FABIANO JOSÉ PERINA

ÓLEOS ESSENCIAIS E FRAÇÕES MAJORITÁRIAS ATIVAS NO CONTROLE DA MANCHA MARROM DE ALTERNARIA (*Alternaria alternata*) EM TANGERINA PONKAN

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/ Fitopatologia, área de concentração em Controle de Doenças e Microscopia Eletrônica, para a obtenção do título de Doutor.

Orientador

Dr. Eduardo Alves

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RESUMO GERAL

Este trabalho objetivou investigar e compreender a natureza da atividade de produtos à base de óleo essencial (OE) para o controle da mancha marrom de alternaria (MMA), causada por *Alternaria alternata*. Foram avaliados os OEs de tomilho (*Thymusvulgaris* L.) - TEO e de canela (*Cinnamomun zeylanicum* L) - CEO e suas respectivas frações majoritárias ativas. Para o TEO foi avaliada a capacidade em inibir o crescimento e a viabilidade de *A. alternata*; em interferir nos eventos iniciais de penetração do patógeno por meio de microscopia eletrônica de varredura e consequências no progresso da doença em frutos; e, por meio da microscopia eletrônica transmissão, a atividade celular e subcelular do TEO e sua fração ativa thymol. Já para o CEO foi avaliada a capacidade em inibir o crescimento do patógeno; em reduzir o progresso da MMA em campo; e, finalmente, a capacidade do CEO e sua fração *trans*-cinnamaldeido (CNE) em induzir a resistência das plantas contra *A. alternata*. A atividade antifúngica do TEO foi atribuída principalmente à substância thymol, que demonstrou alta percentagem de controle da doença em frutos, inibição do crescimento micelial e viabilidade de conídios de *A. alternata*. A aplicação direta de thymol ocasionou o atraso do processo de infecção e impediu a penetração, já a aplicação direta do TEO causou apenas um atraso no processo infeccioso. A análise ultraestrutural demonstrou que a substância thymol ocasiona a ruptura da parede celular e membrana plasmática, desorganização do citoplasma da célula conidial, com possível alvo a organelas específicas no citoplasma. A atividade antifúngica direta de CEO foi atribuída às substâncias eugenol e CNE. Essa demonstrou alta atividade antifúngica superando CEO, que por sua vez, reduziu o progresso da doença à campo. A aplicação preventiva de CEO e da substância CNE induziu o sistema de defesa da planta contra o ataque do patógeno.

Palavras-chave: Indução de resistência. Citros. Modo de ação. Microscopia eletrônica.

GENERAL ABSTRAC

This work aimed at investigating and comprehending the activity of essential oil (EO) based products for the control of Alternaria brown spot (ABS) caused by *Alternaria alternata.* The EOs of thyme (*Thymus vulgaris* L.) – TEO and cinnamon (*Cinnamonun zeylanicum* L.) – CEO and their respective major active substances were evaluated. For the TEO, it were evaluated: the ability to inhibit the growth and viability of *A. alternata*; to affect the early events of pathogen penetration by means of scanning electron microscopy and the consequences of the progress of the disease in fruits, and; the cellular and subcellular activity of the TEO and its active thymol fraction by means of transmission electron microscopy. For the CEO, the ability to inhibit pathogen growth; to reduce the progress of the ABS in the field; and, finally, the capacity of the CEO and its trans-cinnamaldehyde (CNE) fraction in inducing plant resistance against *A. alternaria*, were evaluated. The antifungal activity of the TEO was attributed mainly to the thymol substance, which demonstrated a high percentage of disease control in fruits, inhibition of the mycelial growth and viability of *A. alternata* conidia. The direct application of thymol led to a delay in the infection process and prevented penetration, while the direct application of the TEO only caused a delay in the infectious process. The ultrastructural analysis demonstrated that the thymol substance causes the rupture of cellular wall and plasma membrane, disorganization of the cytoplasm of the conidia cell, with possible targeting of specific organelles in the cytoplasm. The direct antifungal activity of the CEO was attributed to eugenol and CNE substances. The latter showed high antifungal activity, overcoming the CEO, which, in turn, reduced the progress of the disease in field. The preventive application of CEO and CNE substance induced the plant's defense system against the pathogen attack.

Keywords: Induction of resistance. Citrus. Mode of action. Electron microscopy.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Um dos grandes problemas enfrentados pelos produtores de tangerina brasileiros diz respeito às doenças de origem fúngica. Para controlá-las, faz-se necessário o emprego de alta carga de fungicidas que, além de aumentar os custos de produção e, por vezes, impossibilitar a comercialização do fruto no mercado externo, contaminam o ambiente com substâncias de elevada toxicidade. Especificamente no Estado de Minas Gerais, destaca-se o fungo *Alternaria alternata* patótipo tangerina, agente etiológico da mancha marrom de alternaria, o qual representa um sério problema para citricultores em várias regiões no Brasil e em outros países (TIMMER et al., 2003; PEEVER et al., 2004; PRATES, 2007; VICENT et al., 2000). Uma das possíveis alternativas para superar tal problema diz respeito ao emprego de produtos de origem vegetal, já que as plantas são capazes de produzir substâncias com atividade antifúngica. Em vista disso, foi avaliada a eficácia de óleos essenciais que apresentaram atividade antifúngica direta e indireta, na indução de resistência comprovada em pesquisas anteriores (PEREIRA et al., 2011) bem como suas frações ativas isoladas. Visando ao desenvolvimento de um novo método de controle de *A. alternaria* em citros, realizou-se um projeto de ampla abrangência incluindo testes de campo para quantificar a capacidade de produtos naturais à base de óleos essenciais e suas frações em controlar a doença, elucidar a natureza bioquímica e estrutural da resposta de defesa de plantas de citros tratadas ou não com esses produtos contra *Alternaria alternata* patótipo tangerina.

2 REFERENCIAL TEÓRICO

2.1 A importância da citricultura nacional

O Brasil é o maior produtor de laranja (FOOD AND AGRICULTURE ORGANIZATION OF UNITED NATIONS, 2014) e exportador de suco, possuindo cerca de 165 milhões de árvores produtivas, contra cerca de 60 milhões de árvores existentes na região produtora da Flórida dos Estados Unidos (NEVES et. al, 2011), segundo maior produtor de laranja (FOOD AND AGRICULTURE ORGANIZATION OF UNITED NATIONS, 2014). Com isso, a citricultura brasileira é líder mundial e tem-se destacado por alavancar o crescimento socioeconômico, contribuindo com a elevação da balança comercial e, principalmente, com a geração de empregos diretos e indiretos na área rural. Dados do último levantamento detalhado demonstram que o PIB do setor citrícola foi de US$ 6,5 bilhões em 2009, sendo US$ 4,39 bilhões no mercado interno e US$ 2,15 bilhões no mercado externo. Contudo, a cadeia citrícola arrecada US$ 189 milhões em impostos para o Brasil (NEVES et al., 2011).

O cultivo nacional de tangerina e seus híbridos cresceram, sendo o Brasil o terceiro maior produtor mundial (FOOD AND AGRICULTURE ORGANIZATION OF UNITED NATIONS, 2014). Dessa forma, a produção de tangerinas e seus híbridos no Brasil ocupam o segundo lugar em importância econômica entre as espécies cítricas e entre as tangerinas, a variedade 'Ponkan' (*Citrus reticulata* Blanco) é a de maior importância, uma vez que corresponde a 60% das variedades cultivadas (NEVES et al., 2011). A produção brasileira de tangerinas da última safra contabilizada no país foi da ordem de 1.270.108 toneladas, as quais foram produzidas, principalmente, na região Sudeste, seguida pela região Sul (ANUÁRIO DA AGRICULTURA BRASILEIRA, 2010).

Dentre os estados que se destacam na produção citrícola, Minas Gerais ocupa o quarto lugar entre os maiores produtores do país. Este fato, aliado ao seu tamanho em área e a variedade agroclimática, possibilita uma citricultura diversificada e, de certo modo, regionalizada, com produção de ótimas frutas frescas. Tais características propiciaram o desenvolvimento da citricultura empresarial em pólos como o Triângulo Mineiro, e a produção voltada para de frutas frescas como é o caso das regiões Sul de Minas, Norte de Minas e Vale Médio Paraopeba, as quais se destacam no cultivo de tangerina e seus híbridos (ANUÁRIO DA AGRICULTURA BRASILEIRA, 2010).

Apesar da constante evolução desse setor, a citricultura é seriamente afetada pela incidência de pragas e doenças. Os últimos levantamentos ressaltam que a incidência de pragas e doenças foi responsável pela erradicação de 40 milhões de árvores nesta década, onde a mortalidade de árvores saltou de 4% para preocupantes 7,5% (NEVES et al., 2011). Assim, as doenças contribuem, em grande escala, para que a produção de frutas cítricas de boa qualidade seja um enorme desafio para o agricultor brasileiro, de maneira especial para àqueles que se localizam em áreas em que a ocorrência de certas doenças é favorecida por fatores climáticos.

2.2 A Mancha marrom de alternaria (MMA)

Dentre as doenças que afetam a atividade da produção de tangerinas no Brasil, destaca-se a mancha marrom de alternaria (MMA), que tem como agente etiológico o fungo *Alternaria alternata* patótipo tangerina (Fr: Fr) Keissl (PEEVER et al., 2004), sendo considerada a principal doença das tangerinas e seus híbridos (SPÓSITO, 2007). Esta enfermidade vem causando preocupação desde 2001, quando foi relatada pela primeira vez no Brasil, no Estado do Rio de Janeiro, ocasionando severos danos provocados à produção de tangerinas e de

seus híbridos (GOES; MONTESDE OCA; REIS, 2001). O aparecimento dessa doença nos pomares tem elevado, significativamente, os custos de produção, em função do manejo adotado na tentativa de conter a devastação provocada pela doença (SPÓSITO et al., 2003).

No Brasil, até meados de 2006, a mancha marrom de alternária havia sido constatada causando danos severos aos cultivos comerciais de tangerina variedade Dancy, Tangor e Murcott, com maior severidade e, em plantios comerciais de tangerina variedade Ponkan com menor severidade (SPÓSITO, 2006). Entretanto, o cenário atual revela que plantios comerciais de tangerinas Ponkan, situados no Sul do Estado de Minas Gerais e no Estado de São Paulo, estão sofrendo danos altamente severos da doença, inclusive, inviabilizando a continuidade do cultivo desta tangerina em certos municípios.

O alto custo dos fungicidas específicos para o controle desse fungo, somado à necessidade de diversas aplicações sequenciais para o controle, tem levado produtores de algumas regiões a desistirem do cultivo de tangerinas, principalmente, das variedades mais suscetíveis (SPÓSITO, 2006).

2.3 Etiologia da mancha marrom de alternária

A doença mancha marrom de alternária (MMA) foi relatada pela primeira vez em 1903, na Austrália (KIELY, 1964) e, no Brasil em 2001, acometendo pomares de tangerinas "Dancy" no Estado do Rio de Janeiro (GOES; MONTESDE OCA; REIS, 2001). Dois anos mais tarde, foi constatada nos municípios de Campanha (Sul de Minas Gerais) e Montenegro (Rio Grande do Sul) e em outros sete municípios de São Paulo (SPÓSITO et al., 2003) e, mais recentemente, relatada no estado da Paraíba (LOPES; SCHWAN-ESTRADA, 2006). Esta doença, também, é preocupante em vários países como:

África do Sul, Austrália, Colômbia, Cuba, Espanha, EUA, Israel e Turquia (PEEVER et al., 2002).

A MMA tem como agente etiológico o fungo *Alternaria* a*lternata* (Fr:Fr) Keissl patótipo tangerina, que acomete, principalmente, as folhas e frutos de tangerina e seus híbridos, resultando em grandes perdas na produção, além de compor uma doença de difícil controle (CARVALHO et al., 2011). Este patógeno produz uma toxina hospedeiro-específica, denominada ACT, que consiste num fator de virulência para este patógeno, propiciando o aparecimento dos sintomas característicos em tangerinas e seus híbridos.

Nas folhas jovens, os sintomas iniciais aparecem como lesões necróticas e pequenas, de coloração marrom ou preta, as quais se expandem tomando grandes áreas do tecido do hospedeiro, que é necrosado pela toxina seletiva ao hospedeiro ACT (KOHMOTO; AKIMITSU; OTANI, 1991), podendo tomar grandes áreas das superfícies das folhas, estas podem ser distinguidas facilmente das lesões em folhas maduras, que, geralmente, aparecem circundadas por um halo amarelado (AKIMTSU; PEELVER; TIMMER, 2003). Além disso, as folhas novas são mais suscetíveis que as maduras (FEITCHENBERGER et al., 2005). Nos frutos aparecem como lesões marrom-escuras, variando de minúsculas pontuações a grandes manchas e em algumas vezes tomam um aspecto corticoso e saliente, formando camadas de abscisão, deixando grandes orifícios na superfície do fruto (FEITCHENBERGER et al., 2005). Os sintomas desta doença, também, são evidentes em ramos novos, aparecendo, inicialmente, em forma de lesões necróticas, de tamanho variável e coloração marrom-escura. Com a evolução da doença, pode ocorrer queda de folhas e subsequente seca de ramos (SPÓSITO, 2006).

O ciclo da MMA tem início pela sobrevivência do patógeno nos tecidos vegetais afetados, presentes na planta ou caídos no solo (TIMMER et al., 1998). Os conídios, esporos assexuais, são produzidos na superfície das lesões e

liberados após chuvas ou molhamento dos tecidos por orvalho e pronunciadamente, em locais onde ocorrem mudanças na umidade relativa, uma vez que essas mudanças bruscas estimulam a esporulação (AKIMITSU; PEEVER; TIMMER, 2003).

Os conídios são disseminados a longas distâncias pela ação do vento e depositados na superfície de tecidos jovens. A disseminação do fungo está altamente condicionada às condições ambientais e são favoráveis à infecção quando ocorre um período de molhamento de 10 a 12 horas e temperatura entre 20 e 30 °C (TIMMER; SOLEIL; OROZCO-SANTOS, 2000). A temperatura ótima para a infecção é de 27 °C (CANIHOS; PEEVER; TIMMER, 1999). Com o declínio da temperatura, períodos de molhamento mais longos são requeridos para que a infecção ocorra e, segundo Akimitsu; Peever e Timmer (2003) a 32 °C, a infecção ocorre mesmo em condições de baixa umidade.

Durante a germinação, os conídios liberam a toxina ACT, específica para tangerinas e seus híbridos, que matam as células antes da infecção (AKIMITSU; PEEVER; TIMMER, 2003). A penetração do patógeno na folha pode ocorrer diretamente ou por meio de estômatos, uma vez que foi associada à formação de apressórios em estudos realizados em Israel (SOLEL; KIMCHI, 1998), entretanto, não associada à formação destes em estudos realizados na Flórida (AKIMITSU; PEEVER; TIMMER, 2003). A expressão dos sintomas ocorre entre 24 e 36 h após a infecção (SPÓSITO, 2006). Por fim, a esporulação ocorre, predominantemente, em folhas maduras e relativamente pouco em frutos ou galhos (AKIMITSU; PEEVER; TIMMER, 2003)

A liberação de conídios é estimulada pelo impacto das gotas de chuva sobre a superfície foliar e, também, por quedas acentuadas na umidade. Em condições de campo, os conídios são dispersos após chuvas, quando a umidade abundante está presente para possibilitar a infecção. Alternativamente, a liberação de conídios em épocas secas pode ser iniciada quando da ocorrência de

orvalho na parte da manhã, com subsequente dispersão por ocasião da presença de ventos durante o dia, que, neste caso, sucede a infecção com a ocorrência de orvalho à noite. Sendo assim, o primeiro método de dispersão é o mais comum em épocas ou áreas chuvosas e são característicos de infecção precoce, e o segundo em regiões semiáridas e infecções tardias (AKIMITSU; PEEVER; TIMMER, 2003).

Contudo, a MMA é uma doença altamente destrutiva em áreas com alta umidade, sendo necessárias várias aplicações com fungicidas para reduzir a severidade da doença (TIMMER et al., 2003). Porém, mesmo em áreas semiáridas, esta doença pode ser um problema significativo. Nesse contexto, Canihos, Erkilic e Timmer (1997) e Solel (1991) ressaltaram que, em grande parte da área do Mediterrâneo, as chuvas são raras após a floração e frutificação, entretanto, a ocorrência de orvalhos proporciona umidade suficiente para possibilitar a infecção. Condições como essa aumentam, significativamente, os custos de produção, o que pode tornar inviável o cultivo de variedades suscetíveis nessas regiões (TIMMER; SOLEIL; OROZCO-SANTOS, 2000).

2.4 O fitopatógeno *A. alternata* patótipo tangerina

Os fungos do gênero *Alternaria* são cosmopolitas, acometem vários hospedeiros vegetais e são considerados saprofíticos facultativos, sobretudo, são mais evoluídos quando envolvidos com patogenicidade (WALTON, 1996). Esses fungos, denominados anamorfos/imperfeitos em virtude da ausência da fase sexual, na maioria de suas espécies, são morfológica e geneticamente similares; entretanto, apresentam diferenças patológicas (KUSABA; TSUGE, 1994). Por esse fato, linhagens de certas espécies, principalmente *A. alternata*, têm sido referidas como *forma specialis* ou patótipos, em função do seu hospedeiro (KOHMOTO; OTANI, 1995).

A espécie *A. alternata*, incita essencialmente duas enfermidades distintas em plantas de citros, a saber: a MMA (*A. alternata* patótipo tangerina); e a mancha foliar de alternaria (MFA), que é atribuída ao fungo *A. alternata* patótipo limão rugoso, a qual é caracterizada por afetar especificamente as folhas de limoeiros 'Rugoso' (*Citrus jambhiri* Lush.) e 'Cravo' (*Citrus limonia* Osb.) e por sintetizar a toxina hospedeiro-específica ACR (AKIMITSU; PEELVER; TIMMER, 2003; PEEVER et al., 2004).

O agente etiológico da MMA foi inicialmente descrito como *Alternaria citri* Ellis & Pierce (WHITESIDE, 1976), em razão da similaridade morfológica, entretanto, mais tarde, em pesquisas demonstrou-se que os isolados apresentavam características bioquímicas (produção de toxinas seletivas ao hospedeiro) distintas, como consequência disto, foi designada como *A. alternata* (KOHMOTO; SCHEFFER; WHITESIDE, 1979) e, em seguida, referida de forma mais apropriada, como *Alternaria alternata* patótipo tangerina (Fr: Fr) Keissl (PEEVER et al., 2004).

Assim sendo, este patótipo causa doença, especificamente, em tangerinas e seus híbridos. De tal forma, adota-se a designação de patótipo no presente projeto, considerando o fato de que existem claras diferenças biológicas, bioquímicas e genéticas entre esses fungos, os quais podem ser diferenciados por testes de patogenicidade, ensaios de toxinas ou marcadores moleculares (PEEVER et al., 2002).

Dentro do gênero *Alternaria* existem, ainda, outras duas espécies que incitam doenças em citros, a saber: *Alternaria citri* e *Alternaria limícola*. A espécie *A. citri* incita a Podridão Negra de Alternaria (PNA) que acomete laranjas doces e limões, causando podridão de frutos (AKIMITSU; PEELVER; TIMMER, 2003). Enquanto a *A. limícola* é a responsável pela doença conhecida como mancha foliar em limão mexicano (*Citrus aurantifolia* Swingle) que, por

sua vez, é mais restrita a essa variedade de citros amplamente cultivada no México (AKIMITSU; PEELVER; TIMMER, 2003).

O fungo *A. Alternata* produz conídios de forma e tamanhos variáveis (20-63 μm de comprimento x 9-18μm de largura), de cor oliva a marrom-escuro, dotados de 4 a 6 septos transversais e 1 ou mais septos longitudinais (ELLIS, 1993). O patógeno se desenvolve saprofiticamente em tecidos cítricos mortos ou em outros substratos, produzindo grande número de conídios, que podem ser carregados pelo vento até a superfície de frutos, folhas e outros órgãos suscetíveis (AKIMITSU; PEELVER; TIMMER, 2003) cumprindo, assim, um importante papel no ciclo da doença.

2.5 Controle da mancha marrom de alternaria

Visando à utilização de um método de controle menos agressivo ao ambiente, o manejo da MMA foi fundamentado na aplicação de fungicidas à base de cobre, os quais são recomendados para o controle da doença no Brasil (SOLEIL; OREM; KIMCHI, 1997). Todavia, como a disseminação do fungo está altamente condicionada às condições ambientais, os quais, nas condições dos campos de produção de tangerina brasileiros facilitam a dispersão e a infecção do patógeno, são necessárias várias aplicações de fungicidas dos mais variados grupos químicos, podendo alcançar um total de quinze aplicações para reduzir a severidade da doença, (TIMMER et al., 2003). Este fato eleva, consideravelmente, os custos de produção tornando a atividade antieconômica, além de inviabilizar a exportação do produto em função de possíveis acúmulos de resíduos nos frutos e de promover o desequilíbrio ambiental e nutricional decorrente do excesso de aplicação de cúpricos na área de cultivo.

Sendo assim, segundo Spósito (2006), para se obter um controle adequado dessa doença, é necessário adotar estratégias envolvendo o manejo do

pomar em sincronia com os tratamentos por fungicidas. Dentre as principais medidas de manejo recomendadas para a MMA, destacam-se o plantio de mudas sadias; a seleção de áreas para plantio evitando-se áreas de má circulação de ar; evitar-se plantios adensados; reservar as áreas mais altas e arejadas do pomar para o cultivo das variedades mais suscetíveis; a realização de podas de limpeza na parte interna da copa das plantas visando à melhor aeração; à manutenção da nutrição e de sanidade das plantas, evitando o uso excessivo de fertilizantes e irrigação; e a pulverização das plantas com fungicidas que apresentam ação contra o fungo (AKIMITSU; PEEVER; TIMMER, 2003).

Entre os grupos de fungicidas efetivos contra a MMA, citam-se as estrobilurinas, dicarboximidas, os triazóis, ditiocarbamatos e produtos à base de cobre (AZEVEDO, 2005). Vale ressaltar, que os fungicidas do grupo dos benzimidazóis não apresentam eficácia no controle da doença, podendo até agravá-la em algumas situações (FEITCHENBERGER et al., 2005).

Para o manejo sustentável da doença, além das táticas supracitadas, preconiza-se o plantio de variedades resistentes. As variedades de tangerina que possuem certo grau de resistência à mancha marrom são 'Thomas' e a 'Fremont' (FUNDO DE DEFESA DA CITRICULTURA, 2005), além de alguns híbridos e variedades que mostraram ser, potencialmente, resistentes em pesquisas e podem ser consultadas, respectivamente, nos trabalhos conduzidos por Chagas et al. (2007) e Azevedo et al. (2010).

Apesar das medidas de manejo recomendadas acima serem importantes para o manejo da doença, tem-se observado, muitas vezes, no campo que o controle da mesma não tem sido eficiente, levando diversos produtores a realizarem aplicações excessivas de fungicidas, o que pode levar ao acúmulo substancial de elevadas quantidades de substâncias tóxicas no ambiente. Além disso, medidas de controle, baseadas apenas em aplicações de fungicidas, podem propiciar a seleção de isolados do patógeno insensíveis ao princípio ativo,

ocasionando possíveis excessos de aplicações e acúmulos, ainda, maiores de substâncias tóxicas no meio ambiente. Dessa forma, faz-se necessário buscar por alternativas sustentáveis, para o controle da doença, por meio de pesquisas envolvendo substâncias, oriundas de produtos de origem natural, que possibilitem a exploração de produtos que forneçam vantagens comparativas em relação ao modo de ação dos fungicidas convencionais.

2.6 Uso de produtos de origem vegetal como medida de controle de doenças fundamentado na atividade direta e na indução de resistência

Atualmente o Brasil é o maior consumidor de agrotóxicos do mundo (MINISTÉRIO DO MEIO AMBIENTE, 2014). Este fato, somado à crescente exigência do mercado internacional por produtos vegetais de qualidade, livres de contaminação por microrganismos e resíduos químicos, leva a imprescindível intensificação de pesquisas visando à exploração de produtos que possuam frações ativas sobre fitopatógenos e, ao mesmo tempo, sejam menos agressivos ao meio ambiente. Assim, como é de indubitável importância, compreender melhor o modo de ação envolvido no controle exercido por essas substâncias, na interação planta-patógeno.

Visando desenvolver métodos de controle menos agressivos ao meio ambiente, muitos pesquisadores estão procurando demonstrar a eficácia de produtos de origem vegetal em diferentes interações, como alternativa menos onerosa ao ambiente para emprego na agricultura. Cientes do grande potencial desses produtos para o controle de doenças, vários autores têm buscado encontrar substâncias ativas contra fitopatógenos, isolar e identificar as substâncias responsáveis pelo controle e, em uma abordagem mais minuciosa, elucidar o modo de ação envolvido no controle da doença.

A respeito de produtos naturais e suas frações, Hammond-Kosack e Jones (2002) ressaltaram que, entre os incontáveis metabólitos produzidos por plantas, predominam-se, principalmente, três classes de produtos naturais: alcaloides, terpenoides e compostos fenólicos, os quais podem estar envolvidos diretamente na inibição do patógeno ou indiretamente, na resposta de defesa de plantas contra fitopatógenos e pragas. Desta forma, pesquisas com a finalidade de determinar componentes ativos em óleos essenciais (OE) de plantas, revelam que estes são constituídos por uma grande variedade de moléculas, dentre as quais predominam-se terpenos e terpenoides, compostos fenólicos, compostos aromáticos, derivados dos fenilpropanoides e componentes alifáticos (BAKKALI et al., 2008).

Em virtude disso, estudos realizados com OEs, obtidos com base em plantas têm indicado o potencial destes no controle de fitopatógenos, tanto por sua ação fungitóxica direta, como pela capacidade de indução de fitoalexinas, indicando a presença de compostos capazes de induzir a resposta de defesa da planta (SCHWAN-ESTRADA, 2003). Assim sendo, a exploração da atividade biológica de compostos presentes em OEs de plantas pode se constituir ao lado da indução de resistência, em mais uma forma potencial de controle de doenças em plantas.

Com o propósito de exemplificar o controle direto de OEs sobre fitopatógenos, pode-se citar a pesquisa feita por Pereira et al. (2011) que evidenciaram uma atividade direta na inibição do crescimento micelial de *Cercospora coffeicola* por parte de OEs de cravo-da-índia, canela, nim, tomilho e capim-limão, esses autores ressaltam que os OEs de canela e citronela, também, reduziram a incidência e a severidade da cercosporiose, além de apresentar o referido efeito fungitóxico. De forma semelhante, ao investigar o modo de ação de OEs, por meio da análise em microscopia eletrônica de transmissão, Roswalka (2010) relataram uma ação fungitóxica notória sobre

conídios de *Colletotrichum gloeosporioides* e *Colletotrichum musae*, causando danos severos relacionados à desorganização e degradação celular e consequente impossibilidade de germinação desses conídios. Similarmente, resultados promissores foram encontrados enquanto ao efeito fungitóxico do OE de cravo-da-índia, o qual demonstrou inibição de 100% no crescimento micelial de *Glomerella cingulata* e *C. gloeosporioides* (ROZWALKA et al., 2008).

Ainda, a respeito do efeito direto de produtos naturais, pode-se citar, também, o estudo realizado por Carvalho et al. (2011) com vistas à exploração de produtos de origem vegetal com atividade contra o agente etiológico da mancha marrom de alternaria (MMA), onde extratos vegetais de 105 espécies de plantas foram avaliados enquanto a atividade direta *in vitro* e *in vivo* sobre *Alternaria alternata*. Os autores obtiveram o extrato obtido, com base em *Anadenanthera colubrina* como promissor, o qual foi capaz de reduzir a doença em frutos de tangor Murcott em níveis comparáveis aos obtidos com fungicidas comerciais além de apresentar-se, como o extrato mais ativo *in vitro* em relação aos demais extratos pesquisados. Esta pesquisa foi aprofundada recentemente, com vistas a isolar as substâncias responsáveis pelo controle obtido por parte do extrato de *A. colubrina* além de determinar o possível modo de ação de tais substâncias. Foi assim que Campos et al. (2014) atingiram resultados bastante promissores com o isolamento das substâncias ativas e identificaram por meio de estudos *in silico* algumas proteínas do fungo que são alvo dessas substâncias. Dessa forma, os autores isolaram as substâncias ativas β-Sitosterol e β-sitosteryl linolato, com base no extrato de angico branco (*A. colubrina*), contra *A. alternata* e sugeriram que tais substâncias agem por meio da ligação com as proteínas de ligação ao oxysterol (*oxysterol-binding proteins*), as quais estão envolvidas na biossíntese de esteróis, impedindo, portanto, o transporte do ergosterol, essencial à integridade da membrana plasmática fúngica. Analogamente, em pesquisas realizadas com o intuito de verificar o efeito de

OEs sobre células eucarióticas revelou-se que estes atuam como pró-oxidantes, afetando, principalmente, as membranas celulares e o interior de organelas como as mitocôndrias, conforme exposto numa importante revisão sobre o assunto (BAKKALI et al., 2008). Segundo esses autores, os efeitos citotóxicos de OEs em células podem estar associados a mudanças no potencial de oxirredução intracelular decorrente da atividade de exposição aos OEs.

A indução de resistência em plantas contra patógenos representa um método alternativo no controle de doenças, a qual ativa os mecanismos de defesa que se encontram na forma latente na planta. A resistência induzida pode ser ativada em plantas por uma série de substâncias, evitando ou atrasando a entrada e/ou a subsequente atividade do patógeno em seus tecidos, por meio de mecanismos de defesa característicos. Resende et al. (2004) e Cavalcanti et al. (2005) citam que a resistência induzida em plantas pode ocorrer tanto por meio do tratamento com agentes bióticos (produtos de origem vegetal, microrganismos ou parte desses) ou abióticos (substâncias químicas). Avanços na pesquisa envolvendo a indução de resistência em plantas vêm sendo acompanhados pelo surgimento de novos produtos comerciais que apresentam maior eficácia, estabilidade e menor impacto ao ambiente (RESENDE et al., 2006), sendo capazes de propiciar melhorias na produtividade agrícola, em decorrência da redução de perdas ocasionadas por estresses bióticos e abióticos, dentro do conceito de amplo espectro de ação, conferido por estes indutores.

Pesquisas realizadas com OE visando ao controle de doenças baseados na indução de resistência são incipientes, mas promissoras. Pereira et al. (2011), utilizando OEs das espécies *C.zeylanicum* e *Cymbopogon nardus* (L.) observaram a inibição do desenvolvimento e elongação da hifa do patógeno *Cercospora coffeicola* em folhas de cafeeiro (*Coffea arabica* L.) inoculadas aos dois dias após a aplicação, sugerindo a ativação de respostas de defesa da planta e a atividade preventiva desses OEs. Nesse mesmo contexto Latha et al. (2009)

estudaram a influência da adição do extrato da planta de Zimmu (*Allium cepa* L. x *Allium sativum* L.) a rizobactérias promotoras de crescimento em plantas (PGPR), bem como sua capacidade no incremento da indução de resistência das plantas, demonstrando a influência do extrato da referida planta na promoção de crescimento vegetal e na resistência sistêmica induzida (ISR) resultando no aumento da resistência a doenças em plantas de tomate contra a doença pinta preta (*Alternaria solani* Sorauer), além de demonstrar a atividade direta desse produto natural à base de plantas no crescimento micelial de *A. alternata*.

Resultados promissores, também, foram alcançados por Pereira et al. (2008), ao utilizar extrato de casca de café e óleo essencial de tomilho como indutores de resistência à Cercosporiose em mudas de cafeeiro. Semelhantemente, Balbi-Peña et al. (2006), estudando o uso de extrato bruto de cúrcuma, observaram que estes apresentaram níveis de controle de *Alternaria solani* similares a um fungicida cúprico em tomate, indicando possível indução de resistência.

Em relação à determinação de componentes ativos, presentes em OEs, em estudos realizados com diversas espécies de plantas têm detectado a presença de compostos fungitóxicos por meio de cromatografia de camada delgada (CCD) e cromatografia gasosa acoplada a detector de massas (CG-MS). Nesse ponto de vista, Stangarlin et al. (1999) verificaram a presença de frações nas quais houve inibição do desenvolvimento de *Colletotrichum graminicola*. Observaram, também, a presença de duas frações fungitóxicas bem definidas nos extratos de capim limão e uma nos de cânfora e alfavaca.

Em um estudo realizado por Yen e Chang (2008), envolvendo a avaliação de substâncias isoladas com base em OEs de plantas e sua atividade contra fungos causadores de apodrecimento em espécies florestais, revelou-se uma atividade fungitóxica direta e sinergística por parte dos compostos eugenol e cinamaldeído. Similarmente, Wang et al. (2010) verificaram uma atividade

direta evidente por parte do componente eugenol recorrente de OEs, sobre hifas de *Botrytis cinerea* agente etiológico do mofo cinzento em espécies frutíferas e vegetais.

Contudo, poucos são os relatos no que se refere ao modo de ação desses OEs sobre fitopatógenos (ROSWALKA, 2010). Uma importante descoberta foi realizada recentemente acerca da ação dos produtos naturais: ácido salvianólico A, ácido salvianólico B e ácido caftárico (SPERL; SEIFERT; BERG, 2009). Os autores demonstraram que esses componentes agem como inibidores das interações proteína-proteína os quais formam a base para praticamente todos os aspectos de sinalização molecular de enfermidades (SPERL; SEIFERT; BERG, 2009); os autores ressaltam os componentes como antagonistas potentes e seletivos de interações proteína-proteína. Esta descoberta pode ser extrapolada para estudos, envolvendo a busca de produtos que interfiram na interação planta-patógeno, fornecendo bases para um controle sustentável de enfermidades em plantas para que possa ser disponibilizado para o produtor.

3 CONSIDERAÇÕES GERAIS

Os estudos realizados no patossitema *A. alternata* - Citros apresentaram importantes descobertas no que diz respeito a estratégias de controle da MMA em tangerina ponkan. Os dados reunidos sugerem que a atividade dos óleos essenciais, estudados na redução da doença, está intimamente relacionada com a atividade apresentada pelos compostos ativos presentes em maior quantidade em sua composição. Agem tanto de forma direta, como de forma indireta com consistente capacidade de ativar a indução de resistência da planta contra o ataque do patógeno.

O óleo essencial de tomilho *Thymus vulgaris* L (TEO) assim como o óleo essencial de canela *Cinnamomun zeylanicum* L (CEO) demonstraram apresentar uma importante fonte de substâncias de origem natural, capazes de agir diretamente nas estruturas fúngicas. O TEO demonstrou efetiva interferência nos eventos iniciais de penetração e infecção do patógeno. Já o CEO, além do efeito direto, demonstrou uma atividade indireta por meio da ativação de enzimas do sistema de defesa da planta, resultando em uma proteção de amplo aspecto contra o ataque de patógenos.

Estes resultados servem de base, juntamente com outros resultados de pesquisas no âmbito do uso de produtos naturais como fonte de substâncias ativas, para o aprofundamento de estudos no intuito de determinar as dosagens adequadas de princípios ativos, bem como a capacidade de ação em organelas específicas do patógeno e de indução da expressão de genes específicos relacionados ao mecanismo de defesa da planta. Além disso, possibilita o estudo de uma possível interferência dessas substâncias com a toxina de *A. alternata,* como forma de inibir esse efetor do patógeno, na tentativa de explicar, de forma mais detalhada, o potencial de atividade dessas substâncias e maximizar sua atividade garantindo um controle mais efetivo e duradouro do patógeno.

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ARTIGO 1 *Cinnamomun zeylanicum* **L. essential oil and its active substance** *trans*-**cinnamaldehyde** *against* **Alternaria brown spot in citrus: Direct effects and plant induced-resistance ability**

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1   *Cinnamomun zeylanicum* **L. essential oil and its active substance *trans-***
2   **cinnamaldehyde *against* Alternaria brown spot in citrus: Direct effects and**
3   **plant induced-resistance ability.**

4   Running title: *trans*-cinnamaldehyde [(2E)-3-phenylprop-2-enal] for the control
5   of *Alternaria alternata:* mode of action and induced-resistance activity

6

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17

18   **Abstract**
19   **Cinnamon essential oil (CEO) has evidenced activity against several plant-**

20   **pathogenic fungi and reduced the fungal diseases to levels comparable to**

21   **commercial fungicides. The goals of this work was to identify the active**

22   **substances of CEO, to recognize the direct fungicide activity and a putative**

23   **indirect activity by plant resistance induction against Alternaria brown spot**

24   **(ABS). The Gas chromatography–mass spectrometry (GC-MS) of CEO**

25   **resulted in the identification of eugenol and *trans*-cinnamaldehyde (CNE) as**

1 **actives substances, which had minimal inhibitory concentrations (MICs)**

2 **against *A. alternata* of 250 and 62.5 µg mL^{-1} respectively, while, under the**

3 **same conditions, the MICs for a commercial fungicide and CEO was 1250**

4 **and 500µg mL^{-1} respectively. The fungal grown inhibition assay showed that**

5 **the eugenol and CNE prevent the fungal growth by a direct fungicidal**

6 **action. The CEO reduced the ABS disease incidence to levels comparable**

7 **with a commercial plant activator. Both CEO and its active compound CNE**

8 **showed capability to induce the plant defense response with remarkable**

9 **activation of plant defense enzymes. Therefore, both the CEO and its**

10 **substance have potential use in the development of new structures and**

11 **analogues to control the ABS disease caused by *A. alternata*, offering direct**

12 **and indirect activity**

13 **Keywords:** mode of action; plant defense response; fungicidal activity; indirect

14 activity

15

16 **INTRODUCTION**

17 *Alternaria* brown spot disease (ABS) is the most important disease that affects

18 tangerine plants and its hybrids worldwide (Timmer et al., 2003). In Brazil it

19 occurs mainly in environmental conditions encountered in South and Southeast,

20 which correspond to the main tangerine commercial orchards, leading to severe

21 economic losses. The etiological agent is a necrotrophic fungal *Alternaria*

22 *alternata* (Fr.: Fr) Keissl. tangerine pathotype (Peever et al., 2004; Akimitsu et

al., 2003).

Strategies to control ABS are based in the use of cultural methods, use of cupper fungicides and mixture of triazole and strobilurin fungicides registered for citrus in Brazil orchards, since no resistant cultivars are available and no fungicides are actually specifically registered for ABS control. Although such management might reduce disease severity, the ABS control is still largely dependent on fungicide applications and in some cases, depending on host susceptibility and weather conditions, growers may apply 2 to 15 fungicide sprays during the course of the growing season (Vega and Dewdney; 2014). As pointed by Vicent et al. (2009) due to the short incubation period where necrotic lesions are visible just 24 h after infection was initiated, curative applications are ineffective and the control of ABS depends mostly on the protective action of the fungicides. Therefore, such amount of fungicides applications can make the production of tangerines economically unfeasible (Campos et al., 2014). In addition, the chemical control by certain fungicide used in some cases, might generate control failure (Vega and Dewdney; 2014). Copper compounds and triazole mixed with strobilurin are the fungicides commonly used for ABS control. However there is no fungicide really registered in Brazil for ABS control in conventional production. Furthermore, copper is the only fungicide accepted in organic production worldwide.

As several research groups have evidenced the plants capability to produce

1   metabolites active against fungi (Pereira et al., 2012b; Lucas et al., 2012;

2   Campos et al., 2014), a possible alternative to overcome this problem involves

3   the use of such metabolites to develop new products to control ABS based on

4   their ability to direct control the fungus as well as the stimulation of natural

5   plant defenses against the pathogen.

6   Plants face many threats from the environment through efficient defense systems

7   that protect them from biotic and abiotic stresses Benhamou (1996). One of

8   earliest defense responses to pathogen attacks in plants is the oxidative burst,

9   involving elevated levels of reactive oxygen (ROS) species such as hydrogen

10   peroxide (H_2O_2), superoxide radicals (O2-) and hydroxyl radicals (OH) around

11   the infection site (Greenberg 1997), and including some scavenging enzymes for

12   ROS, such as catalase (CAT), superoxide dismutase (SOD) and ascorbate

13   peroxidase (APX). ROS, such as hydrogen peroxide (H_2O_2), can be produced

14   both in the regular plant metabolism (Mittler, 2002) and in response to invading

15   pathogens (Lamb and Dixon, 1997), and in excess, it can also serve as secondary

16   messengers in the pathogen-response signal transduction pathway (Veal et al.

17   2007).Thus, the activation of natural plant defenses through systemic acquired

18   resistance (SAR) might be permissively used as a strategy in the ABS control,

19   as it elicits molecules that activate systemic resistance, thus protecting tissues

20   against attack from a wide pathogens range (Hammond-Kosack and Parker,

21   2003).

1   Natural substances derived from plants extract and essential oil have notably

2   shown promising results for controlling diseases in different crops (Campos et

3   al., 2014 Teixeira et al., Perina et al., 2013; Pereira et al., 2011; Lucas et al.,

4   2012). Regarding essential oils, the studies aimed at elucidating the mechanisms

5   of disease suppression, suggest that the active compounds of these oils act

6   directly on the pathogens (Teixeira et al., 2013 al., Perina et al., 2013) or

7   stimulates the plant resistance induction resulting in disease reduction (Lucas et

8   al., 2012; Pereira et al., 2012b). Plant defense responses induced by natural

9   compounds extracted from plants have been showed against other *Alternaria*

10 genus fungal specie (Guleria and Kumar, 2004), as well as in other

11 pathosystems. Such natural products might be successful for use in organic

12 production systems and can be submit to regulatory process in order get

13 registration for fruit post-harvest and/or other plant disease control and be used

14 as promisor Generally recognized as safe (GRAS) products (Burdock and

15 Carabin 2004).

16 Several bioactivity of cinnamon (*Cinnamomun zeylanicum* L.) essential oil

17 (CEO) is known currently, such as antioxidant (Bharti et al., 2013, Singh et al.,

18 2007; Jayaprakasha et al., 2007), antimicrobial activity and cytotoxicity (Unlu et

19 al., 2010; Singh et al., 2007) as well as for plant disease control (Pereira et al.,

20 2012b; Teixeira et al.; 2013). Similarly the *trans*-cinnamaldehyde (CNE) one

21 main compound of CEO, was interesting evidenced to exhibit cytotoxic effects

1   against human cancer cells and potential antioxidant activity (Fang et al., 2004).

2   Screening experiments previously developed through 2009-2012 revealed the

3   CEO as one of the most promising results to control ABS among several plant

4   essential oils in a commercial organic tangerine orchards located in South of

5   Minas Gerais State-Brazil. In fact, *Cinnamomun zeylanicum* L. Blume,

6   commonly known as cinnamon, have been widely studied due to their medicinal

7   properties. Even though examples regarding to active substances obtained from

8   plant essential oils against plant disease, are found and demonstrates the direct

9   effects against plant-pathogen fungi (Rozwalka et al., 2008; Pereira et al., 2011;

10 Teixeira et al., 2013; Perina et al., 2013), to the best of the authors knowledge,

11 no studies were found concerning the capability of likely substance from

12 essential oil to induce defense responses in plant against pathogens attack.

13 Therefore, in order to contribute to the development of new products for ABS

14 control, the goals of this work was to identify and compare the ability of CEO

15 and its active substance *trans*-cinnamaldehyde on reducing the growth of *A.*

16 *alternata in vitro* as well as the CEO capability on reducing the disease

17 incidence under field conditions. Finally, a time course experiment under

18 controlled conditions was carried out to identify and compare the capability of

19 CEO and its substance *trans*-cinnamaldehyde to induce ROS-related enzymes as

20 indicative of plant resistance induction.

21

1 **RESULTS AND DISCUSSION**

2 **Antifungal activities of CEO its active substance CNE against *A. alternata*.**

3 The results from qualitative and quantitative of CEO by GC-MS indicated that

4 the essential oil presented 17 different constituents. The peak area was chosen as

5 the analytical signal for the relative content, and the identified components are

6 listed in Table1.

7

8 **Table 1.** Chemical composition of the *Cinnamomun zeylanicum* leaves
9 essential oil.

RT	RI	Compound	Percentage (%)
5,66	0933	*α*-Pinene	0.9
6,96	0974	*β*-Pinene	0.7
7,87	1003	*α*-Phellandrene	0.7
8.59	1023	*ρ*-Cymene	1.3
8,79	1027	1,8 Cineole	0.8
11.39	1095	Linalool	3.8
14.72	1177	Terpinen-4-ol	2.5
15.44	1189	*α*-Terpineol	0.5
18.79	1268	*trans*-Cinnamaldehyde	17.8
22.68	1357	Eugenol	48.3
23.46	1375	*α*-Copaene	1.2
26.42	1443	*trans*-Cinnamyl acetate	7.8
28.72	1498	Viridiflorene	0,1
29.39	1514	*γ*-Cadinene	0,2
29.65	1521	Eugenol acetate	5.7
32.69	1584	Caryophyllene oxide	1.5
38.69	1758	Benzyl benzoate	3.3
Total identified		…	97.1

10 RT: Retention time; RI: Retention index relative to a homologous series of n-
11 alkanes on the DB-5 capillary column**.**
12

13 Such constituents represented 97.1% of CEO composition. The majority of the

1   identified CEO compounds consisted of eugenol and *trans*-cinnamaldehyde

2   (CNE) which represents 48.3% and 17.8% of the total identified substances

3   respectively. Analogously, several authors have been reported eugenol and CNE

4   as the major constituent of CEO, (Unlu et al., 2010; Wang and Yang; 2009,

5   Bhart et al.,2013) the later authors found close as major substance eugenol

6   79.75% and 16.25% of CNE on the *C. zeylanicum* essential oil also extracted

7   from leaves. Thus, this the two major compound were screened in a previous

8   assay measuring the ability of such substances including the CEO to inhibit the

9   growth of *A. alternata* by means of minimal inhibitory concentration (MIC),

10   capable to prevented fungal development according to visual observation.

11   CEO, eugenol and CNE exhibited MIC values equal to 500, 250 and 62.5μg mL^{-}

12   ^{1}, respectively, while the commercial fungicides Recop$^{®}$ and Nativo$^{®}$ under the

13   same conditions, exhibited MIC values equal 1250 and 18,75μg mL^{-1},

14   respectively (Table 2). Except to the ASM treatment, none of the MIC values

15   observed in the liquid medium presented growth after being transferred to a

16   fresh liquid medium, indicating that the action of the substance evaluated at their

17   MIC were essentially fungicidal activity.

18

1 **Table 2.** Minimal inhibitory concentration (MIC) of *Cinnamomun zeylanicum*

2 leaves essential oil against *Alternaria alternata*

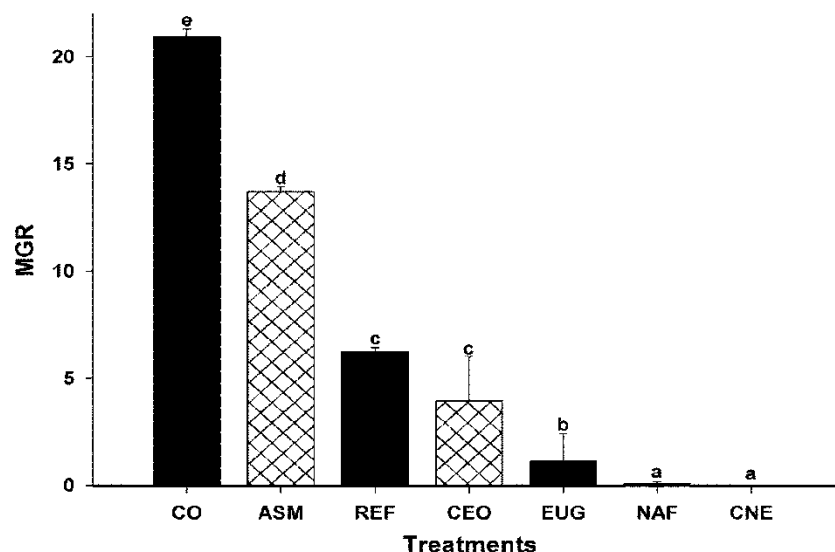| Treatment	MIC values (μg mL^{-1})
C. zeylanicum essential oil (CEO)	500
Eugenol	250
trans-Cinnamaldehyde (CNE)	62.5
ASM[a]	200
Control[b]	1250
Control[c]	18,75

3 [a]ASM - S-methyl ester of benzo [1, 2, 3] thiadiazole-7-carbothioic;[b] Recop®
4 commercial fungicide composed by CuCl$_2$.3Cu(OH)$_2$ at 840g kg^{-1}; [c]Nativo®
5 commercial fungicide composed by trifloxystrobin + tebuconazole (1:2 m/m) at
6 800 g L^{-1}.
7

8 It's worth to point that the high differences among the MIC values for Nativo®

9 and the other substances can occur, as previously highlighted by other authors

10 (Campos et al., 2014), in fact this commercial fungicide comprises the mixture

11 of triazol and strobilurin groups, two active substances which have different

12 fungicide mode of action. Nevertheless, the CNE substance showed a high direct

13 activity against *A. alternata* with at low concentrations. Where compared with

14 the cupper fungicide Recop® the CNE presented MIC 20 fold less, while

15 eugenol and CEO presented 4 and 8 fold less than this largely used fungicide

16 respectively.

17 With respect to mycelial growth rate (MGR), it was observed that, with

18 exception for the medium amended with CNE, the growth happened on all other

19 medium containing the active substance tested in their MIC values obtained in

20 the previous assay. The value of this data comprehends the close environment

1   using that the substance will encounter at field conditions, with solid growth

2   environment. Significantly ($P \leq 0.05$) less mycelial growth were found for CNE

3   and the commercial fungicide Nativo® (Figure 1) which did not differ each

4   other, clarifying a strong fungicide action by CNE when it is exposed to a solid

5   growth media. Under the same conditions, the eugenol showed an inhibition in

6   the MGR but it was less significant than the early substances. On the other hand,

7   it was higher in terms of inhibition than the CEO and the Recop® commercial

8   fungicide that showed similar inhibition. These latter in turn, conferred larger

9   inhibition than ASM which displayed significant inhibition of MGR compared

10   to the control.

11

Figure 1. Graphic representing the effect of *Cinnamomun zeylanicum* L., and its compound *trans*-cinnamaldehyde on mycelial growth rate (MGR) mm.day^{-1} at 25°C. Abbreviations means potato dextrose agar culture medium amended with: CO - Tween 80 at 0,01%; CEO – *C. zeylanicum* leaves essential oil at 500 µg mL^{-1}; REF - Recop$^{®}$ fungicide composed by CuCl2.3Cu(OH)$_2$ 840g kg^{-1} at 1250 µg mL^{-1}; EUG - Eugenol at 250 µg mL^{-1}; CNE - *trans*-cinnamaldehyde at 62.5 µg mL^{-1}; NAF: Nativo$^{®}$ commercial fungicide composed by trifloxystrobin + tebuconazole (1:2 m/m), 800 g L^{-1} at 18.75 µg mL^{-1}. ASM - S-methyl ester of benzo [1, 2, 3] thiadiazole-7-carbothioic at 200 µg mL^{-1}. Bars followed by the same letter do not differ significantly (Tukey P≤0.05). Results are mean ± standard error of the mean from two independent experiments with eight replicates each (n = 16).

The lower result found by CEO which showed a similar performance to the Recop fungicide in solid substrate when compared to liquid medium, can be explained due to the large amount of volatiles substance found in its composition that is better preserved in liquid media than in solid ones. And could be also explicated by the potential molecular interactions among CEO

1   constituents facilitated under liquid medium, which was less significant in solid

2   medium. Regarding the CEO capability to inhibit the MGR, our findings are

3   consistent with those previously reported by others authors highlighting its

4   strong fungicidal activity against other plant pathogenic fungi. As example,

5   Tzortzakis (2009), demonstrated a strong mycelial growth reduction by CEO at

6   500 ppm analogously, such fungicidal activity have been reported against

7   *Stenocarpella maydis* by Teixeira et al. (2013); *Colletotrichum musae*,

8   *Lasiodiplodia theobromae* and *Fusarium proliferatum* by Ranasinghe et al.

9   (2002); and by Simić et al. (2004) whom related strong antifungal activity

10   against some fungi of importance in food poisoning, spoilage fungi, animal and

11   plant pathogens.

12   The antifungal activity found by eugenol and likely the strong activity obtained

13   by CNE, confirmed the strong activity against other plant pathogenic fungi of

14   this CEO components, as previously reported by Goubran and Holmes (1993)

15   for *Monilia* sp. and Botrytis sp. Researchers have used the MIC method as a tool

16   to assess the antifungal and fungistatic activity of substances (Simić et al., 2004;

17   Campos et al. 2014). Concerning the ASM inhibition, our findings are in

18   agreement with those obtained by Ozgonen and Karatas (2013), which

19   demonstrated that the mycelial growth of *Alternaria mali* decreased by

20   increasing ASM concentration, but it was not inhibited completely.

21   Therefore, our results showed that with the exception of the plant activator ASM

1   and the Recop® fungicide, all other substance that showed activity against *A.*

2   *alternata*, demonstrated no revival ability after transferred to a fresh liquid

3   medium. This result revealed a direct fungicidal activity by the substances

4   against the pathogen. However its worth to highlighted, that with respect to

5   effects of pure substances found in essential oils composition against plant

6   pathogenic fungi, it still remain poorly explored. The MIC value found by CNE

7   in addition with the high performance regarding the inhibition of the MGR

8   reinforces the superiority of its substance as a likely active component of the

9   CEO. Therefore the substance CNE was chosen for the trial aiming to assess its

10   capability with respect to activate plant induction activity based in oxidative

11   burst. The MGR result found, highlighted the value of ensure the correlation

12   among such activities encountered under liquid medium conditions in the MIC

13   with the fungicidal activities of the substances in solid medium, approaching the

14   reality that the pathogen will encounter under field and post-harvest conditions

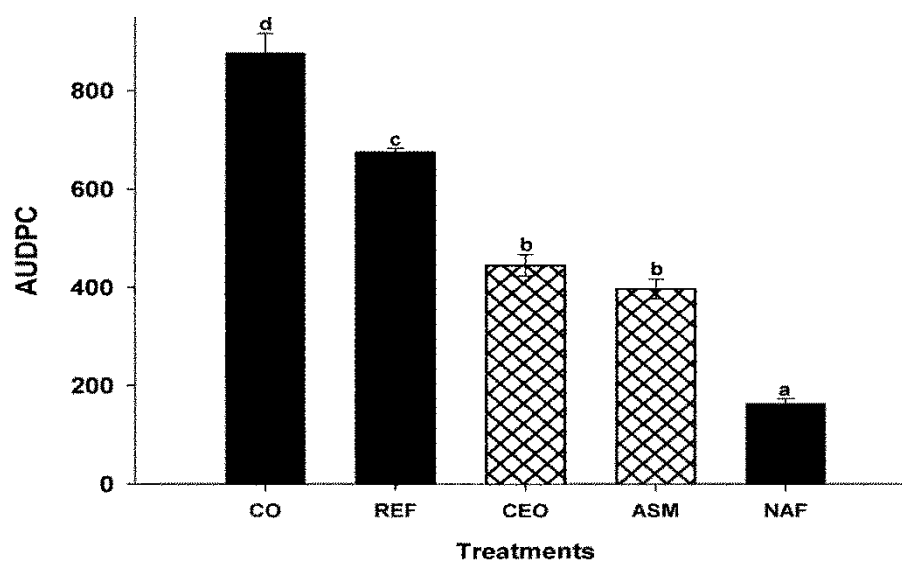15   in plants and fruits.

16

17   **Control of ABS progress in field**

18   The environment conditions registered during the two years of field experiments

19   were: daily average temperature of 23.43°C in 2010 and 24.01°C in 2011 with a

20   rainfall year average of 1232 mm year^{-1} in 2010 and of the 1316 year^{-1} mm in

21   2011 that was most accumulated at summer season.

1 It was observed that all the treatments resulted in significantly lower (Scott-

2 Knott $p \leq 0.05$) area under disease progress curve (AUDPC) compared with the

3 untreated control plants (Figure 2) during the two evaluation periods. The

4 orchards treated by Nativo[®] fungicide resulted in significantly lower AUDPC

5 than all the other treatment sprayed. Interestingly, a significantly similar control

6 in the disease progress was observed for the CEO and the ASM treated plants.

7

8
9 **Figure 2.** Effect of *Cinnamomun zeylanicum* L., on area under disease progress
10 curve (AUDPC). Abbreviations: treatments applied on tangerine plants: CO -
11 control Tween 80 at 0,01%; CEO - *Cinnamomun zeylanicum* leaves essential oil
12 at 1000 µg mL^{-1}; REF - Recop[®] fungicide composed by $CuCl2.3Cu(OH)_2$ 840g
13 kg^{-1} at 2500 µg mL^{-1}; NAF: Nativo[®] commercial fungicide composed by
14 trifloxystrobin + tebuconazole (1:2 m/m), 800 g L^{-1} at 300 µg mL^{-1}. ASM - S-
15 methyl ester of benzo [1, 2, 3] thiadiazole-7-carbothioic at 800 µg mL^{-1}. Bars
16 followed by the same letter do not differ significantly ($p \leq 0.05$) according to the
17 Scott-Knott (1974). Results are mean ± standard error of the two independent
18 experiments with four replicates each (n = 8).
19

1   These overcame the cooper fungicide Recop®, which in turn, showed a

2   significantly control compared to the untreated control plants throughout the

3   whole experiment period evaluated. The significantly similar results obtained

4   with the CEO treatment and the plant activator ASM treatment, this fact

5   generates the idea to investigate the possible resistance induction action by the

6   CEO. Thus, the capability of resistance induction by CEO and its major active

7   substance CNE found in the *in vitro* bioassays were further investigated in the

8   biochemical experiment.

9   Our results demonstrated that at the experiment conditions all the compounds

10   evaluated provided 100 days of protection for the orchards, even after high

11   rainfall conditions recorded during the two summer season in 2010 and 2011.

12   This fact demonstrates the high capability of CEO, ASM in protecting the

13   tangerine trees against ABS even in high rainfall conditions registered,

14   considering that as highlighted by Bassimba et al. (2014), by means of a

15   classification tree analysis in a Mediterranean environment region, indicated that

16   virtually all infections occurred on weeks with rainfall ≥ 2.5 mm and average

17   temperature $\geq 12.5°C$. With regard to protection, these results are in agreement

18   with those obtained by Vicent et al. (2009) which found that different cooper

19   compounds were able to effectively protect 'Fortune' tangerine fruit from *A.*

20   *alternata* infection for a 28 day period evaluated, considering a single spray

21   application used by this authors, contrasting with the two week spray schedule

1 used in the present study. It´s worth to observe that the two weeks application

2 schedule performed in our experiments did not target to curative situations in

3 orchards with high ABS infection have already reached. This schedule was

4 idealized aiming a systemic resistance induction with a protective approach of

5 the orchard. Once with regard to curative fungicide applications, as highlighted

6 by Reis et al. (2006) multiple sprays applied to the same lesion of ABS, did not

7 appear to have a greater effect on sporulation than a single application.

8 Regarding the ASM reduction on branches with leaves and young shoots disease

9 incidence found, the results obtained showed the ability of this compound on

10 protect new expanding leaf and shoots flushes during spring and early summer

11 against the ABS infection. Similarly Graham et al. (2011) found that ASM was

12 highly effective for suppressing foliar canker on young grapefruit and orange

13 trees, although, this effectiveness was encountered by under weather conditions

14 absent of high intensity rains or tropical storms, they also found that sprinkler

15 application of ASM was less effective than soil drench. Researches in respect to

16 the use of essential oils for controlling plant disease are commonly found in the

17 state of art. However, the applications of such compounds on filed conditions

18 were not found to the best of the author's knowledge. Apparently, this is one of

19 the first reports of field application of essential oil in commercial tangerine

20 orchard aiming to control plant disease. Thus, regarding the results obtained

21 with applications of CEO, our data are in accordance with those obtained by

1 Pereira et al. (2011) under greenhouse conditions, where CEO and citronella

2 essential oil reduced the incidence and severity of brown eye spot, in addition,

3 the authors reported the direct toxicity to the pathogen. In addition, the incidence

4 control observed in our experiment are also close related to the results reported

5 by Pereira et al (2012a) also under greenhouse conditions, that showed the

6 ability of CEO in controlling partially the coffee rust disease progress. The close

7 related result observed between CEO and ASM on this research might be related

8 to a possible activation of plant defense response by this essential oil. This fact

9 has proved by Pereira et al (2012b) with the application of citronella essential oil

10 under controlled conditions.

11 **Plant induced-resistance by CEO and CNE**

12 For all products tested, at least one of the factors (products, plant inoculation and

13 time points) was significant. The most relevant data was the deployment of triple

14 interaction (products vs plant inoculation vs time points) which was significant

15 for CAT, APX, PAL and POX activities, while for the SOD and GLU activity

16 the most explanative significant factor was the product and time point (Table 3).

17

1 **Table 3.** Analysis of variance for the effects of products (P), sampling time (T) and plant inoculation (I) on the activity of
2 peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and β -1,3-glucanase.

Sources of variation	df	F values[a]					
		POX	CAT	APX	PAL	SOD	GLU
P	3	32.98	4.80	40.67	0.1786	0.1059*	0.1139*
T	3	11.16	34.36	7.68	0.3304	0.6484*	0.0897*
I	1	14.30	1.09	2.29	0.0011	0.0045 ns	0.0005 ns
Block	2	0.008 ns	0.33 ns	0.13 ns	0.0001 ns	0.0002 ns	0.0006 ns
PxT	9	10.80	0.56 ns	10.37	0.1190	0.0266 ns	0.0014 ns
PxI	3	2.95ns	0.39 ns	1.99	0.0016	0.0062 ns	0.0016 ns
TxI	3	11.26	1.40	15.57	0.0580	0.0470	0.0061
PxTxI	3	4.05*	2.50*	8.66*	0.0185*	0.0135 ns	0.0011 ns

3 [a] Levels of probability: ns: not significant, *: 0.05

1   Thus it was performed a statistical triple interaction deployment for the most

2   important factors that explain the variance in the variable studied for the former

3   case and a separated factor analysis by Scott-Knott (p>0,05) for the latter.

4   Therefore each product within plant inoculation and time point was deployed

5   and also, the plant inoculation within products and sampling time was deployed

6   as factors that better explained the variations obtained by means of Scott-Knott

7   (p>0,05). Thus, allowing inferences on the performance of each product in

8   inoculated or non-inoculated plant at each time point and also for comparisons

9   about the inoculation plants in particular product within each time point.

10 The CAT activity was most effectively increased by CEO and ASM following

11 by CNE treatment for the product factor within each time point and plant

12 inoculation. Where the CEO and ASM increased significantly the activity in

13 non-inoculated at 84 and 144 HPS being most the CEO most effective (Scott-

14 Knott, $p < 0.05$) than ASM in the former time point and regarding the latter time

15 point CEO, ASM and CNE were significantly similar and effective on

16 increasing this enzyme activity (Fig. 3A). For inoculated plants, the CEO and

17 ASM products increased significantly the CAT activity at 96 HPS, overcoming

18 the other treatments for non-inoculated plants and at 96 and 144 HPS for

19 inoculated plants. For the inoculation factor, within each time point and product,

20 it was observed that all time points were similar or significantly higher for

21 inoculated plants, except for the first time point, where the CEO sprayed plants

1   showed a significantly higher increase in CAT activity on non-inoculated plants.

2   In all other time points it was observed a significantly increasing on activity for

3   inoculated plants with significantly higher increase at 96 HPS for CEO and

4   ASM and in the same way, at 120 and 144 HPI for the control plants compared

5   to non-inoculated plants.

6   The SOD and GLU activity did not showed significance for interaction among

7   the factors product, time points and plant inoculation, demonstrating that those

8   factors promoting no influence each other when considered together. Then, a

9   clearly capability to activate both SOD and GLU activity was significantly

10   higher than control plants and similar among ASM, CEO and its compound

11   CNE (Table 4), evidencing the capability of both essential oil and its active

12   substance CNE on plant defense activation. With respect to time point post

13   product spraying, it was observed that for SOD activity, the higher activity was

14   reached by the lower time points collected being 84 HPS followed by 96; 120;

15   and 144 HPS where, each single time point showed significance difference

16   (Scott-Knott $P \leq 0.05$) each other.

17

1 **Table 4.** Enzymatic activity of superoxide dismutase (SOD) and β -1,3-
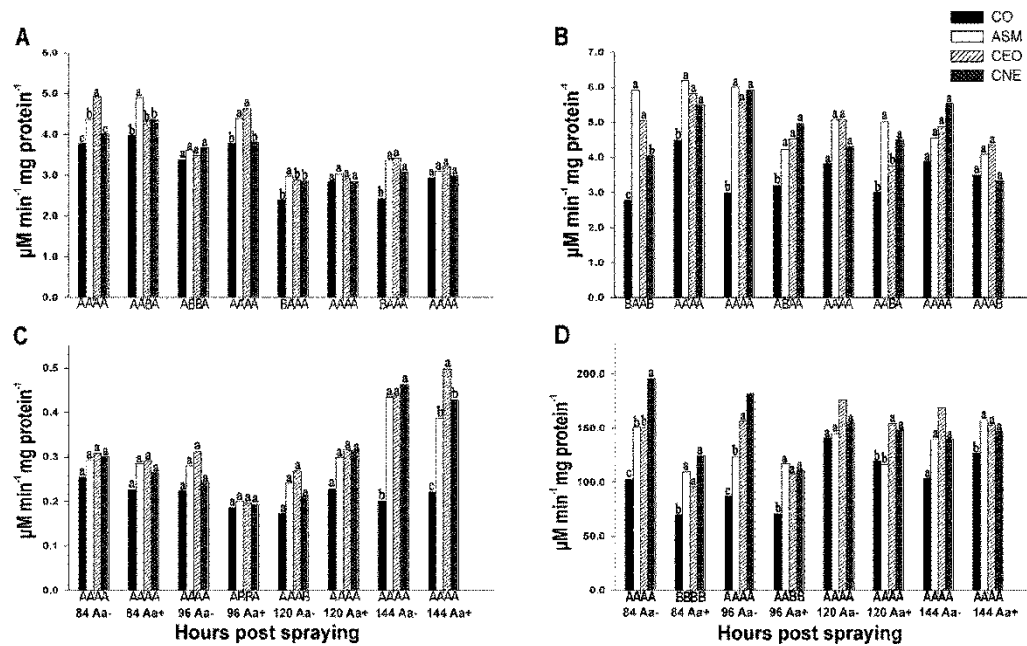2 glucanase (GLU) related to product spraying and time point collected.

Product[a]	Activity		Time point[b]	Activity	
	SOD	GLU		SOD	GLU
CO	0.3321 b	2.7554 b	84	0.5150 a	2.9037 a
CNE	0.3993 a	2.8260 a	96	0.4069 b	2.7918 b
ASM	0.4074 a	2.8376 a	120	0.3277 c	2.7652 c
CEO	0.4160 a	2.8390 a	144	0.3051 d	2.7971 b

3 [a]Products spraying on citrus plant: CO - Control Tween 80 at 0,01%; CNE -
4 *trans*-cinnamaldehyde at 250μg mL^{-1}; ASM - S-methyl ester of benzo [1, 2, 3]
5 thiadiazole-7-carbothioic, at 800 μg mL^{-1}; CEO - Cinnamomun zeylanicum
6 leaves essential oil at 1000 μg mL^{-1}. [b]Time points expressed in hours post
7 product spraying (HPS).
8

9 For the GLU activity, it was obtained that the higher enzyme activity at 84 HPS,

10 followed of 96 and 144 HPS which showed the same potential activity and the

11 lower activity time point reached at 120 HPS. This fact, of decrease at 120 HPS

12 with posterior increase at 144 HPS, might indicate a possible tendency for an

13 increment on GLU activity with latter in time points (higher than 144 HPS).

14 The APX activity for the product factor within each time point and plant

15 inoculation, in general was most effectively increased for the ASM, followed by

16 CNE and CEO (Fig. 3B). Where the ASM treatment exhibited a significantly

17 increase in APX activity in all time points for both inoculated and non-

18 inoculated plants. Similarly, the CNE treatment presented a significant increase

19 on APX activity in all time points for both inoculated and non-inoculated plants,

20 although in the first time point (84 HAS) in non-inoculated plants treated by

21 CNE had a lower increase than plants treated by ASM and CEO, this increase

22 was significantly higher when compared to the control plants. A correspondent

1   situation was observed for plants treated by CEO which also resulted in an

2   increase for all time points within inoculated or non-inoculated plants. Where,

3   except for inoculated plants at 120 HPS, it was observed that CEO spraying

4   resulted in a lower increase than the reached by CNE and ASM treated plants,

5   even though, when compared with control plants, this increase was significantly

6   higher.

7   In respect to the inoculation factor within each time point and product, it was

8   obtained that for the first time point (84 HPS) control plants and CNE treated

9   plants showed an increase significant statistically for inoculated plants, in the

10 second time point (96 HPS), only ASM treated plants and inoculated showed a

11 significantly lower increase on APX activity than non-inoculated plants, while

12 for all other treatments the activity between inoculated or non-inoculated was

13 similar statistically. An analogue situation was observed in the two latter time

14 points, where at 120 HPS the increase in APX activity was significantly lower in

15 inoculated plants only for the CEO treatment; and at 144 HPS the increase in

16 APX activity was lower in inoculated plants only for the CNE treatment.

1
2 **Figure 3.** Activities of A catalase (CAT), B ascorbate peroxidase (APX), C phenylalanine ammonia-lyase (PAL); D
3 peroxidase (POX) in leaves of citrus plants noninoculated and inculated by *Alternaria alternata*, indicated by hours post
4 product spraying (HPS) followed by Aa- and Aa+ respectively. Means within Aa- or Aa+ treatments followed by the
5 same lowercase letter above the graphic bars, for each product, are significantly different+ ($p \leq 0.05$) Scott-Knott test;
6 means from Aa- and Aa followed by the same uppercase letter bellow the graphic bars, for each product, are significantly
7 different. Three experiments were conducted with consistent results and the data presented represent the best experiment.
8 Bars represent the mean of the mean three independent biological experiments with 3 replicates each (n = 9).

1   There was significant increasing in PAL activity for all products evaluated

2   achieved only at the last time point compared with the control. Where for non-

3   inoculated plants, all products stimulated the increasing on PAL activity

4   similarly, when compared with the control plants (Fig. 3C). For inoculated

5   plants, the CNE sprayed plants resulted in higher increasing on PAL activity,

6   followed by CEO and ASM that showed similar increase on PAL activity and

7   higher than control plants sprayed by distilled sterile water in this case.

8   Regarding to the inoculation factor within each time point and product, there

9   lower increase in PAL activity only at 96 HPS for ASM and CEO sprayed and

10   inoculated plants. However at 120 HPS there was an increase on PAL activity

11   observed to inoculated plants that was treated by the product CNE, as a pure

12   compound from the cinnamon essential.

13   The POX activity was most effectively increased by CNE for all evaluated time

14   points with or without plant inoculation, which was followed by CEO and then

15   by ASM sprayed plants (Fig 3D). Where CNE showed a significant increasing

16   on POX activity overcoming all other products independently of time point

17   analyzed and plant inoculation. Similar result was obtained by plants sprayed

18   with CEO that, except in the first time point, 96 HPS within non-inoculated

19   plants. Where it showed an increase on POX activity, which was similar to ASM

20   but lower than the CNE treatment, for all other time points, independently on

21   plant inoculation it presented capability to increase the POX activity at the same

1 level reached by CNE sprayed plants. Although all ASM treated plant had

2 showed a capability on increasing of POX activity on all time points for

3 inoculated or non-inoculated plants if compared with the control, its capability

4 was overcame by CNE and CEO at 84 and 96 HPS non-inoculated plants and at

5 120 HPS inoculated plants. Regarding the inoculation factor, within time point

6 and product, there was not observed significant differences for the last two time

7 point evaluated (120 and 144 HPS) however, there was observed a significant

8 lower increasing on POX activity for all inoculated treatments at 84 HPS. A

9 lower increasing on POX activity also was registered at 96 HPS for the

10 treatments CEO and CNE on inoculated plants when compared the same

11 treatments for non-inoculated plants.

12 Regarding the quantification of defense enzymes in plants treated with natural

13 products, in analogy with the results found by CEO and its compound CNE,

14 some researches examples only could be found in other pathosystems. For

15 example, Pereira et al., (2012b) found increase activity on POX at 336 HPS

16 treated by citronella essential oil in order to control the plant pathogen brown

17 eye spot on coffee plants. Similarly Lucas et al., (2012) found increased activity

18 of peroxidases, chitinase and β -1,3-glucanase in tomato plants sprayed with

19 clove essential oil and challenged with *Xanthomonas vesicatoria*, Also, some

20 promising results on inducing plant resistance by essential oil spraying have

21 been shown by Pereira et al. (2008), whom demonstrated an increased POX

1 activity in coffee plants sprayed with thyme essential oil when challenged with

2 *Cercospora coffeicola* at seven and eleven days post spraying.

3 With respect to the low differences on enzyme activity among inoculated and

4 non-inoculated plants, our results are in agreement with those obtained by

5 Vanackeret al. (1998), which found that although Pathogen-induced increases in

6 the apoplastic antioxidant SOD, CAT, APX, among other enzyme activities

7 were observed, the inoculation had no effect on the total foliar ascorbate pool

8 size or the redox state. One interesting fact was pointed by Jennings et al. (1998)

9 whom demonstrated that *A. alternata* is capable to produce and secrete mannitol

10 as a response to host factors, in order to suppress reactive oxygen species-

11 mediated defenses. Considering that some fungal pathogens may take advantage

12 from an increase in ROS levels, higher SOD activity in reached by plants treated

13 by CEO and CNE could be a strategy to restrict pathogen colonization once the

14 additional ROS can be removed from the plant tissues, as highlighted by Debona

15 et al. (2012).

16 The results obtained by the plant defense enzyme activation capability assay,

17 were consistent with those obtained in the experiments conducted aiming to

18 evaluate the control of ABS progress in field, which also revealed close results

19 between ASM and CEO spraying on plants. Taken together, such results with

20 those proven the antifungal activity by CEO and CNE it was evidenced a mutual

21 effect, characterized by direct antifungal activity and an indirect activity by

1 activation of plant defense response against plant pathogen reached. Such

2 mutual effect direct on pathogen and indirect on plant resistance inducing was

3 also reported by other author before (Pereira et al., 2012b) in coffee plants

4 treated by citronella (*Cymbopogon nardus*) essential oil.

5

6 **MATERIALS AND METHODS**

7 **Essential Oil and *trans*-cinnamaldehyde**

8 The *Cinnamomun zeylanicum* L. essential oil (CEO) was acquired from

9 Professor Accorsi Medicinal Plants[®](Piracicaba, SP, Brazil) previously shown to

10 control other plant diseases. It was stored at 4°C in dark vials until tested. The

11 *trans*-cinnamaldehyde (CNE) purity $\geq$ 98%, was acquired from Sigma-Aldrich[®]

12 (Cas number 14371-10-9).

13 **Fungal strain**

14 The strain of *A. alternata* tangerine pathotype used in this study was isolated

15 from Ponkan previously obtained from a severely infected tangerine fruits. It

16 was single-spored and identified using conidium morphology as *A. alternata*.

17 Pathogenicity tests on susceptible 'ponkan' tangerine fruits and leaves

18 confirmed it to be the tangerine pathotype of *A. alternata* (Whiteside, 1976). It

19 was stored in the 'Coleção micológica de Lavras' Universidade Federal de

20 Lavras, MG - Brazil culture collection under the register number CML

21 XX2014Aa. Single spore isolates were placed on potato dextrose agar (PDA Hi

22 Media) plates for 7-10 days at 25 ^{o}C under constant illumination to produce

1   conidia (Carvalho et al., 2012) in order to use as an isolate source for the

2   bioassays.

3   **Analysis of the Essential Oil and Compound Identification**

4   The CEO analysis was performed using gas chromatography coupled to mass

5   spectrometer (Schimadzu mod QP2010 Plus), working at 70 eV, with a DB-5

6   capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 µm), ultrapure helium as carrier gas,

7   split 1:15, manual injection, injector at 220º C and 77 minutes of running time. It

8   was used the following program: column heating at 60º C for 2min; heating until

9   240ºC at 3ºC minute^{-1}; stayed at 240ºC for 15 minutes; interface GC-MS at

10   250ºC and ion bean with 200ºC; at 1.0 mL min^{-1}. The identification of the

11   chemical constituents was based on comparisons of their GC their mass spectra

12   with the GC-MS system databank (FF NSC 1.2.lib) and retention indices (RI) on

13   an apolar column (Adams, 2007).

14   **Fungal growth inhibition assays**

15   **Minimum Inhibitory Concentration (MIC)**

16   Aiming to elucidate the most active substance against *A. alternata,* the CEO, its

17   major substances found in the former experiment, eugenol and CNE was

18   dissolved in Tween 80 at 1000 µg mL^{-1} in potato dextrose broth (PDB) liquid

19   medium (Hi Media product number M403) under a sterile test-tube. A sample

20   (100 µL) of each final solution (CEO, eugenol or CNE + Tween 80 + PDB) was

21   added into the first and second rows of 96-well plates, performing serial

1 dilutions, resulting in the concentration of CEO, eugenol and CNE to range from

2 1000 to 31.25 µg mL^{-1} obtained by addition of a suspension at 2.0 x 10^{4}conidia

3 mL^{-1} of *A. alternata* into the wells. The controls for this experiment were: 1)

4 Tween 80 (since it was used for the preparation of all different treatment tested

5 as dispersant); 2) Copper oxychloride (Recop$^®$ CuCl$_2$.3Cu(OH)$_2$ at 840g.kg^{-1};

6 Atar do Brasil Defensivos Agrícolas Ltda), with concentration ranged from 2500

7 to 1.22 µg mL^{-1} in the wells; and 3) Triazole and strobilurin mixture [Nativo$^®$

8 (100 g of trifloxystrobin + 200 g of tebuconazole) L^{-1}; Bayer, Brazil], for which

9 the concentration was in the same range as Recop$^®$ 4) The commercial plant

10 activator S-methyl ester of benzo [1, 2, 3] thiadiazole-7-carbothioic (ASM) with

11 concentration ranged from 800 to 25 µg mL^{-1}. All plates were incubated at 25

12 ºC with a 12h photoperiod for 72 h. The lowest concentration of each product

13 that prevented fungal development according to visual observation was

14 considered to be the minimal inhibitory concentration (MIC). In order to observe

15 whether each treatment were fungistatic or fungicidal against the *A. alternata* the

16 MIC obtained for each treatment and its replicate, were re-suspended in a fresh

17 PDB liquid medium for more 72 hours and then evaluated while a possible

18 revival of mycelial growth by visual observation.

19 **Mycelial growth rate (MGR)**

20 To examine the effect of CEO, CNE and eugenol on mycelial growth under solid

21 growth substrate, PDA (Hi Media product number M096) were prepared and

1   autoclaved at 121 °C for 20 min. Then 15 mL of PDA amended with CEO,

2   CNE, Tween 80, commercial fungicides Recop® and Nativo® and the plant

3   activator ASM, at final concentrations equivalents to the value of their MIC

4   obtained in the previous assay (section 2.4.1), were poured into 9 cm diameter

5   Petri dishes. Eight replicate dishes of each treatment were inoculated centrally

6   with a 5 mm mycelial disc cut from a 10-day-old colony of *A. alternata* grown

7   on PDA and incubated at 25º C with 12h of photoperiod. Two black lines were

8   drawn across the bottom of the dishes at right angles through the center using a

9   marker pen. In the next step, the mycelial growth was evaluated daily recording

10   the radial mycelial growth across the four axes using a digital caliper rule

11   (*Pittsburgh code* 47260 8"). This trial was conducted twice, comprising two

12   different experiments under the same conditions.

13   **Control of ABS disease progress in field**

14   All experiment was conducted in a commercial orchard located in Pouso Alegre,

15   MG South Minas Gerais State (Brazil) at 22º13'48"S, 45º56'11"W and 900 m of

16   altitude, with natural incidence of ABS through 2010 and 2011spring and early

17   summer season. CEO at 1000 µg mL^{-1} ASM at 800 µg mL^{-1} Copper

18   oxychloride fungicide (Recop®) at 2500 µg mL^{-1}, triazole and strobilurin

19   mixture fungicide (Nativo®) at 300 µg mL^{-1} were evaluated in both experiments.

20   All products were added by Tween 80 at 1000 µg mL^{-1}.The control treatment

21   consisted by plots without any product spray with the same replicate number.

1 Trees were sprayed to runoff with 6.0 L plant^{-1} at 3000 KPa through a motorized

2 sprayer (Backpack Stihl Sr420, USA, Cifarelli engine), equipped with a hand-

3 held spray gun. It were performed a two week spray schedule (Vicent et al.,

4 2009) aiming to protect expanding leaf and shoots releases during spring and

5 early summer. Thus, a total of 4 sprays was performed. The experimental plot of

6 120 m^2 was used for each treatment replicate, comprising a total experimental

7 area of 2400 m^2, where guard trees were located between plots within rows and

8 one guard row was located between treated rows, arranged in a completely

9 randomized design. The orchard was planted in 1999 with tangerine cv. Ponkan

10 (*C. reticulata* Blanco) in *Citrus limonia* Osbeck cv. Cravo rootstock on a 3-by-

11 4-m spacing. The orchard had rows in an east-west orientation and was managed

12 with fertilization; pruning and irrigation followed recommended practices,

13 except for pest control, which were not applied in the area during the two years

14 experimental period.

15 The incidence of ABS was evaluated counting the number of branches that had

16 infected leaves and young shoots in the useful experimental plot (six plants for

17 each replicate) within 14 points previously marked in a 1.5 m wide range along

18 the entire plant canopy radius, according to the following scoring scale: 1 = no

19 leaves or young shoots symptoms; 2 = 1-2 symptomatic points (SP) 3 = 3-4 SP;

20 4 = 5-6 SP; 5 = 7-8 SP; 6 = 9-10 SP; 7 = 11-12 SP; 8 = 13-14 SP in leaves and

21 young shoots throughout marked points on the tree canopy radius. A total of five

1   ABS incidence evaluations were performed every 20 days after first product

2   spray. It was used five replications and experimental units comprising of 3 trees.

3   In each experiment, differences between the untreated control and other

4   treatments were analyzed with the Scott-Knott test and p values ≤ 0.05.

5   **Plant induced-resistance by CEO and CNE**

6   In order to characterize the biochemical mechanisms of resistance in Ponkan

7   tangerine plants to tangerine pathotype of *A. alternata* induced by stimulation of

8   CEO and its compound CNE, three-month old plants of Ponkan tangerine (*C.*

9   *reticulata*) were treated with: 1) CEO at 1000 μg mL^{-1}; 2) CNE at 250μg mL^{-1}; 3

10 ASM at 800 μg mL^{-1} and; 4) Distilled sterile water added to Tween 80 at 1000

11 μg mL^{-1} was used as a control. All the treatments included plants with and

12 without inoculation and were added to Tween 80 at 1000 μg mL^{-1} as dispersant.

13 A randomized block design was used with three replications and experimental

14 units consisting of two plants. For inoculation, conidial suspension (10^6 conidia

15 mL^{-1}) was produced and sprayed onto leaves at three days after application

16 (DAA), 14 leaves were collected per replication for each treatment throughout

17 the time course at 84, 96, 120 and 144 hours post spraying (HPS). Thus, samples

18 were immediately frozen in liquid nitrogen and then stored at $-80°C$ for further

19 analysis. Fresh leaf material (mix of the 14 leaves collected per treatment) from

20 treated and untreated tangerine plants were ground into a fine powder using a

21 pestle and mortar in liquid nitrogen. The entire assay was conducted in

1 triplicates for each time point collected.

2 For the protein extract targeting to determine the peroxidase (POX; EC

3 1.11.1.7), phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and β -1,3-glucanase

4 (GLU; EC 3.2.1.6) activity, 200 mg of leaf tissue leaf powder was homogenized

5 in an ice bath with 1.5 ml of a sodium phosphate buffer (50 mM; pH 6.5). To

6 examine the activity of superoxide dismutase (SOD; EC 1.15.1.1), catalase

7 (CAT; EC 1.11.1.6) and L-ascorbate peroxidase (APX; EC 1.11.1.11), 200 mg

8 of leaf tissue leaf powder was homogenized in an ice bath with 1.5 ml of

9 potassium phosphate buffer (400 mM; pH 7.8), containing EDTA (10 mM)

10 ascorbic acid (200mM) and 1% (wt/vol) polyvinylpolypyrrolidone (PVP). For

11 all enzyme activity, the homogenate was centrifuged at $13,000 \times g$ for 25 min at

12 4°C, and the supernatant was used as a crude enzyme extract. All steps were

13 carried out at 0-4 °C. The protein content of crude extracts was determined using

14 the Bradford (1976) protein assay, with bovine serum albumin (BSA) as a

15 standard.

16 The POX activity was examined according to Kar and Mishra (1976). The

17 reaction mixture contained sodium phosphate buffer (100 mM; pH 7.0), guaicol

18 (50 mM), and H_2O_2 (125 mM) in a final amount of 1.5 ml added of 10 μL of the

19 crude enzyme extract to the reaction mixture. Thus the reaction was started and

20 then the POX activity was determined through the absorbance of colored

21 purpurogallin recorded at 420 nm for 2 min at 25°C measured in an EIA reader

1 (Wirth and Wolf, 1990). It was used an extinction coefficient of 2.47 mM^{-1}cm $^{-}$

2 ^{1}(Chance and Maehley, 1955) to calculate the POX activity, which was

3 expressed as micromoles of purpurogallin produced per minute per milligram of

4 protein.

5 The PAL reaction was started upon addition of 5 µL extract to a mixture

6 containing 145 µL of buffer 50 mM Tris-HCl (pH 8.8) and µL of 50 mM L^{-1}

7 phenylalanine. Thus the absorbance of the end-product of *trans*-cinnamic acid

8 was measured in an EIA reader spectrophotometer at 290 nm. To estimate the

9 PAL activity, it was used a molar extinction coefficient of 10^{4} mM^{-1} cm^{-1}

10 (Zucker, 1965), which was expressed as micromoles per minute per milligram of

11 protein.

12 The GLU activity of β -1,3-glucanase (GLU; EC 3.2.1.6) was measured

13 CMCurdlan-RBB (4.0 mg mL^{-1}) a specific substrate for β -1,3-glucanase,

14 supplied by Loewe Biochemica GmbH and adjusting the amount of enzyme

15 extract to 100 µL (reducing the volume of acetate buffer to adjust the final

16 volume to 310 µL per well, in a 96-well microplate, with a capacity of 350

17 µL. To promote the hydrolytic action of β -1,3-glucanase, it was used an

18 incubation step for 100 minutes at 35º C. The samples were then measured

19 photochemically with a 620 nm filter in an EIA reader.

20 The SOD activity was determined by measuring its ability to photochemically

21 reduce the p-nitrotetrazole blue (NTB) (Del Longo et al., 1993). The reaction

1   was started after the addition of 30 µl of the crude enzyme extract to 1.5 ml of a

2   mixture containing potassium phosphate buffer at 100 mM (pH 7.8), methionine

3   (70mM), *p*-Nitrotetrazolium blue at 1mM, EDTA (10 µM) and riboflavin (0.2

4   mM). The reaction was incubated at 25°C under 15W daylight fluorescent lamps

5   (Philips 15W, T5) for 10 min. In the next step, the production of formazan blue,

6   which resulted from the photo-reduction of NTB, which was monitored by the

7   increase in absorbance at 560 nm measured in an EIA reader. Values obtained

8   were subtracted from the values obtained from the samples of the replications

9   for each treatment exposed to light. One unit of SOD was defined as the amount

10 of enzyme necessary to inhibit NBT photoreduction by 50% (Beauchamp and

11 Fridovich, 1971).

12 The CAT activity was determined following the method of Cakmak and

13 Marschner (1991). The reaction mixture was composed by potassium phosphate

14 buffer at 200 mM (pH 7.0) and 250 mM H_2O_2 in a total volume of 1.5 ml. After

15 the addition of 10 µl of the crude enzyme extract to the reaction mixture, the

16 CAT activity was measured by means of the rate of H_2O_2 decomposition at 240

17 nm for 1 min at 25°C measured in an EIA reader. To calculate the CAT activity,

18 it was used an extinction coefficient of 36 M^{-1} cm^{-1}(Anderson et al., 1995)

19 where it was expressed as milimoles per minute per milligram of protein.

20 The APX activity was determined according to Nakano and Asada (1981). The

21 reaction mixture comprised of potassium phosphate buffer at 200 mM (pH 7.0),

1   H_2O_2 (2 mM) and ascorbic acid (10mM) in 1.5 mL of final volume, achieved by

2   the addition of 10 µl of the crude enzyme extract performing the reaction. The

3   APX activity was measured by the rate of ascorbic acid oxidation at 290 nm for

4   1 min at 25°C in an EIA reader. It was used an extinction coefficient of 2.8 mM^-

5   ^{1} cm^{-1} (Nakano e Asada, 1981) to calculate the APX activity, which was

6   expressed as millimoles $minute^{-1}$ per milligram of protein.

7   **CONCLUSIONS**

8   The activity of *C. zeylanicum* essential oil (CEO) against the fungus *A. alternata*

9   was confirmed and mainly attributed to the substance *trans*-cinnamaldehyde

10   (CNE), which demonstrated high activity against *A. alternata* growth

11   outperforming the CEO and eugenol. The CEO was capable to reduce the

12   disease incidence progress in tangerine commercial orchards at high levels than

13   the cooper fungicide and comparable to a commercial plant activator. The CEO

14   and its mainly active substance CNE stimulate the plant defense system,

15   providing significant increases in enzyme levels at some point. The CEO was

16   most effective on enzyme activation than its compound CNE.

17   **ACKNOWLEDGEMENTS**

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(VERSÃO PRELIMINAR)

ARTIGO 2 *Thymus vulgaris* **essential oil and thymol against** *Alternaria alternata* **(Fr.) Keissler: effects on growth, viability, early infection, and cellular activity**

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Thymus vulgaris essential oil and thymol against Alternaria alternata (Fr.) Keissler: Effects on growth, viability, early infection, and cellular activity

Running title: Thymol (2-isopropyl-5-methylphenol) for the control of
Alternaria alternata: mode of action

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Abstract

BACKGROUND: In initial assays *Thymus vulgaris* essential oil (TEO) has demonstrated activity against several plant-pathogenic fungi and has reduced the fungal diseases to levels comparable to commercial fungicides. Thus, the goal of this work was to identify the major active substance in this essential oil and recognise its putative cellular activity in fungi using an ultrastructure approach. RESULTS: Gas chromatography-mass spectrometry (GC-MS) analysis of TEO from leaves resulted in the identification of 2-isopropyl-5-methylphenol (thymol, henceforth called TOH) as a major compound. This compound had minimal inhibitory concentrations (MICs) of 250 µg mL^{-1} against *A. alternata*; under the same conditions, MICs for commercial fungicides and TEO were 1250 and 500 µg mL^{-1}, respectively. Ultrastructure analysis showed that this monoterpene phenolic substance prevented fungal growth, decreased fungal viability and prevented the penetration in fruits by a cell wall/plasma membrane interference mode of action with organelles targeted for destruction in the cytoplasm. CONCLUSION: These findings suggest that TOH was responsible for the anti-fungal activity of TEO. Therefore, both the essential oil and its major substance have potential for use in the development of new phenolic structures and analogues to control Alternaria brown spot disease caused by *A. alternata*.

Keywords: early infection, mode of action, ultrastructure, cellular activity.

1 INTRODUCTION

Alternaria brown spot (ABS), caused by *Alternaria alternata* (Fr.) Keissler tangerine pathotype,[1–3] is one of the most important diseases that affects tangerine plants and their hybrids. In addition to tangerine, the pathogen also infects grapefruit and their hybrids, occasionally causing serious economic losses in commercial tangerine fields in Brazil. The pathogen infects young tissues of leaves, branches, and fruits. Symptoms can appear as quickly as 24 h after infection.[1] Producing the ACT-toxin, it causes rapid necrosis in plant tissues, leading to the featured ABS symptoms.[4] Affected fruits experience accelerated maturation, which may cause them to drop before harvest, increasing loss. Formerly, ABS control in Brazil involved copper-based fungicides.[5] However, the spread and infection of the pathogen is highly subject to environmental conditions. Because such conditions are commonly found in Brazilian tangerine fields, numerous applications can be necessary to control ABS, resulting in production of tangerines that is economically unfeasible[4] and harmful to the environment.

Thus, one alternative to overcome such problem involves the development of new products to control this disease based on natural products. Of the countless metabolites produced by plants, three classes of natural products are prevalent: alkaloids, terpenoids, and phenolic compounds, which possibly act directly against fungi. Such metabolites may serve as promising

sources of additional products to control plant disease. Thus, much research has been conducted regarding the capability of natural products against plant pathogens.[6–14] Regarding essential oils against plant pathogens, numerous research has demonstrated their effectiveness,[8–17] and *T. vulgaris* and *Thymus* spp. regularly have been demonstrated to exhibit the best performances,[7,10,12] which have occasionally shown levels of control comparable to commercial fungicides.[7] Although examples concerning composition of active substances obtained from plant essential oils against microorganisms are easily found in food and medicine,[12,13] such studies rarely are found in plant-pathogenic fungi.[10] Hence, little is known about the precise cellular mechanism of their antimicrobial action.[17] Also, no study to date has demonstrated the effects of TEO and its main constituent activities against plant-pathogenic fungi at the cellular level.

Thymus oils have been extensively investigated due to their popularity and medicinal properties. Reports indicate that the oils of thyme and their species are among the main essential oils used in the food industry in addition to those used in cosmetics as preservatives and antioxidants.[18] In effect, the TEO from *Thymus vulgaris* L. (Lamiaceae), which is native to the western Mediterranean region of Europe, has long been used due its medicinal properties compared to the other species.[19] It consists of a mixture of phenolic compounds, with the main compound being the natural monoterpene phenolic TOH.[20]

Therefore, to contribute to the development of new products for the control of fungal plant diseases, the goals of this work were to identify and compare the abilities of TEO and its main substance, TOH, to reduce the growth and viability of *A. alternata in vitro* as well as the implications of the direct effects in the early infection stages associated with the disease progression. Ultimately, a transmission electron microscopy (TEM) ultrastructure analysis was performed to identify and compare the activities of TEO and its main substance at the cellular level.

2 MATERIALS AND METHODS

2.1 Essential oil and its major substance, TOH

The TEO was acquired from Professor Accorsi Medicinal Plants® (Piracicaba, SP, Brazil) due to its ability to control other diseases.[21] It was stored at 4°C in dark vials until tested. The TOH was acquired from Sigma-Aldrich (CAS number 98-83-8; product Sigma-T0501), and its purity was $\geq 99.5\%$.

2.2 Fungal strain

The strain of *A. alternata* tangerine pathotype used in this study was isolated from Ponkan tangerine fruits and grown on potato dextrose agar (PDA) plates for seven days at 25°C under constant illumination using Philips daylight fluorescent lamps (20W, TLT, 75RS) to produce conidia.[6]

2.3 Analysis of the essential oil and compound identification

The TEO analysis was performed using gas chromatography coupled to a mass spectrometer (Schimadzu model QP2010 Plus) running at 70 eV with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm) using ultrapure helium as a carrier gas, split 1:15 with manual injection, using an injector at 220ºC for 77 min of running time. The following program was used: column heating at 60ºC for 2 min; heating until 240ºC at 3ºC min^{-1}; hold at 240ºC for 15 min; interface GC-MS at 250ºC and ion beam at 200ºC at 1.0 mL min^{-1}. The identification of the chemical constituents was based on comparisons of their GC mass spectra with the GC-MS system databank (FF NSC 1.2.lib) and retention indices (RI) on an apolar column.[22]

2.4 Fungal growth and viability assays

2.4.1 Minimum inhibitory concentration (MIC)

The TEO and TOH were dissolved in Tween 80 at 1000 μg mL^{-1} final concentration in a liquid medium potato dextrose broth (PDB) (Hi Media product number M403) in a sterile test tube. A sample (100 μL) of each final solution (TEO or TOH + Tween 80 + PDB) was added to the first and second rows of 96-well plates, with 100 μL of PDB added to the contents of the second-row wells. After homogenisation, 100 μL of the well contents of the second row were transferred to the third row, and this process was repeated to the fifth row.

Finally, a 100-µL aqueous suspension containing 2.0×10^4 conidia mL^{-1} of *A. alternata* was added to the wells, resulting in the concentrations of TEO and TOH ranging from 31.25–1000µg mL^{-1}. The controls for this experiment included the following: 1) Tween 80 (which was used as dispersant for all treatments); 2) the commercial fungicide Recop® (CuCl$_2$.3Cu(OH)$_2$ at 840g kg^{-1}; Atar of Brazil Agrochemicals, Ltd), with concentration ranging from 1.22–2500 µg mL^{-1} in the wells; and 3) the commercial fungicide Nativo® ([100 g trifloxystrobin + 200 g tebuconazole] L^{-1}; Bayer, Brazil), for which the concentration was in the same range as Recop®. All plates were incubated at 25ºC with a 12-h photoperiod for 72 h. The lowest concentration of each product that prevented fungal development according to visual observation was considered to be the minimal inhibitory concentration (MIC).

2.4.2 Mycelial growth rate (MGR)

To examine the effects of TEO and TOH on mycelial growth, potato dextrose agar PDA (Hi Media product number M096) was prepared and autoclaved at 121°C for 20 min. Then, 15 mL of PDA amended with TEO, TOH, Tween 80, and the commercial fungicides Recop® and Nativo® at final concentrations equivalent to their final MIC values (observed in the previous assay [section 2.4.1]) were poured into 9-cm diameter Petri dishes. Two black lines were drawn across the bottom of the dishes at right angles through the centre using a

marker pen. Eight replicate dishes of each treatment were inoculated centrally with a 5-mm mycelial disc cut from a 10-day-old colony of *A. alternata* grown on PDA and incubated at 25ºC with a 12-h photoperiod. The mycelial growth was evaluated daily by recording the radial mycelial growth across the four axes using a digital calliper rule (Company: Pittsburg). This trial was conducted twice, comprising two different experiments under the same conditions.

2.4.3 Conidial viability

The conidia of *A. alternata* comprise the structure responsible for epidemic complications caused by ABS disease. In order to investigate the conidial viability when treated with TEO and its major pure substance, a viability assay was performed. The conidial cell viability was determined by measuring the germination capability when exposed to different TEO and TOH concentrations. Conidia were considered viable if they had a germ tube equivalent to a half-length of their own size, with normal structure preserved. This assay was conducted in sterile 96-wells plates. The concentrations of TEO, TOH, and the commercial fungicide Recop® ranged from 31.25−1000 µg mL^{-1}, while the commercial fungicide Nativo® ranged from 18.75−600 µg mL^{-1}. A control was used under similar experimental conditions with the dispersant Tween 80 (at 1000 µg mL^{-1}) in the absence of TEO and TOH. An inoculum of 1.0×10^4 conidia mL^{-1} was incubated with TEO and TOH in PDB growth media at 25ºC

under illumination (daylight fluorescent lamps; Philips 20W, TLT, 75RS) for 12 h. The total amount of germinated conidia was determined microscopically using a haemocytometer (Neubaeur cell-counter chamber), and the results were expressed as percentage of germination. One hundred conidia per concentration with six replicates and blocks containing four replications were counted (2400 conidia in total). Germination was observed and quantified in a Zeiss Axio Observer Z.1 microscope coupled with an AXIO CAM MRm-ZEISS digital camera. The trial was conducted twice, consisting of biological replicates in different time periods in the same conditions.

2.5 Effects of early infection events inferred by scanning electron microscopy (SEM) and *in-vivo* effects on disease progress

An assay was conducted to evaluate the early infection events of *A. alternata* affected by TEO and TOH *in vivo* in a time-course experiment. Fresh, ripe, and healthy Ponkan tangerine fruits were used. The fruits were first washed in sterile water and dried for 60 min under aseptic conditions in a laminar flux chamber. Four locations around the plant insertion point were marked using a permanent marker. Then, 30 µL of TEO (500 µg mL^{-1}) and TOH (250 µg mL^{-1}), obtained in an aqueous suspension of 1.0×10^6 conidia mL^{-1} containing Tween 80 at 1000 µg L^{-1}, were added to each selected location. This experiment was performed with eight fruits per treatment using the following as controls: 1) the conidial suspension in Tween 80 (1000 µg L^{-1}); 2) the mixture of conidial suspension and

the fungicide Recop® (CuCl$_2$.3Cu(OH)$_2$ at 840 g kg^{-1} at 2500 µg mL^{-1}; and 3) the mixture of conidial suspension and the fungicide Nativo® ([100 g of trifloxystrobin + 200 g of tebuconazole] L^{-1}; Bayer, Brazil) at 300 µg mL^{-1}. A forth control consisted of eight fruits treated only with Tween 80 (1000 µg L^{-1}) containing no conidia, TEO, TOH, or fungicide. All fruits were kept in a humidity chamber at 25°C under a 12-h photoperiod. Four fruits were collected 6, 12, 15, 18, and 24 hours post inoculation (HPI) to prepare samples for analysis via SEM. Samples were collected using a circular puncher cut (5-mm diameter) and prepared for SEM analysis according to Campos et al.[4] The SEM tracking analysis was conducted in a LEO EVO 40XVP microscope at a 20-kV accelerating voltage and 10.0-mm working distance using the Zeiss Smart SEM User Interface software, version 5.4. All images were edited with the software Photopaint (Corel Draw 12® software suite).

For the ABS progress monitoring throughout the disease progression and comparisons with non-treated fruits and fungicide controls, an assay was conducted with the other four fruits out of eight used in this trial that were kept under the same experimental conditions. At 7 and 10 days post inoculation (DPI), the lesion diameter around each inoculation point was measured with a digital calliper rule (Company: Pittsburg). Average values of spots of each fruit were converted into spot development rates (ABS dr) according to Carvalho et. al.[6]

2.6 The TEO and TOH cellular activity by TEM

To elucidate and compare the cellular mode of action involved in TEO and its main chemical constituent TOH, a TEM bioassay was conducted on the *A. alternata* ultrastructure to observe direct effects of TEO and TOH. The TEO and TOH as well as the control concentrations applied were obtained using the same methodology cited in the previous section (2.5). The substances were diluted by a 1.0×10^6 conidia mL^{-1} *A. alternata* suspension in sterilised-distilled water. All the treatments were added separately in 100-mL glass Erlenmeyer flasks and kept in a shaker at 100 rpm at 25°C under illumination for 24 h. Then, 2.0-mL aliquots of these solutions were quickly placed in microtubes and fixed in a modified Karnovsky fixative solution (0.026 g mL^{-1} glutaraldehyde and 0.025g mL^{-1} paraformaldehyde in 0.05 M sodium cacodylate buffer [pH 7.2] containing 0.001 M $CaCl_2$) for 24 h. The microtubes were centrifuged for 5 min at 6000 rpm and the supernatant discarded. Next, 0.5 mL of a low-melting agarose gel (Sigma Aldrich CAS Number 39346-81-1) at 1.0% was added and carefully mixed with a sterile spatula, generating a gel pellet containing the adhered *A. alternata*. The samples were then prepared for TEM analysis. Briefly, they were fixed in 1% (v/v) osmium tetroxide, dehydrated in ethanol (once at 25, 50, 75, and 90% and three times at 100%), and then infiltrated in increasing Spurr/acetone gradients of 30% (8 h), 70% (12 h), and 100% twice (24 h each). Then, ultrathin (< 100 nm) sections were cut by a Reichrt-jung (ultracut)

ultramicrotome using a diamond knife, collected on copper 300-mesh grids, and allowed to dry on Formvar. Sections were then quickly post-stained with 2% uranyl acetate followed by 3% lead citrate for 3 min. Finally, the samples were examined using a Zeiss EM 109 transmission electron microscope operating at 80 kV. The images generated were digitally recorded and edited in Photopaint.

3 RESULTS AND DISCUSSION

3.1 Antifungal activities of TEO and its main chemical substance TOH against A. alternata

To guarantee the TEO used in this study contained the active substance, GC-MS analysis was performed. The qualitative and quantitative analyses by GC-MS revealed 95.9% of the TEO composition, which is summarised in Table 1. Such analyses revealed the monoterpene phenol TOH (2-isopropyl-5-methylphenol) to be the main constituent (46.4%). Several authors have reported TOH as the major constituent of TEO,[23–25] which agrees with results we obtained for essential oil from leaves of *T. vulgaris*. Thus, this compound was screened in a assay measuring the ability of such substances (including the TEO) to inhibit the growth of *A. alternata* by means of MIC values capable of preventing fungal development according to visual observation.

The TEO and TOH exhibited MIC values equal to 500 and 250 μg mL^{-1}, respectively, while the commercial fungicides Recop[®] and Nativo[®] under the same conditions showed MIC values equal to 1250 and 18.75 μg mL^{-1},

respectively (Table 2). With respect to the larger differences among the MIC values for Nativo® and the other isolated substances, as noted by other authors,[4] it is important to highlight that this commercial fungicide is composed by a combination of triazol and strobilurin groups, two active substances that have different fungicidal modes of action.

Table 1. Chemical composition of *Thymus vulgaris* leaf essential oil.

RT	RI	Compound	Percentage (%)
8.33	1016	α-Terpinene	1.1
8.56	1023	ρ-Cymene	12.1
8.70	1025	Limonene	1.1
9.81	1056	γ-Terpinene	10.7
11.03	1098	Terpinolene	0.2
11.35	1096	Linalool	4.4
13.30	1142	Camphor	1.1
13.85	1154	Isoborneol	3.9
15.32	1190	α-Terpineol	0.4
17.69	1244	Carvacrol, methyl ether	1.9
18.73	1266	Geranial	1.3
19.98	1294	Thymol	46.4
20.35	1302	Carvacrol	0.4
23.88	1384	β-Bourbonene	0.2
24.22	1391	β- Elemene	0.1
25.43	1417	Caryophyllene	6.2
26.76	1452	α-Humulene	0.3
28.82	1496	Valencene	0.3
28.93	1503	α-Muurolene	0.1
29.13	1507	β- Bisabolene	0.1
29.42	1513	γ-Cadinene	0.70
29.98	1525	δ-Cadinene	1.3
32.33	1583	Caryophyllene oxide	1.0
34.17	1631	γ-Eudesmol	0.3
34.67	1642	α-Epi muurolol	0.4
Total identified			95.9

RT: Retention time; RI: Retention index relative to a homologous series of n-alkanes on the DB-5 capillary column.

Table 2. Minimal inhibitory concentration (MIC) of *Thymus vulgaris* leaf essential oil against *Alternaria alternata*.

Treatment	MIC ($\mu g\ mL^{-1}$)
Thymus essential oil (TEO)	500
Thymol (TOH)	250
Control RF[a]	1250
Control NF[b]	18.75

[a]Recop® commercial fungicide composed of $CuCl_2.3Cu(OH)_2$ at 840 g kg^{-1}; [b]Nativo® commercial fungicide composed of trifloxystrobin + tebuconazole (1:2 m/m) at 800 g L^{-1}.

In order to mimic conditions that the substance will encounter in field conditions, a mycelial growth assay was performed to simulate a solid growth environment. Our results showed that the mycelial growth occurred on all media containing the active substance tested, at the same concentration found to inhibit the mycelial growth in the MIC obtained in the previous assay. Significantly ($P \leq 0.05$) slower mycelial growth was found for TOH and the commercial fungicide Nativo® (Figure 1), revealing a strong performance by TOH exposed to a solid growth medium. Under the same conditions, TEO inhibited the MGR, but it was less significant than the commercial fungicide Recop®.

This lower result could be explained due to the large amount of volatile substance found in the TEO composition that is better preserved in liquid media than in solid ones. In fact, GC-MS analysis has shown TEO composition includes diverse substances that could be acting directly on the fungal growth that have low boiling points, a characteristic of volatile compounds.
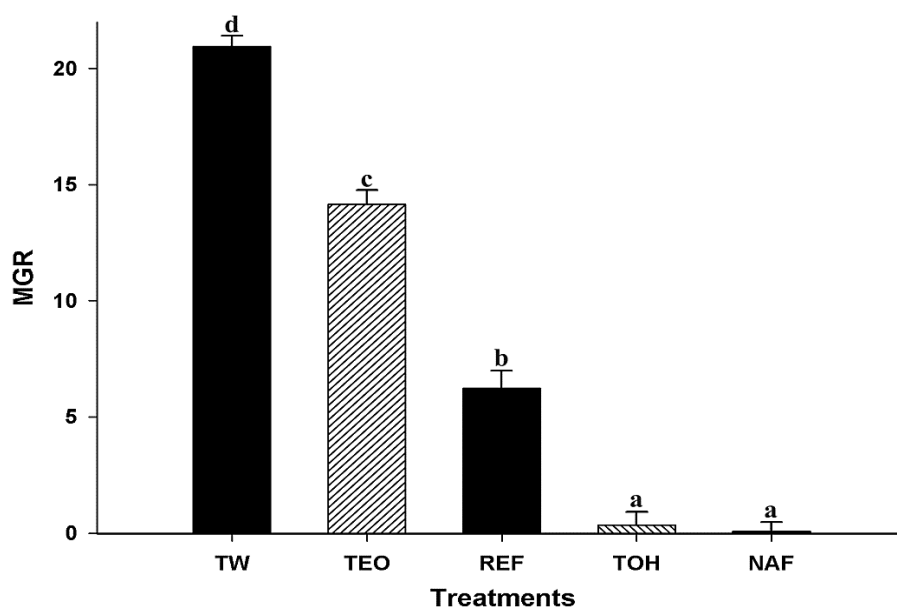
Figure 1. Effect of *Thymus vulgaris* and thymol on mycelial growth rate (MGR) (mm day^{-1}) at 25°C. TW: Tween 80 at 0.01%; TEO: *T. vulgaris* leaf essential oil at 500 µg mL^{-1}; REF: Recop® commercial fungicide composed of CuCl2.3Cu(OH)$_2$ at 840 g kg^{-1}; TOH: thymol at 250 µg mL^{-1}; NAF: Nativo® commercial fungicide composed of trifloxystrobin + tebuconazole (1:2 m/m) at 800 g L^{-1}. Bars followed by the same letter do not differ significantly (P ≤ 0.05) according to the Scott-Knott test (1974). Results are means ± standard error of the mean from two independent experiments with eight replicates each (n = 16).

This is intuitive because TOH has a boiling point of 232°C (450°F). Another potential explanation for the lower performance of TEO observed on MGR might be the possibility of molecular interface interactions among substances found in TEO being more prevalent in the liquid medium, resulting in a higher activity than exists in solid medium. Thus, it is worth noting that in the MIC assay the TEO showed a better performance than the latter commercial

fungicide, highlighting the importance of knowledge of volatile properties of the essential oils as a challenge for practical use in field situations.

Regarding the use of essential oils, several studies have demonstrated the ability of essential oils to reduce fungal plant pathogenic mycelial growth. Teixeira et al.[10] demonstrated that TEO inhibited all mycelial growth of *Stenocarpella maydis* (Berk.) Sutton at concentrations ranging from 1000–20000 µg mL^{-1}. The capability of TEO to decrease mycelial growth was also reported by other authors.[26] They observed a high performance by the TEO among several essential oils against mycelial growth of *Cercospora coffeicola* Berk & Cooke. Such studies usually showed that the *in-vitro* concentrations are at least twice as much than those found *in vivo* or in field concentrations necessary to control plant diseases. However, the effects of mainly pure components against plant pathogenic fungi have been poorly explored. In an attempt to understand the activities of some main substances, such as monoterpenes, a study investigating the capacity of such substances to reduce growth of *Fusarium verticilioides* showed that TOH and limonene exhibited the highest inhibitory effects.[27] Our results are in accordance with those obtained by these authors, who reported promising MIC concentrations for TOH (250 µg mL^{-1}). Regarding the activity of the main substance of TEO on the mycelial growth, our findings are consistent with those obtained by other authors,[28] who found that TOH at concentrations of 200 µg mL^{-1} caused a reduction in the

growth of *Penicillium citrinum*.

The capability of TEO and its isolated substance TOH to reduce the conidial viability produced quadratic dose-response behaviour as the TEO and TOH concentrations increased (Figure 2).
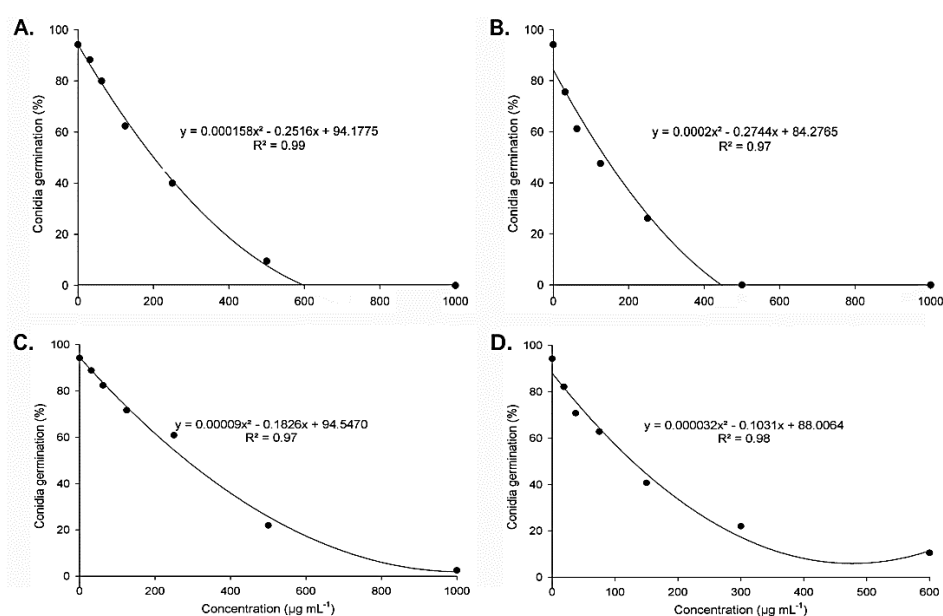

Figure 2. Fitted quadratic regression for different concentrations of *Thymus vulgaris* essential oil, thymol, and commercial fungicides against *Alternaria alternata* conidial germination. Concentrations ranged from 31.25−1000 µg mL^{-1} for A) *T. vulgaris* leaf essential oil; B) thymol; and C) the Recop$^{®}$ commercial fungicide composed of CuCl2.3Cu(OH)$_2$ at 840 g kg^{-1} and ranged from 18.75−600 µg mL^{-1} for D) the Nativo® commercial fungicide composed of trifloxystrobin + tebuconazole (1:2 m/m) at 800 g L^{-1}. Bars followed by the same letter do not differ significantly (P ≤ 0.05) according to the Scott-Knott test (1974). Results are means from two independent experiments with six replicates each (n = 12).

The regression analysis allowed a better understanding of the difference

between tested concentrations. For all evaluated products, the conidial viability correlated negatively with product concentration. TEO concentrations that inhibited all conidial viability were 1000 µg mL^{-1} compared to 500 µg mL^{-1} for TOH. It is also worth noting that total inhibition of germination was not reached using the concentrations tested for the two commercial fungicides (Figure 2). Despite this, the MGR results demonstrated a stronger action by the TOH substance on mycelial structures of *A. alternata* compared to conidia. In the MGR assay, the concentration of 250 µg mL^{-1} was able to reduce the MGR to values that were statically equal to the commercial fungicide Nativo®, which is composed of two different substances, compared with the concentration of 500 µg mL^{-1} that was necessary to inhibit all conidia germination in the other assay. These results support the probable action pronounced in solid substrates by this substance. Regarding conidial viability, other studies also have demonstrated the ability of different essential oil species to prevent conidial germination,[7,12–14] which may be due to certain substances. However, these works did not obtain absolute correlations between these changes and the chemical substance demonstrated to lead to this inhibition.

3.2 Early infection of *A. alternata in vivo* influenced by TEO and TOH effects tracking by SEM and the disease progress implication

The structures of *A. alternata* exposed to the control (Tween 80) were morphologically intact, with ostensible development of the germ tube when

evaluated at 6 HPI (Figure 3). However, exposure to TEO or TOH caused delayed development of the germ tube, where no germinated conidia were found at 6 HPI (Figure 3f and l, respectively). Moreover, both substances showed apparent damage to the conidial cell wall at this stage of infection. This observation is in strong accordance with the results of the structural mode of action investigated by TEM (section 3.3), which suggests the action by TEO and, in a more pronounced way, TOH on the conidial cell wall. At 12 HPI, our results showed an evidenced delay in early infection steps caused by TEO and were more evident with TOH. Conidia treated with TEO were still starting to elongate their germ tubes, while conidia treated with TOH were still un-germinated (Figure 3m) and, with few exceptions, showed an initial germination process. In contrast, at the same stage, the control showed a well-developed hypha that was already starting the ramification process. Such observations are in agreement with the conidial viability study that demonstrated the ability to completely inhibit conidia germination by TEO and TOH after 12h at concentrations higher than 1000 and 500 μg mL^{-1}, respectively.
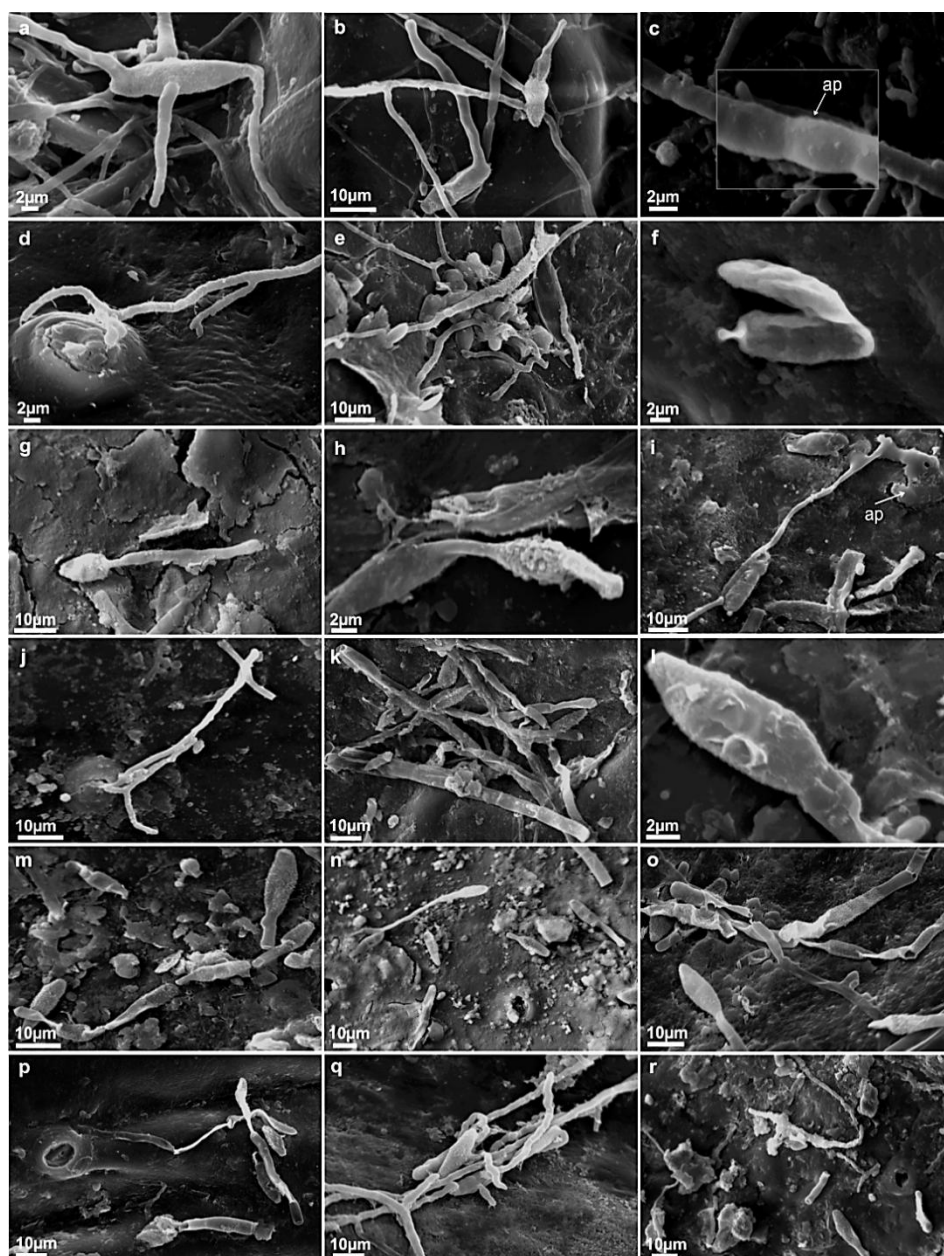
Figure 3. Scanning electron micrographs with details of *Alternaria alternata* early infection after exposed to: (a–e) Tween 80 at 1000 µg mL^{-1} after 6, 12, 15, 18 and 24 hours post infection (HPI), respectively; (f–k): *Thymus* essential oil at 500 µg mL^{-1}; damaged conidia at 6 HPI (f); the beginning of the germination process at 12 HPI (g); a hyphae still beginning the ramification process at 15

HPI (h); delayed appressorium formation at 18 HPI (i); delayed penetration process through the stomata (j); and mycelial layer formed by damaged hyphae at 24 HPI (k). Thymol (at 500 µg mL^{-1})-treated fruits: (l) after 6 HPI showing damaged conidia at; (m) 12 HPI showing still un-germinated conidia among few initial germ tubes started; (n) 15 HPI showing conidia still beginning germ tube elongation; (o) 18 HPI representing the hyphae still in the elongation process with evident damage and no appressorium formation; (p) and 24 HPI representing the prevention of the of the hyphae elongation and the penetration of the fungus, with no appressorium or mycelial layer formed. (q) Structures exposed to Recop$^®$ fungicide (2500 µg mL^{-1}) at 24 HPI showing a mycelial layer formation. (r) Structures of *A. alternata* exposed to Nativo$^®$ (at 2500 µg mL^{-1}) at 24 HPI demonstrating the prevention of the penetration process as a consequence of the damaged conidia and hyphae.

The analogous ability of delaying the early infection of *A. alternata* was evidenced during all other time points by the TEO-, TOH-, and commercial fungicide-treated samples (Figure 3). Thus, at 15 HPI the control samples (Tween 80-treated) exhibited appressoria formation (Figure 3c), and at 18 HPI they demonstrated the beginning of the penetration process throughout the stomata (Figure 3d). Later, at 24 HPI, the beginning of the mycelial layer formation occurred (Figure 3e). At the same time point, the TEO samples analysed exhibited damaged conidia at 6 HPI (Figure 3f); the beginning of the germination process at 12 HPI (Figure 3g); the hyphae still beginning the ramification process with strong damage to these structure at 15 HPI (Figure 3h); delayed appressoria formation at 18 HPI (Figure 3i); and delayed penetration process throughout the stomata with a mycelial layer formed by damaged hyphae at 24 HPI (Figures 3j and k). On the other hand, at the same time points the TOH samples showed conidia still beginning the elongation of

the germ tube among some unsuccessfully germinated conidia at 15 HPI (Figure 3n). The hyphae elongation process with evident damage was noted at 18 HPI, and, in contrast to the TEO samples, no appressoria formation was observed (Figure 3o) at this or any time point. Such apparent damage observed in conidia and hyphae structures subsequently prevented the elongation and penetration of the fungus to the inner tissue fruits (Figure 3p). Regarding the control fungicides analysed, a very similar behaviour to TEO was observed with the fungicide Recop®, where a delayed infection process was observed with damage to the hyphae and mycelial layer formation at 24 HPI (Figure 3q). Furthermore, the fungicide Nativo® demonstrated a similar delay and analogous behaviour to TOH, resulting in a few germinated conidia and non-mycelial layer formation, consequently preventing penetration in the inner fruit tissues up to 24 HPI (Figure 3r).

Structural SEM time-course analysis of early infection steps has been extensively studied in other plant pathogenic fungal species.[29,30] Studies have been developed involving the influence of active substances and products,[14,21] and the effects of active natural substances on fungal structural injuries have been extensively reported.[2,5,11−13,20] Such authors also have reported conidia damage[6,13] and conidial germination interference by SEM analysis.[14,21] Regarding *A. alternata* tangerine pathotypes, severe conidia shrinkage caused by exposure to *Ruta graveolens* extract has been demonstrated.[6] However,

regarding the penetration interference by the active substance, those studies did not show a direct correlation with the isolated chemical substance that purportedly causes it. A recent study[4] demonstrated the direct action of a plant extract-isolated substance and accomplished the interaction of the putative substance with fungal substances; however, the authors did not report the early infection steps in the host-pathogen interaction.

The SEM results are in accordance with the influence of TEO and TOH on the *in-vivo* ABS disease progress performed in fruits, which aimed to correlate the early infection process and its implication in terms of disease progress. Our result showed that TOH was able to reduce the ABS progress measured as ABS disease rate (ABSdr) to values equal ($P \leq 0.05$) to the commercial fungicide Nativo® at both time points evaluated at 7 and 10 DPI (Table 3).

Table 3. Effect of *Thymus* essential oil, thymol, and fungicides on Alternaria brown spot disease progress (ABSdr) in Ponkan tangerine fruits.

Treatment	Concentration (µg mL^{-1})	[a]ABSdr (Spot mm)			
		7 DPI[b]	Control	10 DPI	Control
Thymus essential oil (TEO)	1000	0.9117 bB	8.8%	0.6617 bA	33.8%
Thymol (TOH)	500	0.7066 aB	29.3%	0.5100 aA	49.0%
Control RF[c]	2500	0.9033 bB	9.7%	0.6533 bA	34.7%
Control NF[d]	300	0.6001 aB	40.0%	0.4350 aA	56.5%
Tween 80	100	1.0000 bB	…	1.0000 cB	…
CV	--	11.74%	…	13.45%	…

[a]Means with the same lowercase letter within a column and the same capital letter within a line do not differ (P ≤ 0.05) (Tukey test, P ≤ 0.05). [b]DPI: days post infection. [c]Recop® commercial fungicide composed of CuCl2.3Cu(OH)$_2$ at 840 g kg^{-1}; [d]Nativo® commercial fungicide composed of trifloxystrobin + tebuconazole (1:2 m/m) at 800 g L^{-1}.

On the other hand, TEO-treated fruit showed equivalent reduction in ABSdr to the result found for the commercial fungicide Recop®, and they exhibited no differences to the Tween 80 at 1000 μg mL^{-1} treatment at 7 DPI. However, at 10 DPI, both TEO- and Recop®-treated fruits showed similar ABSdr, revealing partial control of these treatments, which where outperformed by TOH and Nativo®. This demonstrates that these results agree with the SEM results, considering their influence at early stages of fungal infection. Therefore, our results made evident the superiority by TOH to TEO, the latter which outperformed Recop® with respect to ABS disease progress reduction.

Some studies have demonstrated that TOH has the capability to reduce the fungal ability to produce toxin.[27,28] This may be possible based on the fact that *A. alternata* produces the host-selective ACT-toxin, which is well known to have a unique importance in pathogenicity.[29] Our results suggests that TOH also may have potential action in the production of ACT, which is implicated by the failed early infection shown by SEM analysis. The effect of plant substances against the ABSdr also has been previously reported.[6] The authors found that although the best results by plant extracts were statistically similar to those of the commercial fungicides, the efficiency of these extracts was reduced over the time, where the ABSdr was significantly higher (0.38 at 8 DPI and 0.49 at 12 DPI). Our results showed that TOH, TEO, and the commercial fungicides evaluated showed similar behaviour regarding ABSdr over time. All of them

showed a statistically significant reduction in disease rate, indicating a reduction in the progress of ABS over time. Such results are required for products that have fungicidal properties once they tend to lead to disease control.

3.3 Cellular activity of TEO and TOH by TEM

Of the five treatments used in TEM analysis, only the commercial fungicide Nativo® (used as one of the positive controls) did not present a clear structure of *A. alternata* in the attempting to explain the mode of action in either of the two experiments. The samples representing the negative control, which were treated with Tween 80 at 1000 µg µL^{-1}, showed a well-developed conidia cell wall with an intact plasma membrane and cytoplasm organisation that showed a normal structure of organelles in most of the images (Figure 4a); it was possible to demonstrate the beginning of germ tube formation (Figure 4b) and the hyphae ultrastructure (Figure 4c). Such germ tube structures were not observed using the other substances tested. As a consequence of the TEO treatment, the conidia cell showed clear damage to the fungal cell wall and plasma membrane (Figures 4d–f), which later resulted in cytoplasm disorganisation and organelle destruction (Figure 4d). In general, conidia exposed to TOH (Figures 4g and h) showed strong cell wall and membrane disruption, supported by the mode of action also reported to be starting in conidia treated with TEO. Those effects resulted in clear cytoplasm disorganisation and an abnormality and destruction
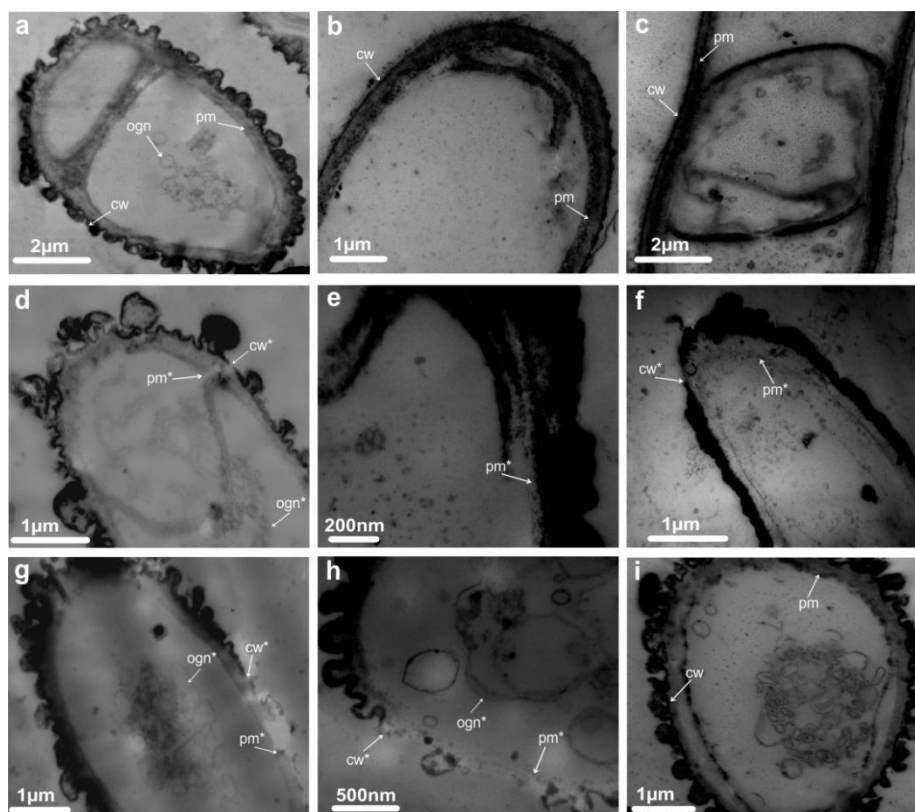
of organelles (Fig. 4g and h).

Figure 4. Transmission electron micrographs of *Alternaria alternata* conidia exposed to Tween 80 (a–c); *Thymus* essential oil at 500 μg L^{-1} (d–f); thymol (g and h); and Recop$^{®}$ commercial fungicide at 2500 μg mL^{-1}. Arrows indicate pm: plasma membrane; cw: cell wall; ogn: organelles; pm*, cw*, and ogn* indicate damaged plasma membrane, cell wall, and organelles, respectively. (a) Shown are normal cells, cell wall with intact plasma membrane, cytoplasm organisation, and structure of organelles; (b) hyphae at beginning of the germ tube formation; (c) normal structure in a hyphae originated from a conidia; (d) arrows indicate the cell wall, plasma membrane, and organelle damage; (e) arrow indicates plasma membrane disorganisation; (f) arrow indicates cell wall and plasma membrane injury; (g and h) general and detailed views of the damage to the cell wall, plasma membrane, and organelles indicated by the arrows; (i) arrows represent the normal cell structures obtained with the commercial fungicide Recop$^{®}$.

Despite the evident organelle effects, the isolated action could not clearly be correlated to specific organelle morphology due the strong damage caused by TEO and TOH.

Injury effects of TEO on fungal structures previously have been reported using TEM.[15,16] The latter authors showed that essential oils of *T. eriocalyx* (Ronniger) Jalas and *T. x-porlock* resulted in plasma membrane rupture and destruction of the mitochondria and organelles in cells of *Aspergillus niger* Van Tieghem. The former authors demonstrated that TEO at 200 μg μL^{-1} caused plasma membrane disruption aside from cytoplasmic and mitochondrial disorganisation. Such effects would be attributed to the isolated substance presented. However, the authors did not obtain absolute correlation regarding the substance that leads to such effects. Regarding the action by TOH on the plasma membrane, cell wall, and organelles, our results suggest that this substance may be responsible for such effects. This is because this substance is the main component of TEO, and closely related actions by TEO were noted earlier.[15,16]

Based on the results obtained by SEM, TEM, and their correlations with those achieved by MIC, MGR, and conidial viability, it is believed that the TEO activity on the conidia cell wall, plasma membrane, and cytoplasm disorganisation later results in organelle destruction of *A. alternata*. Such cellular actions subsequently result in decreased efficiency of pathogen infection and the subsequent disease progress. This suggests that the TEO damage and

effects on disease control are due to its main substance TOH. The TOH was demonstrated to be highly responsive when applied under the same conditions; it resulted in improved control over TEO and was found to be statistically similar to high-efficacy commercial fungicides composed of combinations of triazol and strobilurin groups, as observed in our assays. The use of TEO containing high TOH concentrations may be a possible strategy in the integrated management of disease, providing satisfactory results for reducing ABS disease in Ponkan tangerine orchards and as a control strategy for postharvest fruits, especially in organic production systems where pesticides are banned. Therefore, this isolated substance has potential for use in the development of new fungicides alone or as combinations to control numerous plant-pathogenic fungi.

4 CONCLUSIONS

The activity of essential oil from *T. vulgaris* against the fungus *A. alternata* was confirmed and is likely attributed to the substance thymol. This substance demonstrated high percentage of disease control in fruits and activity against *A. alternata* growth and viability, outperforming TEO. The direct application of TOH resulted in a delayed early infection process, preventing fungal penetration, while direct TEO only caused a delay in the infection process. The ultrastructure analysis demonstrated that TOH acts at the cellular level against fungi by cell wall and plasma membrane disruption with subsequent cytoplasm disorder that

may target specific organelles in the cytoplasm. Ultrastructure data suggest that TEO activity is closely related to TOH, its major constituent.

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