Chemical composition and in vitro inhibitory effects of essential oils from fruit peel of three Citrus species and limonene on mycelial growth of Sclerotinia sclerotiorum

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Abstract

Essential oils (EO) from aromatic and medicinal plants generally perform a diverse range of biological activities because they have several active constituents that work in different mechanisms of action. EO from Citrus peel have an impressive range of food and medicinal uses, besides other applications. EO from Citrus reticulata, C. sinensis and C. deliciosa were extracted from fruit peel and analyzed by GC-MS. The major constituent of EO under evaluation was limonene, whose concentrations were 98.54%, 91.65% and 91.27% for C. sinensis, C. reticulata and C. deliciosa, respectively. The highest potential of inhibition of mycelial growth was observed when the oil dose was 300 µL. Citrus oils inhibited fungus growth in 82.91% (C. deliciosa), 65.82% (C. sinensis) and 63.46% (C. reticulata). Anti-Sclerotinia sclerotiorum activity of 90% pure limonene and at different doses (20, 50, 100, 200 and 300 µL) was also investigated. This monoterpane showed to be highly active by inhibiting 100% fungus growth even at 200 and 300 µL doses. This is the first report of the in vitro inhibitory effect of natural products from these three Citrus species and its results show that there is good prospect of using them experimentally to control S. sclerotiorum, in both greenhouse and field conditions.

Keywords: Citrus reticulata, Citrus sinensis, Citrus deliciosa, white rot, limonene, white mold.

Composição química e efeito inibitório in vitro dos óleos essenciais das cascas dos frutos de três espécies de Citrus e do limoneno sobre o crescimento micelial de Sclerotinia sclerotiorum

Resumo

Óleos essenciais de plantas aromáticas e medicinais geralmente exibem uma gama diversificada de atividades biológicas, pois possuem vários constituintes ativos que atuam por meio de vários mecanismos de ação. Os óleos essenciais das cascas de Citrus têm uma variedade impressionante de usos em alimentos, medicamentos entre várias outras aplicações. Os óleos essenciais (OE) de Citrus reticulata, C. sinenses e C. deliciosa foram extraídos das cascas dos frutos e analisados por CG-EM. O limoneno foi o constituinte majoritário encontrado nos óleos essenciais avaliados, nas concentrações de 98,54%, 91,65% e 91,27% para C. sinensis, C. reticulata e C. deliciosa, respectivamente. Os maiores potenciais de inibição do crescimento micelial foi observado na dose de 300 µL dos óleos. Os óleos de Citrus inibiram em 82,91% (C. deliciosa), 65,82% (C. sinensis) e 63,46% (C. reticulata) o crescimento do fungo. A atividade anti-Sclerotinia sclerotiorum do limoneno 90% puro e em diferentes doses (20, 50, 100, 200 e 300 µL) foi também investigada. Este monoterpeno demonstrou-se altamente ativo inibindo 100% o crescimento do fungo inclusive nas doses de 200 e 300 µL. Este é o primeiro relato sobre o efeito inibitório in vitro dos óleos essenciais destas três espécies de Citrus e os resultados deste estudo mostram que existe uma boa perspectiva de uso destes produtos naturais experimentalmente para controlar o S. sclerotiorum tanto em condições de estufa como em condições de campo.

Palavras-chave: Citrus reticulata, Citrus sinensis, Citrus deliciosa, podridão branca, limoneno, mofo branco.
1. Introduction

The fungus Sclerotinia sclerotiorum, one of the most common pathogens, causes white mold and severe damage to several economically important cultures, such as beans and soybeans, thus, making Brazilian producers face great losses (Haddad et al., 2017). Controlling this disease with the use of chemicals is not only ineffective but also contradictory, since it does not follow the current tendency which searches for ecologically balanced and stable agricultural systems that do not release toxic waste (Milan et al., 2015).

White mold development is favored by certain conditions, such as high humidity and low/moderate temperatures. It may be controlled, mainly by fungicide application, which depends on several factors, such as soil inoculum density, phases of the epidemic, fungicide coverage of plants, number of pulverization steps, fungitoxicity doses, application time, volume and equipment, plant spacing, besides disease incidence and severity (Silva et al., 2017).

Regarding the fungus S. sclerotiorum, it develops resistant structures called sclerotia, which can survive in soil for several years even if there are no hosts (Silva et al., 2011). Sclerotia play a very important role in the life cycle of this phytopathogen, since they are precursors of apothecia, where ascospores are formed. In ideal conditions, ascospores may infect cultures and start infection by spores (Silva et al., 2011). In fact, several factors, such as nutrients of the substrate in which sclerotia develop, sclerotium age and environmental factors (humidity, temperature, light, soil pH, soil aeration and burial depth), influence germination of this fungus sclerotia (Gomes et al., 2017).

Regarding problems caused by white mold, several studies have shown that natural products have promising activities, for instance, the bioactivity of essential oils (EO) against different phytopathogens, such as S. sclerotiorum (Al-Taisan et al., 2014). EO, mainly the ones extracted from Citrus species, exhibit a broad spectrum of biological activity and activity against Gram-positive and Gram-negative bacteria, yeast and mycotoxigenic and deteriorating filamentous fungi (Qadir et al., 2018).

In order to keep carrying out studies of determination of chemical composition of EO and their activity against white mold (Valadares et al., 2018) and considering the interest of our research group in EO from Citrus species (Estevam et al., 2016; Lemes et al., 2018), the study reported by this paper addresses the chemical composition of EO extracted from Citrus reticulata, C. sinensis and C. deliciosa fruit peel (Figure 1) and their in vitro inhibitory effect on mycelial growth of S. sclerotiorum.

2. Material and Methods

2.1. Plant material

Plant material was collected in Rio Verde (17°99.4’63.2”S and 51°05.2’44.6”W), a city located in Goiás state, Brazil, on January 2nd, 2018, at 9 a.m. The plant was identified by the botanist Luzia Francisca de Souza and voucher specimens of Citrus reticulata, Citrus sinensis and Citrus deliciosa were deposited in the herbarium in Rio Verde, at the Instituto Federal Goiano (IFGOIANO) under identification number #4488, #4489 and #4490, respectively.

2.2. Essential oil extraction

EO from Citrus were extracted from fruit peel by hydrodistillation – performed in triplicate – in a Clevenger-type apparatus for 2 h. The plant material was divided into three 500-g samples and 500 mL distilled water was added to each sample. After manual collection of EO, water traces which remained in the oil were removed with anhydrous sodium sulfate. The next step was filtration. EO were then stored in an amber bottle and kept in a refrigerator at 4 °C until analysis. Calculation of EO yield was based on the weight of the fruit peel; it was expressed as the average of the triplicate analyses.

2.3. Chemical analysis of essential oils

The analysis of the chemical constituents of EO from the fruit peel of both plants was carried out using a Shimadzu QP 5000 GC gas chromatograph equipped with a fused-silica capillary column OPTIMA-5 (30 m x 0.25 mm x 0.250 µm) and a helium carrier gas (He) detector and electron impact ionization (EI) (70 eV) (Table 1). The initial temperature was maintained at 150 °C for 3.0 min, programmed to 280 °C at 10 °C/min and left at this temperature for an additional 4.0 min. The others parameters were as follows: injector temperature 250 °C, detector temperature 280 °C, injection pressure 100 kPa, due to Split 30, mass spectrometer detection range 43-500 m/z, start time (cut team solvent) 2.0 min and flow 1.1 mL/min. The identification of oil components was based on the linear retention index (Kovats Index – KI) calculated with respect to the retention times of a homologous series of n-alkanes (C-14 to C-26, C-28 and C-30) and the fragmentation pattern observed in the mass spectra and by comparing these values with the literature data (Adams, 2007) and NIST/EPA/NIH Mass Spectral Library (NIST 08).

2.4. Antifungal assay

The isolate of Sclerotinia sclerotiorum Ss12 (BRM 29673) was provided by the Embrapa Arroz e Feijão, whose headquarters is in Santo Antônio de Goiás, GO, Brazil. Assays were carried out in the agricultural microbiology laboratory at IF Goiano – Campus Rio Verde and the antifungal activity of EO from fruit peel of Citrus species

![Figure 1. Citrus reticulata (1), Citrus sinensis (2) and Citrus deliciosa (3).](image-url)
Anti-Sclerotinia sclerotiorum activity of essential oils from Citrus peel was evaluated by the disc-diffusion method described by Xavier et al. (2016) – with modifications – whose EO doses were 20 µL (diluted in 480 µL of H₂O + 100 µL of Tween), 50 µL (diluted in 450 µL of H₂O + 100 µL of Tween), 100 µL (diluted in 400 µL of H₂O + 100 µL of Tween), 200 µL (diluted in 300 µL of H₂O + 100 µL of Tween) and 300 µL (diluted in 200 µL of H₂O + 100 µL of Tween) for three Citrus species, respectively (Table 2). The anti-Sclerotinia sclerotiorum activity of limonene alone and at the same doses of EO (20 - 300 µL; with dilutions equal to those of the EO) was also evaluated. In the analyses, limonene (90% purity) was purchased from Sigma-Aldrich®, Castle Hill, NSW, Australia. Negative controls were dishes with no addition of EO (witness) whereas the positive control was the fungicide Frowncide 500 SC, at 10 µg/mL of active ingredient. Petri dishes were sterilized and prepared with PDA culture medium. After medium solidification, EO, at previously mentioned doses, were added and smeared on the surface of the dish with the help of a Drigalski spatula. Afterwards, 5 mm diameter PDA medium discs with 10-day-old mycelium were placed in the center of the dishes. Then, they were incubated at 28 ± 2 °C and mycelial growth was measured daily, up to full growth of the fungus on control dishes. The treatment was carried out in quadruplicate and the experimental design was thoroughly randomized. Data were submitted to the analysis of variance (ANOVA) and resulting means of all treatments were evaluated by the Scott-Knott test at 5% significance by the ASSISTAT software.

Percentage of inhibition of mycelial growth (IMG) was calculated by the following Formula (1):

\[ IMG(%) = \left( \frac{\text{control growth} - \text{treatment growth}}{\text{control growth}} \right) \times 100 \]

3. Results and Discussion

Extraction of EO from Citrus sinensis, C. reticulata and C. deliciosa fruit peel yielded 0.8%, 0.6% and 0.7%, respectively. GC-MS identified four chemical constituents of EO from C. sinensis (total was 99.80%), four from C. reticulata (total was 98.88%) and eleven from C. deliciosa (total was 99.14%). The major constituent of the three EO under analysis was limonene, which was found at the following concentrations: 98.54% (C. sinensis), 91.65% (C. reticulata) and 91.27% (C. deliciosa). Components,

| Compounds       | RI     | RA% | Literature | C. sinensis | C. reticulata | C. deliciosa |
|-----------------|--------|-----|------------|-------------|---------------|--------------|
| Tricyclene      | 921    | 0.25|           | -           | -             | -            |
| α-Thujene       | 924    | 923 |           | 0.13        | -             | -            |
| Sabinene        | 969    | 968 |           | 1.06        | 1.06          | 1.06         |
| β-Pinene        | 974    | 976 |           | 0.74        | 0.93          | 1.27         |
| n-Octanol       | 998    | 996 |           | -           | -             | 0.19         |
| δ-2-Carene      | 1001   | 1000| 0.27      | -           | -             | -            |
| Limonene        | 1024   | 1023| 98.54     | 91.65       | 91.27         | 91.27        |
| (E)-β-Ocimene   | 1044   | 1044| -         | -           | -             | 0.09         |
| γ-Terpineol     | 1054   | 1055| -         | 6.17        | 0.12          | 0.12         |
| Linalool        | 1095   | 1097| -         | -           | 4.04          | -            |
| 3-Thujanol      | 1164   | 1164| -         | -           | -             | 0.28         |
| α-Terpineol     | 1186   | 1185| -         | -           | -             | 0.37         |
| cis-Cadina-1(6),4-diene | 1461 | 1461| -         | -           | 0.39          | -            |
| β-Macrocarpene  | 1499   | 1498| -         | -           | 0.06          | -            |
| **Total**       |        |     | 99.80     | 98.88       | 99.14         |              |

RI: Retention index; RA%: relative area.

![Table 1](image1)

![Table 2](image2)
retention indexes and relative percentages (%) are shown in Table 1.

Comparison between compounds identified and listed in Table 1 and others reported by similar studies of Citrus species showed that compounds of EO from C. sinensis and C. reticulata fruit peel had little chemical variability, i.e., only four constituents were identified in each oil. However, these oils exhibited a very high content of the monoterpene limonene, which was found at concentrations of 98.54% in C. sinensis oil and 91.65% in C. reticulata oil. Kamal et al. (2011) reported higher chemical variability in compounds of fruit peel of the same species, but lower concentrations of limonene, by comparison with findings of this study. Concerning the chemical composition of C. deliciosa oil, this study found high chemical variability, since eleven chemical constituents were identified in EO from C. deliciosa fruit peel. Limonene was also identified at significant concentration – 99.14% –, which is higher than the one reported by El-hawary et al. (2013), who found 77.55% in EO from fruit peel. In this study, the chemical composition was also similar to the one that was previously reported in the case of EO from twenty Citrus species found in China. Limonene, α-pinene, sabine and terpinene were the characteristic compounds of metabolic profiles of all Citrus under evaluation (Jing et al., 2015).

In vitro antifungal activity of EO from C. sinensis, C. reticulata and C. deliciosa fruit peel was evaluated against the phytopathogenic fungus Sclerotinia sclerotiorum. Percentages of inhibition of mycelial growth (IMG) of EO from Citrus fruit peel are shown in Table 2.

The highest inhibitory potential against mycelial growth of the fungus S. sclerotiorum exhibited by oils under study was found when the oil dose was 300 µL. It inhibited 82.91%, 65.82% and 63.46% in the cases of EO from C. deliciosa, C. sinensis and C. reticulata, respectively (Table 2). It should be highlighted that all three EO from these three Citrus species, at the highest concentration under investigation, inhibited more than 50% of fungal growth. In addition, it is worth mentioning that EO from C. deliciosa fruit peel were the most active ones, a fact that may be explained by the high chemical variability of their compounds, which may act synergistically and increase their biological activity (Sriwattanachai et al., 2018).

The antifungal activity of EO from Citrus found by this study may be related to the high concentration of the chemical constituent limonene, whose antifungal activity has been widely described in the literature. Chee et al. (2009) reported the promising activity of this monoterpene against the fungus Trichophyton rubrum. Hamdani et al. (2015) studied EO of four Citrus species and reported their potential in the biological control of phytopathogens, such as Fusarium oxysporum, Penicillium sp., Alternaria sp. and Fusarium sp. They also highlighted the high concentration of limonene in the oils under study.

Special attention was given to the major constituent limonene. As a result, in this study, the anti-Sclerotinia sclerotiorum activity of this 90% pure monoterpene and at doses of 20, 50, 100, 200 and 300 µL (with dilutions) was also investigated (Table 2). At all doses under evaluation, total inhibition of white mold occurred at 200 and 300 µL, thus representing 100% of inhibition of mycelial growth. When limonene (90% pure) was tested individually, it inhibited S. sclerotiorum growth in 100%. It should be highlighted that limonene has already been identified at high concentrations in EO extracted from several Citrus species. This monoterpene – in its pure form – exhibited promising antifungal activity against other phytopathogenic fungi, such as Aspergillus niger, Phytophthora digitatum, Rhizoctonia solani, Fusarium oxysporum and Fusarium verticillioides, even against S. sclerotiorum itself (Jing et al., 2014; Ma et al., 2015). In addition, the therapeutic effects of limonene, such as anti-inflammatory, antioxidant, antiinocceptive, anticancer, antidiabetic, antihyperalgesic, antiviral and gastroprotective ones, have been deeply studied (Vieira et al., 2018).

The mechanism of action of EO which exhibit some kind of biological activity is not very clear. Many studies suggest that cell membranes of microorganisms are the targets of bioactive volatile compounds since EO are complex mixtures of apolar molecules that bestow them high hydrophobicity. Therefore, EO cause degradation of the cell wall, disruption of cytoplasmic membrane, cytoplasmic leakage, cell lysis and, eventually, cell death (Jing et al., 2014). As a result, EO from Citrus deserve the distinction they have got lately, as well as their broad applicability to several areas, such as chemical, pharmaceutical, food and agronomical ones (Palazzolo et al., 2013).

4. Conclusion

In short, EO extracted from Citrus sinensis, C. reticulata and C. deliciosa fruit peel exhibit antifungal activity against the phytopathogen Sclerotinia sclerotiorum by inhibiting fungal growth in about 50% at 300 µL. The monoterpene limonene was the chemical constituent that was identified at high concentration in EO from Citrus under investigation. When limonene (90% pure) was tested in its isolated form and at doses under evaluation (200 and 300 µL – with dilutions), its high activity against white mold was proven (inhibition of 100%). In addition, the antifungal activity of EO from Citrus species may result from synergism among the compounds that constitute the oils. Results of this study show that there is good prospect of using these EO from Citrus species experimentally to control phytopathogens in both greenhouse and field conditions. In sum, the monoterpene limonene proved to be an excellent natural alternative for the control of S. sclerotiorum.

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