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### Article

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

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22

## 23 ABSTRACT

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

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

44

45 **1. Introduction**

46

47 An important disease of citrus in the Americas is the postbloom 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

72 photochemical process that may produce several reactive oxygen species (ROS) leading  
73 to non-specific oxidative damage and causing the subsequent death of the microbial  
74 cells without significant damage to host tissues [14,15,18,19,22,23].

75         When produced at the plant surface, either via natural plant-produced PS or via  
76 applied PS, reactive species can interact with pathogens such as fungi and bacteria and  
77 even with insect ovipositors [24,25]. The multiple targets of ROS reduce the chance of  
78 selecting tolerant microorganisms. In addition, the PS used in APT are usually less toxic  
79 to humans and animals and are less aggressive to the environment than most of the  
80 currently used fungicides [14-16,22]. Also, unlike many conventional fungicides or  
81 antibiotics that kill only metabolically active cells, APT is able to kill both  
82 metabolically active and inactive dormant or quiescent structures such as fungal conidia  
83 [14,15,20,23,26] and bacterial spores [27,28]. The disadvantage of APT compared to  
84 conventional fungicides, the main strategy for control of fungal plant diseases, is that it  
85 does not work at night.

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

93         The stable covalent photoconjugation of furocoumarins with DNA was thought  
94 to bear sole responsibility for the lethal effect of this group of PS. Psoralens are capable  
95 of forming either monofunctional (single strand) or bifunctional adducts (interstrand  
96 cross-links) with DNA [35,36]. The oxygen-dependent mechanism responsible for the

97 photodynamic action of psoralens was discovered later. Joshi and Pathak [37]  
98 demonstrated the *in vitro* production of singlet oxygen ( $^1\text{O}_2$ ) and superoxide radical by  
99 several linear and angular furocoumarins. It was postulated that both forms of active  
100 oxygen contribute to the *in vitro* phototoxicity of the agents, possibly at the level of the  
101 cell membrane. The photosensitizing action of furocoumarins on membrane  
102 components was reviewed by Dall'acqua and Martelli [38]. It is currently accepted that  
103 damage by furocoumarins might result from dual or even multiple processes [39,40].

104 Photoinactivation of plant pathogenic fungi were performed using different types  
105 of PS against species of several genera [14,15,33,41-50]. So far little attention has been  
106 paid to some important aspects of APT that are crucial for its commercial use under  
107 field conditions. For example, most of the studies were performed *in vitro* and only a  
108 few of them evaluated the effects of APT on the plant host or in the environment  
109 [14,15,51]. We have demonstrated that APT under solar radiation with phenothiazinium  
110 PS, such as methylene blue derivatives, coumarins and furocoumarins efficiently kill  
111 conidia of *Colletotricum acutatum* without damaging the plant host *Citrus sinensis*  
112 [14,15].

113 The aim of the current study was to evaluate the efficacy of APT under solar  
114 radiation with pure furocoumarins and coumarins and also with extracts rich in these  
115 compounds obtained from 'Tahiti' acid lime (*Citrus latifolia*) and grapefruit (*Citrus*  
116 *paradisi*) on conidia of *C. acutatum*. All the extracts and the coumarins 7-  
117 hydroxycoumarin and 5,7-dimethoxycoumarin were obtained in the present study. The  
118 furocoumarins 5,8-dimethoxypsoralen and the mixture of the two coumarins were  
119 obtained and identified previously and their phototoxicity to fungal conidia was already  
120 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.

123

## 124 **2. Material and methods**

125

### 126 *2.1. Mass spectrometry*

127

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<sup>-1</sup>. 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<sup>-1</sup>. 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.

142

### 143 *2.2. Nuclear magnetic resonance spectroscopy*

144

145 <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> and CD<sub>3</sub>OD at 500 MHz on a Bruker  
146 Advanced DRX-500 spectrometer (Bruker, Darmstadt, Germany). <sup>13</sup>C NMR spectra

147 were acquired at 125 MHz on a Bruker Advanced DRX-400 spectrometer (Bruker,  
148 Darmstadt, Germany).

149

150 *2.3. Collection of crude extracts from 'Tahiti' lime peel and its essential oil; collection*  
151 *of grapefruit essential oil*

152

153 'Tahiti' lime peel essential oil, red and white grapefruit essential oils are  
154 produced during the industrial processing of *Citrus latifolia* and *C. paradisi*,  
155 respectively. The 'Tahiti' lime oils were kindly provided by Citrusuco Company  
156 (Fischer Group, Matão, SP, Brazil). The red and white grapefruit essential oils were  
157 obtained from Dierberger (Dierberger Óleos Essenciais, S.A., Barra Bonita, SP, Brazil).  
158 In order to isolate coumarins and furocoumarins, 100 mL of 'Tahiti' lime peel oil were  
159 basified to pH 13 with a 5 mol L<sup>-1</sup> NH<sub>4</sub>OH solution. Then, the mixture was partitioned  
160 three times with 1.8 L of ethyl acetate, and the remaining alkaline aqueous phase was  
161 acidified to pH 1 with a 5 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> solution, followed by three times partition  
162 with 1.8 L of ethyl acetate. The ethyl acetate fractions from the acidified water fraction  
163 were combined and concentrated under vacuum to afford 79.37 g of crude extract  
164 (named crude extract 1). To verify the presence of coumarin compounds, thin layer  
165 chromatographic (TLC) analysis (Kieselgel 60 F<sub>254</sub> 20 × 20 cm, Merck, Germany) was  
166 undertaken with a mobile phase of hexanes/ethyl acetate (7:3 v/v) and hexanes/acetone  
167 (7:3 v/v). TLC spots were visualized under a 254 and 366 nm UV lamp before and after  
168 sprinkling the plates with a hydroalcoholic solution of KOH 1 M. Then, an aliquot of  
169 61.5 g of the crude ethyl acetate extract was submitted to classical open column liquid  
170 chromatography (silica gel 0.060 × 0.200 mm, 60 A, Merck, Germany) using a mobile  
171 phase of hexanes/ethyl acetate at increasing gradient of polarity. All fractions obtained



172 were concentrated under vacuum and analyzed by TLC using hexanes/ethyl acetate (7:3  
173 v/v) and hexane/acetone (7:3 v/v) as previously described. The fraction of 0.97 g eluted  
174 in hexanes/ethyl acetate (75:25 v/v) showed the characteristic fluorescence emission of  
175 the furocoumarins and coumarins. For the other essential oils, 50 mL of each one were  
176 basified to pH 13 with a 5 mol L<sup>-1</sup> KOH hydroalcoholic solution (70% of KOH solution  
177 and 30% of ethanol). The mixtures were partitioned three times with 900 mL of ethyl  
178 acetate, and the remaining alkaline aqueous phases were acidified to pH 1 with a 5 mol  
179 L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> solution, followed by three times partition with 900 mL of ethyl acetate. The  
180 ethyl acetate fractions of the acidified fraction were combined and dried under vacuum  
181 to afford 23.11 g, 16.77 g, and 11.89 g of crude extracts of ‘Tahiti’ lime (crude extract  
182 2), red grapefruit (crude extract 3) and white grapefruit (crude extract 4) essential oils,  
183 respectively. To verify the presence of coumarin compounds, thin layer  
184 chromatographic (TLC) analysis (Kieselgel 60 F<sub>254</sub> 20 × 20 cm, Merck, Germany) was  
185 undertaken with a mobile phase of hexanes/ethyl acetate (7:3 v/v) and hexanes/acetone  
186 (7:3 v/v). TLC spots were visualized under a 254 and 366 nm UV lamp before and after  
187 sprinkling the plates with a hydroalcoholic solution of KOH 1 M. The crude ethyl  
188 acetate extract obtained from red grapefruit essential oil was submitted to liquid  
189 chromatography (silica gel 0.040 × 0.063 mm, 60 A, Merck, Germany) using mobile  
190 phase of hexanes/ethyl acetate at increasing gradient of polarity. All fractions obtained  
191 were concentrated under vacuum and analyzed by TLC as previously reported. Four  
192 fractions eluted with hexanes/ethyl acetate (70:30, 65:35, 60:40 and 55:45 v/v) showed  
193 the same characteristic fluorescence emission of furocoumarins and coumarins. Then,  
194 they were combined to afford 0.34 g, and submitted to preparative HPLC.

195

196 *2.4. Collection of crude extract of ‘Tahiti’ lime peel*

197

198 For comparison with the oils obtained from the citrus industry, an extract from  
199 'Tahiti' lime peel was obtained. The limes were peeled and the peels were dried in an  
200 oven at 45 °C with circulating air for 3 h. After drying, the peels were milled in a small  
201 coffee mill (Cadence MDR 301, Brazil), resulting in a powdered biomass of 20.44 g of  
202 powder. Then, the powder was macerated three times with 200 mL of hexanes for 48 h,  
203 the extract was filtered through filter paper and the solvent was concentrated under  
204 vacuum furnishing 5 mL of the oily extract. This was then basified to pH 13 with a 5  
205 mol L<sup>-1</sup> KOH hydroalcoholic solution (70% of KOH solution and 30% of ethanol). The  
206 mixture was partitioned three times with 90 mL of ethyl acetate, and the remaining  
207 alkaline aqueous phase was acidified to pH 1 with a 5 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> solution, followed  
208 by three times partitions with 90 mL of ethyl acetate. The ethyl acetate fractions of the  
209 acidified fraction were combined and dried under vacuum to afford 0.172 g of crude  
210 extract (crude extract 5). To verify the presence of coumarin compounds, thin layer  
211 chromatographic (TLC) analysis was carried out as previously described.

212

### 213 *2.5. Isolation and identification of coumarins and furocoumarins*

214

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

222 (Synergi, Phenomenex Inc., CA, USA) with pore sizes 4  $\mu\text{m}$ , 150  $\times$  4.60 mm  
223 dimension. The solvents used as mobile phase were methanol/water chromatographic  
224 grade (Merck, Germany) in gradient starting with 31% of methanol, linearly increasing  
225 to 100% of methanol at 40 min of analyses, keeping 100% till 45 min, and linearly  
226 down to 31% at 47 min and keeping 31% till 52 min. The detection was undertaken at  
227 270 and 360 nm. The volume of injection was 20  $\mu\text{L}$  (1  $\text{mg mL}^{-1}$ ), and the flow rate was  
228 1  $\text{mL min}^{-1}$ . After HPLC analyses, the separation of the compounds was performed in a  
229 Shimadzu preparative HPLC (Shimadzu, Kyoto, Japan) equipped with manual injector,  
230 UV-Vis detector (SPD-20A), binary solvent system (LC-6AD), degasser (DGU-20A5)  
231 and an automatic sample collector (FRC-10A). The column used for the separation was  
232 a preparative column Polar-RP (Synergi, Phenomenex Inc., CA, USA) with pore sizes 4  
233  $\mu\text{m}$ , 250  $\times$  21.20 mm dimension. The samples were diluted in methanol/acetonitrile  
234 (1:1) chromatographic grade (Merck, Germany) and 500  $\mu\text{L}$  of a – 60  $\text{mg mL}^{-1}$  solution  
235 was injected several times. The mobile phase used was the same described above for  
236 HPLC analyses and the flow rate was 15  $\text{mL min}^{-1}$ . After separation, the samples were  
237 analyzed by HPLC under the same conditions described above and four fractions were  
238 obtained. The fractions were named as fractions 1.1, 1.2, 2.1 and 2.2, and were analysed  
239 by GC-MS,  $^1\text{H}$  RNM and  $^{13}\text{C}$  RMN.

240

## 241 2.6. Photosensitizers

242

243 The compound 8-Methoxypsoralen (8-MOP; cat # M3501-1G) was purchased  
244 from Sigma–Aldrich, Inc. (St. Louis, MO, USA) (Fig. 1C). Coumarin (2*H*-chromen-2-  
245 one; cat # 00C1067.06.AF) was purchased from Synth (Synth, SP, Brazil) (Fig. 1D).  
246 5,7-dimethoxycoumarin ( $\text{C}_{11}\text{H}_{10}\text{O}_4$ ) (Fig. 1A) and 7-hydroxycoumarin ( $\text{C}_9\text{H}_6\text{O}_3$ ) (Fig.

247 1F) were extracted and purified from ‘Tahiti’ lime peel oil and red grapefruit essential  
248 oil, respectively. Isopimpinellin (5,8-dimethoxypsoralen, C<sub>13</sub>H<sub>10</sub>O<sub>5</sub>) (Fig. 1E), and a 3:1  
249 mixture of 7-methoxycoumarin (C<sub>10</sub>H<sub>8</sub>O<sub>3</sub>) (Fig. 1F) and citropten (5,7-  
250 dimethoxycoumarin, C<sub>11</sub>H<sub>10</sub>O<sub>4</sub>) (Fig. 1A) were obtained previously [15].

251 The absorption spectra of the pure compounds and crude extracts (1 to 5) were  
252 measured using an Ultrospec™ 2100 pro UV–visible spectrophotometer (GE  
253 Healthcare) (see Supplementary Fig. S1). The fluorescence emission spectra of the  
254 compounds were measured using a Hitachi Fluorescence Spectrometer (Hitachi,  
255 Hitachi, Japan). The excitation wavelengths were 303, 312, 324, 328, 318, 320, 317,  
256 319 and 336 nm for 8-MOP, coumarin, 7-hydroxycoumarin, 5,7-dimethoxycoumarin  
257 and crude extracts 1, 2, 3, 4 and 5, respectively (see Supplementary Fig. S1). Solutions  
258 of the compounds were prepared just prior to use. All compounds were dissolved in  
259 dimethyl sulfoxide (DMSO) (Sigma), and appropriate dilutions were prepared with  
260 0.01% (v/v) Tween 80 solution (Sigma–Aldrich).

261

## 262 2.7. Fungal isolate, colony growth and conidia production

263

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

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

277

#### 278 *2.8. Evaluation of the effect of APT on conidial survival*

279

280 In two-milliliters microtubes (Polypropilen, Axygen Scientific, CA, USA) was  
281 added 1.3 mL of the conidial suspension and solution of: (1) 8-MOP; (2) coumarin; (3)  
282 5,7-dimethoxycoumarin; (4) 7-hydroxycoumarin; (5) isopimpinellin; (6) mixture of two  
283 coumarins; (7) crude extract 1; (8) crude extract 2; (9) crude extract 3; (10) crude  
284 extract 4; (11) crude extract 5. Final concentration of conidia in the mixtures was  $2 \times$   
285  $10^6$  conidia mL<sup>-1</sup>. Final concentrations of pure compounds were 50 μM. Concentrations  
286 in mg L<sup>-1</sup> of coumarin, 8-MOP, isopimpinellin, 5,7-dimethoxycoumarin and 7-  
287 hydroxycoumarin were 7.31, 10.8, 12.3, 10.3 and 5.7, respectively. Final concentration  
288 of the mixture of coumarins and extracts was 12.5 mg L<sup>-1</sup>. Final concentration of  
289 DMSO was 1 % in all the mixtures. The tubes were held in the dark for 30 min at 25 °C  
290 and 1 mL of each suspension were transferred to a 24-well flat-bottomed microtitre  
291 plates (Polystyrene, TPP, Switzerland). Plates were covered with a 0.13-mm-thick  
292 cellulose diacetate film (JCS Industries, Le Mirada, CA) to avoid contamination and  
293 exposed to solar radiation for 1 h floating in water at  $25 \pm 2$  °C. Three different types of  
294 control-plates were prepared in parallel in all the experiments: (1) control-plates in

295 which conidia were exposed to solar radiation but not treated with the PS; (2) control-  
296 plates in which conidia were treated with the PS and protected from solar radiation  
297 during the exposure (plates were wrapped in aluminum foil) and (3) control-plates in  
298 which conidia were not treated with the PS and were protected from solar radiation.  
299 Temperatures of the conidial suspensions were recorded during the experiments. After  
300 light exposure, conidial suspensions were collected and serially diluted 10-fold in a  
301 0.01% (v/v) Tween 80 solution to provide dilutions of  $10^{-1}$  to  $10^{-2}$  times the original  
302 concentration, and 50  $\mu$ L were spread on the surface of 5 mL of PDAY medium  
303 containing 0.08 g L<sup>-1</sup> of deoxycholic acid sodium salt (Fluka, Italy) in Petri dishes (60  $\times$   
304 15 mm). Three replicate dishes were prepared for each treatment in each experiment.  
305 The dishes were incubated in the dark at 25 °C. After 24 h, colony-forming units (CFU)  
306 were counted daily at 8 $\times$  magnification for up to 7 days. Solar radiation effect and PS  
307 effect (dark toxicity) were expressed as a ratio of CFU of conidia treated only with light  
308 and only with PS to CFU of conidia treated with neither. APT effects were expressed as  
309 a ratio of CFU of conidia treated with light and PS to CFU of conidia treated with  
310 neither. As we know that exposure only to light reduces conidial survival, the  
311 significance of APT with each photosensitizer was calculated in relation to conidia  
312 exposed only to solar radiation. All the light exposures were carried out between 11 and  
313 12 h under clear sky. Pure compounds were evaluated on October 14<sup>th</sup>, 15<sup>th</sup> and 29<sup>th</sup> of  
314 2014, crude extracts were evaluated on September 8<sup>th</sup>, 9<sup>th</sup> and 10<sup>th</sup> of 2014, and 8-MOP  
315 and coumarin were evaluated on all the six days. Experiments were undertaken in  
316 Ribeirão Preto, SP, Brazil (21° 10' 39" S latitude, 546 m altitude).

317

318 *2.9. Evaluation of 8-MOP stability under solar radiation*

319

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  $\mu$ M 8-MOP solution was placed into Petri dishes (60  $\times$  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  $25 \pm 2$  °C. Experiments were performed under  
328 clear sky on March 24<sup>th</sup>, April 17<sup>th</sup> and July 13<sup>th</sup> 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  $\mu$ M 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-bottomed 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 22<sup>th</sup> with the solution that were exposed on March 24<sup>th</sup>, April 17<sup>th</sup> and July 13<sup>th</sup>  
336 2015, respectively.

337

#### 338 *2.10. Solar radiation measurements*

339

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*

346

347 The three *C. sinensis* plants employed in the study were approximately 1.7 m  
348 tall. Five  $\mu\text{L}$  of 8-MOP ( $50 \mu\text{M}$ ;  $10.8 \text{ mg L}^{-1}$ ), 5,7-dimethoxycoumarin ( $50 \mu\text{M}$ ;  $10.3 \text{ mg}$   
349  $\text{L}^{-1}$ ), crude extract 1, crude extract 5 and the mixture of coumarins (all at  $12.5 \text{ mg L}^{-1}$ )  
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.

355

356 *2.12. Statistical analysis*

357

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*

367

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



370 grapefruit essential oil (fraction 2) were elucidate, as following: Fraction 1.1 was  
371 identified as 5,7-dimethoxycoumarin (Fig 1A), the  $^1\text{H}$  RMN data was obtained in 500  
372 MHz using  $\text{CDCl}_3$  as solvent. The data showed doublets at 6.2 ppm ( $d$ ,  $J= 9.5$ ) and 7.9  
373 ppm ( $d$ ,  $J= 9.5$ ), corresponding to H-3 and H-4, respectively, wich are hydrogens of a  
374 pyrone moiety in Z configuration. Chemical shifts corresponding to aromatic hydrogens  
375 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  
376 3.8 and 3.9 ppm showed the presence of two methoxyl groups. The  $^{13}\text{C}$  RMN data was  
377 obtained in 125 MHz also using  $\text{CDCl}_3$  as solvent and the chemical shifts showed  
378 signals at 161.9 ppm, 111.2 ppm, 139.2 ppm, 104.4 ppm, 157.4 ppm, 95.2 ppm, 164.1  
379 ppm, 93.1 ppm, 157.2 ppm, 55.8 ppm and 55.9 ppm. These chemical shifts correspond  
380 to C-2, C-3, C-4, C-4a, C-5, C-6, C-7, C-8, C-8a,  $\text{OCH}_3$  and  $\text{OCH}_3$ , respectively. EI-  
381 MS/z calculated for the fraction 1.1 was 206 ( $\text{C}_{11}\text{H}_{10}\text{O}_4$ ). Fraction 2.1 was identified as  
382 7-hydroxycoumarin (Fig.1B), and the  $^1\text{H}$  NMR data was obtained in 500 MHz using  
383  $\text{CD}_3\text{OD}$  as solvent. The data showed doublets at 6.2 ppm ( $d$ ,  $J= 9.5$ ) and 7.8 ppm ( $d$ ,  $J=$   
384 9.5), corresponding to H-3 and H-4, respectively, wich are hydrogens of a pyrone  
385 moiety. Chemical shifts corresponding to aromatic hydrogens were detected at 6.7 ppm  
386 ( $d$ ,  $J = 2.3$  Hz, H-8), 7.3 ppm ( $d$ ,  $J = 8.5$  Hz, H-5) and at 6.8 ppm ( $dd$ ,  $J = 2.3$  and  $J =$   
387 8.5 Hz, H-6). The chemical shift at 5.0 ppm showed the presence of one hydroxyl  
388 group. The  $^{13}\text{C}$  NMR data was obtained in 125 MHz also using  $\text{CD}_3\text{OD}$  as solvent and  
389 the chemical shifts showed the the signals at: 163.7 ppm, 112.3 ppm, 146.1 ppm, 113.1  
390 ppm, 130.7 ppm, 114.5 ppm, 163.2, 103.4 ppm, 157.3 ppm. These chemical shifts  
391 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  
392 calculated for the fraction 2.1 was 162 ( $\text{C}_9\text{H}_6\text{O}_3$ ). Chemical shifts for both coumarins  
393 were compared with the data previously reported [52].

394 All the crude extracts were analyzed by GC-MS. The data obtained were  
395 compared with the similarity index expressed as a percentage by the equipment  
396 standards library. Moreover, linear retention index (LRI) calculations were carried out  
397 and the indices were compared with literature data, making possible the identification of  
398 the crude extracts components. All the crude extracts, except crude extract 4, obtained  
399 from white grapefruit essential oil, showed the presence of coumarins and/or  
400 furocoumarins. From the crude extract 1, obtained from Tahiti lime peel oil, only 5,7-  
401 dimethoxycoumarin (9.98%) was identified and isolated. From the crude extract 2,  
402 obtained from Tahiti lime essential oil, coumarins 5,7-dimethoxycoumarin (19.84%)  
403 and 7-methoxycoumarin (10.19%), and the furocoumarins isopimpinellin (7.51%) and  
404 bergapten (4-methoxy-7*H*-furo[3,2-*g*]chromen-7-one) (8.83%) were identified. From  
405 the crude extract 3, obtained from red grapefruit essential oil, it was identified and  
406 isolated 7-hydroxycoumarin (5.28%), 7-hydroxy-6-methoxy-4-methyl-coumarin  
407 (0.50%) and 7-methoxy-8-(2-oxo-3-methyl-butyl)coumarin (10,82%). The crude extract  
408 5, obtained from Tahiti lime peel oil showed the highest variety and percentage of  
409 coumarins in comparison to all the others. Among the six compounds present in the  
410 extract, four of them were coumarins or furocoumarins, such as 5,7-dimethoxycoumarin  
411 (45.23%), 7-methoxycoumarin (8.19%), bergapten (22.83%) and isopimpinellin  
412 (15.73%). In the majority of the studied extracts it was found the terpenoids commonly  
413 found in citrus essential oils such as limonene, linalool,  $\alpha$  and  $\gamma$ -terpineol,  $\alpha$  and  $\beta$ -  
414 bisabolene and  $\gamma$ -terpinene commonly found in citrus essential oils [53,54].

415

416 *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<sup>th</sup>) to 29.64% (Oct 14<sup>th</sup>) ( $P < 0.05$  for Sept 8<sup>th</sup>, 9<sup>th</sup> and Oct 14<sup>th</sup> and  $P > 0.05$  for Sept  
423 10<sup>th</sup> and Oct 15<sup>th</sup> and 29<sup>th</sup>) (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  $\mu$ M of 8-MOP, coumarin, 5,7-dimethoxycoumarin,  
434 7-hydroxycoumarin or isompimpinellin, or with 12.5 mg L<sup>-1</sup> 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<sup>th</sup> and 10<sup>th</sup> and Oct 15<sup>th</sup>  
437 and 29<sup>th</sup> and 99.99 and 99.56% on Sept 9<sup>th</sup> and Oct 14<sup>th</sup>, 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<sup>th</sup>) and 82.80% (Oct 29<sup>th</sup>) of the

442 conidia (thus, a reduction of less than 1 log in conidial survival) ( $P < 0.05$  for all days)  
443 (Figs. 3A-C). APT with the coumarin was much less effective and killed between  
444 29.28% (Oct 29<sup>th</sup>) and 47.85% (Sept 9<sup>th</sup>) of conidia ( $P < 0.05$  for Sept 8<sup>th</sup>, 9<sup>th</sup> and 10<sup>th</sup>  
445 and  $P > 0.05$  for Oct 14<sup>th</sup>, 15<sup>th</sup> and 29<sup>th</sup>) (Figs. 3A-F). APT with 5,7-dimethoxycoumarin  
446 had little effect and killed between 23.33% (Oct 29<sup>th</sup>) and 38.40% (Oct 15<sup>th</sup>) of the  
447 conidia ( $P > 0.05$  for all days) as well as APT with 7-hydroxycoumarin which killed  
448 between 21.96% (Oct 15<sup>th</sup>) and 38.06% (Oct 14<sup>th</sup>) ( $P > 0.05$  for all days) (Figs. 3A-C).  
449 APT with the mixture of the two coumarins was very effective and killed between  
450 99.53% (Oct 14<sup>th</sup>) and 99.72% (Oct 29<sup>th</sup>) of the conidia (reduction of approximately 3  
451 logs in conidial survival) ( $P < 0.05$  for all days) (Figs. 3A-C).

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

458

### 459 *3.3. Evaluation of 8-MOP stability under solar radiation*

460

461 Exposure of 8-MOP to solar radiation change its absorption and fluorescence  
462 emission spectra (see Supplementary Fig. S1) and reduce its effectiveness in APT (Fig.  
463 4). All the changes were dependent of the exposure time. When APT were performed  
464 using 8-MOP previously exposed to solar radiation for 12 h, mortality ranged from  
465 83.87 to 98.8% insted of 100% archived with the non exposed photosensitizer (Fig. 4).

466

### 467 3.4. Evaluation of APT effects on leaves of *Citrus sinensis*

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

471

## 472 4. Discussion

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

490 The effects of APT under solar radiation with the five pure compounds (two  
491 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 compounds 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 (citraopten) (reduction of approximately 3 logs). As APT with citraopten 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 little 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 efficacy of the photosensitization. 8-MOP penetrates the conidia of *C. acutatum* and  
510 accumulates in numerous cytoplasmatic vesicles [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 of *C. acutatum* and  
513 *Aspergillus nidulans* conidia with 8-MOP at 50  $\mu$ M 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

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

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

533         The residual effect of the antimicrobial is usually desirable because it allows a  
534 reduction in the number of applications required to control the target pathogen. It is  
535 known that exposure to high irradiance causes photobleaching of the PS and the loss of  
536 its photosensitizing activity [14,59]. The exposure of 8-MOP to solar radiation changed  
537 its photophysical properties and reduced its effectiveness in APT; i.e., APT with 8-MOP  
538 previously exposed to solar radiation killed between 85 and 98% of the conidia instead  
539 of the 100% achieved with the nonexposed PS. Despite the inactivation caused by solar  
540 radiation, 8-MOP was still able to kill at least 85% of the conidia even after 12 h of  
541 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



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

568

## 569 **5. Conclusion**

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

576

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582

583

## 584 **Appendix A. Supplementary material**

585

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770 **Figure legends:**

771

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

777

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

780

781 **Figure 3.** Photo inactivation of *Colletotrichum acutatum* conidia, with the pure  
782 compounds and with the mixture of the coumarins (A, B and C), and with crude extracts  
783 1 to 5 (D, E and F). Conidia were incubated with the PS for 30 min before solar  
784 radiation exposures. Error bars are standard deviations of three replicates. † No  
785 survivals were observed.

786

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

791

792 **Figure 5.** Photodynamic treatment of sweet orange (*Citrus sinensis*) leaves with 8-MOP  
793 (50  $\mu\text{M}$ ; 10.8  $\text{mg L}^{-1}$ ), 5,7-dimethoxycoumarin (50  $\mu\text{M}$ ; 10.3  $\text{mg L}^{-1}$ ), crude extract 1,  
794 crude extract 5 and the mixture of coumarins (all at 12.5  $\text{mg L}^{-1}$ ). Five  $\mu\text{L}$  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

801 **Figure S1.** Visible absorption spectra (A, B and C) and fluorescence spectra (D, E and  
802 F) of the pure compounds (50  $\mu\text{M}$ ) and crude extracts (12.5  $\text{mg L}^{-1}$ ) employed in the  
803 study.

804 **Figure S2.** Visible absorption spectra (A, B and C) and fluorescence spectra (D, E and  
805 F) of 8-MOP (50  $\mu\text{M}$ ) after exposures to full-spectrum solar radiation.

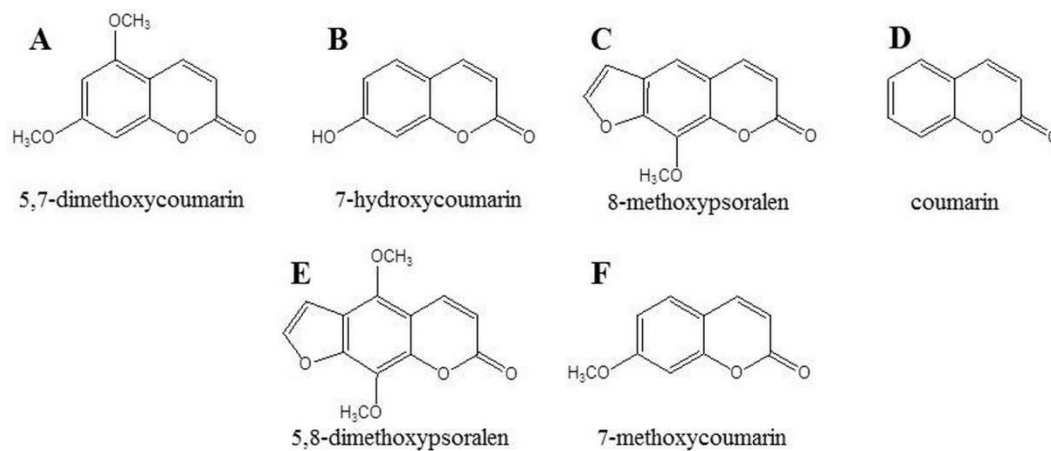
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809 **Figure 1**

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813 **Fig. 1.** Chemical structures of the coumarins (A) 5,7-dimethoxycoumarin (citraopten) (B)814 7-hydroxycoumarin, (D) 2*H*-chromen-2-one (coumarin) and (F) 7-methoxycoumarin

815 and furocoumarins (C) 8-methoxypsoralen (8-MOP) and (E) 5,8-dimethoxypsoralen

816 (isopimpinellin). The coumarins 5,7-dimethoxycoumarin and 7-hydroxycoumarin were

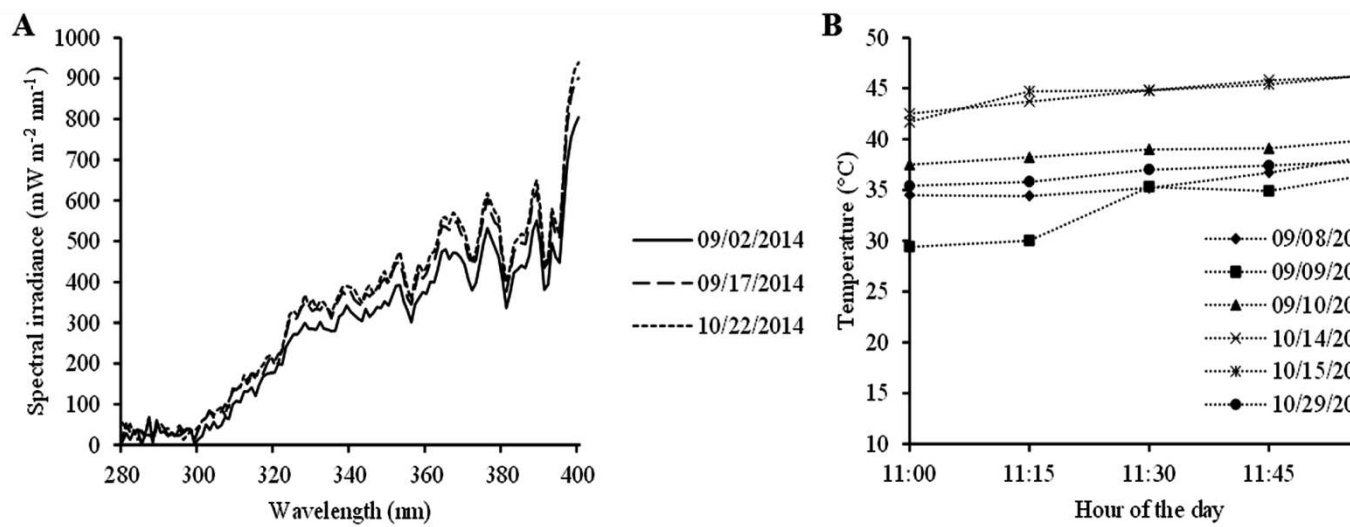
817 isolated in the present study.

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820 **Figure 2**

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

825 the APT experiments.

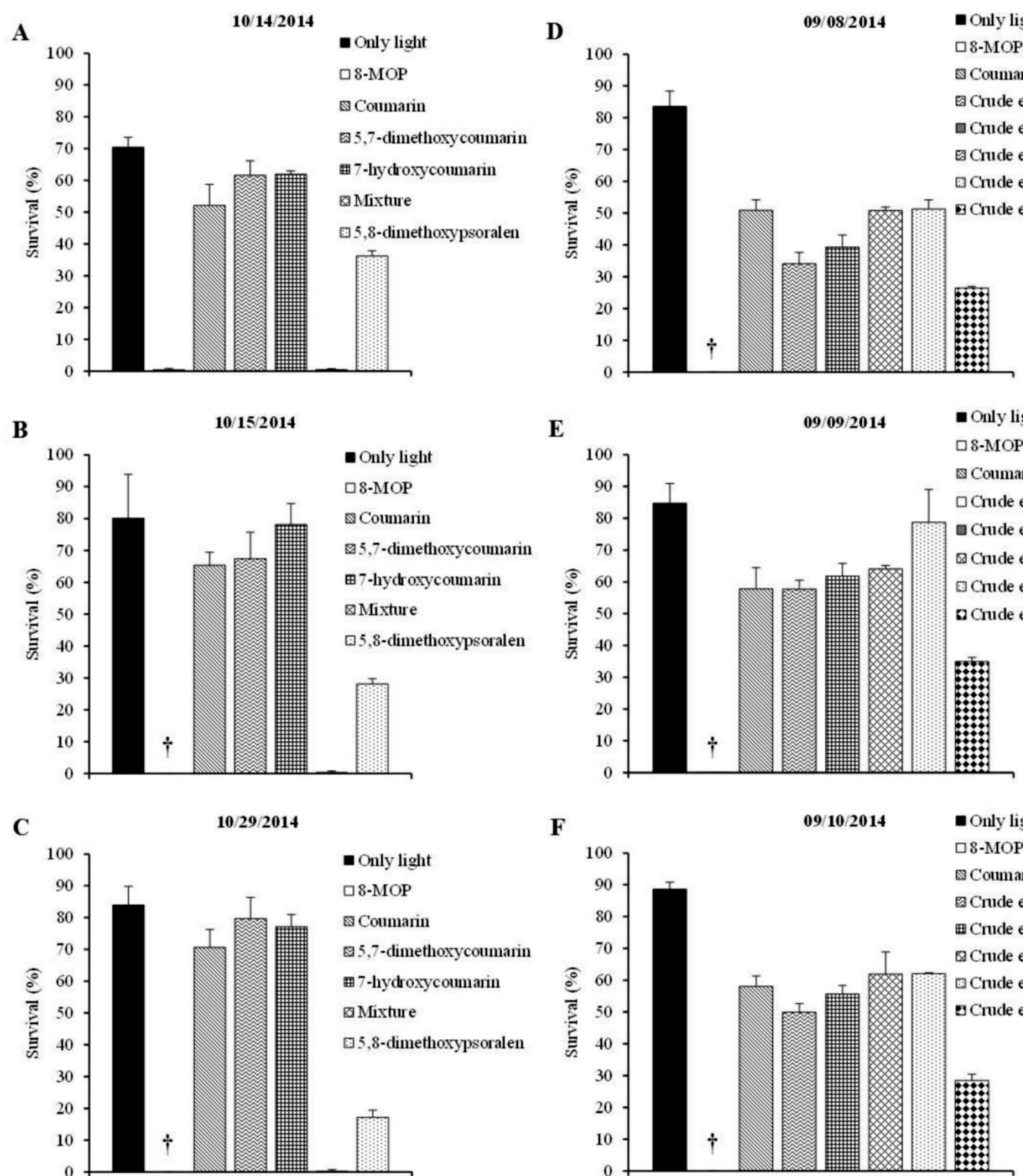
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829 **Figure 3**

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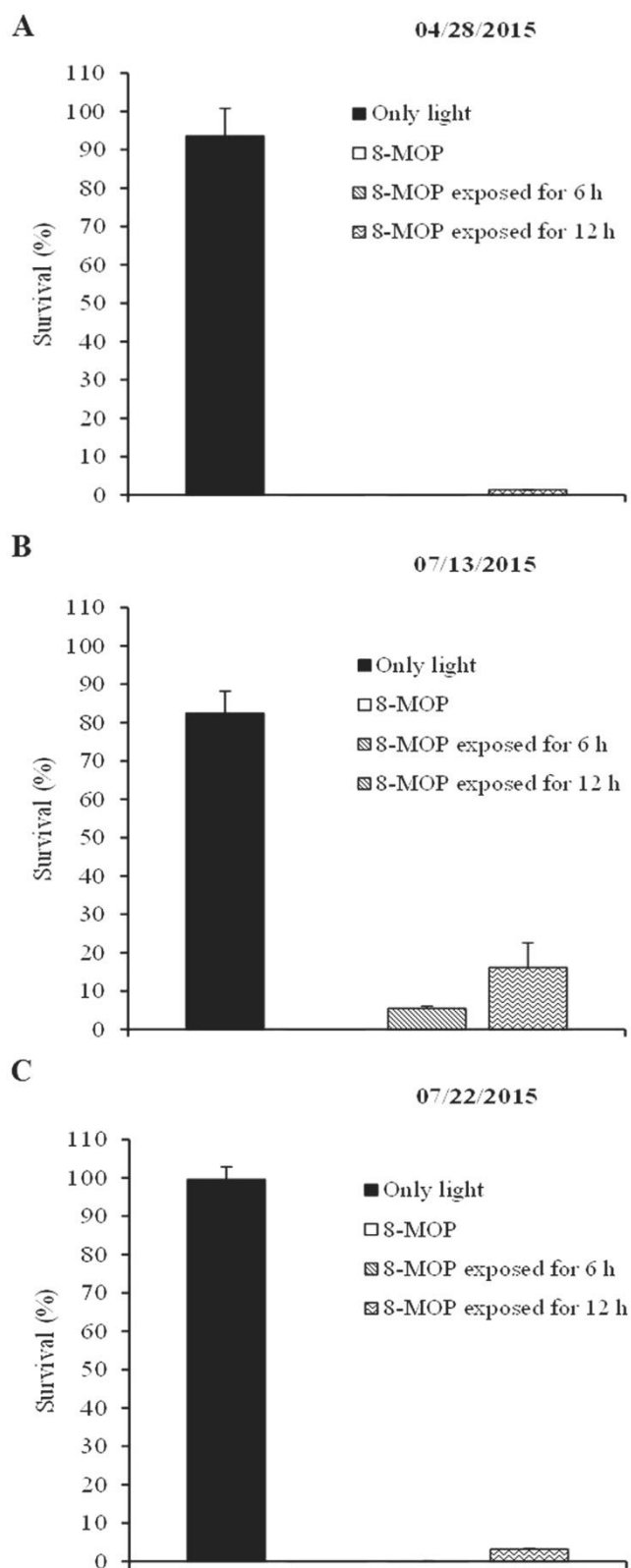


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833 **Fig. 3.** Photo inactivation of *Colletotrichum acutatum* conidia, with the pure compounds  
834 and with the mixture of the coumarins (A, B and C), and with crude extracts 1 to 5 (D,  
835 E and F). Conidia were incubated with the PS for 30 min before solar radiation  
836 exposures. Error bars are standard deviations of three replicate dishes.



837 **Figure 4**

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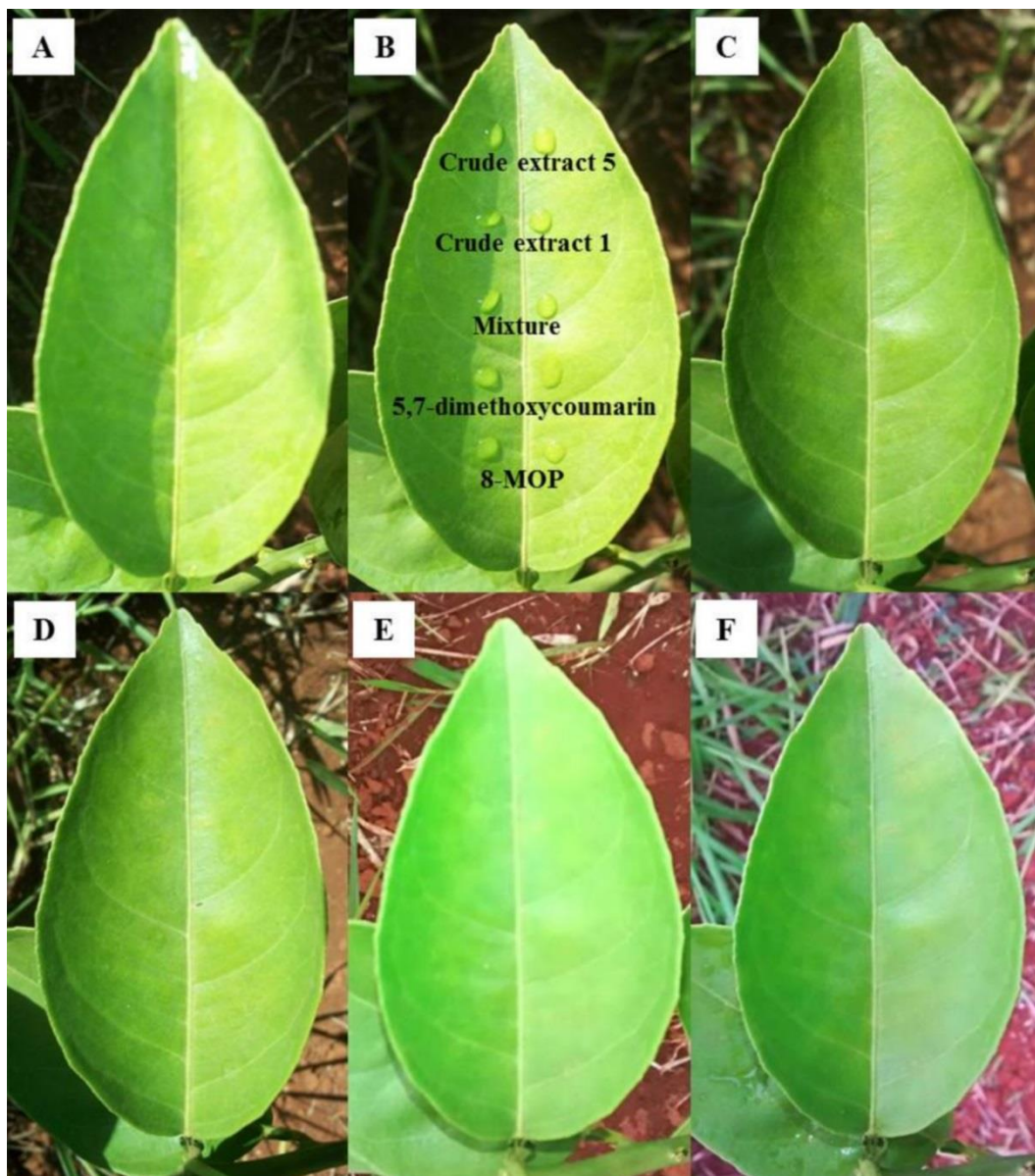
839

840 **Figure 4.** Photo inactivation of *Colletotrichum acutatum* conidia with solar radiation (1  
841 h) and 8-MOP previously exposed to full-spectrum solar radiation. Error bars are  
842 standard deviations of three replicate dishes.

843

844 **Figure 5**

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847

848 **Fig. 5.** Photodynamic treatment of sweet orange (*Citrus sinensis*) leaves with 8-MOP  
849 ( $50 \mu\text{M}$ ;  $10.8 \text{ mg L}^{-1}$ ), 5,7-dimethoxycoumarin ( $50 \mu\text{M}$ ;  $10.3 \text{ mg L}^{-1}$ ), crude extract 1,  
850 crude extract 5 and the mixture of coumarins (all at  $12.5 \text{ mg L}^{-1}$ ). Five  $\mu\text{L}$  were spotted  
851 every three days for 21 days on the adaxial surface of the leaves of each citrus young  
852 tree. After application of the compounds, trees were kept outdoors under a natural  
853 sunlight regime. Leaves were photographed (A) before treatment, (B) immediately after

854 the first treatment, (C) two hours later, (D) 3 days later, (E) two weeks later and (F)  
855 three weeks later.

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