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 coumpounds or extracts.

Keywords: microbial photo inactivation; photoantimicrobial; fungal photodynamic inactivation; *Colletotrichum acutatum;* coumarins; furocoumarins.

1. Introduction

47 An important disease of citrus in the Americas is the postblom fruit drop (PFD)
48 or blossom blight [1,2] caused by *Colletotrichum acutatum sensu lato* and
49 *Colletotrichum gloeosporioides sensu lato* [3,4]. However, *C. acutatum* is much more
50 important than *C. gloeosporioides* in all citrus growing areas. Recent studies have
51 proposed several cryptic species within the *C. acutatum* complex based on multilocus
52 phylogeny [5,6]. Although a thorough study about the ethiology of PFD has not been
53 done yet, *C. abscissum* was recently identified by molecular data as a causal agent of
54 PFD within the *C. acutatum* complex [7]. Typical PFD symptoms are orange-brown
55 lesions on petals and small peach-brown to dark-brown necrotic spots on the stigma and
56 style [8,9]. Flower infection leads to hormonal changes and causes fruit abscission [10].
57 *C. acutatum* produces acervuli on both sites of the petals with abundant unicellular
58 hyaline conidia surrounded by a mucilaginous matrix [9]. It has been proposed that
59 conidia are dispersed by rain splash after the mucilage has been dissolved by water
60 [1,2]. However, recent results have shown that dispersal of the pathogen may be also
61 related to a mechanism other than splash dispersal [11]. The control of PFD is based on
62 fungicide sprays during the entire bloom period, particularly after rain events [12].
63 There are only two fungicides groups (strobilurins and triazoles) available for PFD
64 control in sweet orange commercial orchards to juice production in São Paulo state,
65 Brazil [12]. This limited availability of compounds has stimulated the development of
66 new strategies for control of fungal pathogens [13-15].

67 The light-based approach antimicrobial phototreatment (APT) is a promising
68 antifungal alternative that can be used to control fungi that cause diseases in humans
69 and plants [14,15,16-22]. APT mode-of-action is based on the use of a photosensitizer
70 (PS) that preferentially accumulates in the target microbial cells [14,15,18,19,21,23].
71 Subsequent exposure of the PS to light of an appropriate wavelength starts a

photochemical process that may produce several reactive oxygen 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 eficently 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 coumpounds 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 photoxicity to fungal conidia was already demonstrated [15]. The stability of commercial furocoumarin 8-MOP under solar

121 radiation was evaluated. The effects of APT with the pure compounds and extracts on
122 the leaves of the plant host *C. sinensis* were also determined.
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124 **2. Material and methods**
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126 *2.1. Mass spectrometry*
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128 For identification of coumarins and furocoumarins, gas chromatography-mass
129 spectrometry (GCMS) analyses were performed using a Shimadzu QP2010 Plus
130 (Shimadzu Corporation, Kyoto, Japan) system equipped with a AOC-20i autosampler
131 under the following conditions: Restek Rtx-5MS fused silica capillary column (30 m ×
132 0.25 mm i.d. × 0.25 μm film thickness), composed of 5%-phenyl–95%-
133 methylpolysiloxane operating in the electron ionization mode at 70 eV. Helium
134 (99.99%) was used as carrier gas at a constant flow of 1 mL min^{-1}. The injection volume
135 was 0.1 μL (split ratio of 1:20), the injector temperature was 240 °C, and the ion-source
136 temperature was 280 °C. The oven temperature was programmed to increase from 60 °C
137 to 240 °C at 3 °C min^{-1}. Mass spectra were taken with mass range from 40 to 600 Da.
138 Identification of coumarins and furocoumarins was performed by comparing the
139 obtained mass spectra with Wiley 7, NIST 08 and FFNSC 1.2 spectra databases, as well
140 as by comparison of their mass spectra with those reported in the literature. Percentage
141 content was estimated by internal normalization.
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143 *2.2. Nuclear magnetic resonance spectroscopy*
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145 ^{1}H NMR spectra were recorded in $CDCl_3$ and CD_3OD at 500 MHz on a Bruker
146 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) 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. The crude ethyl acetate extract obtained from red grapefruit essential oil was submitted to liquid chromatography (silica gel 0.040 × 0.063 mm, 60 A, Merck, Germany) using mobile phase of hexanes/ethyl acetate at increasing gradient of polarity. All fractions obtained were concentrated under vacuum and analyzed by TLC as previously reported. Four fractions eluted with hexanes/ethyl acetate (70:30, 65:35, 60:40 and 55:45 v/v) showed the same characteristic fluorescence emission of furocoumarins and coumarins. Then, they were combined to afford 0.34 g, and submitted to preparative HPLC.

2.4. Collection of crude extract of 'Tahiti' lime peel

For comparison with the oils obtained from the citrus industry, an extract from 'Tahiti' lime peel was obtained. The limes were peeled and the peels were dried in an oven at 45 °C with circulating air for 3 h. After drying, the peels were milled in a small coffee mill (Cadence MDR 301, Brazil), resulting in a powdered biomass of 20.44 g of powder. Then, the powder was macerated three times with 200 mL of hexanes for 48 h, the extract was filtered through filter paper and the solvent was concentrated under vacuum furnishing 5 mL of the oily extract. This was then basified to pH 13 with a 5 mol L^{-1} KOH hydroalcoholic solution (70% of KOH solution and 30% of ethanol). The mixture was partitioned three times with 90 mL 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 partitions with 90 mL of ethyl acetate. The ethyl acetate fractions of the acidified fraction were combined and dried under vacuum to afford 0.172 g of crude extract (crude extract 5). To verify the presence of coumarin compounds, thin layer chromatographic (TLC) analysis was carried out as previously described.

2.5. Isolation and identification of coumarins and furocoumarins

The sample obtained from the crude extract of 'Tahiti' lime peel oil (fraction 1) and from the crude extract of red grapefruit essential oil (fraction 2) were diluted in methanol/acetonitrile 1:1 (chromatographic grade, Merck, Germany) and subjected to HPLC analysis in a Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler (SIL-10AD), diode array detector (DAD) (SPD-M10CA), ternary solvent system (LC-10AD) and degasser (DGU-14A). The analysis was undertaken with the help of the Software Class-VP. The column used was an analytical column Polar-RP

(Sinergy, Phenomenex Inc., CA, USA) with pore sizes 4 μm, 150 × 4.60 mm dimension. The solvents used as mobile phase were methanol/water chromatographic grade (Merck, Germany) in gradient starting with 31% of methanol, linearly increasing to 100% of methanol at 40 min of analyses, keeping 100% till 45 min, and linearly down to 31% at 47 min and keeping 31% till 52 min. The detection was undertaken at 270 and 360 nm. The volume of injection was 20 μL (1 mg mL^{-1}), and the flow rate was 1 mL min^{-1}. After HPLC analyses, the separation of the compounds was performed in a Shimadzu preparative HPLC (Shimadzu, Kyoto, Japan) equipped with manual injector, UV-Vis detector (SPD-20A), binary solvent system (LC-6AD), degasser (DGU-20A5) and an automatic sample collector (FRC-10A). The column used for the separation was a preparative column Polar-RP (Synergi, Phenomenex Inc., CA, USA) with pore sizes 4 μm, 250 × 21.20 mm dimension. The samples were diluted in methanol/acetonitrile (1:1) chromatographic grade (Merck, Germany) and 500 μL of a – 60 mg mL^{-1} solution was injected several times. The mobile phase used was the same described above for HPLC analyses and the flow rate was 15 mL min^{-1}. After separation, the samples were analyzed by HPLC under the same conditions described above and four fractions were obtained. The fractions were named as fractions 1.1, 1.2, 2.1 and 2.2, and were analysed by GC-MS, ^{1}H RNM and ^{13}C RMN.

2.6. Photosensitizers

The compound 8-Methoxypsoralen (8-MOP; cat # M3501-1G) was purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA) (Fig. 1C). Coumarin (2*H*-chromen-2-one; cat # 00C1067.06.AF) was purchased from Synth (Synth, SP, Brazil) (Fig. 1D). 5,7-dimethoxycoumarin ($C_{11}H_{10}O_4$) (Fig. 1A) and 7-hydroxycoumarin ($C_9H_6O_3$) (Fig.

1F) were extracted and purified from 'Tahiti' lime peel oil and red grapefruit essential oil, respectively. Isopimpinellin (5,8-dimethoxypsoralen, $C_{13}H_{10}O_5$) (Fig. 1E), and a 3:1 mixture of 7-methoxycoumarin ($C_{10}H_8O_3$) (Fig. 1F) and citropten (5,7-dimethoxycoumarin, $C_{11}H_{10}O_4$) (Fig. 1A) were obtained previously [15].

The absorption spectra of the pure compounds and crude extracts (1 to 5) were measured using an Ultrospec™ 2100 pro UV–visible spectrophotometer (GE Healthcare) (see Supplementary Fig. S1). The fluorescence emission spectra of the compounds were measured using a Hitachi Fluorescence Spectrometer (Hitachi, Hitachi, Japan). The excitation wavelengths were 303, 312, 324, 328, 318, 320, 317, 319 and 336 nm for 8-MOP, coumarin, 7-hydroxycoumarin, 5,7-dimethoxycoumarin and crude extracts 1, 2, 3, 4 and 5, respectively (see Supplementary Fig. S1). Solutions of the compounds were prepared just prior to use. All compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma), and appropriate dilutions were prepared with 0.01% (v/v) Tween 80 solution (Sigma–Aldrich).

2.7. Fungal isolate, colony growth and conidia production

Colletotrichum acutatum sensu lato isolated CA 142 was obtained from blossom blight symptoms of sweet orange petal collected in commercial orchard in Santa Cruz do Rio Pardo, São Paulo, Brazil. Monosporic culture was made and preserved on filter paper. This isolate is stored at the Plant Pathogenic Fungi Collection of the Department of Plant Pathology and Nematology (Escola Superior de Agricultura ''Luiz de Queiroz'', University of São Paulo, Piracicaba, Brazil).

The fungus was grown on 25 mL Acumedia™ Potato Dextrose Agar (Acumedia Manufacturers, Inc. Lansing, MI, EUA) supplemented with 1 g L^{-1} Bacto™ Yeast Extract (BD) (PDAY) in Petri dishes (90 × 10 mm) at 28 °C for 5 days with 12 h (dark/light) photoperiods. Conidia were carefully scraped from the colonies and suspended in a 0.01% (v/v) Tween 80 solution. Conidia concentration was determined with a hemocytometer (Improved Neubauer, Boeco, Germany) and appropriate dilutions were made with the same solution.

2.8. Evaluation of the effect of APT on conidial survival

In two-milliliters microtubes (Polyropilen, Axygen Scientific, CA, USA) was added 1.3 mL of the conidial suspension and solution of: (1) 8-MOP; (2) coumarin; (3) 5,7-dimethoxycoumarin; (4) 7-hydroxycoumarin; (5) isopimpinellin; (6) mixture of two coumarins; (7) crude extract 1; (8) crude extract 2; (9) crude extract 3; (10) crude extract 4; (11) crude extract 5. Final concentration of conidia in the mixtures was 2 × 10^6 conidia mL^{-1}. Final concentrations of pure compounds were 50 µM. Concentrations in mg L^{-1} of coumarin, 8-MOP, isopimpinellin, 5,7-dimethoxycoumarin and 7-hydroxycoumarin were 7.31, 10.8, 12.3, 10.3 and 5.7, respectively. Final concentration of the mixture of coumarins and extracts was 12.5 mg L^{-1}. Final concentration of DMSO was 1 % in all the mixtures. The tubes were held in the dark for 30 min at 25 °C and 1 mL of each suspension were trasnferred to a 24-well flat-bottomed microtitre plates (Polystyrene, TPP, Switzerland). Plates were covered with a 0.13-mm-thick cellulose diacetate film (JCS Industries, Le Mirada, CA) to avoid contamination and exposed to solar radiation for 1 h floating in water at 25 ± 2 °C. Three different types of control-plates were prepared in parallel in all the experiments: (1) control-plates in

which conidia were exposed to solar radiation but not treated with the PS; (2) control-plates in which conidia were treated with the PS and protected from solar radiation during the exposure (plates were wrapped in aluminum foil) and (3) control-plates in which conidia were not treated with the PS and were protected from solar radiation. Temperatures of the conidial suspensions were recorded during the experiments. After light exposure, conidial suspensions were collected and serially diluted 10-fold in a 0.01% (v/v) Tween 80 solution to provide dilutions of 10^{-1} to 10^{-2} times the original concentration, and 50 µL were spread on the surface of 5 mL of PDAY medium containing 0.08 g L^{-1} of deoxycholic acid sodium salt (Fluka, Italy) in Petri dishes (60 × 15 mm). Three replicate dishes were prepared for each treatment in each experiment. The dishes were incubated in the dark at 25 °C. After 24 h, colony-forming units (CFU) were counted daily at 8× magnification for up to 7 days. Solar radiation effect and PS effect (dark toxicity) were expressed as a ratio of CFU of conidia treated only with light and only with PS to CFU of conidia treated with neither. APT effects were expressed as a ratio of CFU of conidia treated with light and PS to CFU of conidia treated with neither. As we know that exposure only to light reduces conidial survival, the significance of APT with each photosensitizer was calculated in relation to conidia exposed only to solar radiation. All the light exposures were carried out between 11 and 12 h under clear sky. Pure compounds were evaluated on October 14th, 15th and 29th of 2014, crude extracts were evaluated on September 8th, 9th and 10th of 2014, and 8-MOP and coumarin were evaluated on all the six days. Experiments were undertaken in Ribeirão Preto, SP, Brazil (21° 10′ 39″ S latitude, 546 m altitude).

2.9. Evaluation of 8-MOP stability under solar radiation

320 The exposure of PS to high irradiances can reduce their activity. We evaluated
321 the effects of exposures to full-spectrum solar radiation on 8-MOP absorption spectra
322 and on its efficacy in killing *C. acutatum* conidia in APT. The efficacy of APT with 8-
323 MOP was evaluated after PS had been previously exposed to solar radiation for up to 12
324 h. Ten mL of a 50 µM 8-MOP solution was placed into Petri dishes (60 × 15 mm)
325 whose lids were replaced by a 0.13-mm-thick premium cellulose triacetate film (Liard
326 Plastics, Salt Lake City, UT, USA). Plates were exposed to solar radiation for 0, 0.5, 1,
327 2, 4, 6, 8, 10 and 12 h floating in water at 25 ± 2 °C. Experiments were performed under
328 clear sky on March 24th, April 17th and July 13th 2015. At the end of the exposures, the
329 volumes of the solutions were readjusted to 10 mL with distilled water. In 2 mL-
330 microtubes, conidia were suspended in 50 µM solutions of 8-MOP not exposed to solar
331 radiation, exposed for 6 h and exposed for 12 h. Tubes were held in the dark for 30 min
332 at 25 °C. Next, 1 mL of the suspensions were placed into a 24-well flat-bottoned plate
333 and exposed to solar radiation for 1 h. The effect of APT on conidial survival was
334 determined as described previously. Experiments were performed on April 29, July 20
335 and 22th with the solution that were exposed on March 24th, April 17th and July 13th
336 2015, respectively.
337
338 *2.10. Solar radiation measurements*
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340 Both solar spectral irradiance and solar UV (290–400 nm) irradiance were
341 measured using a cosine-corrected irradiance probe (CC-3-UV, Ocean Optics, Dunedin,
342 FL, USA) screwed onto the end of an optical fiber coupled to an USB4000
343 spectroradiometer (Ocean Optics, Dunedin, FL) as previously described [14,15].
344

345 *2.11. Evaluation of the effects of APT on leaves of* Citrus sinensis

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347 The three *C. sinensis* plants employed in the study were approximately 1.7 m
348 tall. Five µL of 8-MOP (50 µM; 10.8 mg L^{-1}), 5,7-dimethoxycoumarin (50 µM; 10.3 mg
349 L^{-1}), crude extract 1, crude extract 5 and the mixture of coumarins (all at 12.5 mg L^{-1})
350 were spotted every three days for 21 days on the adaxial surface of three leaves of each
351 plant. After application of the compounds, plants were kept outdoors under a natural
352 sunlight regime. Plants were visually evaluated daily up to 21 days for damage to the
353 leaves. Experiments were conducted in November and December 2014 in Ribeirão
354 Preto, São Paulo state, Brazil.

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356 *2.12. Statistical analysis*

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358 Comparisons between the different treatments were made by analysis of
359 variance (ANOVA). Data from each day were analyzed separately. Tukey's test for
360 multiple comparisons was used and *P* values of <0.05 were considered significant. All
361 analyses were carried out using PROC GLM in SAS/STAT version 9.2 (SAS Institute,
362 2011. Version 9.2. SAS Institute Inc., Cary, NC, USA).

363

364 **3. Results**

365

366 *3.1. Isolation and characterization of the coumarins and furocoumarins*

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368 The compounds present in fractions 1.1 and 2.1, which were obtained from
369 crude extract of 'Tahiti' lime peel oil (fraction 1) and from the crude extract of red

grapefruit essential oil (fraction 2) were elucidate, as following: Fraction 1.1 was identified as 5,7-dimethoxycoumarin (Fig 1A), the ^{1}H RMN data was obtained in 500 MHz using $CDCl_3$ as solvent. The data showed doublets at 6.2 ppm (*d*, *J*= 9.5) and 7.9 ppm (*d*, *J*= 9.5), corresponding to H-3 and H-4, respectively, wich are hydrogens of a pyrone moiety in Z configuration. Chemical shifts corresponding to aromatic hydrogens were detected at 6.3 (*d*, *J* = 2.1 Hz, H-8), 6.3 (*d*, *J* = 2.1 Hz, H-7). The chemical shifts at 3.8 and 3.9 ppm showed the presence of two methoxyl groups. The ^{13}C RMN data was obtained in 125 MHz also using $CDCl_3$ as solvent and the chemical shifts showed signals at 161.9 ppm, 111.2 ppm, 139.2 ppm, 104.4 ppm, 157.4 ppm, 95.2 ppm, 164.1 ppm, 93.1 ppm, 157.2 ppm, 55.8 ppm and 55.9 ppm. These chemical shifts correspond to C-2, C-3, C-4, C-4a, C-5, C-6, C-7, C-8, C-8a, OCH_3 and OCH_3, respectively. EI-MS/*z* calculated for the fraction 1.1 was 206 ($C_{11}H_{10}O_4$). Fraction 2.1 was identified as 7-hydroxycoumarin (Fig.1B), and the ^{1}H NMR data was obtained in 500 MHz using CD_3OD as solvent. The data showed doublets at 6.2 ppm (*d*, *J*= 9.5) and 7.8 ppm (*d*, *J*= 9.5), corresponding to H-3 and H-4, respectively, wich are hydrogens of a pyrone moiety. Chemical shifts corresponding to aromatic hydrogens were detected at 6.7 ppm (*d*, *J* = 2.3 Hz, H-8), 7.3 ppm (*d*, *J* = 8.5 Hz, H-5) and at 6.8 ppm (*dd*, *J* = 2.3 and *J* = 8.5 Hz, H-6). The chemical shift at 5.0 ppm showed the presence of one hydroxyl group. The ^{13}C NMR data was obtained in 125 MHz also using CD_3OD as solvent and the chemical shifts showed the the signals at: 163.7 ppm, 112.3 ppm, 146.1 ppm, 113.1 ppm, 130.7 ppm, 114.5 ppm, 163.2, 103.4 ppm, 157.3 ppm. These chemical shifts correspond to C-2, C-3, C-4, C-4a, C-5, C-6, C-7, C-8 and C-8a, respectively. EI-MS/*z* calculated for the fraction 2.1 was 162 ($C_9H_6O_3$). Chemical shifts for both coumarins were compared with the data previously reported [52].

All the crude extracts were analyzed by GC-MS. The data obtained were compared with the similarity index expressed as a percentage by the equipment standards library. Moreover, linear retention index (LRI) calculations were carried out and the indices were compared with literature data, making possible the identification of the crude extracts components. All the crude extracts, except crude extract 4, obtained from white grapefruit essential oil, showed the presence of coumarins and/or furocoumarins. From the crude extract 1, obtained from Tahiti lime peel oil, only 5,7-dimethoxycoumarin (9.98%) was identified and isolated. From the crude extract 2, obtained from Tahiti lime essential oil, coumarins 5,7-dimethoxycoumarin (19.84%) and 7-methoxycoumarin (10.19%), and the furocoumarins isopimpinellin (7.51%) and bergapten (4-methoxy-7*H*-furo[3,2-*g*]chromen-7-one) (8.83%) were identified. From the crude extract 3, obtained from red grapefruit essential oil, it was identified and isolated 7-hydroxycoumarin (5.28%), 7-hydroxy-6-methoxy-4-methyl-coumarin (0.50%) and 7-methoxy-8-(2-oxo-3-methyl-butyl)coumarin (10,82%). The crude extract 5, obtained from Tahiti lime peel oil showed the highest variety and percentage of coumarins in comparison to all the others. Among the six compounds present in the extract, four of them were coumarins or furocoumarins, such as 5,7-dimethoxycoumarin (45.23%), 7-methoxycoumarin (8.19%), bergapten (22.83%) and isopimpinellin (15.73%). In the majority of the studied extracts it was found the terpenoids commonly found in citrus essential oils such as limonene, linalool, α and γ-terpineol, α and β-bisabolene and γ-terpinene commonly found in citrus essential oils [53,54].

3.2. *Evaluation of the effect of APT on conidial survival*

417 Midday solar spectral irradiance and hourly recorded solar UV (290–400 nm)
418 irradiance along with the temperatures of the conidial suspension during the exposures
419 to full-spectrum sunlight are shown in Figs. 2A and B, respectively.

420 Exposure for 1 h to solar radiation in the absence of PS killed part of the conidia
421 and the effect varied according to the day. Conidial mortality ranged from 11.37% (Sept
422 10^{th}) to 29.64% (Oct 14^{th}) ($P < 0.05$ for Sept 8^{th}, 9^{th} and Oct 14^{th} and $P > 0.05$ for Sept
423 10^{th} and Oct 15^{th} and 29^{th}) (Fig. 3A-F).

424 In the absence of light, pure compounds, the mixture of the two coumarins (7-
425 methoxycoumarin and 5,7-dimethoxycoumarin) and the extracts had no significative
426 effects (i.e. dark toxicity) on the conidial survival (Fig. 3A-F). Treatments only with 8-
427 MOP, coumarin, 5,7-dimethoxycoumarin, 7-hydroxycoumarin, the mixture of the two
428 coumarins and isopimpinellin killed an average of 8.52%, 8.39%, 10.65%, 4,87%,
429 8.39% and 4.07% of the conidia, respectively ($P > 0.05$ for all compounds and days).
430 Treatments only with the crude extracts 1, 2, 3, 4 and 5 killed an average of 6.61%,
431 9.87%, 4.52%, 10.77% and 5.65% of the conidia, respectively. ($P > 0.05$ for all extracts
432 and days).

433 The effects of APT with 50 µM of 8-MOP, coumarin, 5,7-dimethoxycoumarin,
434 7-hydroxycoumarin or isompimpinellin, or with 12.5 mg L^{-1} of the mixture of the two
435 coumarins and crude extracts 1 to 5 on the conidia of *C. acutatum* are shown in Figs.
436 3A-F. APT with 8-MOP killed 100% of the conidia on Sept 08^{th} and 10^{th} and Oct 15^{th}
437 and 29^{th} and 99.99 and 99.56% on Sept 9^{th} and Oct 14^{th}, respectively ($P < 0.05$ for all
438 days) (Figs. 3A-F). In log values, APT with 8-MOP resulted in an approximately 5-log
439 reduction in the survival of the conidia (which is the maximum reduction that could be
440 determined with the experimental design). APT with the furocoumarin isopimpinellin
441 was also effective and killed between 63.37% (Oct 14^{th}) and 82.80% (Oct 29^{th}) of the

conidia (thus, a reduction of less than 1 log in conidial survival) ($P < 0.05$ for all days) (Figs. 3A-C). APT with the coumarin was much less effective and killed between 29.28% (Oct 29th) and 47.85% (Sept 9th) of conidia ($P < 0.05$ for Sept 8th, 9th and 10th and $P > 0.05$ for Oct 14th, 15th and 29th) (Figs. 3A-F). APT with 5,7-dimethoxycoumarin had little effect and killed between 23.33% (Oct 29th) and 38.40% (Oct 15th) of the conidia ($P > 0.05$ for all days) as well as APT with 7-hydroxycoumarin which killed between 21.96% (Oct 15th) and 38.06% (Oct 14th) ($P > 0.05$ for all days) (Figs. 3A-C). APT with the mixture of the two coumarins was very effective and killed between 99.53% (Oct 14th) and 99.72% (Oct 29th) of the conidia (reduction of approximately 3 logs in conidial survival) ($P < 0.05$ for all days) (Figs. 3A-C).

APT with all the crude extracts killed the conidia of the fungi ($P < 0.05$ for all the extracts and days). Crude extract 3 (from Tahiti lime peel) was the most effective among the extracts tested and killed between 65.05% and 73.54% of conidia. The conidia mortality in APT with the other crude extracts ranged from 21.31% to 65.94% and were lower than the obtained with crude extract 3 (Figs. 3D-F) (reduction of less than 1 log in conidial survival for all the extracts).

3.3. Evaluation of 8-MOP stability under solar radiation

Exposure of 8-MOP to solar radiation change its absorption and fluorescence emission spectra (see Supplementary Fig. S1) and reduce its effectiveness in APT (Fig. 4). All the changes were dependent of the exposure time. When APT were performed using 8-MOP previously exposed to solar radiation for 12 h, mortality ranged from 83.87 to 98.8% insted of 100% archived with the non exposed photosensitizer (Fig. 4).

3.4. Evaluation of APT effects on leaves of Citrus sinensis

APT with 8-MOP, APT with the mixture of the two coumarins, and APT with crude extracts 1 and 5 did not cause any visual damage to the adult leaves of *C. sinensis* until 21 days after application of these compounds (see Supplementary Fig. S2).

4. Discussion

The need to overcome deficiencies in conventional strategies to control plant-pathogenic fungi has stimulated the investigation of alternative approaches, such as light-based APT. The use of APT in agriculture to control plant pathogens may require the application of the PS over large areas. Thus we are involved in the development of processes to obtain natural PS in large amounts and at low cost. PS such as coumarins and furocoumarins may be obtained directly from plants or from by-products generated during the processing of fruits such as 'Tahiti' acid lime and grapefruit. In a previous study, we found that coumarins and furocoumarins are present in products, such as peel oil, by-products, such as bagasse, and in wastewater generated during the processing of 'Tahiti' to juice production [15]. A liquid waste that contains coumarins and furocoumarins is the outlet water from the concentrator centrifuge. This effluent has no commercial value, is generated in large amounts during the processing of 'Tahiti', and is discarded after treatment. Both the mixture of the two coumarins and the furocoumarin isopimpinellin (Fig. 1) was previously isolated from the outlet water from the concentrator [15]. In the present study we extracted two coumarins, 5,7-dimethoxycoumarin from 'Tahiti' peel oil and 7-hydroxycoumarin from red grapefruit essential oil.

The effects of APT under solar radiation with the five pure compounds (two furocoumarins and three coumarins), a mixture of two coumarins and five crude extracts

492 on the survival of *C. acutatum* conidia were determined. As expected, exposures only to
493 solar radiation reduced the survival of the conidia. Conidial killing after 1 h of exposure
494 reached up to 30%. The detrimental effect of solar radiation, particularly of solar UVB
495 and UVA to fungal conidia is very well established and depends on the UV irradiance
496 [55,56]. Additionally, the high irradiances in visible and UV spectra enable solar
497 radiation to excite both visible-light-activated and UV-activated PS, such as the
498 coumarins and furanocoumarins [14,15]. The efficacy of different coumarins and
499 furocoumarins as photosensitizers varies widely in different biological systems
500 [15,57,58]. We observed differences in the efficacy of APT with pure coupounds and
501 crude extracts. 8-MOP was the most effective photosensitizer in conidial
502 photoinactivation (approximately 5 log reduction in conidial survival) followed by the
503 mixture 3:1 of the coumarins 7-methoxycoumarin and 5,7 dimethoxycoumarin
504 (citropten) (reduction of approximately 3 logs). As APT with citropten has little effect
505 on conidial survival, most likely 7-methoxycoumarin was responsible for conidial
506 photoinactivation of the mixture. APT with the coumarin 2*H*-chromen-2-one and with
507 the 7-hydroxycoumarin also had litle effect on conidial survival. The close interaction
508 or the accumulation of the PS in the target-cell is one of the factors important to the
509 eficacy of the photosensitization. 8-MOP penetrates the conidia of *C. acutatum* and
510 accumulates in numerous cytoplasmatic vesicules [15]. *In vitro* APT with different PS
511 has already proved effective for conidia of several taxonomically diverse fungi
512 including plant-pathogenic species [14-17,26,33,41-48]. APT af *C. acutatum* and
513 *Aspergillus nidulans* conidia with 8-MOP at 50 µM under solar radiation resulted in a
514 reduction of approximately 4 logs in the survival of conidia for both species and APT
515 with the furocoumarin isopimpinellin resulted in a reduction of less than 2 logs for *C.*
516 *acutatum* and 4 logs for *A. nidulans* conidia [15]. Both PS were also evaluated in the

present study. APT with 8-MOP and UV-A also reduced the survival of conidia of the plant-pathogenic fungi *Fusarium oxysporium*, *F. solani*, *Penicillium italicum* and *P. digitatum* by 4, 3, 1 and 1 log, respectively [33]. APT using the phenothiazinium photosensitizers new methylene blue N (50 µM) or S137 (10 µM) under solar radiation reduced the survival of *Colletotrichum gloeosporioides*, *C. acutatum* and *Asperigillus nidulans* conidia by 5 logs [14]. APT of *Colletotrichum graminicola* conidia with cationic porphyrins (1-2.5 µM) and visible light resulted in complete conidia inactivation [17]. APT with different types of cationic porphyrins (50 µM) was also effective for *Penicillium chrysogenum* conidia and the most effective porphyrin caused a 4.1 log reduction in conidial viability [26].

The use of citrus extracts rich in PS may be useful for APT of plant pathogens since they are cheaper and easier to obtain than pure coumarins and furocoumarins. The most effective extract was obtained from 'Tahiti' peel. This extract presented the highest variety and percentage of coumarins and furocoumarins, such as 5,7-dimethoxycoumarin (45.23%), 7-methoxycoumarin (8.19%), bergapten (22.83%) and isopimpinellin (15.73%).

The residual effect of the antimicrobial is usually desirable because it allows a reduction in the number of applications required to control the target pathogen. It is known that exposure to high irradiance causes photobleaching of the PS and the loss of its photosensitizing activity [14,59]. The exposure of 8-MOP to solar radiation changed its photophysical properties and reduced its effectiveness in APT; i.e., APT with 8-MOP previously exposed to solar radiation killed between 85 and 98% of the conidia instead of the 100% achieved with the nonexposed PS. Despite the inactivation caused by solar radiation, 8-MOP was still able to kill at least 85% of the conidia even after 12 h of exposure to full spectrum sunlight in a tropical site. However, as observed for

542 conventional fungicides, applications at 7 days interval or less for *C. acutatum* control
543 [12] may also be required for the photosensitizers.

544 APT must not damage the host plant. APT with the pure compounds, with the
545 mixture of coumarins and crude extracts applied repeatedly did not cause any damage to
546 sweet orange tree leaves and could be used for blossom blight control. Previously we
547 have shown that APT with coumarins and furocoumarins, including 8-MOP, did not
548 damage the leaves of *Citrus* species but damaged the leaves of strawberry plants [15] -
549 we observed lesions in the strawberry leaves after two weeks of daily application of 8-
550 MOP. Histological analyses indicated that APT killed cells of the strawberry epidermis
551 and parenchyma and caused oxidation of leaf pigments [15]. As photodamage to other
552 *Colletotrichum* plant hosts may occur the adverse effect of APT with coumarins and/or
553 furocoumarins should be carefully determined for each pathosystem. Much of what is
554 known about plant tolerance to photosensitizers comes from the understanding of how
555 plants deal with singlet oxygen generated in chloroplasts by chlorophyll [61-63].
556 However tolerance of plants to other endogenous photosensitizers such as coumarins,
557 furocoumarins, hypericin, thyophenes, curcumin and acetylenes is still not understood.
558 Application of PS in the environment and in large areas will require the use of
559 environmentally safe PS. Coumarins and furocoumarins are suggested here as they are
560 natural compounds produced by several commercial crops, including the citrus species
561 in which they would be used as PS.

562 The APT with several photosensitizers in combination with artificial light
563 sources or solar radiation was highly effective in killing conidia of plant-pathogenic
564 fungi. However, further studies are necessary to evaluate the efficacy of APT under
565 field conditions and the impact of this new antifungal approach to the environment as

well as to establish the appropriate formulation and applications programmes for the selected photosensitizers in each pathosystem.

5. Conclusion

Phototreatment with coumarins, furocoumarins and 'Tahiti' lime peel extract rich in these compounds kills conidia of the plant-pathogenic fungus *C. acutatum* without damaging the plant host *Citrus sinensis*. These are essential prerequisites for the use of APT in the field. The use of natural PS extracted from a by-product of the citrus processing industry to control a major citrus pathogen makes the approach even more interesting.

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Appendix A. Supplementary material

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Figure legends:

Figure 1. Chemical structures of the coumarins (A) 5,7-dimethoxycoumarin (citrpten) (B) 7-hydroxycoumarin, (D) 2*H*-chromen-2-one (coumarin) and (F) 7-methoxycoumarin and furocoumarins (C) 8-methoxypsoralen (8-MOP) and (E) 5,8-dimethoxypsoralen (isopimpinellin). The coumarins 5,7-dimethoxycoumarin and 7-hydroxycoumarin were isolated in the present study.

Figure 2. Midday solar spectral irradiance (A) and hourly recorded temperature (B) during the APT experiments.

Figure 3. Photo inactivation of *Colletotrichum acutatum* conidia, with the pure compounds and with the mixture of the coumarins (A, B and C), and with crude extracts 1 to 5 (D, E and F). Conidia were incubated with the PS for 30 min before solar radiation exposures. Error bars are standard deviations of three replicates. † No survivals were observed.

Figure 4. Photo inactivation of *Colletotrichum acutatum* conidia with solar radiation (1 h) and 8-MOP previously exposed to full-spectrum solar radiation. Experiments were carried out on: (A) 04/28/2015, (B) 07/13/2015 and (C) 07/22/2015. Error bars are standard deviations of three replicates.

792 **Figure 5.** Photodynamic treatment of sweet orange (*Citrus sinensis*) leaves with 8-MOP
793 (50 µM; 10.8 mg L^{-1}), 5,7-dimethoxycoumarin (50 µM; 10.3 mg L^{-1}), crude extract 1,
794 crude extract 5 and the mixture of coumarins (all at 12.5 mg L^{-1}). Five µL were spotted
795 every three days for 21 days on the adaxial surface of the leaves of each citrus young
796 tree. After application of the compounds, trees were kept outdoors under a natural
797 sunlight regime. Leaves were photographed (A) before treatment, (B) immediately after
798 the first treatment, (C) two hours later, (D) 3 days later, (E) two weeks later and (F)
799 three weeks later.

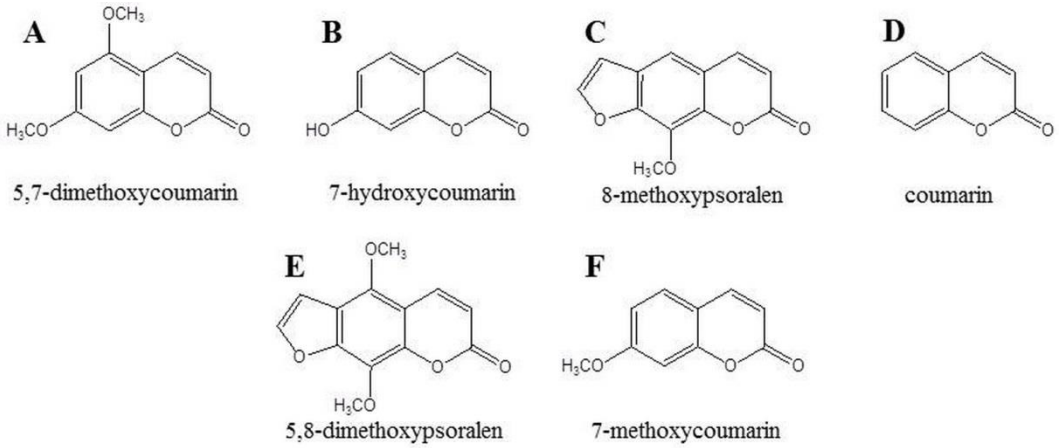
800

Figure S1. Visible absorption spectra (A, B and C) and fluorescence spectra (D, E and F) of the pure compounds (50 µM) and crude extracts (12.5 mg L^{-1}) employed in the study.

Figure S2. Visible absorption spectra (A, B and C) and fluorescence spectra (D, E and F) of 8-MOP (50 µM) after exposures to full-spectrum solar radiation.

809 **Figure 1**

810



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812

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815 and furocoumarins (C) 8-methoxypsoralen (8-MOP) and (E) 5,8-dimethoxypsoralen
816 (isopimpinellin). The coumarins 5,7-dimethoxycoumarin and 7-hydroxycoumarin were
817 isolated in the present study.

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819

820 **Figure 2**

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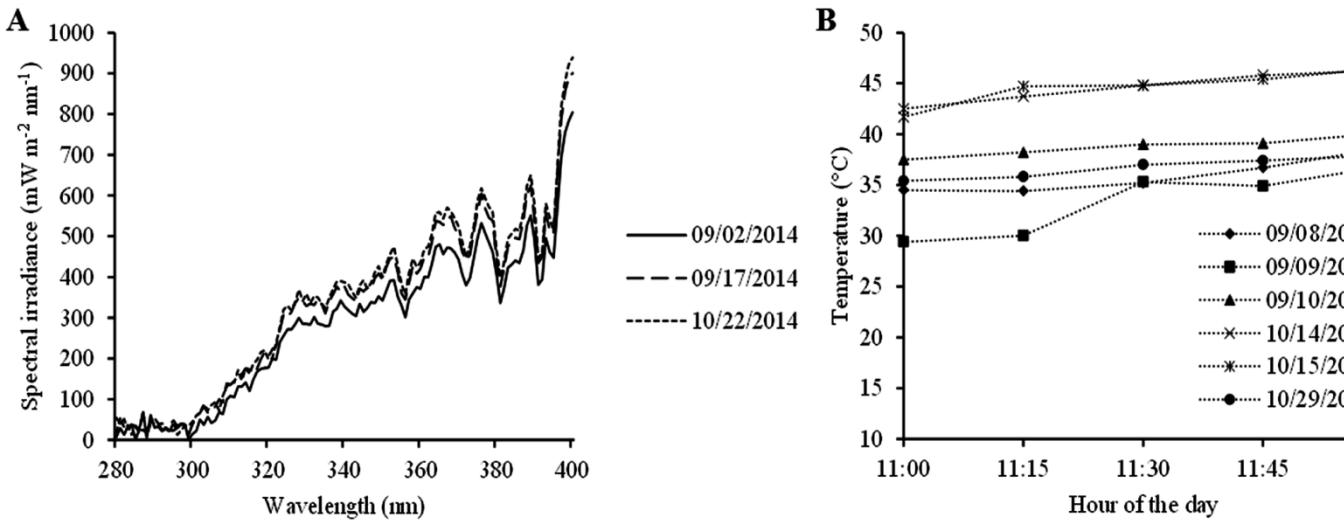

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824 **Fig. 2.** Midday solar spectral irradiance (A) and hourly recorded temperature (B) during

825 the APT experiments.

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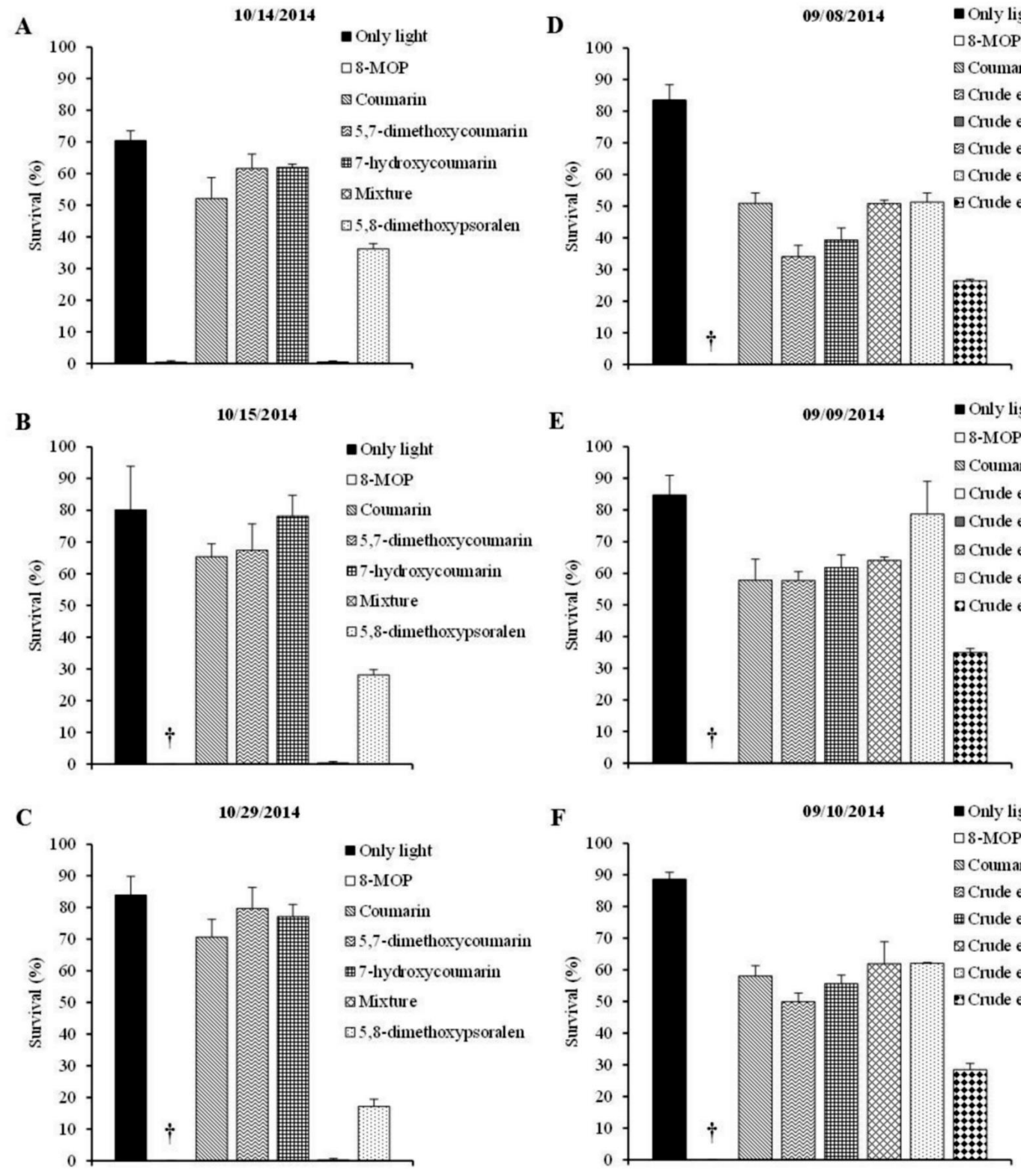
Figure 3


A
10/14/2014
B
10/15/2014
C
10/29/2014
D
09/08/2014
E
09/09/2014
F
09/10/2014
Survival (%)
Only light
8-MOP
Coumarin
5,7-dimethoxycoumarin
7-hydroxycoumarin
Mixture
5,8-dimethoxypsoralen
†

833 **Fig. 3.** Photo inactivation of *Colletotrichum acutatum* conidia, with the pure compounds
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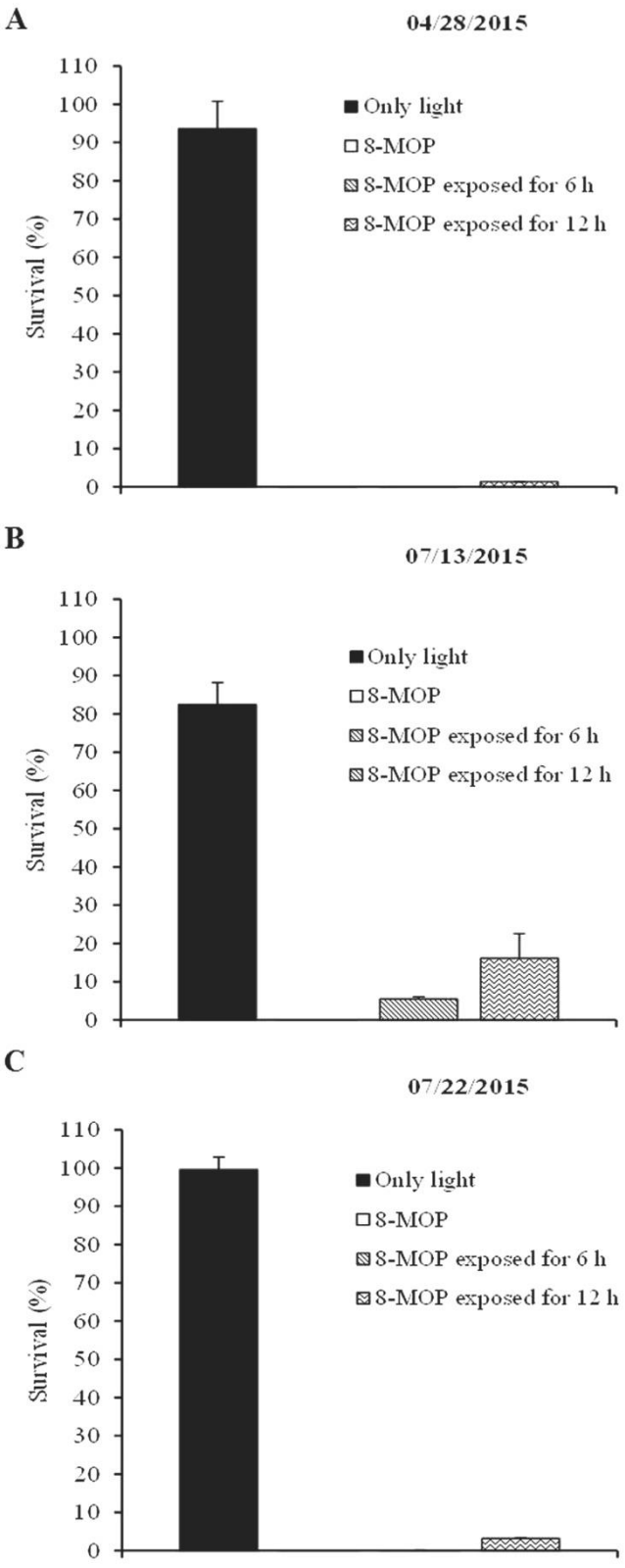
837 **Figure 4**



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842 standard deviations of three replicate dishes.

843

844 **Figure 5**

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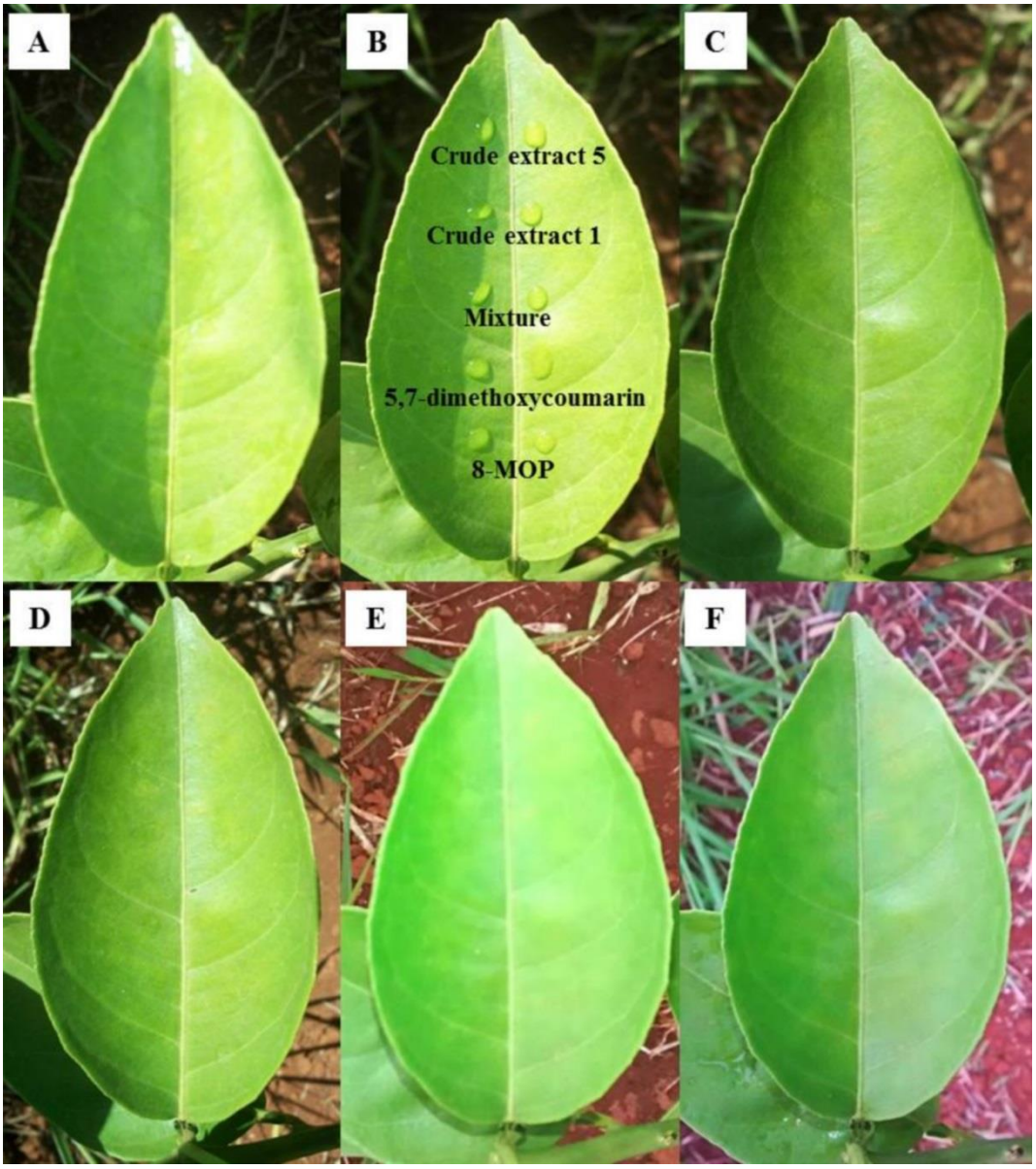

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847

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855 three weeks later.

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