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Inactivation of plant-pathogenic fungus *Colletotrichum acutatum* with natural plant-produced photosensitizers under solar radiation

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ABSTRACT

The increasing tolerance to currently used fungicides and the need for environmentally friendly antimicrobial approaches have stimulated the development of novel strategies to control plant-pathogenic fungi such as antimicrobial phototreatment (APT). We investigated the in vitro APT of the plant-pathogenic fungus *Colletotrichum acutatum* with furocoumarins and coumarins and solar radiation. The compounds used were: furocoumarins 8-methoxypsoralen (8-MOP) and 5,8-dimethoxypsoralen (isopimpinellin), coumarins 2*H*-chromen-2-one (coumarin), 7-hydroxycoumarin, 5,7-dimethoxycoumarin (citropten) and a mixture (3:1) of 7-methoxycoumarin and 5,7-dimethoxycoumarin. APT of conidia with crude extracts from 'Tahiti' acid lime, red and white grapefruit were also performed. Pure compounds were tested at 50 μM concentration and mixtures and extracts at 12.5 mg L^{-1}. The *C. acutatum* conidia suspension with or without the compounds was exposed to solar radiation for 1 h. In addition, the effects of APT on the leaves of the plant host *Citrus sinensis* were determined. APT with 8-MOP was the most effective treatment, killing 100% of the conidia followed by the mixture of two coumarins and isopimpinellin that killed 99% and 64% of the conidia, respectively. APT with the extracts killed from 20% to 70% of the conidia, and the extract from 'Tahiti' lime was the most effective. No damage to sweet orange leaves was observed after APT with any of the compounds or extracts.

1. Introduction

An important disease of citrus in the Americas is the postbloom fruit drop (PFD) or blossom blight [1,2] caused by *Colletotrichum acutatum* sensu *lato* and *Colletotrichum gloeosporioides* sensu *lato* [3,4]. However, *C. acutatum* is much more important than *C. gloeosporioides* in all citrus growing areas. Recent studies have proposed several cryptic species within the *C. acutatum* complex based on multilocus phylogeny [5,6]. Although a thorough study about the etiology of PFD has not been done yet, *C. abscissum* was recently identified by molecular data as a causal agent of PFD within the *C. acutatum* complex [7]. Typical PFD symptoms are orange-brown lesions on petals and small peach-brown to dark-brown necrotic spots on the stigma and style [8,9]. Flower infection leads to hormonal changes and causes fruit abscission [10]. *C. acutatum* produces acervuli on both sites of the petals with abundant unicellular hyaline conidia surrounded by a mucilaginous matrix [9]. It has been proposed that conidia are dispersed by rain splash after the mucilage has been dissolved by water [1,2]. However, recent results have shown that dispersal of the pathogen may be also related to a mechanism other than splash dispersal [11]. The control of PFD is based on fungicide sprays during the entire bloom period, particularly after rain events [12]. There are only two fungicides groups (strobilurins and triazoles) available for PFD control in sweet orange commercial orchards to juice production in São Paulo state, Brazil [12]. This limited availability of compounds has stimulated the development of new strategies for control of fungal pathogens [13–15].

The light-based approach antimicrobial phototreatment (APT) is a promising antifungal alternative that can be used to control fungi that cause diseases in humans and plants [14–22]. APT mode-of-action is based on the use of a photosensitizer (PS) that preferentially accumulates in the target microbial cells [14,15,18,19,21,23]. Subsequent exposure of the PS to light of an appropriate wavelength starts a photochemical process that may produce several reactive oxygen

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species (ROS) leading to non-specific oxidative damage and causing the subsequent death of the microbial cells without significant damage to host tissues [14,15,18,19,22,23].

When produced at the plant surface, either via natural plant-produced PS or via applied PS, reactive species can interact with pathogens such as fungi and bacteria and even with insect ovipositors [24,25]. The multiple targets of ROS reduce the chance of selecting tolerant microorganisms. In addition, the PS used in APT are usually less toxic to humans and animals and are less aggressive to the environment than most of the currently used fungicides [14–16,22]. Also, unlike many conventional fungicides or antibiotics that kill only metabolically active cells, APT is able to kill both metabolically active and inactive dormant or quiescent structures such as fungal conidia [14,15,20,23,26] and bacterial spores [27,28]. The disadvantage of APT compared to conventional fungicides, the main strategy for control of fungal plant diseases, is that it does not work at night.

Some PS such as coumarins and furocoumarins (psoralens) are naturally produced as secondary metabolites in a variety of plant species, particularly in those belonging to the Umbelliferae, Apiaceae and Rutaceae families [29]. Plants might produce these metabolites to act either via light-dependent or -independent mechanisms, as protectants against microorganisms and insects [30,31]. These compounds are synthesized continuously at low levels and at much higher concentrations when plants are stressed by environmental factors, including bacterial and fungal infections [32–34].

The stable covalent photoconjugation of furocoumarins with DNA was thought to bear sole responsibility for the lethal effect of this group of PS. Psoralens are capable of forming either monofunctional (single strand) or bifunctional adducts (interstrand cross-links) with DNA [35,36]. The oxygen-dependent mechanism responsible for the photodynamic action of psoralens was discovered later. Joshi and Pathak [37] demonstrated the in vitro production of singlet oxygen ($^1O_2$) and superoxide radical by several linear and angular furocoumarins. It was postulated that both forms of active oxygen contribute to the in vitro phototoxicity of the agents, possibly at the level of the cell membrane. The photosensitizing action of furocoumarins on membrane components was reviewed by Dall'acqua and Martelli [38]. It is currently accepted that damage by furocoumarins might result from dual or even multiple processes [39,40].

Photoinactivation of plant pathogenic fungi were performed using different types of PS against species of several genera [14,15,33,41–50]. So far little attention has been paid to some important aspects of APT that are crucial for its commercial use under field conditions. For example, most of the studies were performed in vitro and only a few of them evaluated the effects of APT on the plant host or in the environment [14,15,51]. We have demonstrated that APT under solar radiation with phenothiazinium PS, such as methylene blue derivatives, coumarins and furocumarins efficiently kill conidia of *Colletotricum acutatum* without damaging the plant host *Citrus sinensis* [14,15].

The aim of the current study was to evaluate the efficacy of APT under solar radiation with pure furocoumarins and coumarins and also with extracts rich in these compounds obtained from 'Tahiti' acid lime (*Citrus latifolia*) and grapefruit (*Citrus paradisi*) on conidia of *C. acutatum*. All the extracts and the coumarins 7-hydroxycoumarin and 5,7-dimethoxycoumarin were obtained in the present study. The furocoumarins 5,8-dimethoxypsoralen and the mixture of the two coumarins were obtained and identified previously and their phototoxicity to fungal conidia was already demonstrated [15]. The stability of commercial furocoumarin 8-MOP under solar radiation was evaluated. The effects of APT with the pure compounds and extracts on the leaves of the plant host *C. sinensis* were also determined.

2. Material and Methods

2.1. Mass Spectrometry

For identification of coumarins and furocoumarins, gas chromatography–mass spectrometry (GCMS) analyses were performed using a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with a AOC-20i autosampler under the following conditions: Restek Rtx-5MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), composed of 5%-phenyl–95%-methylpolysiloxane operating in the electron ionization mode at 70 eV. Helium (99.99%) was used as carrier gas at a constant flow of 1 mL min^{-1}. The injection volume was 0.1 μL (split ratio of 1:20), the injector temperature was 240 °C, and the ion-source temperature was 280 °C. The oven temperature was programmed to increase from 60 °C to 240 °C at 3 °C min^{-1}. Mass spectra were taken with mass range from 40 to 600 Da. Identification of coumarins and furocoumarins was performed by comparing the obtained mass spectra with Wiley 7, NIST 08 and FFNSC 1.2 spectra databases, as well as by comparison of their mass spectra with those reported in the literature. Percentage content was estimated by internal normalization.

2.2. Nuclear Magnetic Resonance Spectroscopy

^1H NMR spectra were recorded in $CDCl_3$ and CD_3OD at 500 MHz on a Bruker Advanced DRX-500 spectrometer (Bruker, Darmstadt, Germany). ^{13}C NMR spectra were acquired at 125 MHz on a Bruker Advanced DRX-400 spectrometer (Bruker, Darmstadt, Germany).

2.3. Collection of Crude Extracts From 'Tahiti' Lime Peel and its Essential Oil; Collection of Grapefruit Essential Oil

'Tahiti' lime peel essential oil, red and white grapefruit essential oils are produced during the industrial processing of *Citrus latifolia* and *C. paradisi*, respectively. The 'Tahiti' lime oils were kindly provided by Citrosuco Company (Fischer Group, Matão, SP, Brazil). The red and white grapefruit essential oils were obtained from Dierberger (Dierberger Óleos Essenciais, S.A., Barra Bonita, SP, Brazil). In order to isolate coumarins and furocoumarins, 100 mL of 'Tahiti' lime peel oil were basified to pH 13 with a 5 mol L^{-1} NH_4OH solution. Then, the mixture was partitioned three times with 1.8 L of ethyl acetate, and the remaining alkaline aqueous phase was acidified to pH 1 with a 5 mol L^{-1} H_2SO_4 solution, followed by three times partition with 1.8 L of ethyl acetate. The ethyl acetate fractions from the acidified water fraction were combined and concentrated under vacuum to afford 79.37 g of crude extract (named crude extract 1). To verify the presence of coumarin compounds, thin layer chromatographic (TLC) analysis (Kieselgel 60 F_{254} 20 × 20 cm, Merck, Germany) was undertaken with a mobile phase of hexanes/ethyl acetate (7:3 v/v) and hexanes/acetone (7:3 v/v). TLC spots were visualized under a 254 and 366 nm UV lamp before and after sprinkling the plates with a hydroalcoholic solution of KOH 1 M. Then, an aliquot of 61.5 g of the crude ethyl acetate extract was submitted to classical open column liquid chromatography (silica gel 0.060 × 0.200 mm, 60 A, Merck, Germany) using a mobile phase of hexanes/ethyl acetate at increasing gradient of polarity. All fractions obtained were concentrated under vacuum and analyzed by TLC using hexanes/ethyl acetate (7:3 v/v) and hexane/acetone (7:3 v/v) as previously described. The fraction of 0.97 g eluted in hexanes/ethyl acetate (75:25 v/v) showed the characteristic fluorescence emission of the furocoumarins and coumarins. For the other essential oils, 50 mL of each one were basified to pH 13 with a 5 mol L^{-1} KOH hydroalcoholic solution (70% of KOH solution and 30% of ethanol). The mixtures were partitioned three times with 900 mL of ethyl acetate, and the remaining alkaline aqueous phases were acidified to pH 1 with a 5 mol L^{-1} H_2SO_4 solution, followed by three times partition with 900 mL of ethyl acetate. The ethyl acetate fractions of the acidified fraction were combined and dried under vacuum to afford 23.11 g, 16.77 g, and 11.89 g of crude extracts of 'Tahiti' lime (crude extract 2), red grapefruit (crude extract 3) and white grapefruit (crude extract 4) essential oils, respectively. To verify the presence of coumarin compounds, thin layer chromatographic (TLC) analysis (Kieselgel 60 F_{254} 20 × 20 cm, Merck, Germany) was undertaken with a mobile phase of hexanes/ethyl acetate (7:3 v/v)