaliar *in vitro* a atividade antifúngica do óleo essencial de *Eucalyptus globulus*, *Eucalyptus staigeriana, Foeniculum vulgare, Baccharis trimera e Baccharis dracunculifolia* sobre a germinação de conídios de *Botrytis cinerea* e *Colletotrichum acutatum;*

- Avaliar *in vivo* o controle das podridões da uva no pós-colheita com os óleos essenciais selecionados na etapa *in vitro.*

- Avaliar *in vivo* o controle de *Botrytis cinerea* e *Colletotrichum acutatum* em videiras (*Vitis* spp. *e Vitis vinifera*) com o óleo essencial selecionado na etapa *in vitro.*

- Analisar *in vitro* a qualidade enológica do vinho produzido a partir das uvas tratadas com o óleo essencial selecionado na etapa *in vitro.*

4. RESULTADOS

Os resultados desta dissertação estão apresentados na forma de quatro artigos científicos. O primeiro intitulado "Control of postharvest fungal rots on grapes using essential oil of *Foeniculum vulgare* Mill.", que foi submetido à *Journal of Agricultural Science;* o segundo intitulado "Antifungal activity of *Baccharis trimera* and *Baccharis dracunculifolia* essential oils against postharvest fungal rots in grapes", que será submetido à revista *Journal of Phytopathology;* o terceiro intitulado "Alternative control of fungal rots on wine grapes with essential oil of *Eucalyptus staigeriana* and *Eucalyptus globulus"* que será submetido à revista *Journal of Plant Pathology;* e o quarto intitulado "Effect of *Eucalyptus staigeriana* essential oil against fungal rots on table and wine grapes que será submetido à revista *Food Control Journal.*

4.1. Capítulo 1

Control of postharvest fungal rots on grapes using essential oil of *Foeniculum vulgare* Mill.

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Abstract

Fungal rots, caused by *Botrytis cinerea* and *Colletotrichum acutatum,* are the main postharvest decay of table grapes in Brazil. The aim of this study was to assess the chemical composition and the fungicidal effect of *Foeniculum vulgare* essential oil *in vitro,* on mycelial growth (contact and volatile phase) and conidia germination, and *in vivo* on postharvest grapes against both fungi. The major compounds found in *F. vulgare* essential oil were trans-anethole (79.14%), fenchone (11.94%) and estragole (5.76%). The mycelial growth (contact phase) and conidia germination of *B. cinerea* was inhibited completely at concentrations of 50 and 100 ppm (µL mL^{-1}), respectively. For *C. acutatum* mycelial growth (contact phase) e conidia germination was inhibited completely at concentrations of 100 and 200 ppm, respectively. The volatile phase had a fungistatic effect on mycelial growth of both fungi at different concentrations tested, and the pure essential oil (100%) presented fungicidal effect against *B. cinerea.* In *in vivo* tests, were carried out using grapes of *Vitis* spp. cv. "Isabela" and the concentrations of essential oil tested were efficient, reducing the incidence of disease caused by *B. cinerea* and *C. acutatum,* both in preventive and curative treatment. The concentration 200 ppm completely inhibited the incidence of both fungi. The *F. vulgare* essential oil presented fungicidal action against postharvest fungal rots on grapes.

Keywords: Alternative control, *Botrytis cinerea, Colletotricum acutatum*, fennel, *Vitis* spp..

1. Introduction

Grape is one of the most important fruit crops worldwide. In Brazil, grape production destined for processing (wine, juice and derivatives) was 673.422 million kilos in 2014, representing 46.89% of the national production. The remaining production (53.11%) was intended for consumption *in natura* (Mello, 2014). "Isabela" grape is one of the most important varieties of *Vitis* spp. and it is the most diffused variety in the Serra Gaúcha, the southern viticultural region of Brazil, where it represents near 57% of the total production. "Isabela" grape is used to make red table wine and juice and it is also commercialized as table grape (Mello, 2014; Silveira, Hoffmann & Garrido, 2015).

The economic losses due to fungal infection in fruit and vegetables within the postharvest chain are variable and not well documented and they usually reach any where from 30 to 50% and on some occasions rots can lead to total loss of the produce (Smilanick, Brown & Eckert, 2006; Youssef & Roberto, 2014). *Botrytis cinerea* Pers. Fr. and *Colletotrichum acutatum* Simmonds cause fungal rot on a large number of economically important agricultural crops and they are considered the main cause of great losses of postharvest in table grapes (Pearson & Goheen, 1988; Peres, Kuramae, Dias & Souza, 2002; Steel, Greer & Savocchia, 2007; Whitelaw-Weckert et al., 2007).

By over efficient that it is the phytosanitary treatment made in the field, it is not enough to dismiss it in postharvest (Lichter et al., 2002). As a postharvest treatment, grapes are usually stored with sulfur dioxide fumigation (Droby & Lichter, 2004). However, the use of synthetic fungicides and sulfur dioxide is not allowed on organic grapes (Mlikota Glaber & Smilanick, 2001). In addition, the growing public concerns about health and environmental hazards associated with pesticide use have resulted in a considerable interest in developing alternative non-polluting control methods (Youssef & Roberto, 2014).

Several studies have proven the effect of extracted compounds isolated from essential oils of plants, which act as natural fungicides inhibiting fungal activity (Chao & Young, 2000). Moreover, the majority of essential oils are classified by the FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe), recognized as safe for use in foods, so there has been a growing interest in using them in the treatment of fruits and vegetables (Gonzáles-Aguilar et al., 2008).

Fennel (*Foeniculum vulgare* Mill.) is a small genus of annual, biennial or perennial herbs. It is widely cultivated throughout the temperate and tropical regions of the world for its aromatic fruits, which are used as a culinary spice (Diaz-Maroto, Pearez-Coello, Esteban & Sanz, 2006; Rather, Dar, Sofi, Bhat & Qurishi, 2012). Mature fennel fruit and its essential oil are used as flavoring agents in food products and also as a constituent in cosmetic and pharmaceutical products (Rather et al., 2012; Telci, Demirtas & Sachin, 2009). Moreover there are reports on various biological activities of the essential oil, such as hepatoprotective effect (Ozbek et al., 2003), antidiabetic activity (El-Soud et al., 2011), antitumour activity (Pradhan et al., 2008), antioxidant activity (Ruberto, Baratta, Deans & Dorman, 2000; Singh, Maurya, Lampasona & Catalan, 2006), antithrombotic activity (Tognolini et al., 2007), anti-inflammatory activity (Choi & Hwang, 2004) and acaricidal activity (Lee, 2004). In addition, the essential oil of fennel fruits had significant antifungal activity against various phytopathogens (Singh et al., 2006; S. Soylu, Yigitbas, E.M. Soylu & Kurt, 2007).

The aim of this study was to evaluate the effect of *F. vulgare* essential oil on the mycelial growth and conidia germination of *B. cinerea* and *C. acutatum* and evaluate *in vivo* grape rot control in postharvest using the essential oil.

2. Material and methods

2.1 Isolated fungi

Strains of *B. cinerea* (A58/09) and *C. acutatum* (A009/13) used in this work were isolated from grapes of Caxias do Sul (Serra Gaúcha – RS – Brazil) and preserved in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul - Brazil, on PDA (Potato Dextrose Agar) medium. The molecular confirmation of both fungi was done using Internal Transcribed Sequence (ITS)-PCR identification. The DNA extraction was according to Murray and Thompson (1980) and ITS-PCR amplified the region ITS-5.8S rDNA according to White, Bruns, Lee and Taylor (1990). Sequencing was proceed at the Human Genome Center – USP and the sequences obtained were edited with the software BioEdit Sequence Alignment Editor (1997-2005) and used to search for similar sequences using Blastn at NCBI.

2.2 Plant material

Fruits in the final stage of maturation of *F. vulgare* were collected from plants localized in the city of Caxias do Sul – Brazil. A voucher specimen of the plant species was deposited in the Herbarium of the University of Caxias do Sul with number 44057.

2.3 Essential oils extraction and analysis

The essential oil was extracted by hydrodistillation from dried fruits for 1 hour in a Clevenger-type apparatus according to Agostini et al. (2009). For identification and quantification of compounds in the essential oil, it was used the protocol described in Tomazoni et al. (2016) using a gas chromatograph HP 6890, coupled with a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. The analyses were conducted using a fused silica capillary column HP-Innowax (30 m × 0.25 mm i.d., 0,25 µm film thickness, Hewlett Packard, Palo Alto, USA). The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

2.4 In vitro antifungal assay

2.4.1.Antifungal activity of essential oil on mycelial growth

The antifungal properties of essential oil were assessed for its contact and volatile phase effects towards mycelial growth of phytopathogens. Contact phase effect of essential oil was tested according to Feng and Zheng (2007) with minor modifications. Essential oils concentrations used were 10, 50, 100 and 200 ppm, with the addition of Tween 20 (1:1), diluted on autoclaved and melting PDA (Potato Dextrose Agar) (40ºC) under aseptic conditions. The control treatment was just PDA medium with addition of Tween 20 at concentration 200 ppm (similar to the highest concentration used to emulsify the essential oil). These emulsions were poured into 9 cm (Ø) Petri dishes and, after medium solidification, inoculated with 5 mm (Ø) agar disks colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development.

To assess fungicidal action of the volatile phase of essential oil on the mycelial growth of fungi it was utilized the methodology according to Silva (2012) with minor modifications. Agar disks with 5 mm (Ø) colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development were placed in the center of the Petri dish containing PDA culture medium.

The concentrations of essential oils used were 12.5, 25, 50 with the addition of Tween 20 (0.1%) and 100% (pure essential oil, without addition of Tween 20). A 100 µL sample of pure essential oil and the solutions were applied onto a cotton ball attached to the inner face of a Petri dish lid, thereby preventing direct contact of the oil with the culture medium and the mycelium disk, creating a saturated atmosphere of volatile compounds. The control treatment was just PDA medium and 100 µL of Tween 20 (0.1%) applied in cotton ball.

In both tests, for each concentration, ten replicate plates were used. Incubation was performed at 25º C temperature and 12 hours photoperiod, during fourteen days. Diameter orthogonal measurements by fungus development were performed. Fungal growth was recorded on the 3^{rd}, 5^{th}, 7^{th}, 10^{th} and 14^{th} day by measurement of orthogonal diameter. Transfer experiments were performed to provide a distinction between the fungistatic and fungicidal effects of essential oil on the target microorganisms. For this purpose, plugs that did not grow were transferred to fresh PDA dishes to assess their viability and growth after five days of inoculation at 25° C temperature and 12 hours photoperiod. The residual fungal growth was monitored by measurement of orthogonal diameter.

2.4.2 Antifungal activity of essential oil on conidia germination

Antifungal activity of essential oil on conidia germination was tested according to Badawy and Rabea (2013), with minor modifications. Conidia of *B. cinerea* and *C. acutatum* were harvested from a colony of the fungi 14 days grown in PDA at 25º C temperature and 12 hours photoperiod. Five milliliter of sterile water was added to a Petri plate culture. The conidia were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia mL^{-1}. Aliquots of conidia suspension (50 µL) were placed in microtubes containing 500 µL of PDB (Potato Dextrose Broth) medium treated with essential oils, at the concentrations of 10, 50, 100 and 200 ppm, with the additon of Tween 20 (1:1). The control treatment was just PDB with addition of Tween 20 at concentration 200 ppm (similar to the highest concentration used to emulsify the essential oil). The tubes were incubated at 25º C for 16 hours. The samples were placed on a Neubauer chamber and observed under the microscope for conidia germination. The counting of conidia was done using a light microscopy at 10× magnification. All experiments were conducted in ten replicates and in each replicate were evaluated hundred conidia. The conidia

were considered germinated when the length of the germ tube equaled or exceeded the length of the conidia.

2.5 In vivo antifungal assay

2.5.1 Inoculum preparation

Conidia of *B. cinerea* and *C. acutatum* were harvested from a colony of the fungi 14 days grown in PDA at 25º C temperature and 12 hours photoperiod as described above. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia mL^{-1}.

2.5.2 Fruit

Traditionally grown, freshly harvested, *Vitis* spp. cv. "Isabela" grapes from Bento Gonçalves – RS - Brazil were used in experiments.

2.5.3 Antifungal activity of essential oil on grapes

To evaluate the antifungal activity of the essential oil on grapes it was carried out experiments with curative and preventive treatments. The postharvest curative treatment consisted of inoculation of 10 berries for each cluster (10 fruit / treatment) of cultivating vine cv. "Isabela" (*Vitis* spp.), through wounds, approximately 2 mm deep, with the aid of a syringe (Zahavi et al., 2000). After the injury, the clusters were inoculated by spraying the conidia suspension of *B. cinerea* or *C. acutatum*, according to the methodology described by Romanazzi, Nigro, Ippolito, Di Venere and Salermo (2002) and Thomas, Marois and English (1988) with modifications. After 4 hours, the application of essential oil was carried out with the concentrations based on the *in vitro* test (50, 100 and 200 ppm). Subsequently, in order to evaluate the potential of oil in preventing disease, the grape clusters were sprinkled with essential oils in the same concentrations of the previous test and inoculated after 24 hours with the fungi. For both experiments, the clusters were placed in plastic boxes and kept at 25 ± 1° C / 80-90% relative humidity for a period of five days for those inoculated with *B. cinerea* and seven days for those inoculated with *C. acutatum.* At the end of this period, assessment of the incidence and severity of disease was performed. To evaluate the incidence, ten berries for each bunch of grapes that were inoculated were evaluated and it was used the mean number of berries with symptoms of the disease. For assessing the severity, a scale from 0 to 100% was created in accordance with the berry area affected by the disease.

2.6 Statistical analysis

Data normality was determined by Kolmogorov-Smirnov test and the homogeneity of variances was determined using Levene's test. Data were analyzed by ANOVA and the threshold for statistical significance was set at $p < 0.05$. In the case of statistical significance Dunnett's T3 test was applied to separate the means. All statistics analysis was performed using SPSS 22.0 for Windows.

3. Results

3.1 Chemical composition of the essential oil

A total of 09 components of the essential oil were identified by GC-MS, representing 99.95% of the total amount (Table 1). The most abundant components of the *F. vulgare* essential oil were trans-anethole (79.14%), fenchone (11.94%) and estragole (5.76%). Other components such as limonene (1.01%), anisaldehyde (0.96%), 1,8-cineole (0.39%), α-pinene (0.3%), β-thujene (0.25%) and camphor (0.2%) were present in lower amounts.

Table 1. Chemical composition of essential oil from *Foeniculum vulgare* fruits.

Compounds	**RI**[a]	**Peak area (%)**[b]
α-pinene	8.204	0.3
β-thujene	16.526	0.25
limonene	18.232	1.01
1,8-cineole	18.730	0.39
fenchone	28.908	11.94
camphor	34.540	0.2
estragole	41.320	5.76
trans-anethole	47.721	79.14
anisaldehyde	54.970	0.96

[a] RI, the retention index publyshed by Adams.

[b] Peak area obtained by GC-FID.

3.2 In vitro antifungal effect of F. vulgare essential oil

3.2.1 Antifungal activity of essential oil on mycelial growth

The *in vitro* antifungal activity of essential oil differed for each fungi and concentration tested at contact phase experiments (Table 2 and Figure S1 A and B). The effect of essential oil on the mycelial growth of *B. cinerea* resulted in completely inhibition at concentration 50 ppm and the fungicidal action was observed by the transfer experiment. For the concentration 10 ppm there was a significant inhibition until the 5th day compared to control, also the mycelial growth presented a different morphology. On the other hand, the completely inhibition of the

mycelial growth of *C. acutatum* occurred at a higher concentrations (100 and 200 ppm) with fungicidal action proven by the transfer experiment. The concentration 10 ppm presented a fungistatic action until the 3rd day, and the same was observed for concentration 50 ppm until the 10th day, being significantly different from control. Both concentrations presented a modified morphology for the mycelial growth.

Table 2. Effect of different concentrations of *Foeniculum vulgare* essential oil, added on the solid media, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum acutatum* (contact phase).

	Mycelial growth (mm)				
	B. cinerea				
	0	10	50	100	200 (ppm)
3rd day	47,75 ± 2.07 a	14.80 ± 1.26 b	0 ± 0 c	0 ± 0 c	0 ± 0 c
5th day	90.00 ± 0 a	73.53 ± 2.37 b	0 ± 0 c	0 ± 0 c	0 ± 0 c
7th day	90.00 ± 0 a	81.88 ± 3.58 a	0 ± 0 b	0 ± 0 b	0 ± 0 b
10th day	90.00 ± 0 a	82.53 ± 3.33 a	0 ± 0 b	0 ± 0 b	0 ± 0 b
14th day	90.00 ± 0 a	82.75 ± 3.28 a	0 ± 0 b	0 ± 0 b	0 ± 0 b
	C. acutatum				
	0	10	50	100	200 (ppm)
3rd day	17.11 ± 0,39 a	13.23 ± 0.60 b	0 ± 0 c	0 ± 0 c	0 ± 0 c
5th day	39.99 ± 1.38 a	34.50 ± 0.90 a	6.34 ± 2.25 b	0 ± 0 b	0 ± 0 b
7th day	55.08 ± 3.17 a	50.06 ± 1.71 a	19.9 ± 3.45 b	0 ± 0 c	0 ± 0 c
10th day	72.08 ± 4.35 a	63.67 ± 2.52 a	43.78 ± 3.21 b	0 ± 0 c	0 ± 0 c
14th day	83.09 ± 3.77 a	77.08 ± 1.67 a	69.41 ± 3.12 a	0 ± 0 b	0 ± 0 b

Values are the average of ten replicates per treatment ± SE. The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line). Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

The effect of the volatiles compounds in the mycelial growth of *B. cinerea* showed a total inhibition at 100% concentration (fungicidal action was confirmed by transfer experiment) and for *C. acutatum* the same concentration reduced the mycelial growth but do not complete inhibited it (Table 3 and Figure S2 A and B). At the concentration of 25%, growth was complete inhibited until 7th day, and the concentration of 50% inhibited the growth until 10th day of *B. cinerea*, showing that maybe a reapplication of the essential oil could control the growth of the fungus.

Table 3. Effect of different concentrations of *Foeniculum vulgare* essential oil, apllied on the lid, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum acutatum* (volatile phase).

	Mycelial growth (mm)				
	B. cinerea				
	0.0	12.5	25	50	100 (%)
3rd day	57.94 ± 5.17 a	0 ± 0 b	0 ± 0 b	0 ± 0 b	0 ± 0 b
5th day	84.11 ± 2.53 a	8.47 ± 3.08 b	0 ± 0 b	0 ± 0 b	0 ± 0 b
7th day	85.59 ± 2.32 a	38.53 ± 8.81 b	0 ± 0 c	0 ± 0 c	0 ± 0 c
10 th day	85.92 ± 2.11 a	85.89 ± 2.42 a	47.98 ± 10.2 ab	0 ± 0 b	0 ± 0 b
14 th day	87.88 ± 1.42 a	88.78 ± 0.82 a	77.54 ± 6.03 a	19.29 ± 5.01 b	0 ± 0 b
	C. acutatum				
	0.0	12.5	25	50	100 (%)
3rd day	22.80 ± 1.71 a	12.81 ± 1.50 ab	12.86 ± 0.88 ab	10.91 ± 0.45 ab	3.62 ± 1.85 b
5th day	36.71 ± 1.28 a	26.85 ± 2.46 ab	25.59 ± 1.85 ab	18.56 ± 1.03 ab	15.60 ± 1.30 b
7th day	49.07 ± 1.05 a	39.98 ± 3.18 a	35.65 ± 2.03 ab	23.68 ± 1.82 ab	18.69 ± 1.59 b
10 th day	73.74 ± 4.18 a	59.08 ± 4.08 a	45.30 ± 3.50 ab	28.81 ± 3.02 b	24.60 ± 2.86 b
14 th day	80.43 ± 4.72 a	67.58 ± 6.14 a	58.00 ± 5.57 ab	35.69 ± 4.37 ab	29.38 ± 3.59 b

Values are the average of ten replicates per treatment ± SE. The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line). Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

3.2.2 Antifungal activity of essential oil on conidia germination

Germination of conidia of *B. cinerea* was inhibited completely at concentration 100 ppm, while the complete inhibition for *C. acutatum* was at concentration 200 ppm (Figure 1and Figure S3 A and B). The concentrations of 10 and 50 ppm showed a significant reduction in the germination of conidia of *B. cinerea* and in the lenght of the germ tube (data not shown).

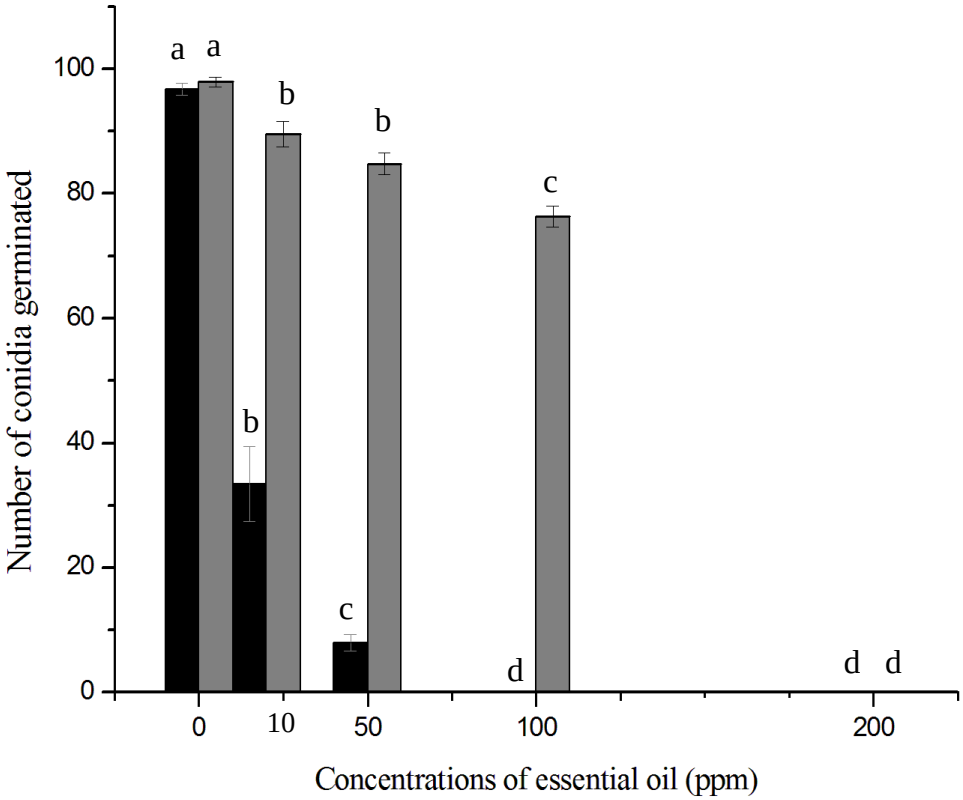


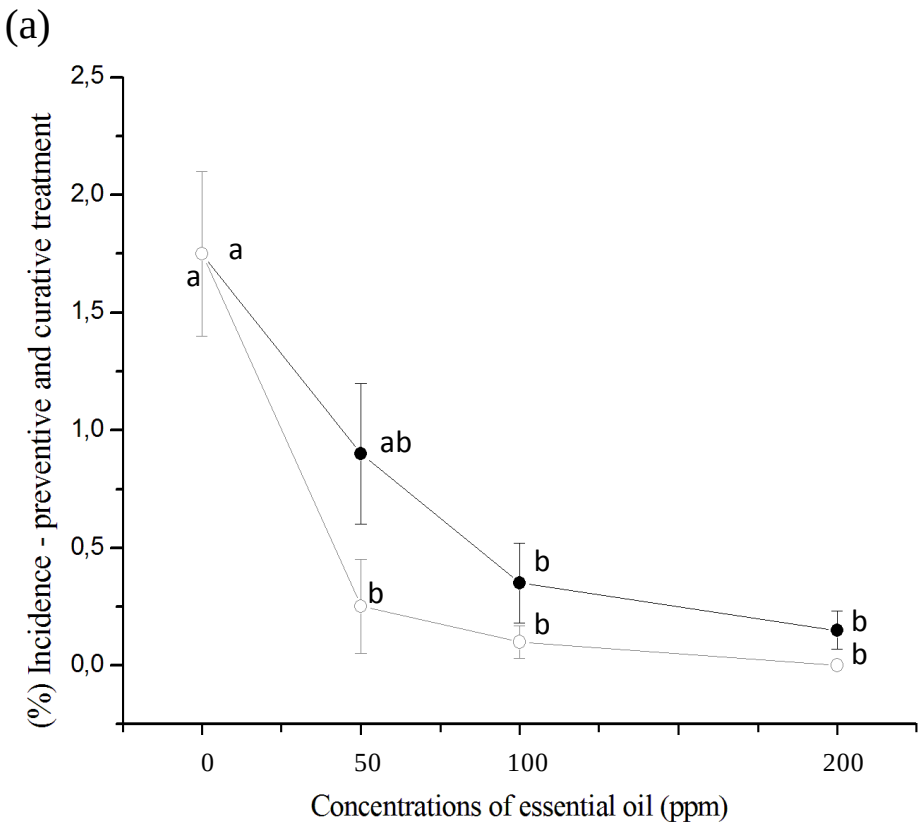
Figure 1. Effect of different concentrations of *Foeniculum vulgare* essential oil on conidia germination of *Botrytis cinerea* (■) and *Colletotrichum acutatum* (□). Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

3.3 Antifungal activity of essential oil in postharvest grapes

Different concentrations of *F. vulgare* essential oil were efficient, reducing the incidence of disease caused by *B. cinerea* and *C. acutatum*, both in preventive and curative treatment. In the preventive treatment of *B. cinerea*, all essential oil concentrations (100 and 200 ppm) were able to reduce the incidence when compared to control. The curative treatment proved to be more efficient and at the concentration 200 ppm no incidence of disease was detected (Figure 2 A and Figure S4 A and B). In the preventive treatment of *C. acutatum* concentration 200 ppm was able to inhibit the incidence of the disease, being different of control. Similarly to the test with *B. cinerea* the curative treatment of *C. acutatum* proved to be more efficient, concentrations 50 and 100 ppm significantly reduced disease incidence, while the concentration 200 ppm presented no incidence of disease (Figure 2 B Figure S4 A and B).

The severity of both diseases, showed no significant difference in concentrations compared to control when the disease was detected (data not shown). The grape clusters treated with

essential oil of *F. vulgare* did not show any obvious signs of phytotoxicity, just showed up brighter.



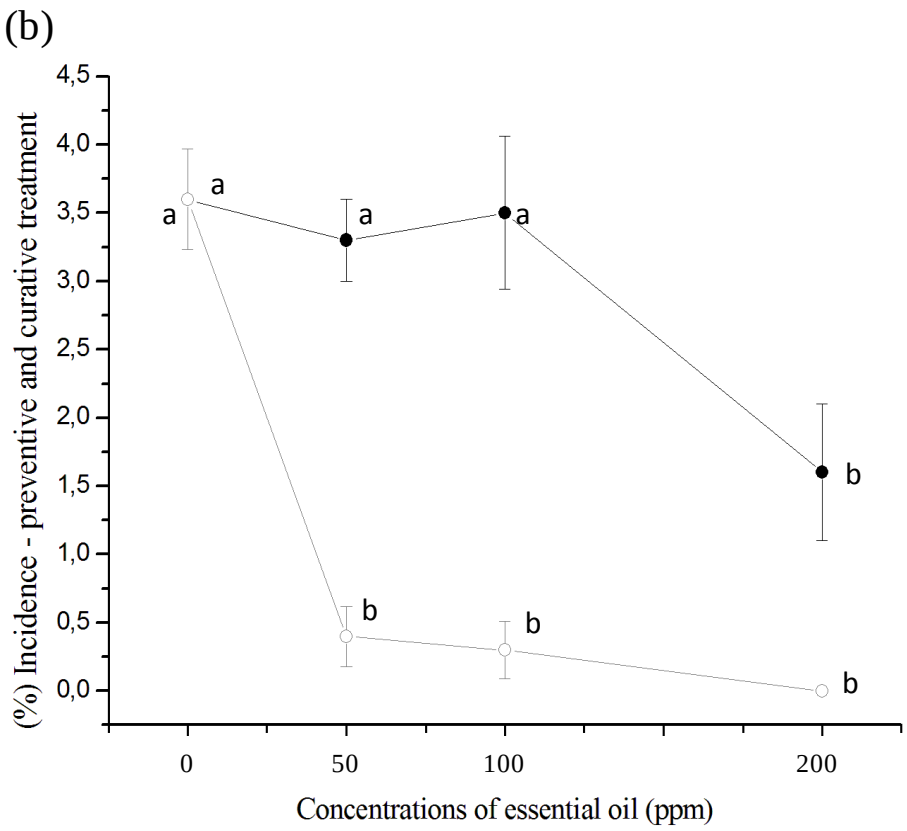


Figure 2. The effects of different concentrations of essential oil of *Foeniculum vulgare* in grapes. Incidence of disease caused by *Botrytis cinerea* (a) and *Colletotrichum acutatum* (b) as to preventive (●) and curative (○) treatment. Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

4. Discussion

The increasing social and economic implications caused by fungi diseases means that there is a constant striving to produce safer food and to develop new antifungal agents (Feng & Zheng, 2007). Essential oils are complex, volatiles and natural compounds of plants, known by its antiseptic, bactericides and fungicides characteristics (Bakkali, S. Averbeck, D. Averbeck & Idaomar, 2008). The antifungal property of several essential oils on postharvest pathogens of fruits and vegetables under *in vitro* and *in vivo* conditions has been investigated (Feng & Zheng, 2007; Zambonelli, Zechini D'Aulerio, Bianchi & Albasini, 1996).

The essential oil of *F. vulgare* used in this experiment contained high levels of trans-anethole (79.14%) as well as fenchone and estragole as major compounds, similarly to the results reported by Roby, Sarhan, Selim and Khalel (2013) that found trans-anethole (65.4%), fenchone (8.26%), estragole (5.2%) and limonene (4.2%) as the major components of *F. vulgare* essential oil.

A literature search revealed that there are variations in the composition and proportion of the major compounds. Kazemi, Rostami and Shafiei (2012) informed that the major components of *F. vulgare* essential oil were trans-anethole and fenchone, while Diao, Hu, Zhang and Xu (2014), Viuda-Martos et al. (2011), Mota et al. (2015) and Telci et al. (2009) found that trans-anethole, estragole, limonene and fenchone in different proportions were the major compounds in the essential oil of Chinese fennel, Egyptian fennel, Portuguese fennel and sweet fennel cultivated in Turkey, respectively. These differences in components and its content of essential oil from fennel may be concerned in the geographical origins (Diaz-Maroto et al., 2006), cultivated varieties and maturity of fennel fruits, as well as extraction methods (Mimica-Dukié, Kujundzié, Sokovié & Couladis, 2003; M.C. Diaz-Maroto, H.I.J. Díaz-Maroto, Sanchez-Polomo & Pérez-Coello, 2005).

The *in vitro* data presented in this study indicated that *F. vulgare* essential oil had a fungistatic effect at low concentrations and fungicidal effect at higher concentrations. The concentration of essential oil required to completely inhibit mycelial growth and conidia

germination of *C. acutatum* was greater than the concentration used for *B. cinerea*. The essential oil of *F. vulgare* has also been reported to reduce the mycelial growth of *Sclerotinia sclerotiorum* and as such could be used as bio fungicide against this phytopathogenic fungus (Soylu et al., 2007). The essential oil of fennel has been reported to show complete inhibition against *Aspergillum niger, Aspergillum flavus, Fusarium graminearum* and *Fusarium moniliforme* (Singh et al., 2006). The effect of essential oils on microbial growth was reported by Fung, Taylor and Kahan (1977) and Tian et al. (2012) who suggested that it might be the result of phenolic compounds and terpenoids present in the essential oils altering microbial cell permeability by interacting with membrane proteins. This would cause deformation of cell structure and functionality and permit the loss of macromolecules from their interior (Pramila et al., 2012). Being lipophilic in nature, essential oil accumulates in plasma membrane, causes swelling of the membrane and makes the membrane proteins inefficient due to increased disorder. This ultimately causes leakage of cell contents and inhibition of cell growth (Helal, Sarhan, Abu Shahla & Abou El-Khair, 2007). Moreover, each essential oils component makes its own contribution to the biological activity of the oil. The volatile phase of essential oil showed a fungistatic action, partially inhibiting the mycelial growth of fungi. Volatile phase of artemisia, peppermint, basil and thyme essential oils were also reported to posses antimicrobial activity against plant pathogenic fungi (Edris & Farrag, 2003; Soylu et al., 2005). Investigators suggested that the antifungal activity resulted from a direct effect of essential oil vapours on fungal mycelium and postulated that the lipophilic nature of essential oils would make possible for them being absorbed by fungal mycelia (Edris & Farrag, 2003; Inouye et al., 2000). In this work, both fungi hyphae grown on media with essential oils revealed alterations in the morphology. Such modifications may be related to the effect of the essential oil on enzymatic reactions regulating wall synthesis for example (Rasooli, Rezaei & Allameh, 2013).

Soylu et al. (2007) reported to reduce the germination of *Sclerotinia sclerotiorum* and Aminifard and Mohammadi (2013) reported that conidia germination and germ tube elongation of *B. cinerea* were inhibited by *F. vulgare* essential oil. Our results corroborated with that as the essential oil of *F. vulgare* also inhibited conidia germination of *B. cinerea* and *C. acutatum.* Besides inhibiting the mycelial growth, phenolic compounds also affect the enzymes responsible for conidia germination and interfere with amino acids that were necessary in germination processes (Nychas, 1995).

The *in vivo* test showed that the essential oil of fennel had a positive effect in controlling the incidence of postharvest fungal rots on grapes caused by *B. cinerea* and *C. acutatum.* Essential oil inhibits postharvest pathogens mainly due to their direct effect on the mycelial growth of the pathogens and conidia germination by affecting the cellular metabolism of the pathogens (Serrano, Martínez-Romero, Castillo, Guillen & Valero, 2005; Tzortzakis, 2007 A; Tzortzakis, 2007 B; Regnier, Combrinck, Du Ploov & Botha, 2010). Prior studies of fennel essential oil has shown better efficacy against postharvest fungi including *Aspergillus* species in different test methods (Singh et al., 2006; Barkat & Bouguerra, 2012; Gemeda, Woldeamanuel, Asrat & Debella, 2014). According to Abdolari, Hassani, Ghosta, Javadi and Meshkatalsadat (2010), fennel essential oil had good inhibitory effects on infection caused by *Alternaria alternata* and *Penicillium digitatum* in postharvest tomato fruits. According to Aminifard and Mohammadi (2013), fennel essential oil inhibited *B. cinerea* growth on plum fruits compared with the control. Lopez-Reyes, Spadaro, Gullinoa and Garibaldua (2010) proved that the essential oil of fennel has antifungal activity as postharvest treatments against *B. cinerea* and *Penicillium expansum* on apples.

Results showed that antifungal activities of essential oils were different under *in vitro* and *in vivo* conditions and these activities were higher under *in vitro* conditions, requiring a higher concentration of the essential oil *in vivo*. Dikbas, Kotan, Dadasoglu and Sahin (2008) noticed that these differences could be attributed to the alternation of site of action of essential oils or alternation in membranes of fungi under *in vivo* condition.

5. Conclusion

Considering the reduction in mycelial growth and germination of *B. cinerea* and *C. acutatum in vitro,* and the reduced incidence of disease symptoms on essential oil treated grapes fruits, we can conclude that *F. vulgare* (fennel) essential oil could be used as possible biofungicide. However, more studies are required before this essential oil can be recommended as commercial and natural antifungal agent to increase the postharvest storage life of grapes.

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Complementary material

Figure S1. Effect of different concentrations of *Foeniculum vulgare* essential oil, added on the solid media, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (contact phase at 14th day). Scale: 2cm.

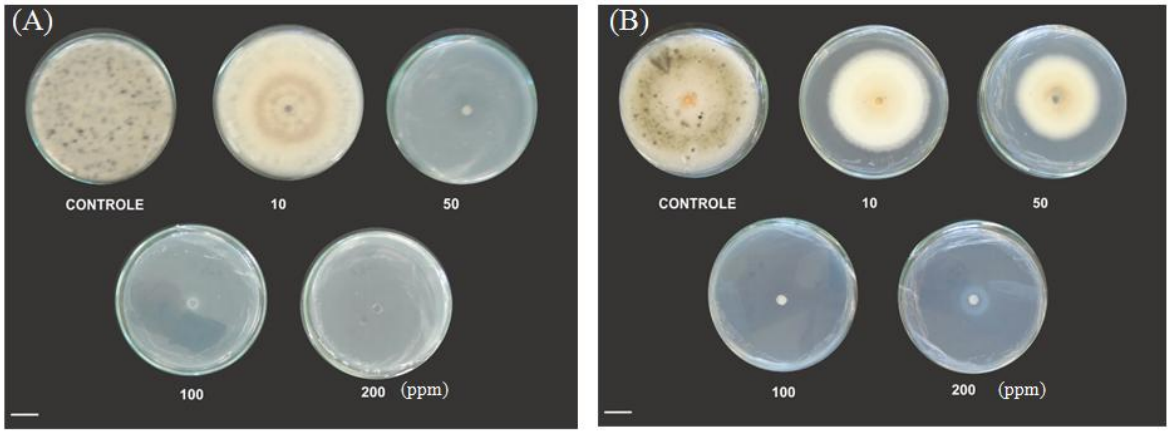


Figure S3. Effect of different concentrations of *Foeniculum vulgare* essential oil on conidia germination of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (10× magnification).

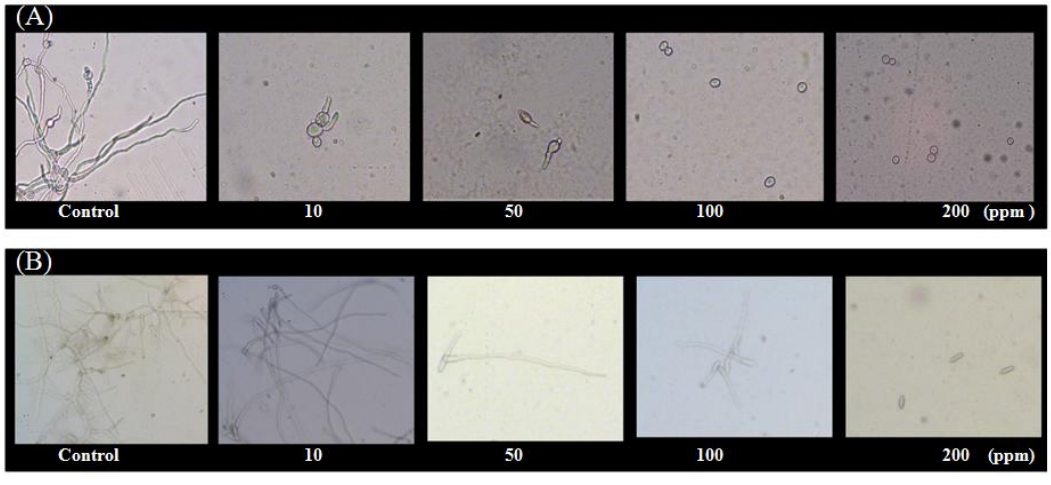
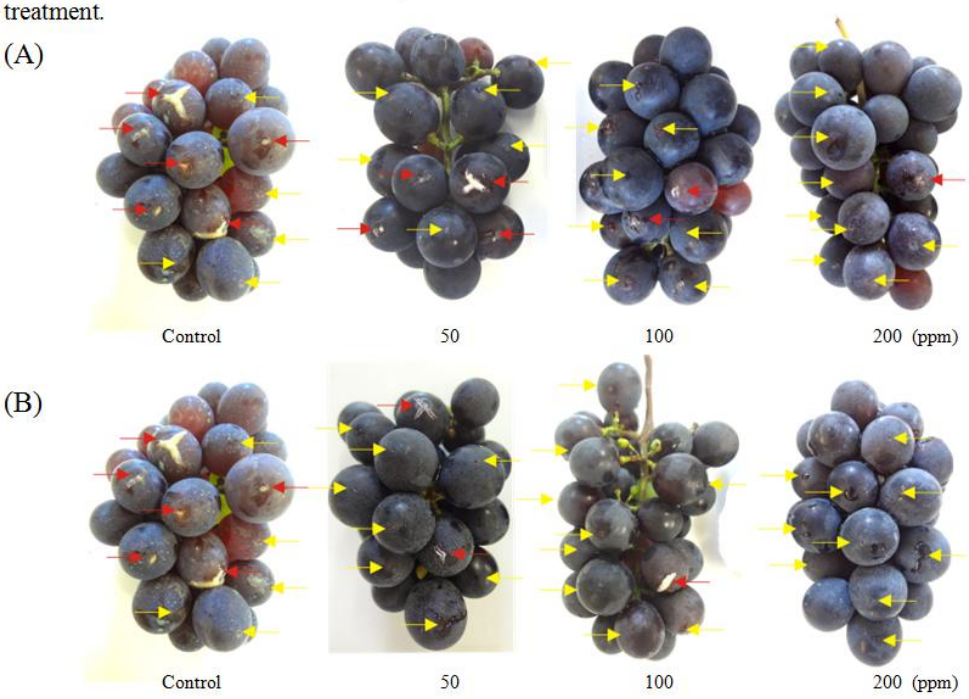
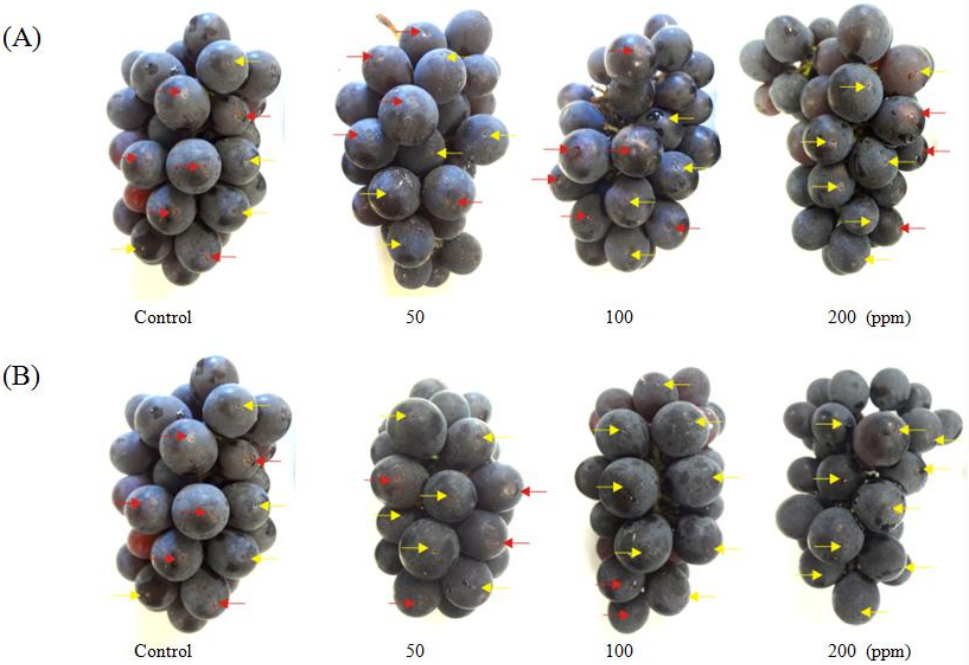


Figure S4. The effects of different concentrations of *Foeniculum vulgare* essential oil in grapes. Incidence of disease caused by *Botrytis cinerea* as to (A) preventive and (B) curative treatment.



The arrows indicate the site of injury.
→ berries with symptoms of the disease (incidence)
→ berries without disease symptoms

Figure S5. The effects of different concentrations of *Foeniculum vulgare* essential oil in grapes. Incidence of disease caused by *Colletotrichum acutatum* as to (A) preventive and (B) curative treatment.



The arrows indicate the site of injury.
→ berries with symptoms of the disease (incidence)
→ berries without disease symptoms

4.2. Capítulo 2

(Será submetido ao *Journal of Phytopathology)*

Antifungal activity of *Baccharis trimera* and *Baccharis dracunculifolia* essential oils against postharvest fungal rots in grapes

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ABSTRACT

Postharvest diseases cause considerable losses to fruits and vegetables during transportation and storage. Synthetic fungicides are primarily used to control postharvest decay loss. However, is necessary the development of new and alternative agrochemicals. The aim of this study was to assess the chemical composition and the antifungal activity of *Baccharis trimera* and *Baccharis dracunculifolia* essential oils. The effect of essential oils against *Botrytis cinerea* and *Colletotrichum acutatum* was determined *in vitro* by mycelial growth (contact and volatile phase) and conidia germination. The *in vivo* efficacy study consisted on spraying the essential oil in harvested grapes of *Vitis* spp. cv. "Isabela" followed by inoculation with the fungus. The major compound found in *B. trimera* essential oil was carquejol acetate (76.53%) and in *B. dracunculifolia* essential oil were β-pinene (19.73%), limonene (16.01%) and spathulenol (9.99%). For *in vitro* tests, *B. dracunculifolia* essential oil showed a fungistatic action, while *B. trimera* essential oil presented fungicidal action and for this, the

later was selected for *in vivo* test. For *in vivo* test, the concentrations of essential oil tested were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum*, both in preventive and curative treatment. These results are promising and indicate that the studied essential oils might be further investigated as natural alternatives to synthetic fungicides for the control of rots on grapes diseases.

Keywords: Alternative control, *Botrytis cinerea*, *Colletotrichum acutatum*, *Vitis* spp..

Introduction

Grape is one of the most important fruit crops worldwide. In Brazil, *Vitis vinifera*, *Vitis labrusca* and *Vitis* spp. are the common species cultivated, for the last species "Isabela" grape is one of the most important varieties. "Isabela" is the most diffused variety in Serra Gaúcha, the southern viticultural region of Brazil, where it represents near 57% of the total production. This variety is used to make red table wine and juice and it is also commercialized as table grape (Mello, 2014, Silveira *et al.*, 2015).

Postharvest decay during the supply chain has been identified as a major factor causing loss which could result in significant economic loss, especially in the fruit marketing chain (Prusky, 2011). Incidences of postharvest diseases may occur during the different stages at harvesting, field handling, packing operations, transportation and storage. *Botrytis cinerea* Pers. Fr. and *Colletotrichum acutatum* Simmonds cause fungal rot and they are considered the main cause of great losses of postharvest in table grapes (Peres *et al*., 2002; Steel *et al.*, 2007; Whitelaw-Weckert *et al.*, 2007).

As a postharvest treatment, grapes are usually stored with sulfur dioxide fumigation (Droby and Lichter, 2004). However, the use of synthetic fungicides and sulfur dioxide is not allowed on organic grapes (Mlikota Gabler and Smilanick, 2001). In addition, growing public concerns about health and environmental hazards associated with pesticide use have resulted

in a considerable interest in developing alternative non-polluting control methods (Youssef and Roberto, 2014). Among the possibilities of alternative control it is the use of essential oils. The essential oils are well known for their antimicrobial and biodegradable properties and for not leave any residual effect on fresh produce (Isman, 2000; Kalemba and Kunicka, 2003; Burt, 2004).

The genus *Baccharis* L. (Asterales, Asteraceae, tribe Asterae, sub-tribe Baccharidinae) comprises around 500 species, with significant popular use in South America mainly as natural medicinal products (Verdi *et al*., 2005). *Baccharis trimera* (Less) is widely distributed in Brazil and is extensively studied regarding its chemical composition and biological activity including antidiabetic (Oliveira *et al*., 2005), antioxidant (Pádua *et al*., 2010; Abad and Bermejo, 2007; Dias *et al.,* 2009), anti-inflammatory (Abad and Bermejo 2007), antiulcerogenic (Dias *et al.,* 2009) schistosomicidal (Oliveira *et al.,* 2012), antibacterial (Avancini *et al*., 2000; Blanco e Benedetti, 2009; Gonçalves, 2010) and antifungal activity (Vieira *et al.,* 2014, Caneschi *et al.,* 2015). *Baccharis dracunculifolia* (D.C.) is also a native plant from Brazil, with a variety of chemical compounds and pharmacological activities attributed to this plant, including antiulcerative (De Barros *et al*., 2007), antibacterial (Pereira *et al.,* 2015), schistosomicidal (Parreira *et al.,* 2010) antivirus (Búffalo *et al*., 2009) and antifungal (Oliveira *et al.,* 2015) properties.

This study evaluates the effectiveness of *B. trimera* and *B. dracunculifolia* essential oils on the inhibition of mycelial growth and conidia germination of *B. cinerea* and *C. acutatum* and evaluates *in vivo* grape rot control in postharvest using them.

Materials and methods

Isolated fungi

Isolates of *Botrytis cinerea* (A58/09) and *Colletotrichum acutatum* (A009/13) used in this work were isolated from grapes of Caxias do Sul (Serra Gaúcha – RS – Brazil) and preserved in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul - Brazil, on PDA (Potato Dextrose Agar) medium. The molecular confirmation of both fungi was done using Internal Transcribed Sequence (ITS)-PCR identification. The DNA extraction was according to Murray and Thompson (1980) and ITS-PCR amplified the region ITS-5.8S rDNA according to White, Bruns, Lee and Taylor (1990). Sequencing was proceed at the Human Genome Center – USP and the sequences obtained were edited with the software BioEdit Sequence Alignment Editor (1997-2005) and used to search for similar sequences using Blastn at NCBI.

Plant material

Leaves of *Baccharis trimera* and *Baccharis dracunculifolia* were collected from plants localized in the city of Bento Gonçalves, RS – Brazil. A voucher specimen of each plant species were deposited in the Herbarium of the University of Caxias do Sul with number 43211 for *B. trimera* and number 43210 for *B. dracunculifolia.*

Essential oils extraction and analysis

Essential oils were extracted by steam distillation from dried plant leaves for 1 hour according to Cassel *et al.* (2009) with modifications. For identification and quantification of compounds in the essential oil, it was used the protocol described in Tomazoni *et al.* (2016), using a gas chromatograph HP 6890, coupled with a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. The analyses were conducted using a fused silica capillary column HP-Innowax (30 m × 0.25 mm i.d., 0,25 µm film thickness, Hewlett Packard, Palo Alto, USA). The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from

literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

In vitro antifungal assay

Antifungal activity of essential oil on mycelial growth

The antifungal properties of essential oils were assessed for its contact and volatile phase effects towards mycelial growth of phytopathogens. Contact phase effect of essential oils was tested according to Feng and Zheng (2007) with minor modifications. Essential oils concentrations ranging between 100 to 700 ppm ($\mu L\ mL^{-1}$), with the addition of Tween 20 (1:1), diluted on autoclaved and melting PDA (Potato Dextrose Agar) (40ºC) under aseptic conditions were used for both fungi. The control treatment was just PDA medium with addition of Tween 20 equal to the highest concentration used to emulsify the essential oil. These emulsions were poured into 9 cm (Ø) Petri dishes and after medium solidification, inoculated with 5 mm (Ø) agar disks colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development.

To assess fungicidal action of the volatile phase of essential oils on the mycelial growth of fungi it was utilized the methodology according to Silva *et al.* (2012) with minor modifications. Agar disks with 5 mm (Ø) colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development were placed in the center of the Petri dish containing PDA culture medium. The concentrations of essential oils used were 12.5, 25, 50 with the addition of Tween 20 (0.1%) and 100% (pure essential oil, without addition of Tween 20). A 100 µL sample of pure essential oil and the solutions were applied onto a cotton ball attached to the inner face of a Petri dish lid, thereby preventing direct contact of the oil with the culture medium and the mycelium disk, creating a saturated atmosphere of volatile compounds. The

control treatment was just PDA medium and 100 µL of Tween 20 (0.1%) applied in cotton ball.

In both tests, for each concentration, ten replicate plates were used. Incubation was performed at 25º C temperature and 12 hours photoperiod, during fourteen days. Fungal growth was recorded on the 3^{rd}, 5^{th}, 7^{th}, 10^{th} and 14^{th} day by measurement of orthogonal diameter. Transfer experiments were performed to provide a distinction between the fungistatic and fungicidal effects of essential oil on the target microorganisms. For this purpose, plugs that did not grow were transferred to fresh PDA dishes to assess their viability and growth after five days of inoculation at 25° C. The residual fungal growth was monitored by measuring the radial growth of the fungi.

Antifungal activity of essential oil on conidia germination

Antifungal activity of essential oil on conidia germination was tested according to Badawy and Rabea (2013), with minor modifications. Conidia of *B. cinerea* and *C. acutatum* were harvested from a colony of the fungi 14 days grown in PDA at 25º C temperature and 12 hours photoperiod. Five milliliter of sterile water was added to a Petri dish culture. The conidia were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia ml^{-1}. Aliquots of conidia suspension (50 µL) were placed in microtubes containing 500 µL of PDB (Potato Dextrose Broth) medium treated with essential oils, at the concentrations of 100, 200, 300 and 400 ppm, with the additon of Tween 20 (1:1). The control treatment was just PDB with addition of Tween 20 at concentration 400 ppm (similar to the highest concentration used to emulsify the essential oil). The tubes were incubated at 25º C for 16 hours. The samples were placed on a Neubauer chamber and observed under the microscope for conidia germination. Conidia counting was done using a light microscopy at 10x magnification. All experiments were

conducted in ten replicates and in each replicate were evaluated hundred conidia. The conidia was considered germinated when the length of the germ tube equaled or exceeded the length of the conidia.

In vivo antifungal assay

Inoculum preparation

Conidia of *B. cinerea* and *C. acutatum* were harvested from a colony of the fungi 14 days grown in PDA as described above. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia ml^{-1}.

Fruit

Traditionally grown, freshly harvested, *Vitis* spp cv. "Isabela" grapes from Bento Gonçalves, RS - Brazil were used in experiments.

Antifungal activity of essential oil in grapes

To evaluate the antifungal activity of *B. trimera* essential oil on grapes it was carried out experiments with curative and preventive treatments. The postharvest curative treatment consists of inoculation of 10 berries for each cluster (10 fruit / treatment) of grape, through wounds, approximately 2 mm deep, with the aid of a syringe (Zahavi, 2000). After the injury, the clusters are inoculated by spraying the conidia suspension of *B. cinerea* or *C. acutatum*, according to the methodology described by Romanazzi *et al*. (2002) and Thomas *et al*. (1988) with modifications. After 4 hours, the application of essential oil was carried out with the concentrations based on the *in vitro* test (200 to 600 ppm). Subsequently, in order to evaluate the potential of the essential oil in preventing disease, the grape clusters were sprinkled with essential oils in the same concentrations of the previous test and inoculated after 24 hours with the fungi. For both experiments, the clusters were placed in plastic boxes and kept at 25

± 1° C / 80-90% relative humidity for a period of five days for those inoculated with *B. cinerea* and seven days for those inoculated with *C. acutatum.* At the end of this period, assessment of the incidence and severity of disease was performed. To evaluate the incidence, ten berries for each bunch of grapes that were inoculated were evaluated and it was used the percentage of mean number of berries with symptoms of the disease. For assessing the severity, a scale from 0 to 100% was created in accordance with the berry area affected by the disease.

Statistical analysis

Data normality was determined by Kolmogorov-Smirnov test and the homogeneity of variances was determined using Levene's test. Data were analysed by ANOVA and the threshold for statistical significance was set at $p < 0.05$. In the case of statistical significance Dunnett's T3 test was applied to separate the means. All statistics analysis was performed using SPSS 22.0 for Windows.

Results

Chemical composition of the essential oil

The number of compounds and their relative amount found in essential oils varied according to plant species and the particular compound (Table 1). The major compound found in *B. trimera* essential oil was carquejol acetate (76.53%) and fourteen other components were present in lower amounts, of which 92.26% of the compounds correspond to monoterpenes (15.55% hydrocarbons and 76.71% oxygenated) and only 7.02% are sesquiterpenes (2.83% hydrocarbons and 4.19% oxygenated). The major compounds found in *B. dracunculifolia* essential oil were β-pinene (19.7%), limonene (16.01%) and spathulenol (9.99%). Seventeen other components were present in lower amounts, of which 35.74% of the compounds

correspond to monoterpenes hydrocarbons and 45.76 % are sesquiterpenes (24.93% hydrocarbons and 20.81% oxygenated). Essential oils extracted from dried leaves of *B. trimera* and *B. dracunculifolia* yielded respectively 1.08 and 0.11% (mL $100g^{-1}$ of dried leaves).

Table 1 Chemical composition of essential oils from *Baccharis trimera* and *Baccharis dracunculifolia*.

Compounds	RI[a]	Peak area (%)[b]	
		B. trimera	*B. dracunculifolia*
Monoterpene Hidrocarbons		13.35	35.74
β-Pinene	12.879	6.25	19.73
β-Phellandrene	13.779	1.17	–
Myrcene	16.528	0.46	–
Limonene	18.240	1.86	16.01
β-Thujene	18.721	1.70	–
β-Ocimene	21.448	1.91	–
Oxygenated monoterpenes		76.71	0
β-Isophorone	29.578	0.18	–
Carquejol acetate	43.394	76.53	–
Sesquiterpene Hidrocarbons		2.83	24.93
Copaene	32.430	–	0.58
α-Gurjunene	34.307	–	0.27
β-Cubebene	34.803	–	0.22
Caryophyllene	37.531	–	5.03
Aromadendrene	37.932	–	0.66
Alloaromadendrene	39.655	–	0.23
α-Caryophyllene	40.735	–	1.09
γ-Muurolene	42.183	0.18	–
Germacrene D	42.674	1.42	5.67
Byciclogermacrene	43.522	–	7.27
Valencene	43.830	0.88	–
δ-Cadinene	44.448	–	2.83
α-Curcumene	45.087	–	0.59
γ-Elemene	47.301	–	0.49
γ-Selinene	56.213	0.35	–
Oxygenated sesquiterpens		4.19	20.81
Caryophyllene Oxide	51.109	–	0.64
Palustrol	51.248	3.12	0.39
Ledol	54.876	0.62	1.34
Nerolidol	54.989	–	6.84
Viridiflorol	56.160	–	1.61
Spathulenol	56.903	–	9.99
β-Eudesmol	58.695	0.45	–

[a] RI, the retention index publyshed by Adams.
[b] Peak area obtained by GC-FID.

In vitro antifungal effect of *B. trimera* and *B. dracunculifolia* essential oils

Antifungal activity of essential oils on mycelial growth

The *in vitro* antifungal activity of essential oils differed for each fungi, essential oil and concentration tested at contact phase experiments (Table 2, Fig. S1 A and B and Fig. S2 A and B). The effect of *B. trimera* essential oil on the mycelial growth of *B. cinerea* resulted in completely inhibition at concentrations 400 ppm and the fungicidal action was observed by the transfer experiment. For concentration 100 ppm there was a significant inhibition at 7^{th} day and concentrations 200 and 300 ppm presented a significant inhibition at 14^{th} day, compared to control. In these three concentrations, it was also observed that the mycelial growth presented a different morphology. On the other hand, the *B. trimera* essential oil had only a fungistatic effect on mycelial growth of *C. acutatum* which varied according to the concentration tested. For concentration 100 ppm there was a significant inhibition until the 10^{th} day compared to control and for concentrations 300, 500 and 700 ppm there were a significant inhibition until the 14^{th} day compared to control. Essential oil of *B. dracunculifolia* had only a fungistatic effect on the mycelial growth of *B. cinerea* and *C. acutatum*. In both fungi it was also observed that the mycelial growth presented a different morphology.

The effect of volatiles of *B. trimera* essential oil in the mycelial growth of *B. cinerea* at the concentrations 50% and 100% presented a significant inhibition until 14^{th} day compared to control. For *C. acutatum,* concentrations 12.5 and 25% presented a significant inhibition until 7^{th} day and concentrations 50 and 100% inhibited the growth until 14^{th} day compared to control. Volatiles of *B. dracunculifolia* essential oil reduced the mycelial growth of *B. cinerea* at concentration 100% from 5^{th} until 14^{th} day compared to control. For *C. acutatum,* all concentrations presented a significant inhibition until 3^{rd} day compared to control, on the

other days did not differ when compared to control (Table 3, Fig. S3 A and B and Fig. S4 A and B).

Table 2 Effect of different concentrations of *Baccharis trimera* and *Baccharis dracunculifolia* essential oils, added on the solid media, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum acutatum* (contact phase).

B. trimera

	Mycelial growth (mm)				
	B. cinerea				
	0	100	200	300	400 (ppm)
3rd day	43.71 ± 2.06 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5th day	86.57 ± 3.43 a	10.93 ± 0.20 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
7th day	87.24 ± 2.76 a	33.78 ± 1.89 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
10th day	90.00 ± 0.00 a	68.24 ± 4.77 a	13.55 ± 2.06 b	0.00 ± 0.00 c	0.00 ± 0.00 c
14th day	90.00 ± 0.00 a	89.56 ± 0.19 a	31.19 ± 4.40 b	13.22 ± 3.27 bc	0.00 ± 0.00 c
	C. acutatum				
	0	100	300	500	700 (ppm)
3rd day	28.02 ± 0.54 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5th day	38.78 ± 0.67 a	10.22 ± 0.40 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
7th day	73.24 ± 1.19 a	29.56 ± 2.62 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
10th day	86.47 ± 1.83 a	42.84 ± 3.24 b	10.44 ± 0.27 c	0.00 ± 0.00 d	0.00 ± 0.00 d
14th day	90.00 ± 0.00 a	66.06 ± 5.81 a	17.81 ± 1.18 b	10.50 ± 0.18 b	8.90 ± 0.54 b

B. dracunculifolia

	Mycelial growth (mm)				
	B. cinerea				
	0	100	200	300	400 (ppm)
3rd day	57.81 ± 1.37 a	22.07 ± 1.99 b	12.06 ± 0.32 b	12.97 ± 1.12 b	11.43 ± 0.62 b
5th day	78.89 ± 2.82 a	32.35 ± 1.05 b	21.88 ± 0.47 c	20.57 ± 0.98 c	17.67 ± 0.42 c
7th day	90.00 ± 0.00 a	53.95 ± 3.56 b	33.80 ± 0.75 c	32.81 ± 1.48 c	30.99 ± 1.74 c
10th day	90.00 ± 0.00 a	68.49 ± 4.37 a	45.22 ± 1.25 b	45.49 ± 3.00 b	39.76 ± 1.97 b
14th day	90.00 ± 0.00 a	80.79 ± 4.25 a	70.51 ± 2.61 ab	64.36 ± 3.23 ab	54.89 ± 3.34 b
	C. acutatum				
	0	100	300	500	700 (ppm)
3rd day	27.40 ± 0.38 a	18.50 ± 0.48 b	14.75 ± 0.72 b	13.30 ± 0.65 b	12.84 ± 0.49 b
5th day	42.80 ± 1.48 a	29.05 ± 3.41 a	25.11 ± 2.19 ab	21.82 ± 1.72 ab	20.08 ± 1.43 ab
7th day	51.30 ± 3.10 a	35.44 ± 4.66 a	29.95 ± 3.13 ab	27.93 ± 1.89 ab	25.42 ± 1.91 ab
10th day	80.15 ± 0.75 a	44.65 ± 5.64 b	37.38 ± 3.79 b	34.62 ± 1.93 b	33.46 ± 1.44 b
14th day	90.00 ± 0.00 a	55.91 ± 6.20 b	46.85 ± 4.58 b	46.38 ± 2.93 b	45.30 ± 1.91 b

Values are the average of ten replicates per treatment ± SE.
The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line).
Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Table 3 Effect of different concentrations of *Baccharis trimera* and *Baccharis dracunculifolia*, essential oils apllied on the lid, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum* acutatum (volatile phase).

B. trimera

	Mycelial growth (mm)				
	B. cinerea				
	0.0	12.5	25	50	100 (%)
3rd day	62.88 ± 5.04 a	41.13 ± 5.50 a	34.34 ± 2.13 ab	26.77 ± 2.28 ab	17.44 ± 1.50 b
5th day	90.00 ± 0.00 a	64.02 ± 4.61 b	54.45 ± 2.66 b	40.85 ± 2.94 b	24.95 ± 1.57 c
7th day	90.00 ± 0.00 a	77.80 ± 3.49 a	70.66 ± 1.54 ab	52.85 ± 2.77 c	35.04 ± 1.63 d
10th day	90.00 ± 0.00 a	80.63 ± 3.58 a	74. 99 ± 2.66 ab	56.79 ± 2.23 c	41.34 ± 1.20 d
14th day	90.00 ± 0.00 a	81.11 ± 3.49 a	75.49 ± 2.45 ab	57.89 ± 2.19 c	43.86 ± 1.79 d
	C. acutatum				
	0.0	12.5	25	50	100 (%)
3rd day	29.11 ± 0.35 a	19.83 ± 0.47 b	17.42 ± 0.83 b	15.28 ± 0.40 bc	10.61 ± 0.44 d
5th day	41.97 ± 0.90 a	28.71 ± 0.79 b	23.58 ± 1.04 b	19.90 ± 0.45 bc	15.31 ± 0.58 d
7th day	66.25 ± 2.41 a	51.49 ± 1.87 b	41.44 ± 1.03 b	32.31 ± 1.76 bc	24.13 ± 1.37 c
10th day	74.96 ± 2.67 a	64.36 ± 2.48 a	55.81 ± 0.94 ab	38.91 ± 1.64 bc	30.89 ± 1.21 c
14th day	83.93 ± 2.93 a	76.03 ± 2.91 a	69.45 ± 2.18 a	45.77 ± 1.59 b	42.71 ± 2.03 b

B. dracunculifolia

	Mycelial growth (mm)				
	B. cinerea				
	0.0	12.5	25	50	100 (%)
3rd day	46.45 ± 4.66 a	44.12 ± 5.32 a	38.71 ± 5.02 a	37.26 ± 4.46 a	28.53 ± 3.72 a
5th day	74.17 ± 1.60 a	67.30 ± 2.45 a	63.01 ± 2.69 a	60.01 ± 2.76 a	52.65 ± 2.06 b
7th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	72.90 ± 1.54 b
10th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	73.95 ± 1.68 b
14th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	76.37 ± 2.45 b
	C. acutatum				
	0.0	12.5	25	50	100 (%)
3rd day	27.77 ± 0.29 a	26.85 ± 0.40 a	26.80 ± 0.15 a	26.16 ± 0.34 a	25.23 ± 0.46 ab
5th day	43.43 ± 0.43 a	38.31 ± 0.50 b	34.41 ± 1.24 b	33.04 ± 0.74 bc	33.51 ± 0.62 bc
7th day	75.53 ± 1.75 a	65.42 ± 1,98 a	61.88 ± 1.79 ab	59.98 ± 2.83 ab	59.82 ± 2.27 ab
10th day	84.53 ± 1.13 a	83.84 ± 1.14 a	76.08 ± 1.52 a	73.61 ± 1.21 ab	72.68 ± 1.02 ab
14th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a

Values are the average of ten replicates per treatment ± SE.
The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line).
Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Antifungal activity of essential oils on conidia germination

B. trimera essential oil inhibited completely the conidia germination of *B. cinerea* at concentration 400 ppm, while the concentrations 100, 200 and 300 ppm showed a significant reduction in the germination of conidia and in the lenght of the germ tube (data not shown). The conidia germination of *C. acutatum* was completely inhibited at the lowest concentration (100 ppm) (Fig. 1 A and Fig. S5 A and B).

B. dracunculifolia essential oil was unable to completely inhibit germination of conidia of both fungi tested. It reduced the conidia germination and the lenght of the germ tube (data not shown) of *B. cinerea* in all tested concentrations when compared to control. However, it could not reduce the conidia germination of *C. acutatum* (Fig. 1 B and Fig. S6 A and B).

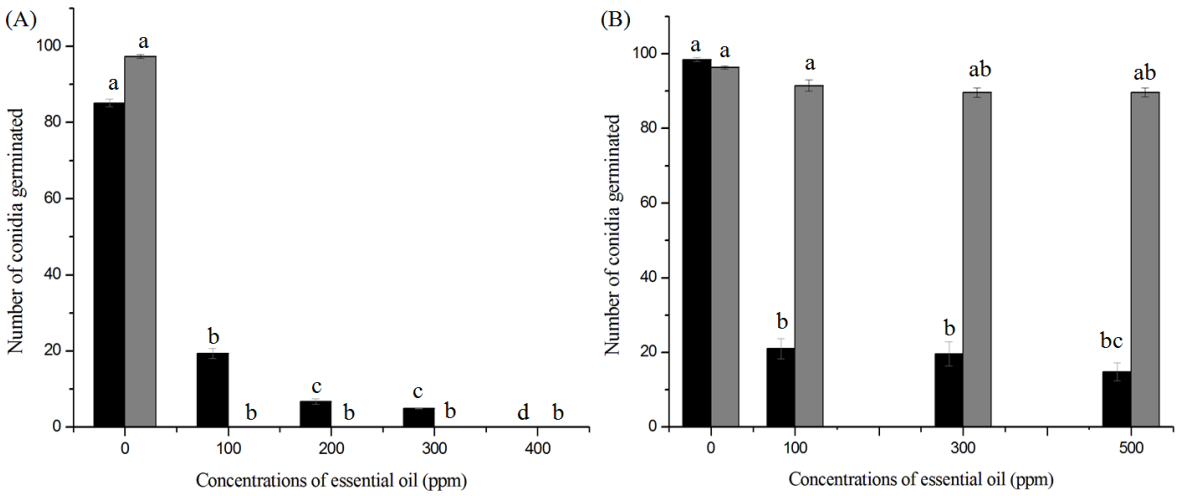

Figure 1. Effect of different concentrations of *Baccharis trimera* (A) and *Baccharis dracunculifolia* (B) essential oils on conidia germination of *Botrytis cinerea* (■) and *Colletotrichum acutatum* (■). Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Antifungal activity of essential oil in postharvest grapes

From the results obtained in *in vitro* tests, *B. trimera* essential oil was selected for *in vivo* tests in postharvest grapes. Different concentrations of the essential oil were efficient, reducing the

incidence and severity of disease caused by *B. cinerea* and incidence of disease caused by *C. acutatum*, both in preventive and curative treatment.

In preventive treatment, the concentrations 400 and 600 ppm were able to reduce the incidence of *B. cinerea* when compared to control and in curative treatment all essential oil concentrations used (200, 400 and 600 ppm) were able to reduce the incidence when compared to control (Fig. 2 A and Fig. S7 A). The preventive treatment reduced the severity of disease only at the highest concentration (600 ppm) and curative treatment, reduced the severity of the disease from the lowest concentration (200 ppm) (Fig. 2 B and Fig. S7 B).

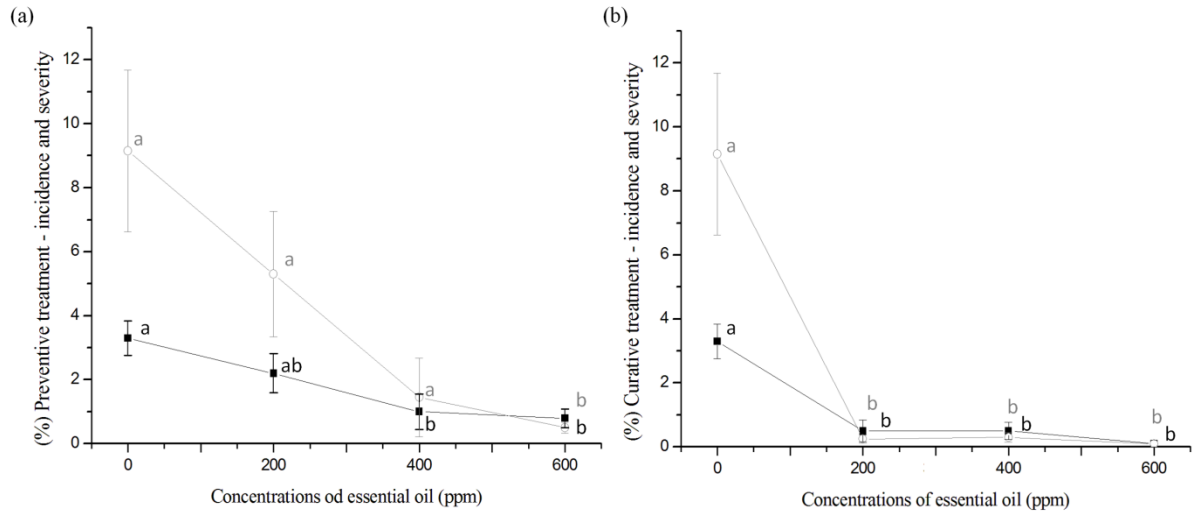

Figure 2 The effects of different concentrations of *Baccharis trimera* essential oil in grapes. Incidence (■) and severity (○) of disease caused by *Botrytis cinerea* as to preventive (a) and curative (b) treatment. Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

In preventive treatment, the concentrations 400 and 600 ppm were able to reduce the incidence of *C. acutatum* when compared to control and in curative treatment all essential oil concentrations used (200, 400 and 600 ppm) were able to reduce the incidence when compared to control (Fig. 3 A and B and Fig. S8 A and B). The severity of disease caused by *C. acutatum*, showed no significant difference in concentrations compared to control when the

disease was detected. The grape clusters treated with *B. trimera* essential oil did not show any obvious signs of phytotoxicity, just showed up brighter.

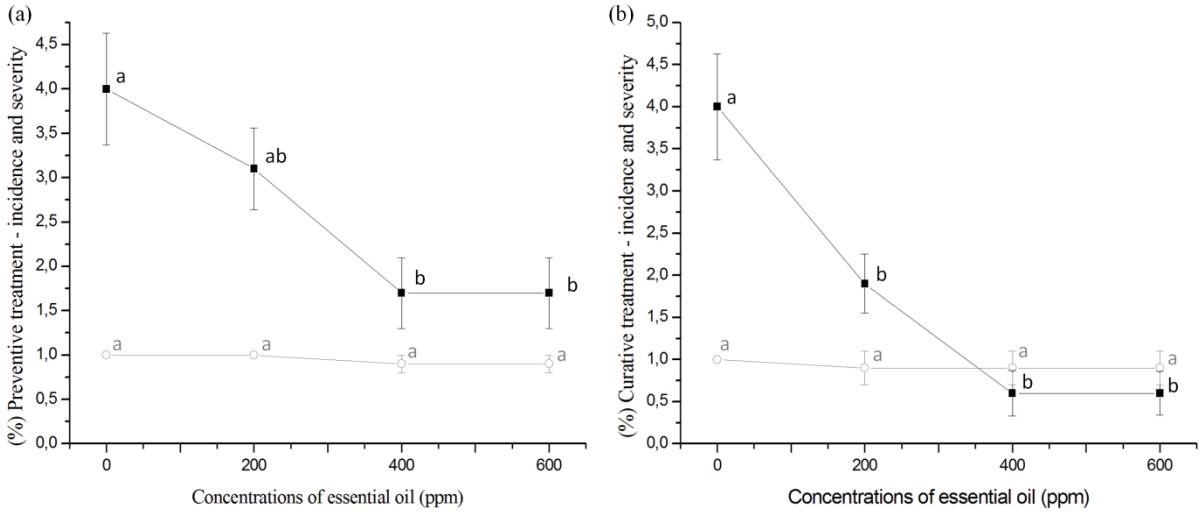

Figure 3 The effects of different concentrations of *Baccharis trimera* essential oil in grapes. Incidence (■) and severity (○) of disease caused by *Colletotrichum acutatum* as to preventive (a) and curative (b) treatment. Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Discussion

Essential oils are complex, volatiles and natural compounds of plants, known by its antiseptic, bactericides and fungicides characteristics (Bakkali *et al*., 2008). In this context, several studies are being conducted to explore the potential of essential oils as antifungal agents (Bowers and Locke, 2004; Arici *et al*., 2011; Sue *et al.*, 2012; Badea and Delian, 2014; Arici and Sanli, 2014) in view of their potential applications as botanical fungicides. The antifungal property of some essential oils on postharvest pathogens of fruits and vegetables under *in vitro* and *in vivo* conditions has been investigated (Zambonelli *et al*., 1996; Feng and Zheng, 2007).

Simões-Pires *et al.* (2005) and Besten *et al.* (2013) found carquejol acetate (35.5 to 68% and 40.7 to 73.5% respectively) as major compound of essential oil from *B. trimera* collected in

different places of southern Brazil, similarly to the results here presented. The analysis of *B. dracunculifolia* essential oil done by Frizzo *et al.* (2008), Massignani *et al.* (2009) and Parreira *et al.* (2010) also found β-pinene, limonene and spathulenol in the composition, but not as major compounds. A literature search revealed that there are variations in the composition and proportion of the major compounds of both essential oils. These differences in components and the content of them in essential oils from species of *Baccharis* may be related to the geographical origins (Simões-Pires *et al.*, 2005; Díaz-Maroto *et al*., 2006; Frizzo *et al.,* 2008), cultivated varieties, male and female specimens (Besten *et al*., 2013), seasons **(**Simões-Pires *et al.*, 2005) as well as extraction methods (Mimica-Dukié *et al*., 2003; Díaz-Maroto *et al.*, 2005).

Oliveira *et al.* (2015) evaluated *B. dracunculifolia* essential oil activity against *Candida albicans* and demonstrated that it presented antifungal activity in high concentrations. On the other hand, Duarte *et al.* (2005) also tested the essential oil against *C. albicans* and demonstrated that it had a low fungicidal activity. Similarly, in this study the *B. dracunculifolia* had a fungistatic effect in high concentrations for both fungi, demonstrating its low fungicidal activity. The *B. trimera* essential oil tested against *Trichophyton rubrum* and *Microsporum canise* exhibited fungicide potential (Caneschi *et al*., 2015), corroborating the results obtained in this study. Fung *et al*. (1977) and Tian *et al.* (2012) suggested that the effect of essential oils on microbial growth might be the result of phenolic compounds and terpenoids present in the essential oils altering microbial cell permeability. This cause deformation of the cell structure, functionality and permits the loss of macromolecules from their interior causing inhibition of cell growth (Sharma and Tripathi, 2006; Helal *et al.,* 2007; Silva *et al.,* 2009; Pramila *et al*., 2012).

Lombardo *et al.* (2016) also assessed the bioactivity of the volatile compounds of *B. trimera* and *B. dracunculifolia* essential oils in control of *Phyllosticta citricarpa* and found similar

results, where *B. trimera* essential oil showed high mycelial growth inhibition while the *B. dracunculifolia* essential oil presented low inhibition. Investigators suggested that the antifungal activity resulted from a direct effect of essential oil vapours on fungal mycelium and postulated that the lipophilic nature of essential oils would make possible for them being absorbed by fungal mycelia (Inouye *et al.,* 2000; Edris and Farrag, 2003). In this work, both fungi hyphae grown on media with essential oils revealed alterations in the morphology. Such modifications may be related to the effect of the essential oil on enzymatic reactions regulating wall synthesis for example (Rasooli *et al*., 2006).

Several studies demonstrate the capabilities of several essential oils to inhibit conidia germination of *B. cinerea* and *C. acutatum* (Bajpai *et al.,* 2008; Alzate *et al.,* 2009; Soylu *et al.,* 2010). Besides inhibiting the mycelial growth, phenolic compounds of essential oils also affect the enzymes responsible for conidia germination and interfere with amino acids that were necessary in germination processes (Nychas, 1995). In *in vitro* tests, *B. trimera* essential oil showed greater inhibition on mycelial growth and conidia germination of *B. cinerea* and *C. acutatum*, this is probably due to the major compound carquejol acetate. This class of substances present in the Asteraceae family is related to antifungal activity (Tabassum and Vidyasagar, 2013).

Essential oil inhibits postharvest pathogens mainly due to their direct effect on the mycelial growth of the pathogens and conidia germination by affecting the cellular metabolism of the pathogens (Serrano *et al.,* 2005; Tzortzakis, 2007a; 2007b; Regnier *et al.*, 2010). According to Tripathi *et al.* (2008) essential oils of *Ocimum sanctum, Prunus persica* and *Zingiber officinale* showed inhibitory effects on infection caused by *B. cinerea* in postharvest grapes fruits. Moreover, Bosquez-Molina *et al.* (2010) and Ali *et al.* (2015) proved that the essential oils of *Citrus aurantifolia, Thymus vulgaris* and *Cymbopogon citratus* have antifungal activity as postharvest treatments against *C. gloeosporioides* on papaya fruit.

These results demonstrate the *in vitro* and the *in vivo* antifungal activities of *B. trimera* essential oil against *B. cinerea* and *C. acutatum* and their potential use as biological fungicide for the control of postharvest fungal rots diseases caused by these micro-organisms on grapes fruits. However, before the development of essential oils as alternatives to synthetic fungicides, their stabilization as well as field studies in different agroecological conditions are required.

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SUPLEMMENTARY MATERIAL

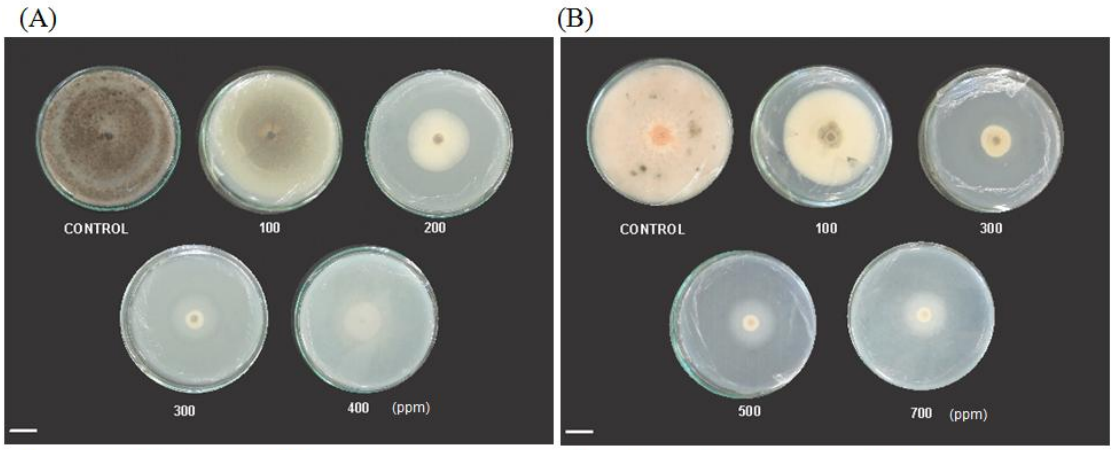

Figure S1: Effect of different concentrations of *Baccharis trimera* essential oil, added on the solid media, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (contact phase to 14th day) Scale: 2cm.

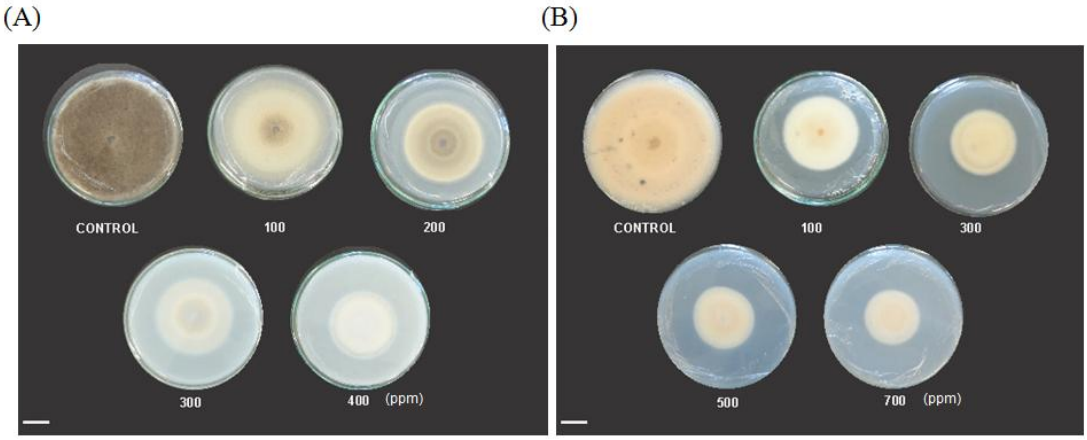

Figure S2: Effect of different concentrations of *Baccharis dracunculifolia* essential oil, added on the solid media, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (contact phase to 14th day) Scale: 2cm.

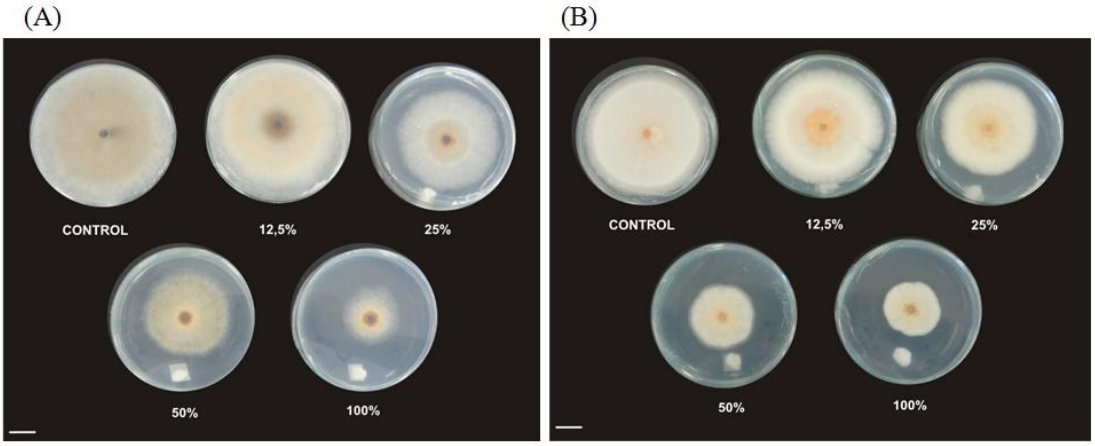

Figure S3: Effect of different concentrations of *Baccharis trimera* essential oil, apllied on the lid, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (volatile phase to 14th day) Scale: 2cm.

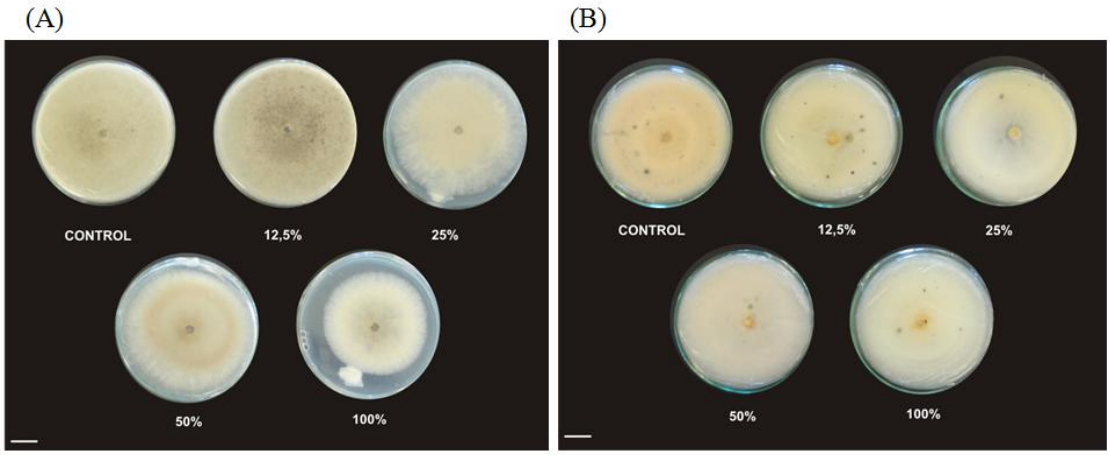

Figure S4: Effect of different concentrations of *Baccharis dracunculifolia* essential oil, apllied on the lid, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (volatile phase to 14th day) Scale: 2 cm.

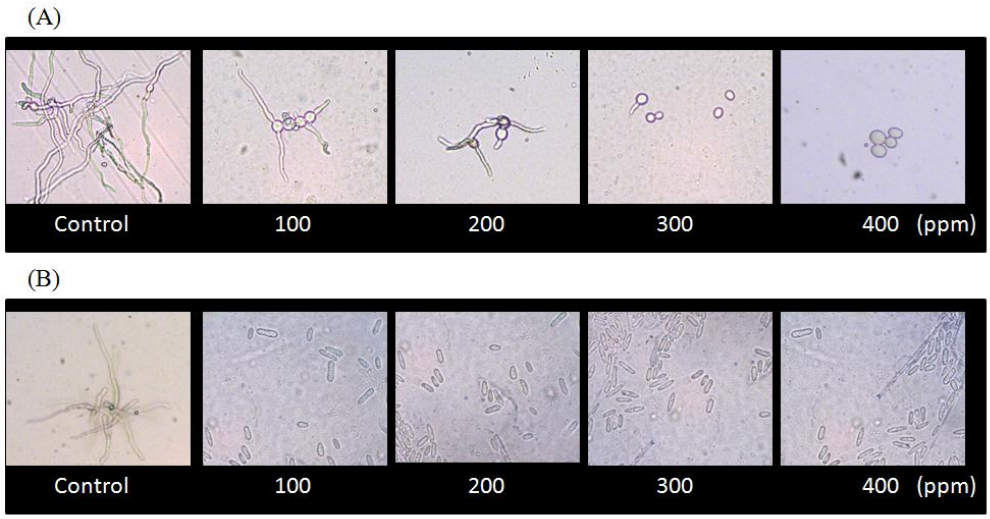

Figure S5: Effect of different concentrations of *Baccharis trimera* essential oil on conidia germination of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum*. (10× magnification).

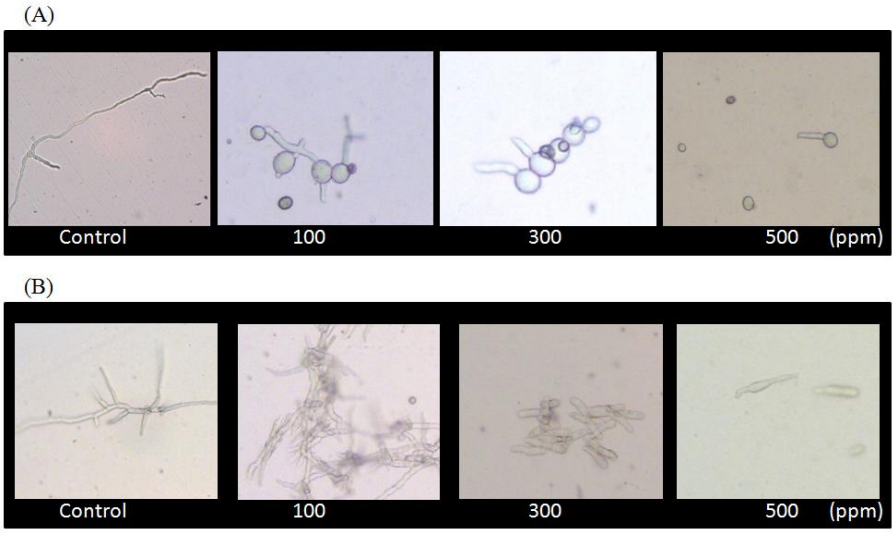

Figure S6: Effect of different concentrations of *Baccharis dracunculifolia* essential oil on conidia germination of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum*. (10× magnification).

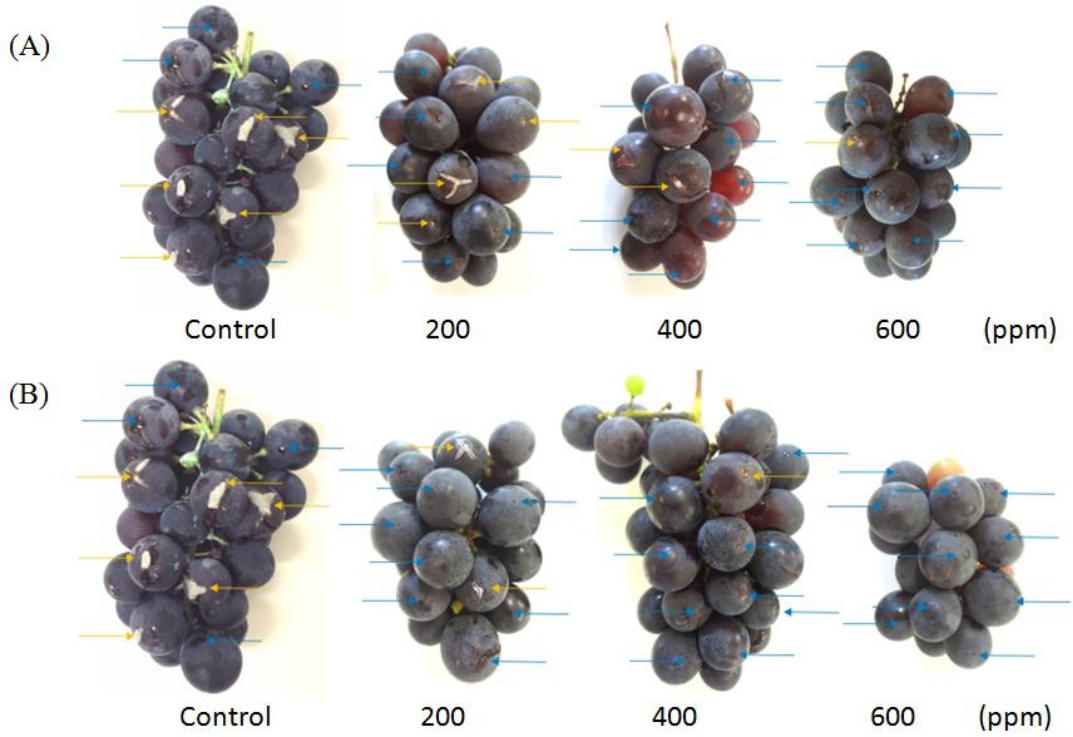

Figure S7: The effects of different concentrations of essential oil of *Baccharis trimera* in grapes. Incidence of disease caused by *Botrytis cinerea* as to (A) preventive and (B) curative treatment.
The arrows indicate the site of injury.
→berries with symptoms of the disease (incidence)
→berries without disease symptoms

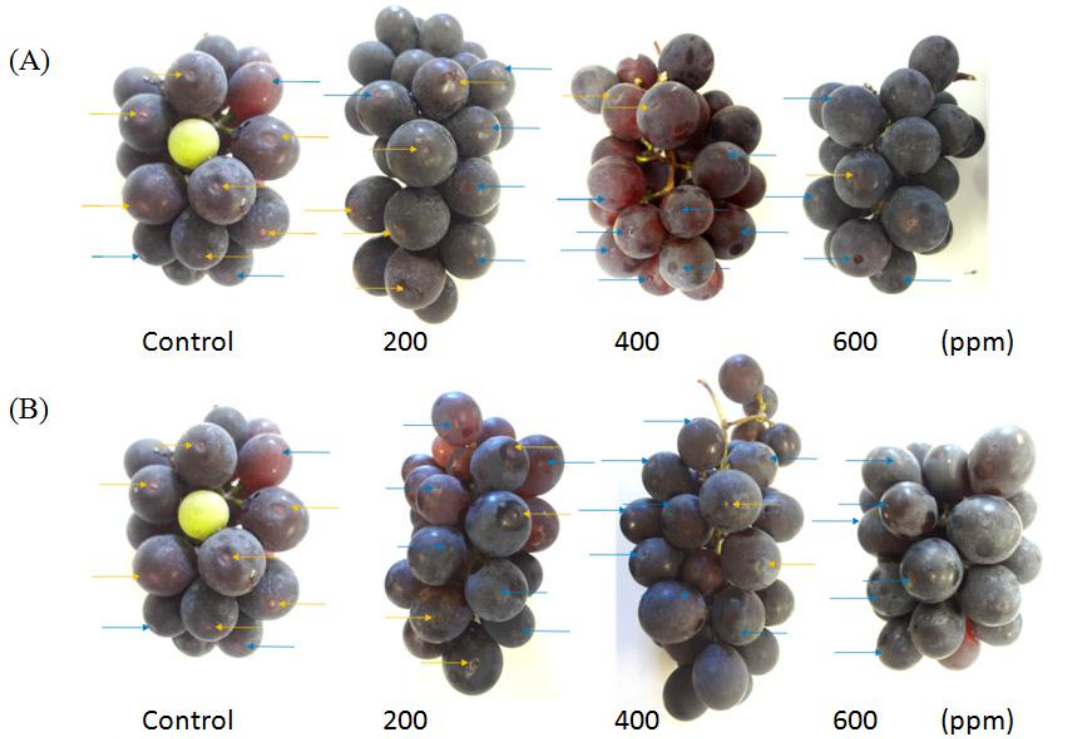

Figure S8: The effects of different concentrations of essential oil of *Baccharis trimera* in grapes. Incidence of disease caused by *Colletotrichum acutatum* as to (A) preventive and (B) curative treatment.
The arrows indicate the site of injury.
→berries with symptoms of the disease (incidence)
→berries without disease symptoms

4.3. Capítulo 3

(Será submetido ao *Journal of Plant Pathology)*

Alternative control of fungal rots on wine grapes with essential oil of *Eucalyptus staigeriana* and *Eucalyptus globulus*

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ABSTRACT

Essential oil are volatile natural compounds produced by plant secondary metabolism have been proven to present antifungal action, enabling their use in phytopathogen control. They also present low environmental impact when compared to conventional pesticides. In the present work, essential oils of *E. staigeriana* and *E. globulus* were tested against *B. cinerea* and *C. acutatum* that causes fungal rots on grapes and are responsible for great economic losses regarding production. The major compound found in *E. staigeriana* essential oil were neral (19.68%) and geranial (28.67%), in *E. globulus* essential oil was 1.8-cineole (64.63%). Essential oils antifungal action was determined *in vitro* on mycelial growth (contact and

volatile phase) and conidia germination and *in vivo* in *Vitis vinifera* cv. "Tannat" for both fungi. Moreover, the grapes were used to evaluate the impact of the alternative control on winemaking. The two essential oils showed a fungicidal action in *in vitro* tests, however the *E. staigeriana* essential oil was more efficient in low concentrations (50 ppm (μL mL^{-1})) and for this, selected for the *in vivo* test. In *in vivo* test, the two concentrations of essential oil tested were more efficient than conventional treatment, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum.* The chemical compounds analyzed in the must and red wine made from grapes treated with *E. staigeriana* essential oil showed that the essential oil has no influence on the characteristic. These results are promising and indicate that *E. staigeriana* essential oil might be further investigated as natural alternative to synthetic fungicides for the control of rots on grapes diseases.

Keywords: *Botrytis cinerea, Colletotrichum acutatum, Vitis vinifera*, biofungicide.

1 Introduction

Grape is one of the most important fruit crops worldwide. In Brazil, "Tannat" grape is one important varieties of *Vitis vinifera*, being one of the most diffused variety in the southern viticultural region of Brazil, where it represents near 9.22% of the total production of vinifera grapes (Camargo 1994; Rizzon and Miele, 2004; Camargo *et al*,. 2008; Mello and Machado, 2014).

The Serra Gaúcha is the leading region, in the south of Brazil, in the produce of grapes for processing. However, it has unfavorable weather conditions due to the high rainfall that affects the development of the culture due to the greater probability of occurring fungal diseases (Westphalen and Maluf, 2000; Conceição *et al.*, 2006). The main cause of great losses in grape is related to *Botrytis cinerea* Pers. Fr. and *Colletotrichum acutatum* Simmonds, the causative agents of fungal rot on a large number of economically important

agricultural crops, including grapes (Pearson and Goheen, 1988; Peres *et al.,* 2002; Steel *et al.,* 2007; Whitelaw-Weckert *et al.,* 2007; Yahia, 2011). Several studies have shown the impact of grapevine pathogens, as *B. cinerea* and *C. acutatum* on wine fermentation, chemical composition and sensory properties of wine (Stummer *et al.,* 2003; Leong *et al.,* 2006; La Guerche *et al.,* 2006; Battilani *et al.,* 2006; Sarrazin *et al.,* 2007; Meunier and Steel, 2009).

Fungicide treatments represent more than half of pesticides applied in viticulture and their use is not without risks to human health. For instance, grapevine growers are directly exposed to pesticides when preparing and spraying them (Flamini and De Rosso 2006; Tsakirakis *et al.,* 2012). These products also affect the physiology of the grapevine itself (Petit *et al.,* 2009) and therefore its production (Jermini *et al.,* 2010). Furthermore, pesticide residues were identified in wine (Cesnik *et al.,* 2008), what can affect natural yeast communities that are necessary for winemaking (Milanovic *et al.,* 2013), as well as for wine aroma (González Álvarez *et al.,* 2012). In addition, growing public concerns about health and environmental hazards associated with pesticide use have resulted in a considerable interest in developing alternative non-polluting control methods (Youssef and Roberto, 2012).

Natural plant protectants, such as essential oils and their major components, that show antimicrobial property activities, low mammalian toxicity and less environmental effects (Isman *et al.,* 2000; Kalemba and Kunicka, 2003; Burt *et al.,* 2004) could be used as alternatives for chemical fungicides. The essential oils are natural antioxidants which are well known for their antimicrobial and biodegradable properties and do not leave any residual effect on fresh produce (Kalemba and Kunicka, 2003).

The *Eucalyptus*, a native genus from Australia, belongs to the Myrtaceae family and comprises about 900 species extensively spread to other countries (Brooker *et al.*, 2004; Marzoug *et al.,* 2011). More than 300 species of this genus contain volatile oils in their leaves

and have been commercially used for the production of essential oils in pharmaceutical, toiletries, cosmetics, and food industries (Pino *et al.*, 2002; Marzoug *et al.*, 2011). Several studies have shown on the antifungal properties of *Eucalyptus* essential oils against phytopathogens *Penicillium digitatum, P. italicum, Didymella bryoniae, Candida albicans, Botrytis cinerea, Colletotrichum graminicola, Phoma sorghina, Fusarium moniliforme, F. oxysporum f. sp. Lycopersici, Alternaria triticina, A. solani and Rhizoctonia solani* (Fiori *et al.*, 2000; Ramezani *et al.*, 2002; Dhaliwal *et al.*, 2004; Somda *et al.*, 2007; Tripathi *et al.*, 2008; Gilles *et al.*, 2010; Jhalegar *et al.*, 2015).

The aim of this study was to evaluate the effect of two species of *Eucalyptus* (*E. staigeriana* and *E. globulus*) essential oils on the mycelial growth and conidia germination of *B. cinerea* and *C. acutatum* and evaluate *in vivo* the rot control of grape in vine using the *E. staigeriana* essential oil. Also, evaluate the wine produced with grapes treated with essential oil.

2 Materials and methods

2.1 Isolated fungi

Strains of *B. cinerea* (A58/09) and *C. acutatum* (A009/13) used in this work were isolated from grapes of Caxias do Sul (Serra Gaúcha – RS – Brazil) and preserved in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul, RS -Brazil, on PDA (Potato Dextrose Agar) medium. The molecular confirmation of both fungi was done using Internal Transcribed Sequence (ITS)-PCR identification. The DNA extraction was according to Murray and Thompson (1980) and ITS-PCR amplified the region ITS-5.8S rDNA according to White, Bruns, Lee and Taylor (1990). Sequencing was proceed at the Human Genome Center – USP and the sequences obtained were edited with the software BioEdit Sequence Alignment Editor (1997-2005) and used to search for similar sequences using Blastn at NCBI.

2.2 Plant material

Leaves of *E. staigeriana* and *E. globulus* were collected from plants localized in the University of Caxias do Sul, Caxias do Sul, RS – Brazil. A voucher specimen of the plant species was deposited in the Herbarium of the University of Caxias do Sul with number 37937 for *E. staigeriana* and number 37972 for *E. globulus.*

2.3 Essential oils extraction and analysis

Essential oils were extracted by steam distillation from dried plant leaves for 1 hour according to Cassel *et al.* (2009) with modifications. For identification and quantification of compounds in the essential oil, it was used the protocol described in Tomazoni *et al.* (2016), using a gas chromatograph HP 6890, coupled with a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. The analyses were conducted using a fused silica capillary column HP-Innowax (30 m × 0.25 mm i.d., 0,25 µm film thickness, Hewlett Packard, USA). The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

2.4 *In vitro* antifungal assay

2.4.1 Antifungal activity of essential oils on mycelial growth

The antifungal properties of essential oils were assessed for its contact and volatile phase effects towards mycelial growth of phytopathogens. Contact phase effect of essential oils was tested according to Feng and Zheng (2007) with minor modifications. Essential oils concentrations ranging between 10 to 700 ppm, with the addition of Tween 20 (1:1), diluted on autoclaved and melting PDA (Potato Dextrose Agar) (40ºC) under aseptic conditions were

used for both fungi. The control treatment was just PDA medium and Tween 20 equal to the highest concentration used to emulsify the essential oil. These emulsions were poured into 9 cm (Ø) Petri dishes and after medium solidification, inoculated with a 5 mm (Ø) agar disks colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development.

To assess fungicidal action of the volatile phase of essential oils on the mycelial growth of fungi was utilized the methodology according to Silva *et al.* (2012) with minor modifications. Agar disks with 5 mm (Ø) colonized by *B. cinerea* or *C. acutatum* mycelium with seven days of development were placed in the center of the Petri dish containing PDA culture medium. The concentrations of essential oils used were 12.5, 25, 50 with the addition of Tween 20 (0.1%) and 100% (pure essential oil, without addition of Tween 20). A 100 µL sample of pure essential oil and the solutions were applied onto a cotton ball attached to the inner face of a Petri dish lid, thereby preventing direct contact of the oil with the culture medium and the mycelium disk, creating a saturated atmosphere of volatile compounds. The control treatment was just PDA medium and 100 µL of Tween 20 (0.1%) applied in cotton ball.

In both tests, for each concentration, ten replicate plates were used. Incubation was performed at 25º C temperature and 12 hours photoperiod, during fourteen days. Fungal growth was recorded on the 3^{rd}, 5^{th}, 7^{th}, 10^{th} and 14^{th} day by measurement of orthogonal diameter. Transfer experiments were performed to provide a distinction between the fungistatic and fungicidal effects of essential oils on the target microorganisms. For this purpose, plugs that did not grow were transferred to fresh PDA dishes to assess their viability and growth after five days of inoculation at 25° C. The residual fungal growth was monitored by measuring the radial growth of the fungi.

2.4.2 Antifungal activity of essential oils on conidia germination

Antifungal activity of essential oils on conidia germination was tested according to Badawy and Rabea (2013) with minor modifications. Conidia of *B. cinerea* and *C. acutatum* were

harvested from a colony of the fungi 14 days grown in PDA at 25º C temperature and 12 hours photoperiod. Five milliliter of sterile water was added to a Petri dish culture. The conidia were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia ml^{-1}. Aliquots of conidia suspension (50 µL) were placed in microtubes containing 500 µL of PDB (Potato Dextrose Broth) medium treated with essential oils, at the concentrations of 10 to 400 ppm, with the additon of Tween 20 (1:1). The control treatment was just PDB with addition of Tween 20 at concentration 400 ppm (similar to the highest concentration used to emulsify the essential oil). The tubes were incubated at 25º C for 16 hours. The samples were placed on a Neubauer chamber and observed under the microscope for conidia germination. Conidia counting was done using a light microscopy at 10 x magnification. All experiments were conducted in ten replicates and in each replicate were evaluated hundred conidia. The conidia were considered germinated when the length of the germ tube equaled or exceeded the length of the conidia.

2.5 *In vivo* antifungal assay

The test was carried out in a commercial vineyard, located in the city of Bento Gonçalves, state of Rio Grande do Sul, southern Brazil, in the geographic coordinates -29.067969, -51.564648 and altitude of 591 meters. The vineyard was implanted in 2010, with spacing of 2.5 m between plants and conducted in trellised cord (horizontal wireframe). It was used *Vitis vinifera* variety "Tannat" grafted on the rootstock 1103 Paulsen. The experimental unit consisted of 15 plants, formed by three lines, each line containing five plants. The spacing between lines is 2.7 m and between plants of 1.70 m.

Treatments consisted of two concentrations of *E. staigeriana* essential oil (100 and 500 ppm), conventional treatment and a control. The plants were sprayed at intervals of seven days and

in case of rain it was done reapplication according Table 1. The treatments began in September 2015 (beginning of bud burst) until February 2016 (harvest). Sprays were directed to the grape clusters and carried out preferably at dawn and to the pour point. It was used a 2 liters hand sprayer of precompression. The control plants were not treated. The parameters evaluated were incidence and severity of diseases. For incidence, it was evaluated all grape clusters of each treatment and used the percentage of mean number of clusters with symptoms of the disease. For assessing the severity, a scale from 0 to 100% was created in accordance with the cluster area affected by the disease.

Table 1. Days in which the different treatments were applied to the *Vitis vinifera* cv. Tannat.

	Treatments				
	Essential oil (100 and 500 ppm)	Thiophanate-methyl (0.7 ppm)	Mancozeb (25 ppm)	Captan (24 ppm)	Tebuconazole (10 ppm)
Application days					
Sep /10 / 2015	X	X		X	
Sep /14 / 2015	X			X	
Sep /18 / 2015	X		X		
Sep /22 / 2015	X			X	
Sep /25 / 2015	X			X	
Out /02 / 2015	X			X	
Out /05 / 2015	X		X		
Out /10 / 2015	X	X		X	
Out /16 / 2015	X			X	X
Out /20 / 2015	X			X	
Out /25 / 2015	X			X	
Nov /01 / 2015	X			X	X
Nov /08 / 2015	X		X		
Nov /12 / 2015	X			X	
Nov /17 / 2015	X			X	
Nov /23 / 2015	X			X	
Nov /27 / 2015	X			X	
Dec /02 / 2015	X		X		
Dec /07 / 2015	X			X	
Dec /13 / 2015	X			X	
Dec /20 / 2015	X			X	
Dec /23 / 2015	X			X	
Jan /05 / 2016	X		X		X
Jan /11 / 2016	X			X	
Jan / 18/ 2016	X	X		X	
Jan /26 / 2016	X			X	
Fev /02 / 2016	X			X	
Fev /08 / 2016	X			X	

2.6 Elaboration of red wine

The wines were produced on a small scale using the grapes harvested in the *in vivo* test and held three microvinifications of 1.5 kg of grapes each treatment. Initially, berry was separated from the rachis and then crushed manually. The wort was placed in a 1 L vessel, fitted with Müller valve and active dry yeast (*Saccharomyces cerevisiae* var. bayanus – lineage PDM - Lafort) at a concentration of 0.20 g L^{-1}. The maceration time was nine days, with two daily reassembly and the fermentation occurred with temperature control (20º C). The wines were filtered and bottled.

2.7 Analyses of red wine

2.7.1 Chemical analyses of must and red wine

The following chemical analyses were carried out according to International Organisation of Vine and Wine (2016) and Embrapa Uva e Vinho (Rizzon and Salvador, 2010). In the must, the parameters evaluated were pH using a pH meter and ºbrix (amount of soluble solids contained in the must) using a densimeter (Mettler Toledo). In the red wine the parameters evaluated were total (TAC) and volatile (VAC) acidity (meq L^{-1} tartaric and acetic acid, respectively) using titration and a distiller; pH using a pH meter; alcoholic content (ALC) (% v/v) and density (DENS) (g L^{-1}) using distillation apparatus and densimeter (Mettler Toledo). The total sugar content (g L^{-1}) was performed using DNS method by spectrometry (Miller, 1959). All the chemical results were obtained in triplicate.

2.8 Chromatographic analyses of volatile compounds in red wine

Terpenes present in wine samples were analyzed according to the methodology proposed by Soares *et al.* (2015) with minor modifications, using solid phase microextraction (SPME) with Polyacrilat fiber (Supelco). In 20 ml of wine it was added 5 g of NaCl and 80 µl of internal standard (3-octanol) 250 mg ml^{-1}. The sample was stirred at 40° C for 5 minutes and then the fiber was inserted into the space above the liquid maintaining agitation for 30 minutes at 40º

C. For identification and quantification of terpenes in wine, it was used the protocol described hereafter: the fiber was injected into gas chromatograph coupled with a mass selective detector (GC-MS); the analysis were performed in an HP 6890 GC using a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data; the column used was HP-Inovax (Polyethylene Glycol – 30 m x 320 µl x 0.50 µl), with helium flow of 2.0 mL min^{-1}; the injector temperature was 250° C and transfer temperature was 260°C; splitless injection with fiber permanency in the injector for 5 minutes and opening splitless valve after 5 minutes. The fiber was in desorption for 5 minutes after removing the injector. The oven was kept at 45° C for 5 min and it was heated up to 180° C at a rate of 3° C min^{-1}, reaching a final temperature of 240° C at 20° C min^{-1}. Analyses were performed in splitless mode and flow rate was 1.0 mL min^{-1}. Identification of the individual components was based on comparison of their GC retention times (R.T.) on polar columns and comparison with mass spectra of components by GC-MS. The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

2.9 Statistical analysis

Data normality was determined by Kolmogorov-Smirnov test and the homogeneity of variances was determined using Levene's test. Data were analysed by ANOVA and the threshold for statistical significance was set at $p < 0.05$. In the case of statistical significance Dunnett's T3 test was applied to separate the means. All statistics analysis was performed using SPSS 22.0 for Windows.

3 Results and discussion

3.1 Chemical composition of the essential oils

Essential oils are complex mixtures of volatile organic substances, consisting of oxygenated compounds and hydrocarbons, such as sesquiterpenes and terpenes, the latter being predominant and are known by its antiseptic, bactericides and fungicides characteristics (Prabuseenivasan *et al.,* 2006; Siqueira *et al.,* 2007; Bakkali *et al.,* 2008; Nerio *et al.,* 2010). The composition of essential oils varies among plant species and even between the same species, due that they may show different types of action (Rakotonirainy and Lavédrine, 2005; Nerio *et al*., 2010). In this study, the number of compounds and their relative amount found in essential oils varied according to plant species and the particular compound (Table 2). The major compound found in *E. staigeriana* essential oil was citral (28.67% geranial and 19.68% neral), similarly to the results reported by Chagas e*t al.* (2002) and Macedo *et al.* (2010). For *E. globulus* essential oil, the major compound was 1,8-cineole (64.63 %), as cited by Sacchetti *et al*. (2005), Combrinck *et al.* (2011) and Mekonnen *et al.* (2016). According to Vitti and Britto (2005), the E. *staigeriana* and *E. globulus* essential oils yielded 1.2 to 1.5% and 1.6 to 2%, respectively. On contrast, we obtained a lower yield of essential oils, being 0.77% and 0.57% ($mL.100g^{-1}$ of dried leaves), for *E. staigeriana* and *E globulus* respectively. The same authors also observed that these essential oils obtained in this work. As reported by McGimpsey and Douglas (1994) and Salgueiro *et al.* (1997), climate, genotype, growth location, rainfall and harvesting regime all can affect the total essential oil content of plants.

Table 2. Chemical composition of *Eucalyptus staigeriana* and *Eucalyptus globulus* essential oils.

Compounds	RI[a]	Peak area (%)[b]	
		E. staigeriana	*E. globulus*
Monoterpene hydrocarbons		**25.84**	**19.34**
α-Pinene	8.171	0.83	14.50
α-Phellandrene	16.268	0.28	–
Myrcene	16.494	0.47	–
Limonene	18.288	17.29	3.52
γ-Terpinene	20.901	0.62	–
cis-β-Ocimene	21.397	0.30	–
o-cimene	22.305	0.44	1.32
δ-Carene	22.944	5.61	–
Oxygenated monoterpenes		**69.58**	**73.89**
1.8-Cineole	18.743	6.16	64.63
Linalool	35.967	1.30	–
Pinocarvone	36.603	–	0.75
Terpinen-4-ol	38.362	0.85	0.39
Trans-Pinocarveol	40.533	–	1.26
Neral	41.837	19.68	–
Methyl Geraniate	42.226	3.78	–
α-Terpineol	42.288	–	7.61
Geranial	43.921	28.67	–
Geranyl Acetate	44.658	2.16	–
Citronellol	45.092	1.31	–
Nerol	46.462	1.72	–
Geraniol	48.243	3.77	–
Eugenol	57.636	0.18	–
Sesquiterpenes hydrocarbons		**0.26**	**2.98**
α-Gurjunene	35.782	–	0.20
Caryophyllene	37.840	0.26	–
Aromadendrene	38.011	–	2.01
Alloaromadendrene	39.714	–	0.29
Ledene	41.634	–	0.19
γ-Selinene	56.172	–	0.29
Oxygenated sesquioterpenes		**0.14**	**2.34**
Epiglobulol	54.192	–	0.45
Globulol	55.997	–	1.89
Spathulenol	56.906	0.14	–
Total of identified compound		95.82	98.55

[a] RI, the retention index publyshed by Adams.

[b] Peak area obtained by GC-FID.

3.2 Antifungal activity of essential oils on mycelial growth and conidia germination

The *in vitro* antifungal activity of essential oils differed for each fungi and concentration tested at contact and volatile phase experiments and conidia germination. The inhibitory effect increased proportionally with concentration and was also affected by treatment duration.

The effect of *E. staigeriana* essential oil on the mycelial growth of *B. cinerea* and *C. acutatum* resulted in completely inhibition at concentration 50 ppm and the fungicidal action was observed by the transfer experiment. (Table 2 and Fig. S1 A and B). For the concentration 10 ppm against *B. cinerea* there was a significant inhibition compared to control until the 7th day and agains *C. acutatum* there was a significant inhibition compared to control until the 3rd day, for both fungi it was also observed that the mycelial growth presented a different morphology. The conidia germination of *B. cinerea* and *C. acutatum* were also inhibited completely at concentration 50 ppm and the concentration 10 ppm showed a significant reduction in the germination and in the lenght of the germ tube (data not shown) (Fig. 1 A and Fig. S5 A and B). Similarly, Gilles *et al.* (2010) demonstrated that the *E. staigeriana* essential oil presented a fungicidal action against *Candida albicans.*

With *E. globulus* essential oil, it was observed that the mycelial growth of *B. cinerea* was completely inhibited at concentration 300 ppm and the fungicidal action was confirmed by the transfer experiment. For the concentration 200 ppm there was a significant inhibition until the 7th day compared to control and the mycelial growth presented a different morphology (Table 3 and Fig. S2 A). To completely inhibit the micelial growth of *C. acutatum*, it was necessary a higher concentration (600 ppm) and the fungicidal action was observed by the transfer experiment. The lower concentrations (400 and 500 ppm) reduced significantly the mycelial growth until the 14th day, compared to control and it was also observed that the mycelial growth presented a different morphology (Table 3 and Fig. S2 B). In the conidia germination,

the *E. globulus* essential oil inhibited completely the germination of *B. cinerea* and *C. acutatum* at concentrations 300 and 200 ppm, respectively. The lower concentrations showed a significant reduction in the germination of conidia and in the lenght of the germ tube for both fungi (data not shown) (Fig. 1 B and Fig. S6 A and B). It was previously reported that *E. globulus* essential oil presented antifungal effect against other fungi: Mota *et al.* (2015) demonstrated the fungicide action against *Candida albicans*, Vilela *et al.* (2009) reported a complete fungal growth inhibition against *Aspergillus flavus* and *A. parasiticus* and Bansod and Rai (2008) reported fungicidal action against *Aspergillus fumigatus* and *A. niger.*

Table 3 Effect of different concentrations of *Eucalyptus staigeriana* and *Eucalyptus globulus* essential oils, added on the solid media, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum acutatum* (contact phase).

E. staigeriana

	Mycelial growth (mm)				
	B. cinerea				
	0	10	50	100	200 (ppm)
3rd day	75.08 ± 1.36 a	39.24 ± 2.20 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
5th day	88.84 ± 0.78 a	57.49 ± 1.98 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
7th day	90.00 ± 0.00 a	73.33 ± 1.68 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
10th day	90.00 ± 0.00 a	88.90 ± 0.73 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
14th day	90.00 ± 0.00 a	89.49 ± 0.34 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
	C. acutatum				
	0	10	50	100	200 (ppm)
3rd day	20.11 ± 0.86 a	14.41 ± 0.67 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
5th day	37.19 ± 1.25 a	31.42 ± 0.76 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
7th day	69.69 ± 5.13 a	70.16 ± 6.64 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
10th day	83.73 ± 2.75 a	78.60 ± 4.19 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
14th day	88.61 ± 1.39 a	87.69 ± 1.57 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b

E. globulus

	Mycelial growth (mm)				
	B. cinerea				
	0	200	300	400	500 (ppm)
3rd day	70.66 ± 1.81 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5th day	90.00 ± 0.00 a	4.45 ± 2.35 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
7th day	90.00 ± 0.00 a	12.12 ± 4.37 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
10th day	90.00 ± 0.00 a	52.28 ± 10.12 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
14th day	90.00 ± 0.00 a	77.12 ± 4.44 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
	C. acutatum				
	0	400	500	600	700 (ppm)
3rd day	26.94 ± 0.38 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5th day	38.54 ± 0.62 a	7.79 ± 0.34 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
7th day	69.46 ± 2.49 a	17.62 ± 1.12 b	9.38 ± 1.83 b	0.00 ± 0.00 c	0.00 ± 0.00 c
10th day	82.26 ± 2.24 a	30.65 ± 0.92 b	19.40 ± 1.54 c	0.00 ± 0.00 d	0.00 ± 0.00 d
14th day	87.26 ± 1.78 a	34.62 ± 1.98 b	32.79 ± 1.54 b	0.00 ± 0.00 c	0.00 ± 0.00 c

Values are the average of ten replicates per treatment ± SE.
The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line).
Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

The effect of volatiles from *E. staigeriana* essential oil in the mycelial growth of *B. cinerea* showed a total inhibition at 50% and 100% (pure essential oil) concentrations (fungicidal action was confirmed by transfer experiment) and concentrations of 12.5 and 25% presented a

significant inhibition until 7th and 14th day respectively compared to control (Table 4 and Fig. S3 A). For the effect on *C. acutatum* mycelial growth, no concentration complete inhibited it, but the concentrations 25, 50 and 100 % presented a significant inhibition until 5th day compared to control (Table 4 and Fig. S3 B). Volatiles from *E. globulus* essential oil reduced the mycelial growth of *B. cinerea* at the concentrations 12.5, 25, 50 and 100% until 3rd, 5th, 7th and 14th day, that order, compared to control. The mycelial growth of *C. acutatum* was reduced at the concentrations 25, 50 and 100 % until 3rd, 7th and 10th day, in that order, compared to control. In both fungi it was also observed that the mycelial growth presented a different morphology. These data suggested that a reapplication of the essential oil could control the growth of the fungus, by renewing the volatiles that act on the inhibition of the fungal growth (Table 4 and Fig. S4 A and B). Similarly, Hua *et al.* (2014) reported that the *E. globulus* essential oil showed mild inhibition on *Aspergillus ochraceus* growth. According Avila-Sosa *et al.* (2012) and Tyagi *et al.* (2014) smaller compounds such as monoterpenes were found to be highly effective when used as headspace volatiles and based on the chemical composition, it can be concluded that the antimicrobial activity of the oil is apparently attributed to its high content of oxygenated monoterpenes.

Table 4 Effect of different concentrations of *Eucalyptus staigeriana* and *Eucalyptus globulus* essential oils, apllied on the lid, on the mycelial growth of *Botrytis cinerea* and *Colletotrichum* acutatum (volatile phase).

E. staigeriana

	Mycelial growth (mm)				
	B. cinerea				
	0.0	12.5	25	50	100 (%)
3rd day	49.89 ± 9.06 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
5th day	59.48 ± 9.28 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
7th day	83.77 ± 2.73 a	5.79 ± 1.69 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
10th day	90.00 ± 0.00 a	71.02 ± 3.75 a	11.03 ± 4.14 b	0.00 ± 0.00 b	0.00 ± 0.00 b
14th day	90.00 ± 0.00 a	84.64 ± 2.26 a	35.35 ± 6.73 b	0.00 ± 0.00 c	0.00 ± 0.00 c
	C. acutatum				
	0.0	12.5	25	50	100 (%)
3rd day	19.30 ± 1.05 a	14.48 ± 0.80 a	11.95 ± 0.61 ab	11.75 ± 0.43 ab	10.45 ± 0.72 ab
5th day	35.21 ± 1.18 a	31.84 ± 0.93 a	25.02 ± 0.89 b	24.57 ± 1.06 b	20.55 ± 1.41 b
7th day	57.77 ± 1.94 a	57.25 ± 2.27 a	55.41 ± 3.63 a	53.14 ± 3.20 a	44.13 ± 3.03 a
10th day	70.28 ± 2.15 a	70.20 ± 3.14 a	69.86 ± 3.22 a	69.07 ± 4.19 a	53.04 ± 3.53 a
14th day	85.01 ± 2.80 a	81.17 ± 3.42 a	80.77 ± 2.44 a	79.46 ± 3.90 a	65.12 ± 5.42 a

E. globulus

	Mycelial growth (mm)				
	B. cinerea				
	0.0	12.5	25	50	100 (%)
3rd day	58.29 ± 2.81 a	29.42 ± 1.21 b	17.35 ± 1.65 c	4.25 ± 1.46 d	0.00 ± 0.00 d
5th day	90.00 ± 0.00 a	73.71 ± 3.81 a	48.60 ± 3.07 b	27.55 ± 3.13 c	0.00 ± 0.00 d
7th day	90.00 ± 0.00 a	90.00 ± 0.00 a	80.00 ± 2.45 a	49.14 ± 3.41 c	0.00 ± 0.00 c
10th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	85.71 ± 2.34 a	5.06 ± 2.18 b
14th day	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	90.00 ± 0.00 a	16.49 ± 1.92 b
	C. acutatum				
	0.0	12.5	25	50	100 (%)
3rd day	27.64 ± 0.51 a	23.90 ± 0.66 ab	20.68 ± 0.38 b	15.81 ± 0.67 c	8.6 ± 0.42 d
5th day	51.19 ± 1.75 a	46.63 ± 1.32 a	41.46 ± 1.35 ab	33.92 ± 1.34 b	22.92 ± 1.22 c
7th day	67.75 ± 2.54 a	62.44 ± 2.08 ab	58.10 ± 1.83 ab	51.00 ± 1.61 b	38.08 ± 1.74 c
10th day	83.74 ± 3.49 a	74.41 ± 3.45 ab	70.75 ± 2.46 ab	65.71 ± 2.48 ab	59.47 ± 1.42 b
14th day	84.30 ± 3.12 a	79.20 ± 3.30 a	76.06 ± 3.04 a	72.92 ± 3.59 a	72.83 ± 3.27 a

Values are the average of ten replicates per treatment ± SE.

The letters indicate the comparison among the different essential oil concentrations evaluated in each day (per line).

Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

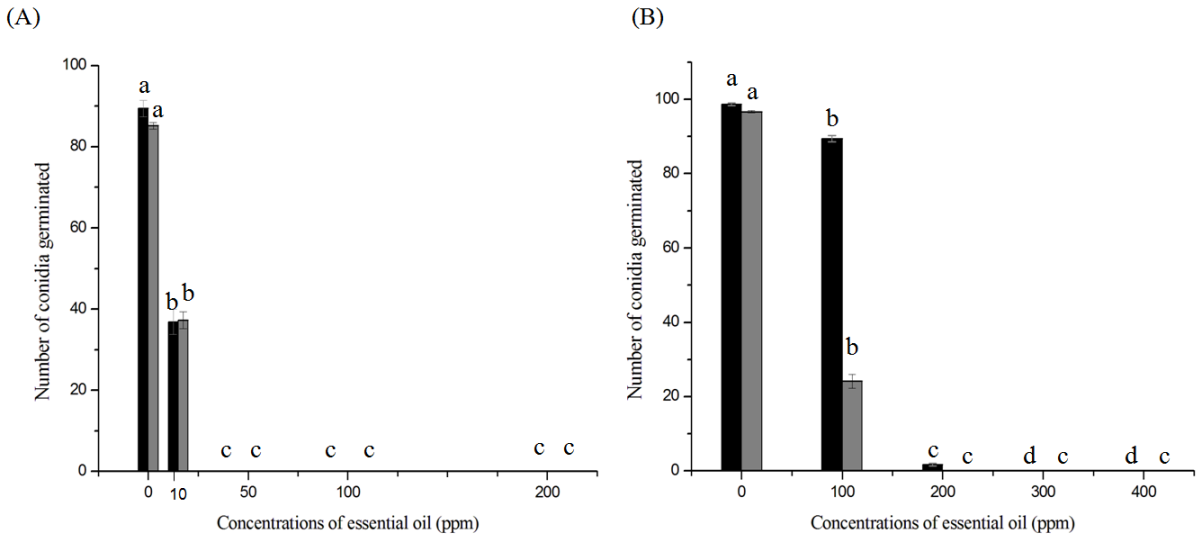

Figure 1. Effect of different concentrations of *Eucalyptus staigeriana* (A) and *Eucalyptus globulus* (B) essential oils on conidia germination of *Botrytis cinerea* (■) and *Colletotrichum acutatum* (■). Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Fung *et al*. (1977) and Tian *et al.* (2012) suggested that the effect of essential oils on microbial growth might be the result of phenolic compounds and terpenoids present in the essential oils altering microbial cell permeability. This cause deformation of the cell structure, functionality and permits the loss of macromolecules from their interior causing inhibition of cell growth (Sharma and Tripathi, 2006; Helal *et al.,* 2007; Silva *et al.,* 2009; Pramila *et al*., 2012). Besides inhibiting the mycelial growth, phenolic compounds also affect the enzymes responsible for conidia germination and interfere with amino acids that were necessary in germination processes (Nychas, 1995). In this work, both fungi hyphae grown on media with essential oils revealed alterations in the morphology, such modifications may be related to the effect of the essential oil on enzymatic reactions regulating wall synthesis for example (Rasooli *et al.,* 2006).

In *in vitro* tests, the *E. staigeriana* essential oil showed greater inhibition on mycelial growth and conidia germination of *B. cinerea* and *C. acutatum,* than *E. globulus* essential oil. This is probably due to the compound citral which was found at high levels and is related to high

antifungal activities against various fungi such as: *Aspergillus niger*, *A. flavus*, *Fusarium oxyisporum*, *F. moniliforme*, *F. pallidoroseum*, *Penicillium digitatum*, *Alternaria alternata*, *Curvularia lunata*, *Phoma sorghina*, *Phytophthora cactorum*, *Cryphonectria parasitica*, *Magnaporthe grisea*, *Gibberella zeae*, *Valsa mali*, *Botrytis cinerea* and *Rhizoctonia solani* (Moleyar and Narasimham, 1986; Pawar and Thaker, 2006; Kishore *et al*., 2007; Lee *et al*., 2008; Hua *et al*., 2014; Li *et al.*, 2015).

3.3 Antifungal activity of essential oil *in vivo* in *Vitis vinifera*

Most of the essential oils have been reported to inhibit phytopathogenic fungi in *in vitro* conditions (Singh *et al.,* 2006; Lopez-Reyes *et al.,* 2010; Bosquez-Molina *et al.*, 2010; Abdolari *et al.,* 2010; Barkat and Bouguerra, 2012; Aminifard and Mohammadi, 2013; Gemeda *et al.*, 2014; Ali *et al.*, 2015). However, to our knowledge the *in vivo* efficacy of the essential oils against the fungal rots of grapes has not been studied in the field. For *in vivo* test we selected the *E. staigeriana* essential oil, due to the results obtained in *in vitro* tests. The two concentrations tested of *E. staigeriana* essential oil were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and severity of disease caused by *C. acutatum* (Table 5). There was no significant differences between the two essential oil concentrations used (100 and 500 ppm). Also there was no significant difference between the control and the conventional treatment. Treatment with essential oil proved to be more effective in controlling the diseases when compared to control and conventional treatment, especially against the phytopathogen *B. cinerea*. The vine and grape clusters treated with essential oil of *E. staigeriana* did not show any obvious signs of phytotoxicity, just showed up brighter in the clusters. Similarly, in *in vivo* conditions, Soylu *et al.* (2010) demonstrated that the essential oil of *Origanum syriacum L. var. bevanii* reduced the severity of disease caused for *B. cinerea* in tomato plants. Al-Reza *et al.* (2010) also showed that the essential oil of *Cestrum nocturnum* reduced the disease caused by *Phytophthora capsici* in pepper plants.

Table 5. *In vivo* evaluation of *Vitis vinifera* cv. "Tannat" grapes with diferents treatments: control, conventional and with *Eucalyptus staigeriana* essential oil.

	B. cinerea		*C. acutatum*	
Treatment	Incidence	Severity	Incidence	Severity
Control	13.33 ± 1.07 a	20.83 ± 1.78 a	15.00 ± 0.63 a	27.08 ± 3.38 a
Convencional treatment	14.42 ± 2.04 a	15.62 ± 1.63 a	14.50 ± 2.77 a	26.04 ± 3.59 a
Essential oil (100 ppm)	2.92 ± 0.73 b	1.79 ± 0.98 b	13.37 ± 1.65 a	15.71 ± 2.68 b
Essential oil (500 ppm)	2.08 ± 0.52 b	0.92 ± 0.83 b	12.00 ± 1.22 a	14.58 ± 1.40 b

Values are the average of twelve replicates per treatment ± SE.
Letters indicate the comparison among the different treatments.
Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

3.4 Chemical and volatile compounds analyzed in the must and red wine

Chemical analyses are performed to explain some sensory changes of wines and are fundamental to understand which compounds influence wine sensory properties and how they affect them (Thorngate, 1997; Colagrande *et al.*, 1988; Girard *et al.*, 2001). The analytical results for the must and red wine samples are shown in Table 6. In the must, the pH does not vary between the different treatments, however the ºbrix was higher at conventional treatment than the other treatments. All samples of wine had values within the reference ranges described by International Organisation of Vine and Wine (2016), with the exception of alcoholic content for samples of conventional treatment and treatments with essencial oil that remained slightly below the minimum (8.5 % v/v) required for this type of wine. The analyzes showed that there was no significant difference between different treatments, showing that the essential oil had no influence on the chemical characteristics of red wine made from grapes treated with *E. staigeriana* essential oil.

Table 6. Chemical parameters evaluated on must and red wines samples of *Vitis vinifera* cv. "Tannat" from treatments: control, conventional and with *Eucalyptus staigerina* essential oil.

	Must		Red wine					
Treatment	pH	ºbrix	pH	TAC (meq L^{-1})	VAC (meq L^{-1})	DENS (g. L^{-1})	ALC (% v/v)	TRSG (g. L^{-1})
Control	2.94 ± 0.74 a	17.18 ± 0.27 b	3.06 ± 0.30 a	88.00 ± 1.00 a	3.00 ± 0.58 a	0.9962 ± 0.00074 a	8.27 ± 0.32 a	1.64 ± 0.58 a
Convencional treatment	3.01 ± 0.62 a	19.74 ± 0.36 a	3.23 ± 0.38 a	90.33 ± 4.98 a	2.33 ± 0.33 a	0.9951 ± 0.00050 a	8.77 ± 0.43 a	2.24 ± 0.22 a
Essential oil (100 ppm)	2.92 ± 0.57a	17.50 ± 0.65 ab	3.07 ± 0.63 a	89.00 ± 3.46 a	1.67 ± 0.33 a	0.9966 ± 0.00094 a	8.20 ± 0.50 a	1.78 ± 0.18a
Essential oil (500 ppm)	2.92 ± 0.23 a	16.61 ± 0.54 b	3.04 ± 0.47 a	87.00 ± 1.76 a	1.67 ± 0.33 a	0.9970 ± 0.00073 a	8.00 ± 0.56 a	1.73 ± 0.23 a

Legend: TAC: total acidity; VAC: volatile acidity; TRSG: total reducing sugars; ALC: alcoholic content; DENS: density.
Values are the average of three replicates per treatment. Letters indicate the comparison among the different treatments.
Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

In all, 54 volatile compounds were identified in the wine produced with the grapes from the four different treatments, including thirteen alcohols, eighteen esters, eight acids, eleven terpenes and four ketones compounds (Table 7). Many of these volatile compounds are commonly found in wines and are derived from grapes and yeast strain fermentation and the winemaking process (Cliff *et al.*, 2002).

Alcohols represented the largest group in terms of the number and concentration of aroma compounds identified. Alcohols are formed from the degradation of amino acids, carbohydrates and lipids (Antonelli *at al.*, 1999; Jiang and Zhang, 2010). The major alcohol found in all the samples was 1-pentanol and the other varied in abundance for each sample, what would bring specific characteristics for each of them. The production of fatty acids has been reported to be dependent on the composition of the must and fermentation conditions (Schreirer, 1979). Acetic, pentanoic, butanoic, hexanoic, heptanoic, octanoic, decanoic and tetradecanoic acids found in the samples, these the octanoic acid were the major fatty acid found. The fatty acids found in the wine samples (i.e. octanoic acid, propanoic acid, butanoic acid, etc.) were also reported by Boido *et al*. (2003) in the analysis of red wine produced with "Tannat" in Uruguay. Esters are secondary aromas, arising during fermentation and resulting from the combination of alcohols and acids (Clarke, 2003). These compounds are important components of wine aroma, due to their fruity and floral notes (Welke *et al.*, 2014). The esters found in all samples are octanoic acid ethyl ester acetate and 1-butanol 3-methyl acetate being responsible for the fruit and floral aroma. Ketones also are largely responsible for the characteristic flavor and aroma of some red wines (Ferreira *et al.*, 1993). The composition of ketones varied among the samples of wines and β-damascenone was found in all samples.

Terpenes are found in grape skins and are transferred to wine during maceration, which is the first step of winemaking. These compounds generally remain unchanged after the fermentation process (Clarke, 2003; Zhang *et al.*, 2011). One of the compounds positively

identified in smaller concentrations in the wine produced from the grapes treated with *E. staigeriana* essential oil, that deserves to be highlighted is eucalyptol (1,8-cineole) as it is not a typical compound of wines (Rocha *et al.*, 2007; Kalua and Boss 2010; Capone *et al*., 2012) and it was not identified in wines produced from grapes of control and conventional treatment. However, eucalyptol has been identified in grapes and wines including Riesling, Cabernet Sauvignon (Kalua and Boss, 2010), Shiraz from Australia (Capone *et al*., 2012) and Fernão-Pires produced in Portugal (Rocha *et al.*, 2007). Moreover, Capone *et al.* (2012) verified that the proximity of *Eucalyptus* trees to grapevines could directly influence concentration of eucalyptol present in the corresponding red wines. With these results we can conclude that, the essential oil used in the treatment of grapes leave a residual compound (1.8-cineole), but that not negatively interfere, since this compound may give minty characteristic to the wine (Capone *et al.*, 2012).

Table 7. Volatile compounds identified in red wine of *Vitis vinifera* cv. Tannat with diferents treatments incluing *Eucalyptus staigerina* essential oil.

Compounds	RI[a]	Peak area (%)[b]			
		Control	Convencional treatment	Essential oil (100 ppm)	Essential oil (500 ppm)
Internal standard					
3-octanol	16.134	12.24	9.40	10.87	9.87
Acids					
Acetic acid	3.340	0.10	–	–	–
Pentanoic acid	6.619	0.01	–	0.05	–
Butanic acid	8.823	0.01	–	0.03	–
Hexanoic acid	16.333	0.43	0.21	–	1.46
Octanoic acid	26.094	4.03	0.94	0.70	4.88
Heptanoic acid	33.594	–	–	0.13	–
Decanoic acid	34.163	1.23	–	–	–
Tetradecanoic acid	44.982	–	–	–	3.39
Alcohols					
2-Methyl -1-Butanol	4.331	5.36	3.94	3.62	6.57
3-Methyl -1-Butanol	4.054	–	39.60	–	35.83
1-Pentanol	4.265	40.31	61.45	40.26	22.48
2,3-Butanediol	7.059	0.23	0.11	0.06	0.10
1-Propanol, 3-ethoxy	7.322	0.07	0.02	0.05	0.09
1,3-Propanediol, 2,2-dimethyl	8.397	0.05	0.13	–	–
1-Pentanol ,3-methyl	8.424	0.12	0.08	0.25	–
1-Hexanol	9.068	3.60	1.59	1.92	1.77
1-Heptanol	14.594	0.11	0.14	–	0.15
1-Hexanol -2-ethyl	17.639	0.12	0.04	0.03	0.08
1-Octanol	19.840	0.29	0.07	0.22	0.14
Phenylethyl alcohol	22.128	6.60	5.72	3.72	5.60
1-Decanol	29.477	0.19			
Terpenes					
β-Mircene	15.570	2.99	–	–	0.02
Limonene	17.362	0.35	0.38	0.42	0.13
1,8-Cineole	17.445	–	–	0.10	0.03
Linalool	21.154	0.98	–	0.14	0.32
Hotrienol	21.169	0.26	–	–	0.33
Nerol oxide	23.812	0.15	–	0.06	0.08
α-Terpineol	25.452	–	0.07	–	0.42
Nerol	27.332	–	–	–	0.15
Citronellol	27.419	0.14	0.52	0.46	0.68
Citronellyl acetate	30.168	0.03	–	–	–
Methyl geranate	31.951	0.08	0.14	–	0.06
Ketones					
2-Butoxy-2-butanone	6.411	0.24	–	–	–
2-Nonanone	20.803	0.05	–	–	0.03
2-Tridecanone	30.365	0.03	–	0.02	0.11
β-damascenone	34.154	0.03	0.05	0.10	0.06
Esters					
Propanoic acid ethyl ester	3.716	0.44	0.33	0.11	0.37
3-Heptanol, 2,4-dimethyl	4.268	2.28	–	–	–
Propanoic acid 2-methyl ethyl ester	4.835	0.09	0.07	–	0.03
Pentanoic acid, 4-methyl	6.974	0.01	–	–	–
Butanoic acid ethyl ester	6.442	0.18	0.47	0.21	0.22
Pentanoic acid, butyl ester	8.151	0.22	–	–	–
2-Butenoic acid ethyl ester	8.510	0.26	–	–	–
1-Butanol, 3-Methyl acetate	9.882	1.64	2.12	1.49	1.10
1-Butanol, 2-Methyl acetate	9.938	0.12	0.15	0.11	0.09
Hexanoic acid ethyl ester	16.220	1.75	2.26	2.02	2.16
Butanedioic acid diethyl ester	25.290	0.30	0.27	0.41	0.21
Octanoic acid ethyl ester	26.042	5.84	7.47	10.16	9.03
Acetic acid 2-phenylethyl ester	28.591	0.46	0.24	0.23	0.09
Ethil-9-decenoate	34.457	0.9	0.30	0.47	0.27
Decanoic acid ethyl esther	35.180	1.37	0.92	1.71	0.84
Dodecanoic acid ethyl ester	40.930	0.16	–	0.22	–
Hexadecanoic acid ethyl ester	49.795	–	0.01	0.05	1.12
Hexanoic acid bis (2-ethylhexil) ester	54.807	0.12	0.7	1.55	0.31

[a] RI, the retention index publyshed by Adams.

[b] Peak area obtained by GC-FID.

The data were mean values of triplicate samples.

4. Conclusion

Considering the results on the inhibition of *B. cinerea* and *C. acutatum* development *in vitro* and *in vivo* and results of winemaking, we can conclude that *E. staigeriana* essential oil could be used as a possible biofungicide. However, additional studies are required before this essential oil can be recommended as commercial and natural antifungal agent in treatment of wine grapes.

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SUPLEMMENTARY MATERIAL

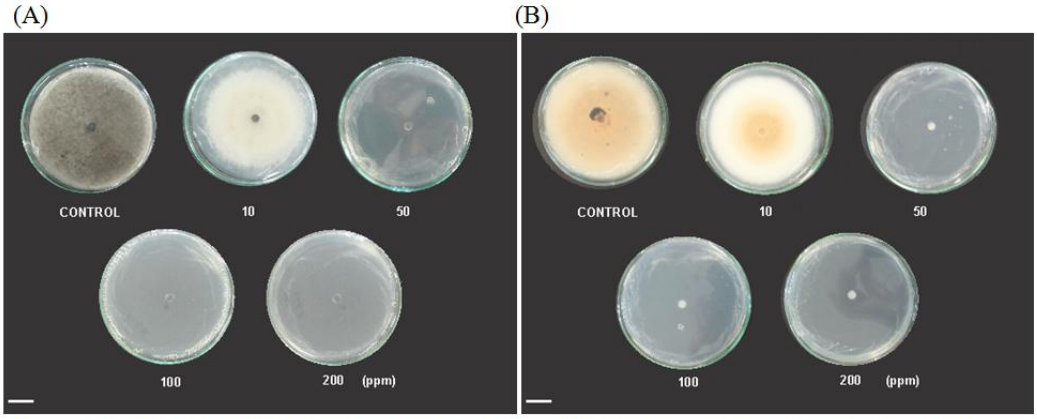

Figure S1: Effect of different concentrations of *Eucalyptus staigeriana* essential oil, added on the solid media, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (contact phase to 14th day) Scale: 2cm.

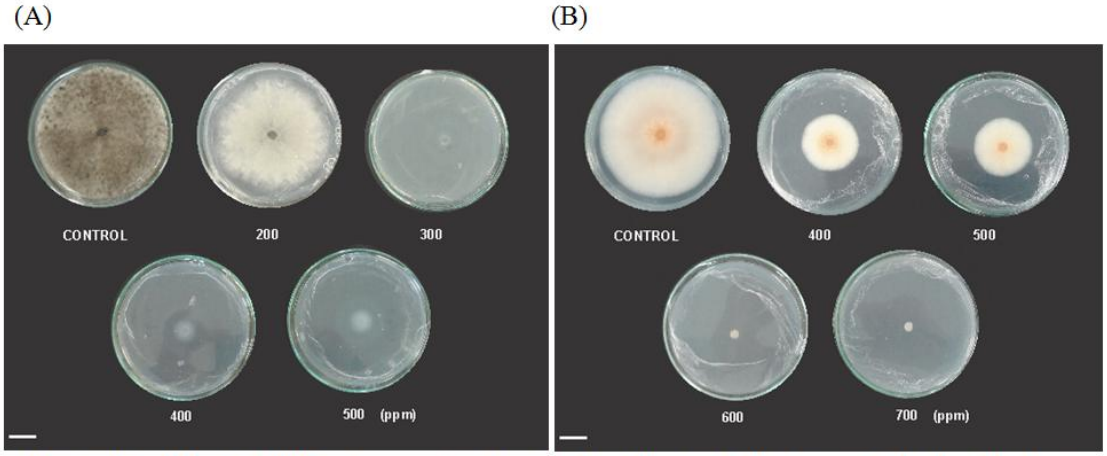

Figure S2: Effect of different concentrations of *Eucalyptus globulus* essential oil, added on the solid media, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (contact phase to 14th day) Scale: 2cm.

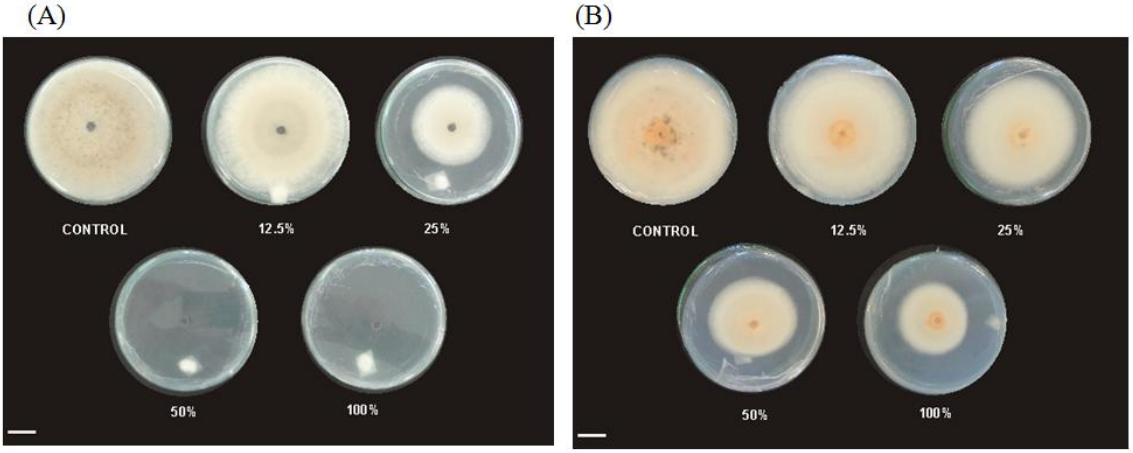

Figure S3: Effect of different concentrations of *Eucalyptus staigeriana* essential oil, apllied on the lid, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (volatile phase to 14th day) Scale: 2cm.

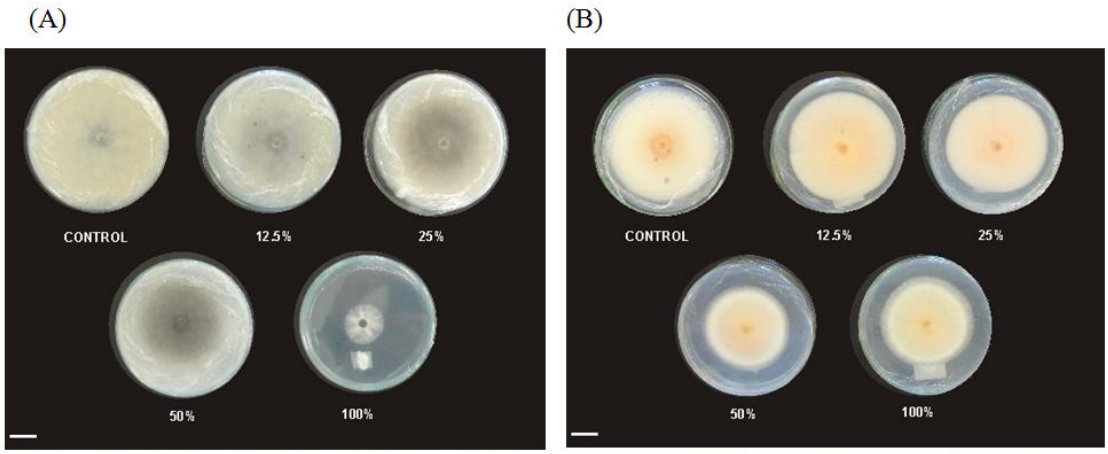

Figure S4: Effect of different concentrations of *Eucalyptus globulus* essential oil, apllied on the lid, on the mycelial growth of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum* (volatile phase to 14th day) Scale: 2cm.

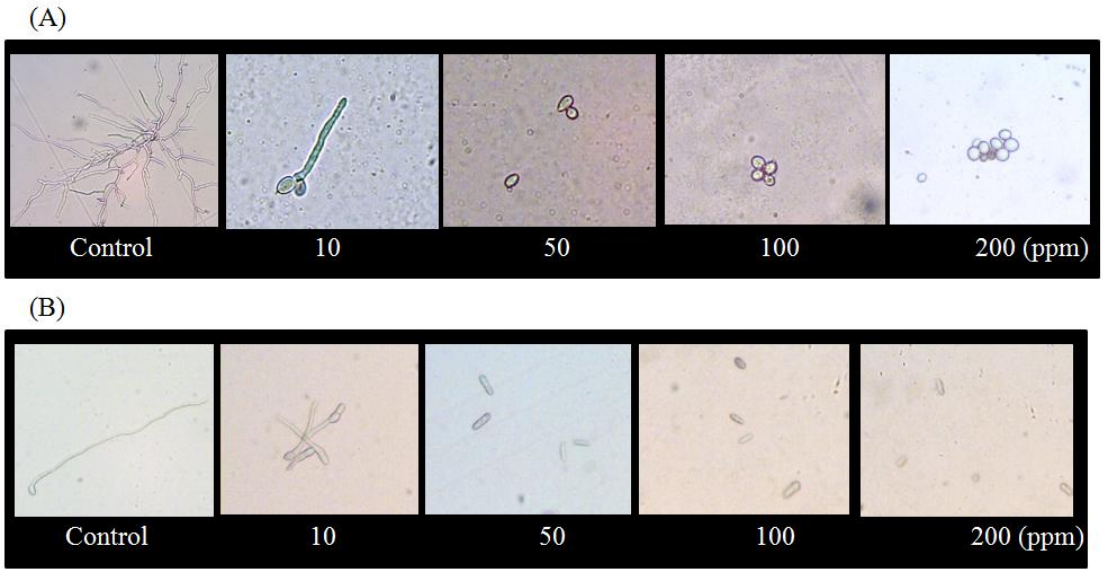

Figure S5: Effect of different concentrations of *Eucalyptus staigeriana* essential oil on conidia germination of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum*. (10× magnification).

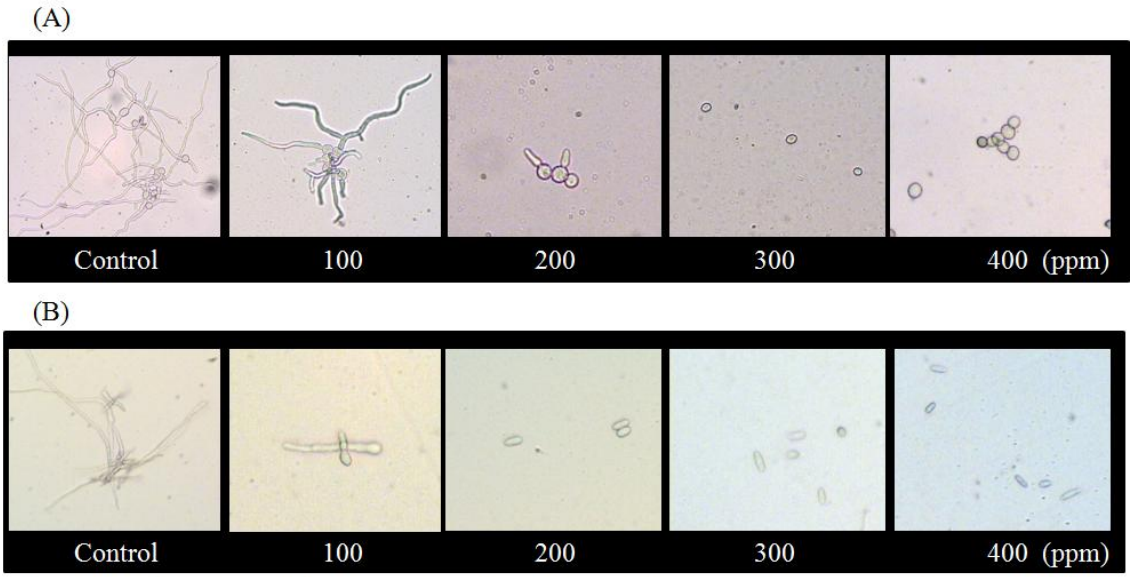

Figure S6: Effect of different concentrations of *Eucalyptus globulus* essential oil on conidia germination of (A) *Botrytis cinerea* and (B) *Colletotrichum acutatum*. (10× magnification).

4.4. Capítulo 4

(Será submetido ao *Food Control Journal)*

Effect of *Eucalyptus* essential oil against fungal rots on table and wine grapes

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ABSTRACT

Several volatile natural compounds produced by plant secondary metabolism have been proven to present antimicrobial action, enabling their use in phytopathogens control.The aim of this study was to find an alternative to synthetic fungicides currently used in the control of fungal pathogens *Botrytis cinerea* and *Colletotrichum acutatum*, the causal agents of fungal rots on table and wine grapes. Antifungal activities of essential oil obtained from *Eucalyptus staigeriana* was investigated in postharvest and vineyard grapes of *Vitis* spp. cv. "Isabela". The major compound found in *E. staigeriana* essential oil were neral (19.68%) and geranial (28.67%). In postharvest test, the concentrations of essential oil tested were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum*, both in

preventive and curative treatment. In vineyard test, the two concentrations of essential oil tested were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum.* The chemical compounds analyzed in must and red table wine made from the grapes treated with *E. staigeriana* essential oil showed two residual compounds (1.8-cineole and citral), but that has no influence on the characteristic of table red wine. This study has demonstrated that *E. staigeriana* essential oil presented potential and promising antifungal action, which could be used as biofungicide in the protection of grapes against *B. cinerea* and *C. acutatum.*

Keywords: *Vitis* spp., *Botrytis cinerea*, *Colletotricum acutatum,* alternative control.

1. Introduction

Grape is one of the most important fruit crops worldwide. In Brazil, *Vitis* spp. is the common specie cultivated, being "Isabela" grape one of the most important varieties. This variety is used to make red table wine and juice and it is also commercialized as table grape (Silveira *et al*., 2015; Mello, 2014).

Botrytis cinerea Pers. Fr. and *Colletotrichum acutatum* Simmonds cause fungal rot and they are considered the main cause of great losses in wine and table grapes (Pearson and Goheem, 1988; Steel *et al.*, 2007; Whitelaw-Weckert *et al.,* 2007). The Serra Gaúcha is one of the most important region in the production of grapes from Brazil, however, it has unfavorable weather conditions due to the high rainfall that affects the development of culture, that results in greater probability of occurring fungal rot diseases (Westphalen and Maluf, 2000; Conceição *et al.*, 2006). Besides the losses observed in the vineyard, there are also losses in postharvest. Fungicide treatments represent more than half of pesticides applied in viticulture and their use is not without risks to human health (Flamini and De Rosso, 2006; Tsakirakis *et al*., 2012).

Some products also affect the physiology of the grapevine itself (Petit *et al.,* 2009) and therefore its production (Jermini *et al.,* 2010). Furthermore, pesticide residues were identified in wine (Cesnik *et al.,* 2008). They can affect natural yeast communities that are necessary for wine making (Milanovic *et al.,* 2013), as well as wine aroma (González Álvarez *et al.,* 2012). By over efficient that it is the phytosanitary treatment made in the field, it is not enough to dismiss it in postharvest (Lichter, 2002). As a postharvest treatment, grapes are usually stored with a sulfur dioxide fumigation (Droby and Lichter, 2004). However, the use of synthetic fungicides and sulfur dioxide is not allowed on organic grapes (Mlikota Gabler and Smilanick, 2001).

There is a growing public concerns about health and environmental hazards associated with pesticide use, what resulted in a considerable interest in developing alternative non-polluting control methods (Youssef and Roberto, 2012). Among the possibilities of alternative control it is the use of essential oils, that are well known for their antimicrobial and biodegradable properties and for not leave any residual effect on fresh produce (Isman, 2000; Kalemba and Kunicka, 2003; Burt, 2004). Moreover, the majority of essential oils are classified by the FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe), recognized as safe for use in foods, so there has been a growing interest in using them in the treatment of fruits and vegetables (Gonzales-Aguilar *et al*., 2008).

Eucalyptus staigeriana is an evergreen, tall tree, or shrub, belonging to Myrtaceae family. Although it is native to Australia, nowadays it has extensively spread to other countries (Vitti e Brito, 2003). Several studies have shown the biological properties of *Eucalyptus staigeriana* essential oil such as antimicrobial activity (Wilkinson and Cavanagh, 2005; Gilles *et al*., 2010), insecticidal activity (Chagas *et al*., 2002; Brito *et al.,* 2006; Batish *et al.,* 2008; Costa *et al.,* 2008; Maciel, 2009), anthelmintic activity (Macedo *et al*., 2010) and antifungal activity (Gilles *et al.,* 2010).

The aim of this study was to evaluate the effect of *Eucalyptus staigeriana* essential oil against *B. cinerea* and *C. acutatum*, causal agents of grape rot in postharvest and vineyard grapes and to investigate the essential oil residue in the wine produced with the grapes treated with it.

2. Materials and methods

2.1. Plant material

Leaves of *E. staigeriana* were collected from plants localized in the city of Caxias do Sul, RS – Brazil. A voucher specimen of plant species was deposited in the Herbarium of the University of Caxias do Sul with number 37973.

2.2. Essential oils extraction and analysis

Essential oils were extracted by steam distillation from dried plant leaves for 1 hour according to Cassel *et al.* (2009) with modifications. For identification and quantification of compounds in the essential oil, it was used the protocol described in Tomazoni *et al.* (2016), using a gas chromatograph HP 6890, coupled with a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. The analyses were conducted using a fused silica capillary column HP-Innowax (30 m × 0.25 mm i.d., 0,25 µm film thickness, Hewlett Packard, USA). The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

2.3. Inoculum preparation

Strains of *B. cinerea* (A58/09) and *C. acutatum* (A009/13) used in this work were isolated from grapes of Caxias do Sul (Serra Gaúcha – RS – Brazil) and preserved in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul, RS -Brazil, on

PDA (Potato Dextrose Agar) medium. The molecular confirmation of both fungi was done using Internal Transcribed Sequence (ITS)-PCR identification. The DNA extraction was according to Murray and Thompson (1980) and ITS-PCR amplified the region ITS-5.8S rDNA according to White, Bruns, Lee and Taylor (1990). Sequencing was proceed at the Human Genome Center – USP and the sequences obtained were edited with the software BioEdit Sequence Alignment Editor (1997-2005) and used to search for similar sequences using Blastn at NCBI. Conidia of *B. cinerea* and *C. acutatum* were harvested from a colony of the fungi 14 days grown in PDA at 25º C temperature and 12 hours photoperiod. Five milliliter of sterile water was added to a Petri dish culture. The conidia were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to obtain a suspension of 1×10^6 conidia ml^{-1}.

2.4. Fruit

Traditionally grown, freshly harvested, *Vitis* spp. cv. "Isabela" grapes from Bento Gonçalves, RS - Brazil were used in experiments.

2.5. Antifungal activity of essential oil in postharvest in grapes

To evaluate the antifungal activity of *E. staigeriana* essential oil on grapes it was carried out experiments with curative and preventive treatments. The postharvest curative treatment consisted of inoculation of 10 berries for each cluster (10 fruit / treatment) of grape, through wounds, approximately 2 mm deep, with the aid of a syringe (Zahavi, 2000). After the injury, the clusters were inoculated by spraying the conidia suspension of *B. cinerea* or *C. acutatum*, according to the methodology described by Romanazzi *et al*. (2002) and Thomas *et al*. (1988) with modifications. After 4 hours, the application of essential oil was carried out with the concentrations 50, 100 and 200 ppm ($\mu L\ mL^{-1}$). Subsequently, in order to evaluate the

potential of the essential oil in preventing disease, the grape clusters were sprinkled with essential oils in the same concentrations of the previous test and inoculated after 24 hours with the fungi. For both experiments, the clusters were placed in plastic boxes and kept at 25 ± 1 ° C / 80-90% relative humidity for a period of five days for those inoculated with *B. cinerea* and seven days for those inoculated with *C. acutatum.* To evaluate the incidence, ten berries for each bunch of grapes that were inoculated were evaluated and it was used the percentage of mean number of berries with symptoms of the disease. For assessing the severity, a scale from 0 to 100 % was created in accordance with the berry area affected by the disease.

2.6. Antifungal activity of essential oil in vineyard

The test was carried out in a commercial vineyard, located in the city of Bento Gonçalves, state of Rio Grande do Sul, southern Brazil, in the geographic coordinates -29.0849531065397 -51.5536597669569 and altitude of 635 meters. The vineyard was implanted in 1982, with spacing of 4.5 × 2.5 m between plants and plants were conducted in trellised cord (horizontal wireframe). It was used *Vitis* spp. variety "Isabela". The experimental unit consisted of 15 plants, formed by three lines, each line containing five plants. The spacing between lines is 3 m and between plants of 2 m.

Treatments consisted of two concentrations of *E. staigeriana* essential oil (100 and 500 ppm), conventional treatment and a control. The plants were sprayed at intervals of seven days and in case of rain it was done reapplication according Table 1. The plants were sprayed at intervals of seven days, and in case of rain it was done reapplication. The treatments began in September 2015 (beginning of bud burst) until February 2016 (harvest). Sprays were directed to the grape clusters and carried out preferably at dawn and to the pour point. It was used a 2 liters hand sprayer of precompression. The control plants were not treated. The parameters

evaluated were incidence and severity. For incidence it was evaluated all grape clusters of each treatment and used the percentage of mean number of clusterss with symptoms of the disease. For assessing the severity, a scale from 0 to 100 % was created in accordance with the cluster area affected by the disease.

Table 1. Days in which the different treatments were applied to the *Vitis* spp. cv. "Isabela"

	Treatments				
	Essential oil (100 and 500 ppm)	Thiophanate-methyl (0.7 ppm)	Mancozeb (25 ppm)	Captan (24 ppm)	Tebuconazole (10 ppm)
Application days					
Sep /10 / 2015	X	X		X	
Sep /14 / 2015	X			X	
Sep /18 / 2015	X		X		
Sep /22 / 2015	X			X	
Sep /25 / 2015	X			X	
Out /02 / 2015	X			X	
Out /05 / 2015	X		X		
Out /10 / 2015	X	X		X	
Out /16 / 2015	X			X	X
Out /20 / 2015	X			X	
Out /25 / 2015	X			X	
Nov /01 / 2015	X			X	X
Nov /08 / 2015	X		X		
Nov /12 / 2015	X			X	
Nov /17 / 2015	X			X	
Nov /23 / 2015	X			X	
Nov /27 / 2015	X			X	
Dec /02 / 2015	X		X		
Dec /07 / 2015	X			X	
Dec /13 / 2015	X			X	
Dec /20 / 2015	X			X	
Dec /23 / 2015	X			X	
Jan /05 / 2016	X		X		X
Jan /11 / 2016	X			X	
Jan / 18/ 2016	X	X		X	
Jan /26 / 2016	X			X	
Fev /02 / 2016	X			X	
Fev /08 / 2016	X			X	

2.7. Elaboration of red table wine

The wines were produced on a small scale using the grapes harvested from the vineyard and held three microvinifications of 1.5 kg of grapes each treatment. Initially, each berry was separated from the rachis and then crushed manually. The wort was placed in a 1 L vessel, fitted with Müller valve and active dry yeast (*Saccharomyces cerevisiae* var. bayanus – lineage PDM - Lafort) at a concentration of 0.20 g L^{-1}. The maceration time was nine days,

with two daily reassembly and the fermentation occurred with temperature control (20º C). The wines was filtered and bottled.

2.8. Chemical analyses of must and red table wine

The following chemical analyses were carried out according to International Organisation of Vine and Wine (2016) and Embrapa Uva e Vinho (Rizzon and Salvador, 2010). In the must, the parameters evaluated were pH using a pH meter and ºbrix (amount of soluble solids contained in the must) using a densimeter (Mettler Toledo). In the red wine the parameters evaluated were (TAC) and volatile (VAC) acidity (meq L^{-1} tartaric and acetic acid, respectively) using titration and a distiller; pH using a pH meter; alcoholic content (ALC) (% v/v) and density (DENS) (g L^{-1}) using distillation apparatus and densimeter (Mettler Toledo). The total sugar content (g L^{-1}) was performed using DNS method by spectrometry (Miller, 1959). All the chemical results were obtained in triplicate.

2.9. Chromatographic analyses of volatile compounds in red table wine

Terpenes present in wine samples were analyzed according to the methodology proposed by Soares *et al.* (2015) with minor modifications, using solid phase microextraction (SPME) with Polyacrilat fiber (Supelco). In 20 ml of wine it was added 5 g of NaCl and 80 µl of internal standard (3-octanol) 250 mg ml^{-1}. The sample was stirred at 40° C for 5 minutes and then the fiber was inserted into the space above the liquid maintaining agitation for 30 minutes at 40º C. For identification and quantification of terpenes in wine, it was used the protocol described hereafter: the fiber was injected into gas chromatograph coupled with a mass selective detector (GC-MS); the analysis were performed in an HP 6890 GC using a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data; the column used was HP-Inovax (Polyethylene Glycol – 30 m x 320 µl x 0.50 µl), with helium flow of 2.0 mL min^{-1}; the injector temperature was 250° C and transfer

temperature was 260° C; splitless injection with fiber permanency in the injector for 5 minutes and opening splitless valve after 5 minutes. The fiber was in desorption for 5 minutes after removing the injector. The oven was kept at 45° C for 5 min and it was heated up to 180° C at a rate of 3° C min^{-1}, reaching a final temperature of 240° C at 20° C min^{-1}. Analyses were performed in splitless mode and flow rate was 1.0 mL min^{-1}. Identification of the individual components was based on comparison of their GC retention times (R.T.) on polar columns and comparison with mass spectra of components by GC-MS. The components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 2005). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks being 100%.

2.10. Statistical analysis

Data normality was determined by Kolmogorov-Smirnov test and the homogeneity of variances was determined using Levene's test. Data were analysed by ANOVA and the threshold for statistical significance was set at $p \leq 0.05$. In the case of statistical significance Tukey's test was applied to separate the means. All statistics analysis was performed using SPSS 22.0 for Windows.

3. Results and discussion

3.1. Chemical composition of the essential oil

Essential oils are complex, volatile and natural plant compounds, known for their antiseptic, bactericide and fungicide characteristics (Bakkali *et al.*, 2008). The composition of essential oils varies among plant species and even between the same species, due that they may show different types of action (Rakotonirainy and Lavédrine, 2005; Nerio *et al*., 2010). In this study, a total of 21 components of the essential oil were identified by GC-MS, representing

95.82% of the total amount (Table 2). The major compound found in *E. staigeriana* essential oil was citral (28.67% geranial and 19.68% neral), similarly to the results reported by Chagas *et al.* (2002) and Macedo *et al.* (2010). According to Vitti and Britto (2005), the *E. staigeriana* essential oil yielded 1.2 to 1.5%. On contrast, we obtained a lower yield of essential oil, being 0.77% (mL $100g^{-1}$ of dried leaves). As reported by Figueiredo *et al.* (2008) climate, genotype, growth location, rainfall and harvesting regime all can affect the total essential oil content of plants. The oxygenated monoterpenes are the most abundant compounds of the *E. staigeriana* essential oil, representing 69.58% of the total amount. Monoterpenes possess many pesticidal activities, including insecticidal (Isman, 2000; Grodnitzky *et al.*, 2002), herbicidal (Duke *et al.*, 2000; Sing *et al.*, 2002), bactericidal (Cristani *et al.*, 2007; Cantore *et al.*, 2009) and fungicidal (Wuryatmo *et al.*, 2003; Cärdenas-Ortega *et al.*, 2005; Marei *et al.*, 2012) properties.

Table 2. Chemical composition of essential oil from *Eucalyptus staigeriana*.

Compounds	RI[a]	Peak area (%)[b]
Monoterpene hydrocarbons		**25.84**
α-Pinene	8.171	0.83
α-Phellandrene	16.268	0.28
Myrcene	16.494	0.47
Limonene	18.288	17.29
γ-Terpinene	20.901	0.62
cis-β-Ocimene	21.397	0.30
o-cimene	22.305	0.44
δ-Carene	22.944	5.61
Oxygenated monoterpenes		**69.58**
1.8-Cineole	18.743	6.16
Linalool	35.967	1.30
Terpinen-4-ol	38.362	0.85
Neral	41.837	19.68
Methyl Geraniate	42.226	3.78
Geranial	43.921	28.67
Geranyl Acetate	44.658	2.16
Citronellol	45.092	1.31
Nerol	46.462	1.72
Geraniol	48.243	3.77
Eugenol	57.636	0.18
Sesquiterpenes hydrocarbons		**0.26**
Caryophyllene	37.840	0.26
Oxygenated sesquioterpenes		**0.14**
Spathulenol	56.906	0.14
Total of identified compound		95.82

[a] RI, the retention index publyshed by Adams.
[b] Peak area obtained by GC-FID.

3.2. Antifungal activity of essential oil in postharvest grapes

The antifungal property of several essential oils on postharvest pathogens of fruits and vegetables under *in vitro* and *in vivo* conditions has been investigated (Zambonelli *et al.*, 1996; Feng and Zheng, 2007). In this study, different concentrations of *E. staigeriana* essential oil were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum*, both in preventive and curative treatment. In the preventive treatment of *B. cinerea*, all oil concentrations (50; 100 and 200 ppm) were able to reduce the incidence and severity when compared to control. The curative treatment proved to be more efficient and at concentration 100 ppm no incidence of disease was detected (Figure 1 A and

B and Figure S1 A and B). In the preventive treatment of *C. acutatum* concentration 100 and 200 ppm were able to inhibit the incidence of the disease, being different of control. Similarly to the test with *B. cinerea* the curative treatment of *C. acutatum* proved to be more efficient, concentrations 100 and 200 ppm significantly reduced disease incidence (Figure 2 A and B and Figure S2 A and B). The grape clusters treated with *E. staigeriana* essential oil did not show any obvious signs of phytotoxicity, just showed up brighter.

Essential oil inhibits postharvest pathogens mainly due to their direct effect on the mycelial growth of the pathogens and conidia germination by affecting the cellular metabolism of the pathogens (Serrano *et al.*, 2005; Tzortzakis, 2007a; 2007b; Regnier *et al.*, 2010). According to Tripathi *et al.* (2008) essential oils of *Ocimum sanctum, Prunus persica* and *Zingiber officinale* showed inhibitory effects on infection caused by *B. cinerea* in postharvest grapes fruits. According to Aminifard and Mohammadi (2013)**,** fennel essential oil inhibited *B. cinerea* growth on plum fruits compared with the control. Moreover, Bosquez-Molina *et al.* (2010) and Ali *et al.* (2015) proved that the essential oils of *Citrus aurantifolia, Thymus vulgaris* and *Cymbopogon citratus* have antifungal activity as postharvest treatments against *C. gloeosporioides* on papaya fruit.

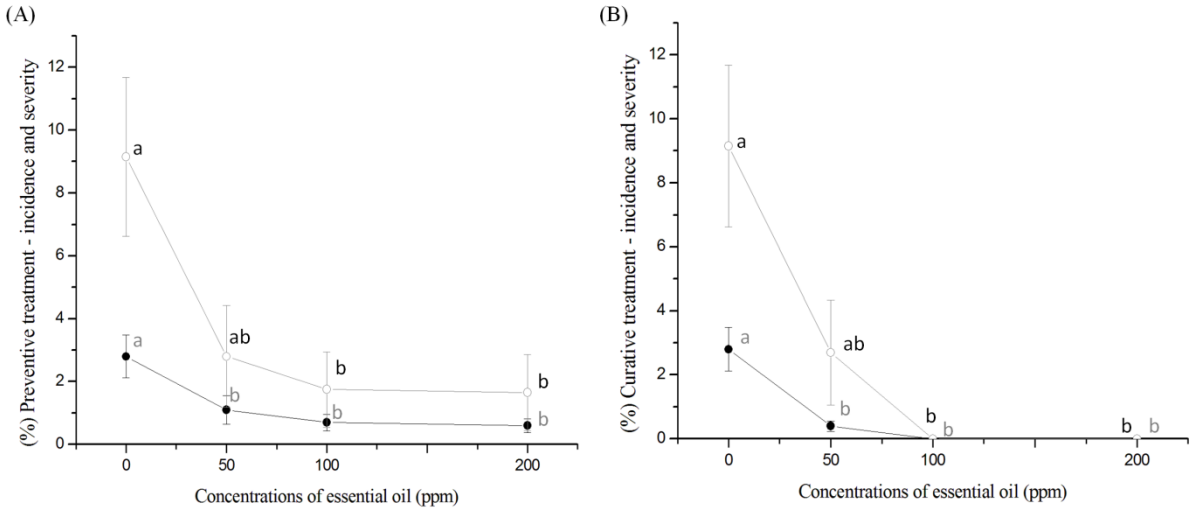

Figure 1. The effects of different concentrations of *Eucalyptus staigeriana* essential oil in grapes. Incidence (●) and severity (○) of disease caused by *Botrytis cinerea* as to preventive (A) and curative (B) treatment. Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Tukey's test ($p \leq 0.05$).

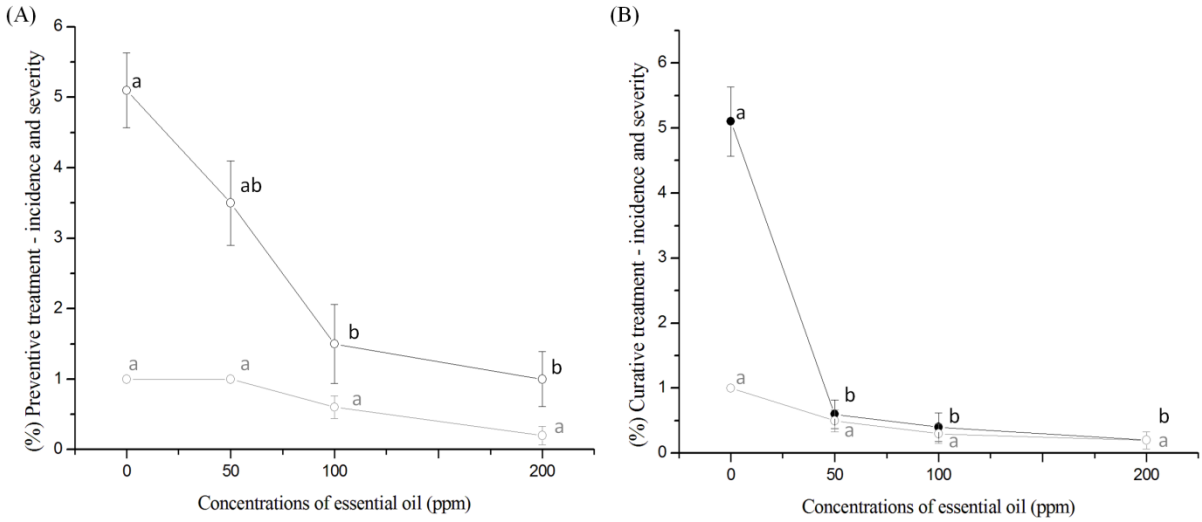

Figure 2. The effects of different concentrations of *Eucalyptus staigeriana* essential oil in grapes. Incidence (●) and severity (○) of disease caused by *Colletotrichum acutatum* as to preventive (A) and curative (B) treatment. Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Tukey's test ($p \leq 0.05$).

3.3. Antifungal activity of essential oil in vines *Vitis* spp. cv. "Isabela"

The widespread use of pesticides has significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food and threat to human health and environment. Public awareness of these risks has increased interest in finding safer alternatives protectant to replace currently used of synthetic chemical pesticides. One such alternative is the use of natural plant protectants with pesticidal activity such as essential oil, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Isman, 2000; Kalemba and Kunicka, 2003; Burt, 2004; Soylu *et al.*, 2010). Unlike to in *in vitro* studies, very few studies have been conducted *in vivo* conditions to show fungicidal properties of essential oils against plant pathogenic fungi (Letessier *et al.*, 2001; Oxenham *et al.*, 2005; Soylu *et al.*, 2007).

In the test in the vineyards, both concentrations tested of *E. staigeriana* essential oil were efficient, reducing the incidence and severity of disease caused by *B. cinerea* and *C. acutatum* (Table 3). There was no significant differences between the two essential oil concentrations used (100 and 500 ppm). The two essential oil concentrations showed a similar result to conventional treatment in the control of both diseases. The grape clusters treated with *E. staigeriana* essential oil did not show any obvious signs of phytotoxicity, just showed up brighter in the clusters. However, it was observed a phytotoxicicity in the most basal leaves of branches in, the vines treated with essential oil at concentration 500 ppm, which caused the fall of them. This may explain the difference between the two essential oil concentrations in the control of disease caused by *C. acutatum*, where the concentration 100 ppm proved to be more efficient than the concentration 500 ppm.

Table 3. *In vivo* evaluation of *Vitis* spp. cv. "Isabela" grapes with diferents treatments incluing *Eucalyptus staigeriana* essential oil.

	B. cinerea		*C. acutatum*	
Treatment	Incidence	Severity	Incidence	Severity
Control	3.80 ± 0.74 a	16.45 ± 4.51 a	26.10 ± 4.10 a	30.00 ± 3.33 a
Convencional treatment	1.25 ± 0.50 b	6.35 ± 3.34 ab	17.80 ± 4.50 ab	26.25 ± 4.35 a
Essential oil (100 ppm)	1.10 ± 0.46 b	2.80 ± 1.62 b	5.40 ± 1.16 bc	4.45 ± 1.76 b
Essential oil (500 ppm)	0.70 ± 0.33 b	1.55 ± 1.22 b	6.50 ± 1.48 b	10.30 ± 2.37 b

Values are the average of twelve replicates per treatment ± SE.
Letters indicate the comparison among the different treatments.
Means followed by same letter do not differ by Tukey's test ($p \leq 0.05$).

3.4. Chemical analyzes in the must and red table wine

Chemical analyses are performed to explain some sensory changes of wines and are fundamental to understand which compounds influence wine sensory properties and how they affect them (Thorngate, 1997; Colagrande *et al.*, 1988; Girard *et al.*, 2001). The analytical results for the must samples, showed that the average values of pH remained between 3.36 and 3.50 and the average values of º brix remained between 15.41 and 16.58 (Table 5). In red table wine, the average values of density of samples remained between 0.9955 and 0.9963. The pH average of all samples were within the optimal pH range for red wines (3.1 to 3.6). The total acidity and volatile acidity average were also kept within the values recommended as standard for this type of wine (55 to 130 meq.L^{-1} and 0.0 to 20 meq.L^{-1}, respectively). The amount of total reducing sugars presented values below 5 g.L^{-1}, that allowed to classify the wine as dry type. The alcoholic content remained slightly below the minimum required for this type of wine. All samples had values within the reference ranges described by International Organisation of Vine and Wine (2016), with the exception of alcoholic content that remained slightly below the minimum (8.5 % v/v) required for this type of wine. The analyzes showed that there was no significant difference between different treatments, showing that the essential oil had no influence on the chemical characteristics of red wine made from grapes treated with *E. staigeriana* essential oil.

Table 4. Chemical parameters evaluation of must and red wines samples of *Vitis* spp. cv. "Isabela" with diferents treatments incluing *Eucalyptus staigerina* essential oil.

Red table wine	Control	Convencional treatment	Essential oil (100 ppm)	Essential oil (500 ppm)
pH	3.19 ± 0.03 a	3.16 ± 0.01 a	3.23 ± 0.03 a	3.08 ± 0.05 a
TAC (meq L^{-1})	63.33 ± 1.20 a	66.67 ± 4.41 a	65.87 ± 2.19 a	65.00 ± 0.0 a
VAC (meq L^{-1})	3.33 ± 0.67 a	4.00 ± 0.58 a	3.00 ± 0.58 a	2.67 ± 0.33 a
DENS (g. L^{-1})	0.9960 ± 0.00025 a	0.9954 ± 0.00066 a	0.9963 ± 0.00042 a	0.9955 ± 0.00007 a
ALC (% v/v)	7.10 ± 0.15 a	7.60 ± 0.35 a	7.93 ± 0.26 a	7.93 ± 0.47 a
TRSG (g. L^{-1})	1.96 ± 0.20 a	2.09 ± 0.22 a	2.47 ± 0.10 a	2.22 ± 0.20 a
Must				
pH	3.36 ± 0.30 a	3.49 ± 0.07 a	3.50 ± 0.20 a	3.41 ± 0.21 a
° Brix	15.41 ± 0.37 a	16.26 ± 0.11 a	15.76 ± 0.45 a	16.58 ± 0.80 a

Legend: TAC: total acidity; VAC: volatile acidity; TRSG: total reducing sugars; ALC: alcoholic content; DENS: density. Values are the average of three replicates per treatment. Letters indicate the comparison among the different treatments. Means followed by same letter do not differ by Tukey's test ($p < 0.05$).

3.5. Volatile compounds analyzes in the red table wine

In all, 69 volatile compounds were identified in wine produced with the grapes from the four different treatments, including seventeen alcohols, twenty esters, seven acids, seventeen terpenes, five ketones, one acetaldehyde, one sulfur compound and one phenol compounds (Table 5). Many of these volatile compounds are commonly found in wines and are derived from grapes and yeast strain fermentation and the winemaking process (Cliff *et al.*, 2002).

Alcohols are formed from the degradation of amino acids, carbohydrates and lipids (Antonelli *at al.*, 1999; Jiang and Zhang, 2010) and represented the largest group in terms of the number and concentration of aroma compounds identified. The major alcohols found in all the samples were 3-methyl-1-butanoland, 1-pentanol phenylethyl alcohol and 2-methyl-1-butanol, the other varied in abundance for each sample, what would bring specific characteristics for each of them. The production of fatty acids has been reported to be dependent on the composition of the must and fermentation conditions (Schreirer, 1979). Octanoic acid was the compound found in all samples, the remaining varied with the sample. Esters are secondary

aromas, arising during fermentation and resulting from the combination of alcohols and acids (Clarke, 2003). These compounds are important components of wine aroma, due to their fruity and floral notes (Welke *et al.,* 2014). The most abundant esters found in all samples were octanoic acid ethyl ester and the hexanoic acid ethyl ester that are responsible for fruit and floral aromas. Ketones also are largely responsible for the characteristic flavor and aroma of some red wines (Ferreira *et al.,* 1993). The composition of ketones varied among the samples of wines and β-damascenone was found in all samples. The sulfur compound found in some samples was dihydro-2-methyl- 3(2H)-thiophenone, according Nicolli *et al.* (2015) this compound may negatively contribute to aroma (odor described as "burned", "burned rubber", or "roasted coffee"). Volatile phenols can be produced by a biochemical degradation of grape phenolic acids by yeast and bacteria (Etievant, 1991; Chatonnet *et al.,* 1992; Chatonnet *at al.,* 1993). The phenol compound found in some samples was 2,4-bis(1,1-dimethylethyl) phenol. The acetal found in all samples was 1-(1-ethoxyethoxy)-pentane and according to Perestrelo *et al.* (2011) acetals are formed during fermentation and their content increases significantly during the oxidative conditions of aging process.

Terpenes are found in grape skins and are transferred to wine during maceration, which is the first step of winemaking. These compounds generally remain unchanged after the fermentation process (Clarke, 2003; Zhang *et al.,* 2011). Two of the compounds identified in the wine produced from the grapes treated with *E. staigeriana* essential oil, that deserves to be highlighted are eucalyptol (1,8-cineole) and citral, as they are not typical compounds of wines (Rocha *et al.,* 2007; Kalua and Boss, 2010; Capone *et al*., 2012) The citral was not identified in wines produced from grapes of control and conventional treatment, however, eucalyptol was found in all samples. Eucalyptol has been identified in grapes and wines including Riesling, Cabernet Sauvignon (Kalua and Boss, 2010), Shiraz from Australia (Capone *et al*., 2012) and Fernão-Pires produced in Portugal (Rocha *et al.,* 2007). According to Saliba *et al.*

(2009) the mechanism by which eucalyptol occurs in finished wine is not well understood. Two mechanisms have been proposed: one proposes that 1.8- cineole is introduced via another source such as nearby *Eucalyptus* trees; a second proposition is that the compound develops from chemical precursors during the winemaking and bottle aging processes. For the first proposal, Capone *et al.* (2012) verified that the proximity of *Eucalyptus* trees to grapevines could directly influence concentration of 1.8-cineole present in the red wines, however it was not observed eucalyptus trees next to the vineyard. For the second explanations, Farina *et al.* (2005) have suggested that significant quantities of 1.8-cineole could be generated from limonene and α-terpineol and that could develop during the aging process. This is the most adequate proposal to explain the eucalyptol levels in wines produced from grapes of control and conventional treatment, though the wines produced from grapes treated with essential oil was observed a larger amount of this compound as well as citral. Citral compound is found in grapes and its concentration can vary according to the stage of maturation and cultivar (Bayonove and Cordonnier, 1971; Hellin *et al.*, 2010), but the presence of this compound has not been reported in wines. With these results we can conclude that, the essential oil used in the treatment of grapes leave residual compounds (1.8-cineole and citral), but that they do not negatively interfere, since 1.8-cineole may give minty characteristic to the wine (Capone *et al.*, 2012) and citral can provide citrus scents (Ahmed *et al.*, 1978).

Table 5. Volatile compounds identified in red table wine of *Vitis* spp. cv. "Isabela" with diferents treatments incluing *Eucalyptus staigeriana* essential oil.

Compounds	RI[a]	Peak area (%)[b]			
		Control	Convencional treatment	Essential oil (100 ppm)	Essential oil (500 ppm)
Internal standard					
3-octanol	16.134	10.17	10.55	10.92	9.10
Acids					
Butanic acid	8.823	–	–	0.23	–
Nonanoic acid	14.665	–	–	0.02	–
Hexanoic acid	16.333	–	0.11	–	0.15
Undecanoic acid	25.119	0.03	–	–	–
Octanoic acid	26.094	4.43	0.59	1.25	2.04
Heptanoic acid	33.594	–	–	0.01	0.05
Tetradecanoic acid	44.982	0.27	–	–	–
Alcohols					
1-Butanol	3.147	–	0.08	–	–
2-Methyl - 1- Butanol	4.331	6.71	4.33	4.72	6.76
3-Methyl-1-Butanol	4.054	40.59	10.30	22.49	42.16
1-Pentanol	4.265	20.17	21.05	14.51	22.71
2,3-Butanediol	7.059	0.05	0.08	0.1	0.15
3-Methyl-1-pentanol	8.424	0.05	0.10	–	–
4-Methyl-1-pentanol	8.015	–	0.01	–	–
1-Hexanol	9.068	0.79	0.64	0.15	–
1-Heptanol	14.594	0.07	0.09	–	0.02
1-Hexanol -2-ethyl	17.639	0.15	0.09	0.13	0.12
1-Octanol	19.840	0.14	0.11	0.11	0.10
Phenylethyl alcohol	22.128	11.10	6.94	3.54	6.67
1-Undecanol	28.807	0.05	–	–	–
2-Hexyl-1-octanol	45.960	0.03	–	–	–
2-Hexyl-1-decanol	46.708	0.02	–	–	–
1-Hexadecanol	47.428	1.11	–	–	–
1-Nonadecanol	51.138	1.75	–	–	–
Terpenes					
β-Mircene	15.570	–	–	0.07	–
Limonene	17.362	0.08	0.14	0.08	0.24
1,8-Cineole	17.445	0.16	0.06	0.86	1.06
Z-β-Ocimene	18.633	–	–	0.08	0.02
Linalool	21.154	0.74	0.45	1.16	0.72
Nerol oxide	23.812	0.07	–	0.05	0.06
Terpinen-4-ol	24.778	0.21	0.38	1.03	0.53
α-Terpineol	25.452	1.16	1.09	1.60	1.58
Nerol	27.332	–	–	0.40	1.19
Citronellol	27.419	0.52	0.28	5.16	2.55
Citral	27.875	–	–	0.54	0.04
Geraniol	28..613	–	–	3.29	1.19
Geranic acid	31.951	0.19	–	2.67	1.77
Nerol acetate	33.477	–	–	0.49	0.46
Geraniol acetate	34.271	–	–	0.61	0.20
Geranil acetone	36.643	0.05	–	0.07	–
Farnesol	39.021	0.02	–	–	0.03

Ketones					
3-Octanone	15.338	0.12	0.15	0.07	–
5-Methyl-3-heptanone	15.359	0.08	–	0.03	–
2-Undecanone	30.345	0.02	–	–	–
2-Tridecanone	30.365	–	–	0.05	0.02
β-damascenone	34.154	0.33	0.05	0.05	0.28
Esters					
Propanoic acid ethyl ester	3.716	0.29	0.38	0.28	0.29
Propanoic acid 2-methyl ethyl ester	4.835	0.13	0.12	0.04	0.08
Butanoic acid ethyl ester	6.442	0.31	0.42	0.32	0.30
2-Butenoic acid ethyl ester	8.510	0.03	0.55	0.62	0.53
3-Methyl 1-butanol acetate	9.882	0.49	0.42	0.21	0.16
2-Methyl 1-butanol acetate	9.938	0.05	0.08	0.04	0.20
Hexanoic acid ethyl ester	16.220	2.21	2.19	2.08	2.06
Ethyl-2-hexenoate	18.415	0.09	0.06	0.08	0.11
Butanedioic acid diethyl ester	25.290	0.45	0.15	0.17	0.47
Octanoic acid ethyl ester	26.042	6.39	7.76	7.35	10.69
Acetic acid 2-phenylethyl ester	28.591	0.33	0.30	0.09	–
Nonanoic acid ethyl ester	30.554	0.1	0.10	0.09	–
Ethil-9-decenoate	34.457	0.32	0.22	1.14	0.15
Decanoic acid ethyl esther	35.180	1.33	1.34	2.56	4.52
Butanoic acid 3-methylethyl ester	36.558	0.04	–	0.02	–
Octanoic acid 3-methylbutyl ester	36.556	0.01	–	0.02	0.03
2-Propenoic acid 3-phenylethyl ester	37.138	0.16	–	0.13	–
2,4-Decadienoic acid ethyl ester E,Z)	37.369	0.22	–	0.28	0.05
Dodecanoic acid ethyl ester	40.930	1.16	0.12	0.54	0.40
Hexadecanoic acid ethyl ester	49.795	0.08	–	0.07	–
Hexanoic acid bis (2-ethylhexil) ester	54.807	2.06	0.15	–	–
Acetal					
1-(1-ethoxyethoxy)- pentane	14.831	0.34	0.22	0.12	0.21
Sulfur compound					
Dhydro-2-methyl-3(2H)-thiophenone	15.108	0.07	0.04	–	0.03
Phenol					
2,4-bis(1,1-dimethylethyl) phenol	38.638	0.04	–	0.04	–

[a] RI, the retention index publyshed by Adams.
[b] Peak area obtained by GC-FID.
The data were mean values of triplicate samples.

4. Conclusion

Considering the results on the inhibition of *B. cinerea* and *C. acutatum* development in postharvest grapes and in vines and results of winemaking, we can conclude that *E.*

staigeriana essential oil could be used as a possible biofungicide. However, additional studies are required before this essential oil can be recommended as commercial and natural antifungal agent in treatment of wine grapes.

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SUPLEMMENTARY MATERIAL

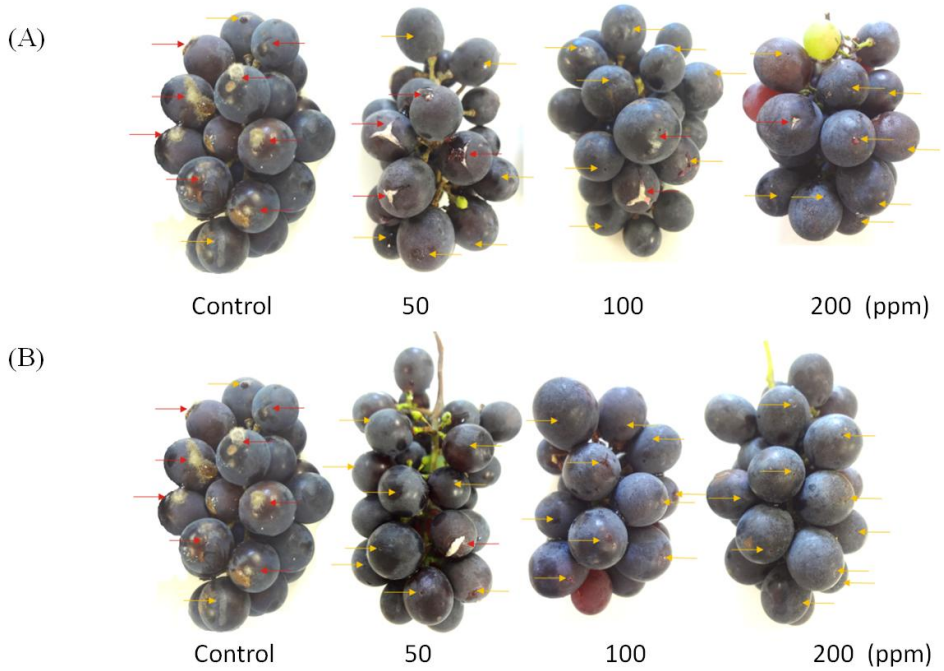

Figure S1: The effects of different concentrations of *Eucalyptus staigeriana* essential oil in grapes. Incidence of disease caused by *Botrytis cinerea* as to (A) preventive and (B) curative treatment.
The arrows indicate the site of injury.
→ berries with symptoms of the disease (incidence)
→ berries without disease symptoms

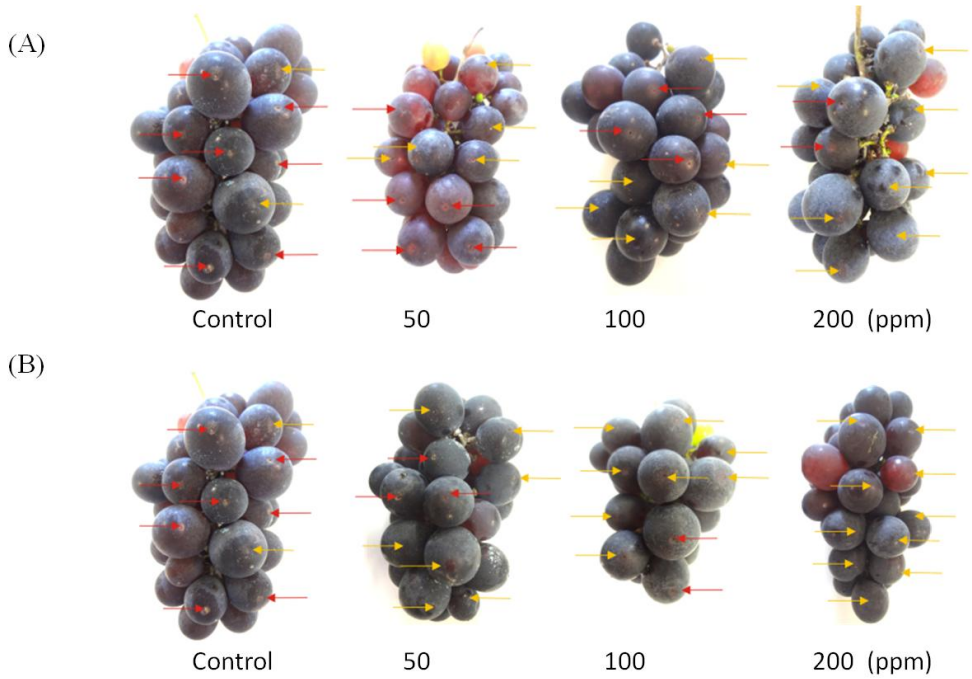

Figure S2: The effects of different concentrations of *Eucalyptus staigeriana* essential oil in grapes. Incidence of disease caused by *Colletotrichum acutatum* as to (A) preventive and (B) curative treatment.
The arrows indicate the site of injury.
→ berries with symptoms of the disease (incidence)
→ berries without disease symptoms

5. DISCUSSÃO GERAL

Objetivando avaliar a atividade antifúngica dos óleos essenciais de *Eucalyptus staigeriana, Eucalyptus globulus, Foeniculum vulgare, Baccharis trimera* e *Baccharis dracunculifolia* sobre os fitopatógenos *Botrytis cinerea* e *Colletotrichum acutatum* em videiras, inicialmente realizou-se a identificação dos compostos desses óleos essenciais. Os compostos majoritários identificados no óleo essencial de *E. staigeriana* foram geranial (28.67%) e neral (19.68%), que misturados formam o monoterpeno citral. Estes compostos também foram encontrados em estudos realizados por Macedo *et al.* (2010) e Chagas *et al.* (2002). O composto majoritário identificado no óleo essencial de *E. globulus* foi 1,8-cineol (64.63%), similarmente Sacchetti *et al.* (2005), Chagas *et al.* (2002) e Mekonnen *et al*. (2016) também encontraram este composto em suas análises. No óleo essencial de *F. vulgare*, os compostos majoritários identificados foram trans-anetol (79.14%), funchone (11.94%) e estragol (5.76%), resultados similares foram reportados por Telci *et al.* (2009), Viuda-Martos *et al.* (2011), Kazemi *et al.* (2012), Roby *et al.* (2013), Diao *et al.* (2014) e Mota *et al.* (2015 a). O óleo essencial de *B. trimera* possui como composto majoritário o carquejol acetato (76.53%) que também foi identificado nas análises de Simões-Pires *et al.* (2005) e Besten *et al.* (2013). No óleo essencial de *B. dracunculifolia* foram identificados os compostos β-pineno (19.73%), limoneno (16.01%) e espatulenol (9.99%) como compostos majoritários, Frizzo *et al.* (2008), Massignani *et al*. (2009) e Parreira *et al.* (2010) também encontraram estes compostos em suas análises, mas não como majoritários.

Diversos trabalhos têm sido conduzidos na utilização destes óleos essenciais como produtos alternativos no controle de fitopatógenos em condições *in vitro*. Gilles *et al.* (2010) demonstraram que o óleo essencial de *E. staigeriana* possui ação fungicida sobre *Candida albicans.* Similarmente, neste trabalho o óleo essencial de *E. staigeriana* apresentou ação fungicida sobre o crescimento micelial (fase de contato) e germinação de conídios de *B.*

cinerea e *C. acutatum.* Os compostos voláteis deste óleo essencial, também apresentaram ação fungicida sobre o crecimento micelial de *B. cinerea* e ação fungistática sobre o crescimento micelial de *C. acutatum.*

Também verificou-se a ação fungicida do óleo essencial de *E. globulus* sobre o crescimento micelial (fase de contato) e germinação de conídios de *B. cinerea* e *C. acutatum.* Este efeito fungicida também foi reportado previamente na literatura por Mota *et al.* (2015 b) que demonstraram que o óleo essencial de *E. globulus* possui ação fungicida sobre *Candida albicans*. Vilela *et al.* (2009) testaram a atividade antimicrobiana deste óleo essencial contra duas espécies de *Aspergillus* e verificou a completa inibição do crescimento micelial de ambos os fungos e Bansod & Rai (2008) demonstraram que o óleo essencial possui ação fungistática contra *Aspergillus fumigatus* e *Aspergillus niger*. Os compostos voláteis deste óleo essencial apresentaram ação fungitática sobre o crescimento micelial de *B. cinerea* e *C. acutatum.* Hua *et al.* (2014) relataram que os compostos voláteis do óleo essencial de *E. globulus* apresentaram uma leve inibição sobre o crescimento micelial de *Aspergillus ochraceus*.

O óleo essencial de *F. vulgare* tem sido descrito por reduzir o crescimento micelial de *Sclerotinia sclerotiorum* (Soylu *et al.,* 2007) e de *Aspergillum niger, Aspergillum flavus, Fusarium graminearum* and *Fusarium moniliforme* (Singh *et al*., 2006). Neste trabalho, a ação fungicida deste óleo essencial foi confirmada contra *B. cinerea* e *C. acutatum* nos testes de crescimento micelial (fase de contato) e germinação de conídios. Os compostos voláteis deste óleo essencial, também apresentaram ação fungicida sobre o crecimento micelial de *B. cinerea* e ação fungistática sobre o crescimento micelial de *C. acutatum.*

Oliveira *et al.* (2015) avaliaram a atividade antifúngica do óleo essencial de *B. dracunculifolia* contra *Candida albicans* e demonstraram que este apresenta uma ação antifúngica em altas

concentrações. Duarte *et al.* (2005) também testaram o óleo essencial contra *C. albicans* e demonstraram que este possui uma ação fungicida muito baixa. Similarmente neste estudo, o óleo essencial de *B. dracunculifolia* apresentou ação fungistática em altas concentrações sobre o crescimento micelial (fase de contato) de *B. cinerea* e *C. acutatum.* Os compostos voláteis deste óleo essencial puro, apresentaram ação fungistática somente sobre o crecimento micelial de *B. cinerea* . O óleo essencial de *B. dracunculifolia* reduziu significativamente a germinação de conídios de *B. cinerea* em todas as concentrações testadas mas não foi capaz de reduzir a germinação de conídios de *C. acutatum.*

A ação fungicida do óleo essencial de *B. trimera* foi confirmada sobre *Trichophyton rubrum* e *Microsporum canise* por Caneschi *et al*. (2015), corroborando com os resultados obtidos neste estudo onde o óleo essencial apresentou ação fungicida sobre o crescimento micelial (fase de contato) de *B. cinerea* e ação fungistática sobre o crescimento micelial (fase de contato) de *C. acutatum.* Sua ação fungicida também foi confirmada sobre a germinação de conídios de *B. cinerea* e *C. acutatum.* Seus compostos voláteis apresentaram ação fungistática sobre o crescimento micelial de ambos os fungos.

Desta maneira, os óleos essenciais das plantas estudadas podem ser uma alternativa para o controle das doenças causadas por *B. cinerea* e *C. acutatum* em videiras. Fung *et al*. (1977) e Tian *et al.* (2012) sugerem que o efeito dos óleos essenciais sobre o crescimento micelial microbiano pode ser o resultado de compostos fenólicos e terpenóides presentes nos óleos essenciais que alteram a permeabilidade da célula microbiana, isto causa a deformação da estrutura da célula, alteração da funcionalidade celular e ocasiona a perda de macromoléculas a partir de seu interior, causando a inibição do crescimento micelial (Sharma & Tripathi, 2006; Helal *et al*. 2007; Silva *et al.*, 2009; Pramila *et al.*, 2012), além disso, os compostos fenólicos também afetam as enzimas responsáveis pela germinação de conídios e interfere na produção de aminoácidos que são necessários nos processos de germinação (Nychas, 1995).

Neste trabalho, ambos fungos cultivados em meios com óleos essenciais (fase de contato e volátil) apresentaram alterações na morfologia. Tais modificações podem ser relacionados com o efeito do óleo essencial sobre a regulação das reações da síntese da parede celular (Rasooli *et al*., 2006).

Os óleos essenciais de *E. staigeriana, F. vulgare* e *B. trimera* foram escolhidos para os ensaios *in vivo* no controle de doenças no pós-colheita de uvas *Vitis* spp. var. "Isabel", pois dentre os três gêneros testados, foram as espécies que demonstraram maior eficiência no controle *in vitro* sobre o crescimento micelial e germinação de conídios de *B. cinerea* e *C. acutatum.* Todos os três óleos essenciais testados foram capazes de reduzir a incidência e severidade da doença causada por *B. cinerea* e a da incidência da doença *C. acutatum*, tanto no tratamento preventivo quanto no curativo, sendo que neste segundo, ambos apresentaram maior eficiência. O óleo essencial inibe patógenos pós-colheita, principalmente devido ao seu efeito direto sobre o crescimento micelial e germinação de conídios, pois afeta o metabolismo celular do patógeno (Serrano *et al*., 2005; Tzortzakis, 2007a; 2007b; Regnier *et al.*, 2010). Vários estudos com óleos essenciais tem apresentado eficácia no controle de diferentes fungos em diferentes frutos no pós-colheita. Segundo Abdolari *et al.* (2010), o óleo essencial de *F. vulgare* possui ação fungicida sobre os fitopatógenos *Alternaria alternata* e *Penicillium digitatum* no pós-colheita de tomates. Aminifard & Mohammadi (2013) confirmaram que este óleo essencial também possui ação fungicida sobre *B. cinerea* em ameixas. Lopez-Reyes *et al.* (2010) provaram que o óleo essencial de *F. vulgare* tem atividade antifúngica nos tratamentos sobre *B. cinerea* e *Penicillium expansum* no pós-colheita de maçãs. Conforme Tripathi *et al.* (2008), os óleos essenciais de *Ocimum sanctum, Prunus persica* e *Zingiber officinale* inibiram os sintomas das doenças causadas por *B. cinerea* no pós-colheita de uvas. Bosquez-Molina *et al.* (2010) e Ali *et al.* (2015) provaram que os óleos essenciais de *Citrus aurantifolia, Thymus*

vulgaris e *Cymbopogon citratus* possuem atividade antifúngica no tratamento pós-colheita de mamão contra *Colletotrichum gloeosporioides.*

Estes resultados evidenciam a ação fungicida dos óleos essências de *E. staigeriana*, *F. vulgare* e *B. trimera* contra *B. cinerea* e *C. acutatum*, e seu potencial uso como fungicida biológico no controle pós-colheita das doenças causadas por estes fungos em uva.

O óleo essencial de *E. staigeriana* foi utilizado para os ensaios *in vivo* no controle de *B. cinerea* e *C. acutatum* em videiras, pois dentre todos os óleos essenciais testados, foi o que demonstrou maior eficiência no controle *in vitro* e *in vivo* no testes pós-colheita. Os tratamentos realizados com as duas concentrações do óleo essencial de *E. staigeriana* reduziram a incidência e a severidade das doenças causadas por *B. cinerea* e *C. acutatum*, tanto em *Vitis* spp. var. "Isabel" quanto em *V. vinifera* var. "Tannat". Similarmente, em condições *in vivo*, Soylu *et al*. (2010) demonstraram que o óleo essencial de *Origanum syriacum L. var. bevanii* reduziu a severidade da doença causada por *B. cinerea* em tomateiros e Al-Reza *et al*. (2010) também demonstraram que o óleo essencial de *Cestrum nocturnum* reduziu os sintomas da doença causada por *Phytophthora capsici* em plantas de pimenta.

Com as uvas de ambas as variedades e dos diferentes tratamentos foram realizadas microvinificações. Análises químicas do mosto e dos vinhos demonstram que não houve diferença significativa entre os diferentes tratamentos. Todas as amostras de vinho apresentaram valores dentro dos intervalos de referência descritos pela Organização Internacional da Vinha e do Vinho (2016) com exceção do teor alcoólico, que permaneceu ligeiramente abaixo do mínimo necessário para estes tipos de vinho.

Nas análises dos compostos voláteis dos vinhos foram identificados diferentes compostos como alcoois, esteres, ácidos, cetonas e terpenos. Um dos compostos positivamente identificado em pequenas concentrações nos vinhos produzidos com as uvas de *V. vinifera*

var. “Tannat” tratadas com o óleo essencial de *E. staigeriana*, e que merece ser destacado é eucaliptol (1,8-cineol), pois não é um composto típico de vinhos (Kalua & Boss 2010; Capone *et al.*, 2012; Rocha *et al*., 2007) e que não foi identificado nos vinhos produzidos com as uvas do controle e do tratamento convencional. Nos vinhos produzidos com as uvas de *Vitis* spp. var. “Isabel” foi identificado o composto eucaliptol (1,8-cineol) em todas as amostras analizadas, além disso, também foi identificado nas amostras de vinho produzidas com as uvas provenientes dos tratamentos com óleo essencial o composto citral. O eucaliptol também foi identificado em uvas e vinhos das variedades Riesling, Cabernet Sauvignon (Kalua & Boss, 2010) e Shiraz da Austrália (Capone *et al*., 2012) e Fernão-Pires produzida em Portugal (Rocha *et al*., 2007). Segundo Saliba *et al.* (2009) os mecanismos pelo qual o eucaliptol ocorre no vinho ainda não são bem compreendidos, mas dois mecanismos têm sido propostos: o primeiro é que o eucaliptol é introduzido através de uma fonte externa, o segundo é de que o composto é derivado de precursores químicos durante o processo de vinificação e envelhecimento. Na primeira proposta, Capone *et al.* (2012) verificaram que árvores de eucalipto próximas ao vinhedo podem sim influenciar diretamente na concentração de eucaliptol presente no vinho. No entanto, não foram observadas árvores de eucalipto próximas ao vinhedo onde foram desenvolvidos os testes *in vivo*. Para a segunda proposta, Farina *et al.* (2005) verificou que quantidades significativas de eucaliptol podem ser gerados a partir do limoneno e α-terpineol durante o processo de fermentação e envelhecimento. Isto pode explicar a presença do eucaliptol nas amostras do controle e tratamento convencional dos vinhos de *Vitis* spp. var. “Isabel”. O composto citral é encontrado na uva e sua concentração pode variar de acordo com o estágio de maturação e a cultivar (Bayonove & Cordonnier, 1971; Hellin *et al.*, 2010) porém, não foi reportada a presença deste composto em vinhos. Com estes resultados, podemos concluir que, o óleo essencial utilizado no tratamento de videiras deixou um composto residual (1,8-cineol), que foi identificado no vinho, mas que não

interfere de forma negativa, uma vez que este composto pode fornecer característica de menta para o vinho (Capone *et al.*, 2012) e o composto citral, que pode fornecer aromas citricos (Ahmed *et al.*, 1978).

Os óleos essenciais avaliados neste trabalho (*E. staigeriana*, *E. globulus*, *F. vulgare*, *B. trimera* e *B. dracunculifolia*) apresentam potencial para serem utilizados como agentes de biocontrole contra os fitopatógenos *B. cinerea* e *C. acuratum*, causadores da podridão cinzenta e podridão da uva madura, respectivamente. Os óleos essenciais são bem conhecidos por suas propriedades antimicrobianas e biodegradáveis e por não deixar qualquer efeito residual em produtos frescos (Isman, 2000; Kalemba & Kunicka, 2003; Burt, 2004). Além disso, a maioria dos óleos essenciais são classificados pela FDA (Food and Drug Administration) como GRAS (Geralmente Reconhecido como Seguro), reconhecidos como seguros para utilização em alimentos, de modo que tem havido um interesse crescente na sua utilização no tratamento de frutas e produtos hortícolas (Gonzales-Aguilar *et al.*, 2008).

Os resultados *in vivo* deste trabalho corfimam e indicam que a utilização desses óleos essenciais pode ser uma alternativa no controle de *B. cinerea* e *C. acutatum* em videiras, além de contribuir para a redução da aplicação de fungicidas e, consequentemente, minimizar seus riscos e perigos ao meio ambiente e a saúde humana, especialmente na produção de frutas para consumo *in natura* e para processamento.

6. CONCLUSÕES

A partir dos resultados obtidos no presente estudo foi possível concluir que:

- Em condições *in vitro,* os óleos essenciais de *E. staigeriana, E. globulus, F. vulgare e B. trimera* adicionados ao meio de cultura, apresentaram ação fungicida sobre o crescimento micelial de *B. cinerea,* enquanto que o óleo essencial de *B. dracunculifolia* apresentou ação fungistática.

- Em condições *in vitro,* os óleos essenciais de *E. staigeriana, E. globulus e F. vulgare* adicionados ao meio de cultura, apresentaram ação fungicida sobre o crescimento micelial de *C. acutatum,* enquanto que os óleos essenciais de *B. trimera e B. dracunculifolia* apresentaram ação fungistática.

- Os compostos voláteis dos óleos essenciais de *E. staigeriana* e *F. vulgare* em altas concentrações apresentaram ação fungicida sobre o crescimento micelial de *B. cinerea* e *C. acutatum,* enquanto que as menores concentrações apresentaram ação fungistática.

- Os compostos voláteis dos óleos essenciais de *E. globulus* e *B. trimera* apresentaram ação fungistática sobre o crescimento micelial de *B. cinerea* e *C. acutatum,* e o óleo essencial de *B. dracunculifolia* apresentou ação fungistática em altas concentrações sobre o crescimento micelial de *B. cinerea.*

- Os óleos essenciais de *E. staigeriana, E. globulus, F. vulgare e B. trimera* inibiram a germinação de conídios de *B. cinerea e C. acutatum* em condições *in vitro*.

- Os óleos essenciais de *E. staigeriana, F. vulgare e B. trimera* foram eficazes no controle da podridão cinzenta e da podridão da uva madura no pós-colheita em uvas da variedade *Vitis* spp. var. “Isabel”, diminuindo a incidência e severidades das doenças em condições *in vivo.*

- O óleo essencial de *E. staigeriana* foi capaz de controlar a incidência e severidade das doenças causadas por *B. cinerea* e *C. acutatum* em videiras das variedades *Vitis* spp. var. "Isabel" e *V. vinifera* var. "Tannat".

- Nas análises químicas dos vinhos produzidos a partir das uvas (*V. vinifera* e *Vitis* spp.*)* tratadas com o óleo essencial de *E. staigeriana* não houve diferenças significativas em comparação com o controle, demonstrando que o óleo essencial não interfere nas características do vinho.

- Na análise dos compostos voláteis dos vinhos, foi identificado o composto 1,8-cineol (eucaliptol) no vinho produzido a partir das uvas de *V. vinifera* var. "Tannat" que foram tratadas com óleo essencial de *E. staigeriana* e os compostos 1,8-cineol (eucaliptol) e citral no vinho produzido a partir das uvas de *Vitis* spp. var. "Isabel" tratadas com o óleo essencial de *E. staigeriana*.

7. PERSPECTIVAS

Para a continuidade deste trabalho, seria importante:

- Avaliar a ação fungicida do óleo essencial de *E. staigeriana* sobre o crescimento micelial e germinação de conídios de outros fitopatógenos da videira;

- Avaliar *in vitro* a ação fungicida de extratos de folhas de *E. staigeriana,* bem como frações do óleo essencial;

- Avaliar *in vivo,* no pós-colheita, outras variedades de uva de mesa (*Vitis* spp, *V. labrusca* e *V. vinifera)*;

- Desenvolver e testar *in vivo* diferentes biosulfactantes;

- Avaliar *in vivo* a ação fungicida do óleo essencial de *E. staigeriana* sobre outros fitopatógenos da videira;

- Avaliar a persistência dos óleos essenciais ao longo do tempo para determinar o tempo de proteção;

- Avaliar o controle de *B. cinerea* e *C. acutatum* com o óleo essencial de *E. staigeriana* em uvas de cultivo protegido;

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