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Citrus species essential oils and their components can inhibit or stimulate fungal growth in fruit

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ABSTRACT

Penicillium digitatum Pers., *Trichoderma viride* Pers. and *Botrytis cinerea* Pers. are typical post-harvested fruits pathogens. The use of essential oils to control fruit fungal diseases is an alternative to synthetic fungicides. The objective of this research was to evaluate the *in vitro* antifungal activity of the hydrodistilled fruit peel essential oils of *Citrus limon* (L.) Burm. f., *C. latifolia* Tanaka ex Q. Jimenez, *C. aurantifolia* (Christm.) Swingle and *C. limonia* Osbeck extracts against the mentioned phytopathogenic fungi. The essential oils were analyzed by gas chromatography-flame ionization detection (GC/FID), gas chromatography-mass spectrometry (GC/MS) and their compositions were determined. Minimum inhibitory concentration assay confirmed only modest antifungal activity for all essential oils, with best results against *B. cinerea* (*C. limonia* and *C. limon* = 312 μg/mL; *C. aurantifolia* and *C. latifolia* = 625 μg/mL). However, according to a volatile activity experiment, which was developed to evaluate the effects of citrus volatiles, citral and other pure chiral components, the essential oils and their main volatile components displayed a differential influence on the growth of the three fungal species tested. While all oils inhibited growth of *B. cinerea* and *T. viride,* the opposite was observed for *P. digitatum,* in which fungal growth was stimulated. A prevalence of inhibitory effects was observed for pure chiral components when tested against *B. cinerea* and *T. viride.* However, with exception to citral, (+)-α-pinene and (+)-β-pinene, all pure chiral volatile compounds stimulated *P. digitatum* growth.

1. Introduction

The losses caused by fruits fungal diseases during storage and transportation are significant. In general, infections may occur throughout all the stages in the field and during harvest, storage and transportation, being caused by injuries which allow entry of pathogens. Diseases such as brown rot, septoria spot, anthracnose, clear rot, green/blue mold, sour rot caused by, respectively, *Phytophthora* spp., *Septoria citri*, *Colletotrichum gloeosporioides*, *Penicillium* spp and *Galactomyces citri-aurantii*, for example, emerge as a result of wounding caused by handling in the field or in the subsequent stages after harvesting.

Alternative strategies for the control of post-harvest diseases seek the integration of low-risk treatments to reduce the use of conventional synthetic fungicides. Success of these strategies will minimize health and environmental risks (Caccioni et al., 1998; Viuda-Martos et al., 2008). Study of plant/pathogen interaction may provide sustainable and environmentally friend solutions for the control of plant disease in agricultural crops.

Essential Oils (EO) have shown satisfactory results in controlling postharvest diseases by providing fungitoxic action. For example, the oils of *Mentha arvensis* L., *Ocimum canum* Sims. and *Zingiber officinale* Roscoe were found to exhibit absolute fungitoxic activity against *P. italicum* (Tripathi et al., 2004). The essential oils from two clonal types of *Thymus vulgaris* L. were characterized and tested as antifungal against *Botrytis cinerea* and *Rhizopus stolonifera,* being highly effective in reducing gray mould and soft rot incidence in strawberry fruits caused by, respectively, *B. cinerea* and *R. stolonifera* (Reddy et al., 1998). Viuda-Martos et al. (2008) demonstrated that citrus essential oils can be considered suitable alternatives in the food industry to control the growth of moulds commonly associated with food spoilage as *Aspergillus flavus* and *Penicillium verrucosum,* amongst others.

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Citrus fruits are produced and consumed in large scale around the world, either *in natura* or as juices (Brown, 1989; Davidson and Harrison, 2002). The fruit peel essential oils are by products of the citrus juice industry and are of great interest as raw materials for pharmaceutical, cosmetics and food industries due to their aromatic properties and antimicrobial activities (Cristani et al., 2007; Viuda-Martos et al., 2008). Moreover, these EO are generally considered safe to use in foods and beverages and are known as generally recognized as safe (GRAS) (Kabara, 1991; Viuda-Martos et al., 2007).

As part of our systematic study on the role of the secondary metabolites of citrus fruits in the chemical defence against their pathogens, the effect of citrus essential oils on cultivated plant pathogenic fungi was evaluated. The results of such evaluation, made with essential oils from four citrus species (limes and lemons) against one pathogenic fungus in citrus fruits (*Penicillium digitatum*), one endophytic fungus in citrus fruits (*Trichoderma viride*) and one general pathogen of fruits (*Botrytis cinerea*) are reported. *T. viride* is an extensively studied saprophytic fungus due to its potential uses in biocontrol (Smitha et al., 2014). It is known to produce enzymes (mainly hydrolytic enzymes) with antagonistic properties against other fungi (Smitha et al., 2014), including *P. digitatum* (Borras and Aguilar, 1990) and *B. cinerea* (Bogumił et al., 2013; Talla et al., 2015).

The effect of some components of these oils, on fungus growth was also evaluated.

2. Material and methods

2.1. Material

Fruits of *Citrus limon* (L.) Burm. f., "siciliano" lemon, *C. latifolia* Tanaka ex Q. Jimenez, "tahiti" lime, *C. aurantifolia* (Christm.) Swingle, "mirim" lime and *C. limonia* Osbeck, "cravo" lime were collected in local markets in Rio de Janeiro, Brazil. The botanical identification was provided by Dr. Rosana Conrado Lopes, and voucher specimens were deposited in the RFA Herbarium, Federal University of Rio de Janeiro/Brazil under the following registration numbers: *C. limonia* (RFA-39493), *C. aurantifolia* (RFA-39492), *C. limon* (RFA-39494) and *C. latifolia* (RFA-39491).

2.2. Sample preparation

Fruit peels (150.0 g) were removed and homogenized in 1 L of distilled water using a laboratory blender. The resulting mixture was immediately subjected to hydro distillation for 2 h in a Clevenger apparatus. Once extracted, the oils were dried over Na_2SO_4 and stored at −18 °C. The average extraction yield was calculated as the mass percentage of oil obtained relative to the mass of peel extracted.

2.3. Pure compounds

All pure compounds used in bioassays or on identification of volatiles were obtained from Sigma-Aldrich Co (Brazil): (+)-α-pinene (99%), (+)-β-pinene (99%), (−)-α-pinene (99%), (+)-β-pinene (99%), citral (neral + geranial − 99%), γ-terpinene (99%), (+)-limonene (99%) and (−)-limonene (99%).

2.4. GC–MS

Analyses were made on a Shimadzu QP2010 Plus (Tokyo, Japan) system using a DB5-MS fused silica capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness). The oven temperature was programmed to rise from 60 °C to 246 °C at 3 °C/min and then hold at this temperature for 20 min. The carrier gas was He (99.999%) with a flow rate of 1.03 mL/min and injector and interface temperatures were maintained at 250 °C and 200 °C, respectively. Oil samples were diluted in $CHCl_3$ and 1 μL aliquots were injected in split mode (split ratio 1:50). Mass spectra were obtained under electron ionization at 70 eV with a mass range *m*/*z* of 40–1000 D.

2.5. Quantitative analysis by GC/FID

A Shimadzu GC 2010 (Tokyo, Japan) equipped with a flame ionization detector on an Agilent DB5 fused silica capillary column (30 m, 0.25 mm i.d., film thickness 0.25 μm) was utilized. The oven temperature was programmed to rise from 60 °C to 246 °C at 3 °C/min, then hold at 246 °C for 20 min. Injector and detector temperatures were maintained at 220 °C and 290 °C, respectively. The oil samples were dissolved in $CHCl_3$, and 1 μL aliquots were injected in split mode with split ratio of 1:50 using H_2 as the carrier gas (1.44 mL/min). Kovats retention indices (KI) of the compounds were determined relative to the retention times of a series of *n*-alkanes (C_7–C_{30}) with linear interpolation. The relative amounts of the components were calculated based on GC peak areas without correction factors.

2.6. Analysis by HS/SPME-GC/FID (Headspace solid/phase micro extraction-gas chromatography with flame ionization detector)

A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used for sampling. Before and after use, the fiber was conditioned by heating on the gas chromatograph injector (300 °C) for 30 min to remove possible contaminants. In addition, prior to each analysis one blank injection was made to confirm absence of contamination. An aliquot of 5 μL of *Citrus* fruit oil was added to a filter paper circle (diameter: 5.0 mm – Whatman (New Jersey, United States)) and the filter paper was placed in an 18 mL amber vial. The vial was tightly closed and, after a 10 min waiting at 23 °C to promote equilibration, the fiber was inserted into the vial and kept in headspace for 20 min. After this time, the fiber was retired and then taken to chromatograph injector where it remained for 60 s to desorption before chromatographic analysis. Chromatographic analysis was performed on a Shimadzu GC 2010 (Tokyo, Japan) equipped with a flame ionization detector. A DB5 silica capillary column (30 m, 0.25 mm i.d., film thickness 0.25 μm) was used. The oven temperature program was: 50 °C (3 min), 50–240 °C at 5 °C/min and then the temperature was maintained at 240 °C for 10 min. Injector and detector temperatures were maintained at 250 °C and 290 °C, respectively. The relative amounts of the components were calculated based on GC peak areas without correction factors.

2.7. HS/SPME-GC/MS

The chemical composition profiles of the substances present in the headspace were obtained GC/MS on a Shimadzu 2010 Plus GC/MS system (Tokyo, Japan). The substances were separated in a fused silica Agilent DB5 capillary column (30m, 0.25 mm id, 0.25 μm.). The oven temperature program was: 50 °C (3 min), 50–240 °C at 5 °C/min and then maintained at 240 °C for 10 min. The mass spectrometer was operated under the following conditions: Interface temperature: 275 °C, electron ionization at 70 eV and scan range from 40 to 1000 Da. Injector and interface temperatures were maintained at 250 °C and 200 °C, respectively. The carrier gas used was He (99.999%) under a flow rate of 1 mL/min.

2.8. HS/SPME-GC/FID enantiomer characterization

The same chromatographic system above was used for **c**hiral analysis. The chromatographic column was replaced by a Restek